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CIÊNCIAS BIOMÉDICAS

Kidney Disease Beyond the Glomerular Filtration Rate: The Role of Mitochondria on the Tubule-Interstitial Crosstalk

Patrícia Crespo Braga

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Tese de candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Categoria – Assistente Hospitalar Graduada Senior de Nefrologia, Professora Associada Convidada com Agregação
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Coorientador – Doutor Marco G. Alves
Categoria – Professor Auxiliar
Afiliação – Departamento de Ciências Médicas, Universidade de Aveiro

De tudo, ficam três coisas:
A certeza de que estamos sempre começando…,
A certeza de que precisamos continuar
A certeza de que seremos interrompidos antes de terminar…
Portanto devemos:
Fazer da interrupção um caminho novo…
da queda um passo de dança,
Do medo uma escada,
Do sonho, uma ponte,
Da procura um encontro…

Fernando Sabino, "III- O Escolhido", in "O Encontro Marcado", Ed Record, ed 68ª, p.145

Of all, three things remain:
The certainty that we are always starting…,
The certainty that we need to continue
The certainty that we will be interrupted before finishing…
Therefore, we must:
Making from interruption a new path…
From the fall a dance step,
From fear a stair
From dream a bridge
From searching a meeting…

Fernando Sabino, "III- The Chosen", in "O Encontro Marcado", Ed Record, ed 68ª, p.145

Declaração de Honra

Declaro que a presente tese é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras de atribuição e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio constitui um ilícito académico.

(Patrícia Crespo Braga)

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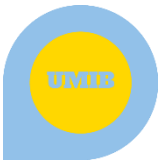

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List of Publications

Publications included in this thesis:

1. **Braga PC**, Alves MG, Rodrigues AS, Oliveira PF (2022). Mitochondrial Pathophysiology on Chronic Kidney Disease. *International Journal of Molecular Sciences,* 23, 1776. https://doi.org/10.3390/ijms23031776

2. Costa J, **Braga PC**, Rebelo I, Oliveira PF, Alves MG (2023). Mitochondria Quality Control and Male Fertility. *Biology* 12,6. https://doi.org/10.3390/biology12060827

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4. Bernardino RL, **Braga PC**, Oliveira PF, Alves MG (2024). Chapter 19 - Determination of glycolysis flux by extracellular flux measurements. In: Ferreira R, Oliveira PF, Nogueira-Ferreira R, eds. *Glycolysis: Academic Press*; 2024: 443-54. https://doi.org/10.1016/B978-0-323-91704-9.00004-5

5. **Braga PC**, Rodrigues AS, Oliveira PF and Alves MG (2024). Chapter 20- Mitochondrial transplantation in the Kidney. In: Ostojic SM, Kubat GB, Ulger O, Gunaydin S, eds. *Mitochondrial Transplantation and Transfer: Academic press*; 2024: 411-434. https://doi.org/10.1016/B978-0-443-18858-9.00020-2

6. **Braga PC**, Vitorino R, Ferreira R, Marques M, Oliveira, PF, Rodrigues AS, Alves MG (2024). Dietary intervention is not able to mitigate the effects of childhood obesity in kidney mitochondria quality control mechanism. *Clinical Nutrition* (submitted).

7. **Braga PC**, Guerra-Carvalho B, Almeida M, Pereira S, Oliveira PF, Alves MG, Rodrigues AS (2024). Metabolic profile of urine of albuminuric and non-albuminuric nephropathic

diabetic patients suggests TCA-cycle related biomarkers. *Diabetes Research and Clinical Practice* (submitted).

8. **Braga PC**, Oliveira PF, Rodrigues AS and Alves MG (2024). Mitochondria quality control as predictor of Diabetic Kidney Disease progression in non-albuminuric patients. (unsubmitted paper).

Abstract

Diabetic kidney disease (DKD) is the dominant cause of end-stage renal disease (ESRD) worldwide. Traditionally, the clinical definition of DKD is a progressive deterioration in kidney function accompanied by chronic albuminuria with an albumin-to-creatinine ratio greater than 30 mg/g and a subsequent decline in the glomerular filtration rate (GFR), eventually leading to dialysis. Beyond the classical clinical classification, it is now well established that a cluster of patients with DKD may exhibit normal levels of albuminuria, referred as NA-DKD patients. This can lead to a silent progression of the disease, potentially delaying diagnosis and appropriate management. Patients with NA-DKD present greater difficulty in clinical management, in the absence of the risk biomarker- albuminuria, and the loss of renal function can progress to advanced stages without adjusted treatment. Thus, there is an urgent need to find a more accurate method to identify metabolic patterns of risk in NA-DKD patients. The kidney is a highly metabolic demanding organ. A critical factor in the development and progression of DKD is a shift in kidney metabolism, specifically within the proximal tubular cells. This metabolic reprogramming involves a shift away from the highly efficient energy production of mitochondrial oxidative phosphorylation towards the less efficient glycolysis and its associated pathways. Therefore, understanding the mitochondria function and the management of its quality control, particularly during the early stages of DKD, holds a great relevance for potentially intervening early in the disease's progression. The main aim of this dissertation was to elucidate the complex metabolic reprogramming occurring in DKD, with a particular focus on the pathophysiological role of mitochondria to identify new opportunities of clinical risk stratification and tailored therapy. This goal was pursued through basic and translational sequential objectives. The initial focus was to investigate how varying levels of glucose stimuli, in combination with insulin resistance (IR) induced through lipotoxicity, can disrupt the metabolic secretome, ultimately impacting mitochondria bioenergetics. For that, we used an immortalized human kidney cell line (HK-2) as model. We exposed the cells to different concentrations of glucose (5 mM, 11 mM and 22 mM) for 24 hours to mimic physiological, mild, and severe hyperglycemic conditions, respectively. In addition, we also evaluated the combined effect of varying levels of glucose and IR induced by lipotoxicity through palmitic acid administration. We assessed the impact of mild and severe hyperglycemia with and without IR on the metabolic secretome of the cells, through proton-nuclear magnetic resonance (^{1}H-NMR), the live-cell mitochondria function using Seahorse Analytics, and we evaluated the mitochondrial membrane

potential, and mitochondrial complexes I and II activities, by enzymatic assays. Furthermore, we measured fatty acid oxidation (FAO) and lipid accumulation. With this work we were able to demonstrate that progression from mild to severe hyperglycemia induced a metabolic shift, where gluconeogenic metabolism played a crucial role in supplying the energy requirements of HK-2. This effect of metabolic reprogramming was reflected in the mitochondrial bioenergetic parameters, where cells cultured under mild hyperglycemic conditions exhibited increased basal respiration, ATP-linked production, maximal respiration capacity, and spare respiration capacity. However, these parameters decreased when cells were cultured under higher glucose concentrations and IR. Our results revealed that in our cell model, lipid accumulation was particularly evident in groups where IR was induced. Furthermore, cells exposed to higher glucose concentrations showed a greater dependence on FAO for metabolism. Notably, in normoglycemic conditions (5 mM), inhibition of FAO led to increased ATP production, suggesting that cells in this state do not rely on FAO as compared to the same glucose condition with induced IR. These findings suggest that the metabolic shifting from mild to severe hyperglycemia coupled with induced IR, impacts HK-2 mitochondria bioenergetics. This reduced capacity for FAO in these cells contributes to intracellular lipid accumulation and mitochondrial dysfunction both of which are critical hallmarks of kidney disease progression. Understanding the role of FAO and its impact on kidney cellular lipid metabolism and mitochondrial function is crucial for identifying potential therapeutic targets to intervene in the progression of DKD. Further research in this area could pave the way for the development of novel treatment strategies aimed at preserving renal function and ameliorating kidney disease in diabetic patients.

This data challenged us to explore the effect of FAO in an animal model and understand how a diet rich in fat (which is associated to obesity and diabetes) and its reversion could influence FAO restoration and its impact in kidney tissue mitochondria function. Nowadays, high-fat diets (HFD) are prevalent and have been linked to non-communicable diseases, including chronic kidney injury. However, the precise mechanisms underlying obesity-related kidney impairment remain elusive. In particular, the metabolic changes that occur in the kidney profile during the transition from an HFD to a healthy/normal diet and the subsequent impact on mitochondria function are still poorly understood. Thus, on the second work, we aimed to characterize the deleterious effects of HFD on kidney mitochondrial quality control mechanisms and associated proteins using a C57BL/6 mouse model. Male mice were divided in 3 groups: a control group fed a standard diet (CTR, n=5), a group fed with a diet high in fat (HFD, n=5) and another group fed with HFD but transitioned back to a standard diet after 60 days post-weaning (HFDt) (n=5). The mice were kept under those diets for 200 days. Biometric data and whole-body metabolism were assessed. Expression of genes associated with mitochondrial dynamics, mitochondrial

complexes and antioxidant defenses in the kidney tissue were analyzed. Kidney homogenates enriched in mitochondria were prepared and characterized by mass spectrometry-based proteomics. In HFD mice, the kidney tissue exhibited a marked reduction in PGC-1α expression. This reduction was accompanied by an imbalance in mitochondrial dynamics, characterized by an increase in fusion markers (elevated MFN1) and a decrease in fission markers (FIS1 and DRP1). Additionally, the activity of mitochondrial CI was increased, while CII activity was decreased in kidney tissue following HFD and HFDt. In the kidneys of HFD fed mice, the enzymatic activity of Manganese Superoxide Dismutase (MnSOD), a key mitochondrial antioxidant enzyme, was significantly reduced, while the activity of Glutathione Reductase (GR), a cytosolic antioxidant enzyme, was elevated. Importantly, both enzyme activities returned to normal levels upon switching back to a standard diet. Proteomic analysis showed alterations in the kidneys of proteins associated with glutathione and glycine metabolism, FAO, and peroxisomal function in HFD and HFDt groups. Notably, HFD negatively affected glutathione-associated proteins in the kidney such as Gsta3 and Gsr, but these changes were reversed with dietary correction. The Acsm3 protein was downregulated in the kidney after HFD and upregulated following dietary correction. Moreover, the interconnected network between mitochondria and peroxisomes, which share some machinery, was compromised in the kidney under HFD-induced stress. HFD impaired kidney FAO in peroxisomes, as evidenced by downregulation of Pecr after HFD and HFDt. This result underscores that dietary correction following obesity can mitigate the systemic metabolic dysfunction and attenuate kidney mitochondria dysfunction. However, it also reveals that such intervention may not be sufficient to fully restore kidney mitochondria dynamics and bioenergetics.

So far it is clear that mitochondria network is a main point for targeted therapeutic strategies aiming at preventing the progression of DKD. Thus, we may hypothesize that mitochondrial-derived metabolites could serve as potential diagnostic biomarkers for kidney disease. Following this translational approach, progressing from a 2D cell model to an animal model, our final challenge was to identify potential diagnostic biomarkers capable of distinguishing between a control population and patients with DKD, either albuminuric (A-DKD) or NA-DKD. For that, 40 patients were selected and divided into a healthy group (n=23), composed of kidney living donors, presenting glycated hemoglobin (HbA1c) < 6.5 %, eGFR > 60 ml/min/1.73 m^2 and non-albuminuric (< 30 mg/g creatinine); and a DKD group (n=17), with eGFR < 60 ml/min/1.73 m^2 and HbA1c > 6.5 %. DKD patients were further split into NA-DKD (n=5, albuminuria < 30 mg/g creatinine) and A-DKD (n=12, albuminuria > 30 mg/g creatinine). The urine of all subjects was analyzed using a non-targeted ^{1}H-NMR metabolomics approach to search for specific metabolic signatures that could characterize both groups. We were able to detect 23 metabolites in urine sample. Citrate, hypoxanthine,

formate and glycine were downregulated in DKD when compared to control group, while glucose, trans-aconitate and glycolate were upregulated. Pearson's correlations with albuminuria, demonstrated that citrate presented the strongest negative correlation, followed by hypoxanthine and formate. On the other hand, glycolate, taurine, and trans-aconitate were positively correlated with albuminuria. Receiver operating characteristic (ROC) analysis was employed to establish a cut-off value for the urinary metabolites that could effectively discriminate between patients with normal and high levels of albuminuria. This analysis indicated that citrate, hypoxanthine, and taurine were the most promising predictors for differentiating between patients with normal and elevated levels of albuminuria, as kidney disease progresses. Among DKD patients, glycine and alanine levels were found to be upregulated and downregulated, respectively, in NA-DKD when compared to A-DKD patients.

In sum, the urine metabolome profile revealed that levels of metabolites related with mitochondria function can potentially serve as a marker to individualize therapeutic choices in DKD, where mitochondria related metabolites can have a key role, especially in the non-albuminuric phenotype.

In conclusion, our findings underscore the role of FAO imbalance in DKD, even when metabolic reprogramming efforts are made to counteract to the effects of diabetes and obesity. Our research further solidifies the idea that mitochondria are key players in DKD, and their functioning shows a correlation with renal hyperfiltration, suggesting that decreased tubular kidney function and dysmetabolism may be potential biomarkers of risk of early detection of disease.

Resumo

A nefropatia diabética (DKD) é a principal causa de doença renal crónica terminal a nível mundial. Tradicionalmente, a definição clínica da DKD é uma deterioração progressiva da função renal acompanhada por albuminúria crónica com uma relação albuminúria/creatinina superior a 30 mg/g, e um subsequente declínio da taxa de filtração glomerular (GFR), que eventualmente leva à necessidade de diálise. Para além da classificação clínica clássica, reconhece-se atualmente que um grupo de doentes com DKD podem apresentar níveis normais de albuminúria, designados de doentes não albuminúricos (NA-DKD). Isto pode levar a uma progressão silenciosa da doença, com consequente atraso no diagnóstico e tratamento adequado. Os doentes com NA-DKD apresentam maior dificuldade de gestão clínica, na ausência do biomarcador de risco – a albuminúria, podendo a perda de função renal evoluir até estádios avançados sem um tratamento ajustado. Assim, existe uma necessidade de encontrar um método mais preciso para identificar padrões metabólicos de risco em doentes NA-DKD. O rim é um órgão altamente exigente em termos energéticos. Um fator crítico no desenvolvimento e progressão da DKD é uma alteração no metabolismo renal, nomeadamente nas células tubulares proximais. Esta alteração metabólica envolve uma reprogramação energética, em que há a mudança da produção de energia altamente eficiente, a fosforilação oxidativa, para uma via menos eficiente, a glicólise e outras vias associadas. Portanto, compreender a função mitocondrial e a gestão dos seus mecanismos, particularmente durante as fases iniciais da DKD, é extremamente importante para intervir na progressão da doença. O principal objetivo desta dissertação foi elucidar o mecanismo de reprogramação metabólica que ocorre na DKD, com um particular enfâse no papel patofisiológico das mitocôndrias, a fim de identificar novas oportunidades de estratificação do risco clínico e terapia personalizada. Este objetivo principal foi estratificado em diversas etapas. O foco inicial foi investigar como diferentes níveis de estímulos de glucose, em combinação com a resistência à insulina (IR) podem perturbar o secretoma metabólico, afetando em última instância, a bioenergética mitocondrial de células de rim. Para isso, foi utilizado uma linha celular humana imortalizada (HK-2) como modelo. As células de rim foram expostas a diferentes concentrações de glucose (5, 11 e 22 mM), durante 24 horas para mimetizar condições fisiológicas, e de hiperglicemia ligeira e severa, respetivamente. Além disso também avaliamos o efeito combinado de diferentes concentrações de glucose e IR, induzido por lipotoxicidade, pela administração de ácido palmítico. Foi avaliado o impacto

da hiperglicemia ligeira e grave, com e sem IR no secretoma metabólico das células HK-2, através da técnica de ressonância magnética nuclear (^{1}H-NMR); a função das mitocôndrias em células vivas foi avaliada utilizando o *Seahorse Analytics* e avaliamos o potencial de membrana mitocondrial. Foi também avaliada a atividade do complexo I (CI) e II (CII), por ensaios enzimáticos. Além disso, medimos a oxidação dos ácidos gordos (FAO) e acumulação de lípidos nas células renais. Com este trabalho conseguimos demonstrar que a progressão da hiperglicemia ligeira para grave induziu uma alteração metabólica, onde o metabolismo gluconeogénico desempenha um papel crucial no fornecimento das necessidades energéticas das células HK-2. Este efeito da reprogramação metabólica refletiu-se nos parâmetros bioenergéticos mitocondriais, onde as células de rim expostas a condições hiperglicémicas ligeiras exibiram um aumento na taxa de consumo de oxigénio relacionado com respiração basal, produção de ATP, capacidade respiratória máxima e capacidade respiratória de reserva. No entanto, estes parâmetros diminuíram quando as células de rim foram expostas a concentrações mais elevadas de glicose e quando a IR foi induzida. Os nossos resultados revelaram ainda que no nosso modelo celular, a acumulação de lípidos foi particularmente evidente nos grupos onde a IR foi induzida. As células de rim expostas a concentrações mais elevadas de glucose apresentaram uma maior dependência de FAO. Em condições fisiológicas (5 mM), a inibição de FAO levou a um aumento da produção de ATP, sugerindo que as células de rim nesta condição, não dependem da FAO, quando comparado com a mesma condição com indução de IR. Estes resultados sugerem que a mudança metabólica de hiperglicemia leve a severa, associada à IR, afeta a bioenergética mitocondrial nestas células. Esta capacidade reduzida de FAO nestas células contribui para a acumulação de lípidos intracelulares e para a disfunção mitocondrial, fatores críticos associados à progressão da doença renal. Compreender o papel da FAO e o seu impacto no metabolismo lipídico celular e na função mitocondrial das células de rim é crucial para identificar potenciais alvos terapêuticos para intervir na progressão da DKD.

Investigações adicionais nesta área poderão ajudar ao desenvolvimento de novas estratégias terapêuticas para intervir na progressão da DKD. Estes dados desafiaram-nos a explorar o efeito da FAO num modelo animal e a compreender como uma dieta rica em gordura (comumente associada à obesidade e diabetes), e bem como a sua reversão poderiam influenciar a restauração da FAO e o seu impacto na função mitocondrial.

Atualmente, as dietas ricas em gordura (HFD) são características das sociedades desenvolvidas e têm sido associadas a doenças não comunicáveis, tal como a doença renal crónica. No entanto, os mecanismos precisos subjacentes à deterioração renal relacionada com a obesidade ainda estão por desvendar. Em particular, as alterações metabólicas que ocorrem no perfil renal durante a transição de uma dieta HFD para uma

dieta saudável/normal e o seu impacto na função mitocondrial são ainda pouco compreendidas.

Assim, no segundo trabalho, visámos caracterizar os efeitos nefastos de uma dieta HFD na qualidade mitocondrial renal, e proteínas associadas utilizando um modelo animal (C57BL/6). Os ratinhos machos foram divididos em 3 grupos: um grupo de controlo, alimentado com uma dieta padrão (CTR, n=5) um grupo alimentado com uma dieta rica em gordura (HFD, n=5) e outro grupo alimentado com HFD (HFDt, n=5), que fez transição de volta para uma dieta padrão após 60 dias. Os ratinhos foram mantidos sob essas dietas durante 200 dias. Foram recolhidos os dados biométricos e avaliados alguns parâmetros de função metabólica. A expressão de genes associados à dinâmica mitocondrial, complexos mitocondriais e defesas antioxidantes foram analisadas em tecido de rim. Os homogeneizados renais enriquecidos em mitocôndrias foram preparados e caracterizados por proteómica baseada em espectrometria de massa. Em ratinhos alimentados com HFD, o tecido renal exibiu uma redução acentuada da expressão de PGC-1α. Esta redução foi acompanhada por um desequilíbrio na dinâmica mitocondrial (MFN1 elevado) e uma diminuição dos marcadores de fissão (FIS1 e DRP1). Além disso, a atividade do CI mitocondrial aumentou, enquanto a atividade do CII diminuiu após HFD e HFDt. Nos rins dos animais alimentados com HFD, a atividade enzimática da Superperóxido Dismutase de Magnésio (MnSOD), uma importante enzima antioxidante mitocondrial foi reduzida, enquanto a atividade da Glutationa Redutase (GR), uma enzima citosólica foi elevada. É importante salientar que ambas as atividades enzimáticas no rim voltaram aos níveis normais após a reversão para uma dieta padrão. A análise proteómica mostrou alterações nas proteínas associadas ao metabolismo da glutationa e da glicina, à FAO e à função peroxissomal no tecido de rim dos grupos HFD e HFDt. HFD afetou negativamente as proteínas associadas à glutationa no rim, como a Gsta3 e a Gsr, mas estas alterações foram revertidas com a correção dietética. A proteína Acsm3 foi regulada negativamente no rim após a HFD e regulada positivamente após a correção dietética. Além disso, a rede mitocôndrias-peroxissomas, que partilham alguma maquinaria celular, foi comprometida em tecido de rim sob stress induzido por HFD. A HFD prejudicou a FAO renal nos peroxissomas, como evidenciado pela regulação negativa de Pecr após HFD e HDFt. Este resultado salienta que a correção dietética após a obesidade, pode mitigar a disfunção metabólica sistémica e a disfunção mitocondrial no rim. No entanto também revela que tal intervenção pode não ser suficiente para restaurar completamente a dinâmica e bioenergética mitocondrial no rim. Com os resultados obtidos podemos concluir que a rede mitocondrial poderá ser um ponto principal para estratégias terapêuticas dirigidas com o objetivo de prevenir a progressão da DKD. Assim podemos formular a hipótese de que os metabolitos derivados das mitocôndrias poderão vir a ser potenciais biomarcadores de

diagnóstico de doença renal. Seguindo esta abordagem translacional, progredindo de um modelo celular 2D para um modelo animal, o nosso desafio final foi identificar potenciais biomarcadores de diagnóstico capazes de distinguir entre uma população controlo e doentes que apresentavam DKD, nomeadamente entre pacientes com (A-DKD) e sem níveis elevados de albuminúria.

Para tal, foram selecionados 40 doentes e divididos em um grupo saudável (n=23), composto por dadores vivos de rim, apresentando hemoglobina glicada (HbA1c) < 6,5%, eGFR > 60 ml/min/1,73 m^2 e não albuminúrico (< 30 mg/g de creatinina) e um grupo DKD (n=17), com eGFR < 60 ml/min/1,73 m^2 e HbA1c > 6,5%. Os doentes com DKD foram divididos em NA-DKD (n=5, albuminúria <30 mg/g de creatinina) e A-DKD (n=12, albuminúria >30 mg/g de creatinina). A urina de todos os indivíduos foi analisada através de ^{1}H-NMR não direcionada para procurar assinaturas metabólicas específicas que pudessem caracterizar ambos os grupos. Foi possível detetar 23 metabolitos na amostra ocasional de urina. O citrato, a hipoxantina, o formato e a glicina foram negativamente regulados no DKD quando comparados com o grupo controlo, enquanto a glicose, o trans-aconitase e o glicolato foram regulados positivamente. As correlações de Pearson com a albuminúria demonstraram que o citrato apresentou a correlação negativa mais forte, seguido pela hipoxantina e pelo formato. Por outro lado, o glicolato, a taurina e o trans-aconitase correlacionaram-se positivamente com a albuminúria. A análise de ROC (*Receiver operating characteristic*) foi realizada para ser possível estabelecer um *cut-off* nos metabolitos da urina que pudessem discriminar eficazmente entre doentes com níveis normais e elevados de albuminúria. Esta análise indicou que o citrato, a hipoxantina e a taurina podem ser os mais promissores para diferenciar os doentes com níveis normais a elevados de albuminuria. Entre doentes com DKD, os níveis de glicina e alanina foram encontrados aumentados e diminuídos, respetivamente, em pacientes NA-DKD quando comparado com pacientes A-DKD.

Em suma, o perfil do metaboloma urinário revela que os níveis de metabolitos relacionados com a função mitocondrial podem potencialmente servir como um marcador para individualizar as escolhas terapêuticas na DKD, onde os metabolitos relacionados com a mitocôndria podem ter um papel fundamental, especialmente no fenótipo não albuminúrico.

Em conclusão, os resultados aqui obtidos salientam o papel do desequilíbrio da FAO na DKD, mesmo quando são feitos esforços de reprogramação metabólica para contrariar os efeitos da diabetes a da obesidade. A nossa investigação solidifica ainda mais a ideia de que as mitocôndrias têm um papel chave na DKD e de que forma o seu funcionamento está correlacionado com a hiperfiltração renal, criando uma janela de oportunidade para aceder à assinatura da diminuição da função tubular renal e do dismetabolismo como biomarcadores de risco.

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List of Abbreviations

1H-NMR	Proton Nuclear Magnetic Resonance
2-DG	2-Deoxy-D-Glucose

A

ACAA2	Acetyl-Coa Acyltransferase 2
ACE	Angiontesnsin Converting Enzyme
ACN	Acetonitrile
ACOX1	Acyl-CoA Oxidase 1
ACSL1	Acyl-CoA Synthetase Long-Chain Family Member 1
ACSM3	Acyl-Coenzyme A Synthetase
A-DKD	Albuminuric Diabetic Kidney Disease
AKD	Acute Kidney Disease
AKI	Acute Kidney Injury
AKT	Protein Kinase B
ATP	Adenosine Triphosphate
AUC	Area Under Curve

B

B2m	Beta 2-Microglobulin
BCA	Bicinchoninic Acid
BCFA	Branched-chain Fatty Acids
BMI	Body Mass Index
BSA	Bovine Serum Albumin

C

CKD	Chronic Kidney Disease
CKD-EPI	CKD Epidemiology Collaboration
CAT	Catalase
CoA	Coenzyme A
CPT1	Carnitine Palmitoyltransferase 1

D

DAG	Diacylglycerol
DAMPS	Damage Associated Molecular Patterns
DCPIP	2,6-Dichlorophenolindophenol
DKD	Diabetic Kidney Disease
DM	Diabetes Mellitus
DN	Diabetic Nephropathy
DRP1	Dynamin Related Protein
DTNB	5,5′-Dithiobis- (2-Nitrobenzoic Acid)

E

ECAR	Extracellular Acidification Rate
ECM	Extracellular Matrix

EMT	Epithelium-Mesenchymal Transition
EGFR	Estimated Glomerular Filtration Rate
ENOS	Endothelial Nitric Oxide Synthase
ER	Endoplasmic Reticulum
ESKD	End - Stage Kidney Disease
ETC	Electron Transport Chain

F

FADH2	Flavin Adenine Dinucleotide
FAO	Fatty Acid Oxidation
FCCP	Carbonyl Cyanide-P-Trifluoro methoxyphenylhydrazone
FFA	Free Fatty Acids

G

G6PDH	Glucose-6-Phosphate Dehydrogenase
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GECs	Glomerular Endothelial Cells
GLP-1	Glucagon Like Peptide-1
GLP-1R	Glucagon-Like Protein 1 Receptor
GLUT	Glucose Transporter
GPX	Glutathione Peroxidase
GR	Glutathione Reductase
GSSG	Glutathione Disulfide
GSH	Glutathione
GSK3β	Glycogen Synthase Kinase 3 Beta

H

HBA1C	Glycated Hemoglobin A1c
HFD	High Fat Diet
HFDt	High Fat Diet Transition
HK-2	Human Kidney-2
H2O2	Hydrogen Peroxide

I

ICAM-1	Intracellular Adhesion Molecule 1
IL	Interleukin
IKBA	NF-Kb Inhibitor Alpha
InsR	Insulin Receptor
IR	Insulin Resistance
IRI	Ischemia-Reperfusion Injury
INSR	Insulin Receptor
IRS	Insulin Receptor Substrate
ITS	Insulin-Transferrin Selenium

J

JAK2	Kanus Kinase 2
JC-1	5,5,6,6’-Tetrachloro-1,1’,3,3’-Tetraethylbenzimi-Dazoylcarbocyanine Iodide

K

KDIGO	Kidney Disease-Improving Global Outcomes
KIM	Kidney Injury Molecule -1

L

LCFA	Long Chain Fatty Acids
LDH	Lactate Dehydrogenase
L-FABP	Liver-Type Fatty Acid-Binding Protein

M

MAPK	Mitogen-Activated Protein Kinase
MCs	Mesangial Cells
MFN	Mitofusin
MRAs	Mineralocorticoid Receptor Antagonist
MMP	Mitochondrial Membrane Potential
MNSOD	Manganase Superoxide Dismutase
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
MTOR	Mammalian Target Of Rapamycin

N

NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide Hydrogenase
NA-DKD	Non-albuminuric Diabetic Kidney Disease
NBT	Nitro Blue Tetrazolium Chloride
NF-κB	Nuclear Factor-Kb
NGAL	Neutrophil Gelatinase-Associated Lipocalin
NRF	Nuclear Factor

O

OCR	Oxygen Consumption Rate
OPA1	Optic Atrophy -1
OS	Oxidative Stress
OXPHOS	Oxidative Phosphorylation

P

PA	Palmitic Acid
PBS	Phosphate Buffer Saline
PCA	Partial Component Analysis
PGC1-α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha
PLS-DA	Partial Least Squares Discriminant Analysis
PI3K	Phosphoinositide 3-Kinase
PKC	Protein Kinase C
PT	Proximal Tubular
PVDF	Polyvinylidene Fluoride

Q

qPCR	Quantitative Polymerase Chain Reaction

R

RAAS	Renin-Angiotensin-Aldosterone System
RIPA	Radioimmunoprecipitation Assay Buffer
RNS	Reactive Nitrogen Species
ROC	Receiver Operating Characteristic
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction

S

SGLT	Sodium Glucose Transporters
SIRT	Sirtuin
SOD	Superoxide Dismutase
SRB	Sulforhodamine B
STAT	Signal Transducer And Activator Of Transcription

T

T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TCA cycle	Tricarboxylic Acid Cycle
TNF-α	Tumor Necrosis Factor Alpha

U

UACR	Urinary Albumin Creatinine Ratio

V

VCAM-1	Vascular Cell Adhesion Protein 1
VEGF	Vascular Endothelial Growth Factor

W

WHO	World Health Organization

Chapter 1

Introduction

This chapter was adapted from the following published works:

Braga PC, Alves MG, Rodrigues AS, Oliveira PF (2022). Mitochondrial Pathophysiology on Chronic Kidney Disease. *International Journal of Molecular Sciences,* 23, 1776. https://doi.org/10.3390/ijms23031776

Costa J, **Braga PC**, Rebelo I, Oliveira PF, Alves MG (2023). Mitochondria Quality Control and Male Fertility. *Biology* 12,6. https://doi.org/10.3390/biology12060827

Braga PC, Rodrigues AS, Oliveira PF and Alves MG (2024). Chapter 20-Mitochondrial transplantation in the Kidney. In: Ostojic SM, Kubat GB, Ulger O, Gunaydin S, eds. *Mitochondrial Transplantation and Transfer: Academic press*; 2024 :411-434. https://doi.org/10.1016/B978-0-443-18858-9.00020-2

Braga PC, Oliveira PF, Rodrigues AS and Alves MG (2024). Mitochondria quality control as predictor of Diabetic Kidney Disease progression in non-albuminuric patients. (unsubmitted paper).

The integral texts are available in the Annex Section of this Dissertation.

1.1.Kidney Diseases

Kidney diseases represent a growing public health concern. The kidney has a high energy demand, due to its role in filtering the blood to remove accumulated toxins along with concentrating the urine to avoid dehydration (Sarko & Georges, 2016). To balance that activity, the kidney has an active repair capacity since they are able to restore in terms of function and structure after episodes of acute lesions. However, as it is a highly demanding organ, it is susceptible to multiple injuries that with time may result in major problems, such as culminating in a partial or even complete kidney failure. At this stage, the only solution is dialysis and ultimately, kidney transplant (Higgins et al., 2019). In 2020, with an update in 2024, an independent organization with the mission to improve care and outcomes of patients with kidney disease worldwide (Kidney disease: Improving Global Outcomes), organized a consensus conference to harmonize existing acute kidney disease (AKD) and chronic kidney disease (CKD) definitions (Stevens et al., 2024). CKD is defined as a progressive and irreversible loss of kidney function.

AKD and CKD are two major categories of kidney diseases, that can be distinguished by the duration of the renal outcomes (Fig 1.1). The term acute defines a condition of a sudden episode (6h to 7 days) of injury that is short and can be reversible, while chronic has a long term and with persistent episodes associated (Weisbord & Palevsky, 2016). On the other hand, acute kidney injury (AKI) is a subset of AKD and is associated with high morbidity and mortality, including an increase in adverse renal outcomes. It is also suggested that AKI is not self-limited, as it serves as an incoming for subsequent AKI episodes and potentially to CKD (Heung et al., 2016). William MacNider used the term AKI for the first time in 1918 to describe a case of acute mercury poisoning. Recently, the term acute renal failure has been replaced by AKI (Bellomo et al., 2004). AKI is characterized by a sudden loss of renal function, uremia, and dysregulation of extracellular volume and electrolytes, resulting from multiple insults, including sepsis, cardiovascular surgery, and ischemia–reperfusion injury (IRI). AKI has additional causes, such as direct kidney injury and urinary tract obstruction, in addition to decreased blood flow (Lameire et al., 2006). Patients with AKI typically have a heterogeneous etiology, involving extrarenal pathology (such as prerenal azotemia and acute postrenal obstructive nephropathy) and particular kidney diseases (like acute interstitial nephritis, acute glomerular, and vasculitic renal disorders) (Chawla & Kimmel, 2012). As previously mentioned, the diagnosis of AKI is predicated on a rapid decrease in GFR, which is also accompanied by a decrease in urine production or an increase in serum creatinine levels (Khwaja, 2012). After this first step of diagnosis, AKI can be classified into three main categories: prerenal, intrinsic, and postrenal AKI. Prerenal

and postrenal AKI result from a decline in GFR caused by extrarenal disease, whereas "intrinsic" AKI represents true kidney disease (Agarwal et al., 2016). Furthermore, an episode of AKI in patients with CKD can cause further renal deterioration to end stage renal disease (ESRD).

The principal hallmarks are an estimated glomerular filtration rate (eGFR) < 60 mL/min per 1.73 m^2, the presence of markers of kidney damage (proteinuria, active urine sediments, structural abnormalities, histological damages), for more than 3 months, and/or a history of kidney transplantation (Lameire et al., 2021). Uremic retention is usually classified according to its removal pattern by dialysis and can be classified in three different groups: (1) small water-soluble compounds with a Molecular weight (MW) < 500 D, (2) middle- to large molecules, mostly peptides; MW > 500 D, and (3) protein-bound compounds with a low MW (<500), these solutes being difficult to remove by any type of dialysis. In this last group, this type of retention is a trigger for kidney fibrosis in CKD (Mutsaers et al., 2015).

AKI is considered an independent risk factor for CKD and ESRD, where severe AKI may be followed by an irreversible loss in kidney function (Bucaloiu et al., 2012; Coca et al., 2012). But currently, it is known to be strongly linked to interrelated syndromes, where CKD predisposes or sensitizes people to AKI (James et al., 2015), and AKI is one of the most notable variables to speed up the progression of CKD (Palomba et al., 2017).

The global prevalence of CKD is estimated at more than 843 million people. Of these, only a small percentage of patients reach the end-stage of the disease, requiring treatments to replace kidney function. Nowadays, more than 2.5 million people are currently on treatment, and it is estimated that the numbers will rise to 5.4 million by 2030 (Vanholder et al., 2021). Multiple factors can contribute to AKI-related renal disease progression, including age, diabetes, hypertension and cardiac problems, severe episodes of AKI, and even delayed renal function recovery from AKI (Yan et al., 2021). Indeed, people with diabetes and CKD are at high risk for kidney failure, cardiovascular disorders and premature mortality.

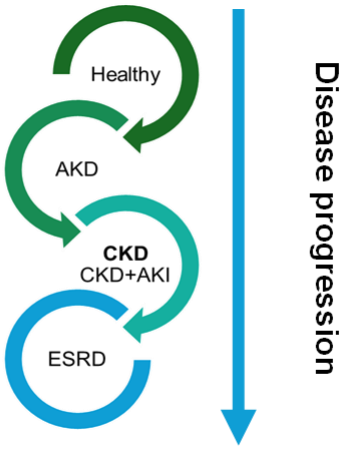

Fig.1.1- Different phases of kidney disease progression from normal kidney function through acute kidney disease (AKD), chronic kidney disease (CKD) to end-stage renal disease (ESRD). Patients at baseline may: (1) suffer from to AKD, (2) recover or progress to CKD, or (3) progress to ESRD following the initial episode of acute kidney injury (AKI). Individuals with AKD may either (1) return to baseline AKD or (2) advance to CKD or ESRD following more AKI occurrences. Similarly, CKD patients exposed to AKI may either (1) regain their pre-AKI level of CKD or (2) advance to ESRD**.**

1.1.1. Diabetes mellitus as the main contributor to development of kidney disease

Diabetes mellitus (DM) is a major public health challenge worldwide. According to International Diabetes Federation, 537 million people worldwide have diabetes in 2021, and this number is expected to increase to 783 million by 2045. The growing burden of DM is secondary to the pandemic of obesity, which in turn has been attributed to increased intake of processed food, reduced physical activity, and increased sedentary behavior. DM is considered the predominant pathophysiologic driver of CKD (Levin et al., 2017), leading to kidney transplantation or dialysis worldwide, although other conditions, such as hypertension, metabolic disorders, sepsis, IRI, and exposure to nephrotoxins may also lead to CKD (Bagshaw et al., 2008; Emlet et al., 2015; Uchino et al., 2005). Over 25% of individuals with diabetes have CKD, and it is estimated that 40% of people with diabetes will experience CKD at some point in their lives (Afkarian et al., 2016). It can be assumed that the prevalence of CKD related to diabetes will be expanded in proportion to the rise in diabetes prevalence (Afkarian et al., 2016) (Fig.1.2).

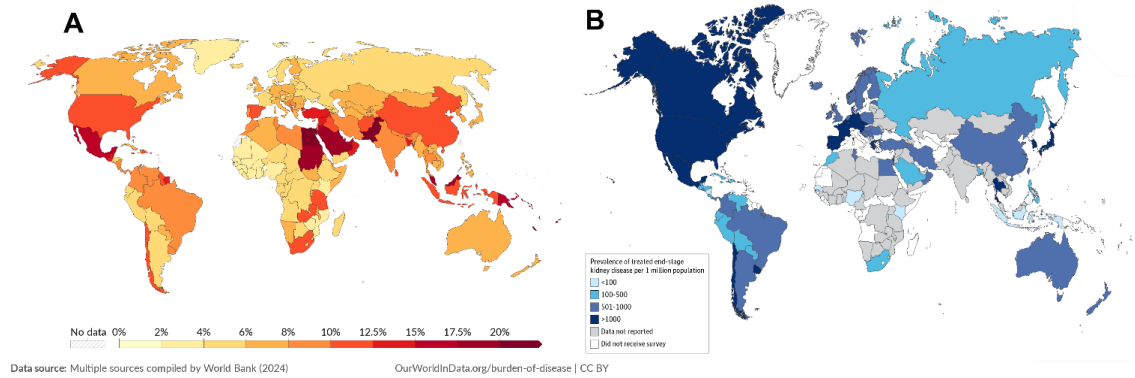

Fig.1.2 - Global prevalence of diabetes disease (A) per percentage of individuals among 20-79 years old who have diabetes and the global incidence of ESKD patients per 1 million of people (B). Data not available indicates that data were either not known or not provided on the questionnaire for countries that received the survey. Image of Panel A was kindly provided by *World Bank (2024) – processed by Our World in Data, accessed on 1st July 2024, ourworldindata.org/grapher/diabetes-prevalence,* and panel B was adapted by (Bello et al., 2017), JAMA**.**

DM is a chronic, multifactorial and polygenic disease typified by a persistent hyperglycemia, which are caused by abnormal insulin secretion, function or both (Punthakee et al., 2018). According to the World Health Organization (WHO) classification, Type 1 (T1DM) and Type 2 (T2DM) are the most common forms of the disease, where T1DM is characterized by the destruction of pancreatic β-cells, leading to absolute insulin insufficiency, while T2DM is associated with insulin resistance (IR) and relative insulin insufficiency or mainly decrease of insulin secretion with or without IR. The two primary metabolic processes that insulin is thought to control are glucose and lipid metabolism. Insulin stimulates glycogenesis and lipogenesis by facilitating the translocation of glucose transporter 4 (GLUT4) to the plasma membrane, which allows glucose to enter the cells (Kersten, 2001; Qaid & Abdelrahman, 2016). Upon insulin binding to the receptor, it undergoes to a conformational change, enhancing tyrosine kinase activity and auto-phosphorylation. Then, the phosphorylation of the insulin receptor substrate occurs, which triggers the Pi3k/akt and MAPK signaling pathways (Boucher et al., 2014; White & Kahn, 2021). The control of insulin clearance/action exerted by the kidney is complex. The differentiation of kidney segments-where insulin governs distinct pathways is the only factor that makes the diversity of insulin effects conceivable. In the kidney, the clearance of insulin (6-8 units) is orchestrated by two main mechanisms: at the glomerular filtration level and post-glomerular. The clearance in glomerular filtration is important for nutrient conservation, an important step to control blood glucose levels (Ritz et al., 2013), while post-glomerular filtration, is responsible for insulin signaling, having an effect on the gluconeogenesis pathway (Rico-Fontalvo et al., 2022).

Insulin is also able to influence renal sodium transport (Molnár et al., 2021). It has been found that in podocytes, the proteins of the insulin signaling canonical pathway, namely the insulin receptor (INSR), both Insulin receptor substrate 1 and 2 (IRS1 and IRS2), being the latter more expressed (Coward et al., 2005). This depicts the effect of insulin in human podocyte, which results in glucose uptake via GLUT1 and GLUT4. Mice with DKD and exhibiting significant levels of albuminuria and histological characteristic such as podocyte foot structure loss and glomerulosclerosis, presented a podocyte-specific deletion of INSR (Welsh et al., 2010). Insulin plays multiple roles in the renal tubule, including in metabolism, regulation of electrolyte and acid-base balance, and absorption of filtered substances. The precise process by which insulin carries out these distinct roles is not entirely understood, yet. Renal proximal tubules are a key organ for gluconeogenesis, producing glucose at levels comparable to the liver (Meyer et al., 2002). Indeed, proximal tubular (PT) cells contribute significantly to endogenous glucose production, accounting for up to 25 % during fasting and 60% after meals (Mutel et al., 2011). Some studies have demonstrated that insulin can suppress gluconeogenesis in PT cells. Nakamura et al., showed that the regulation of sodium reabsorption and gluconeogenesis, depends on the recruitment of specific IRS. That study demonstrated that insulin is able to reduce the expression of gluconeogenic genes in isolated human and rat PT cells, indicating that insulin directly suppresses gluconeogenesis, independently of IRS1/AKT2/mTORC1/2 (Nakamura et al., 2020). Furthermore, Tiwari et al., showed that abnormalities were further defined based on the section targeted by INSR deletion in kidney tubular cells in a mouse model with INSR deletion. In a case of decreased INSR at PT cells, animals had a mild diabetic phenotype, without increased IR when compared to control. In addition, these animals showed higher activity of gluconeogenic enzymes (Tiwari et al., 2013). Conversely, higher blood pressure and poorer sodium excretion were observed in mice whose INSR deletion was limited to the distal tubule (Tiwari et al., 2008). These alterations have an impact on the tubular function. Among other factors, insulin can play a role in the absorption of albumin by PT cells in diabetic and in non-diabetic conditions. Renal homeostasis depends on PT cells retrieving albumin from ultrafiltration. IR is frequently present in CKD patients, even in the absence of diabetes. In fact, IR can be detected at very early stages, when eGFR is still within the normal range, which may be related to triggering CKD (Fliser et al., 1998). Furthermore, IR can also be associated with widespread CKD and rapid decline in renal function in elderly, non-diabetic individuals (Cheng et al., 2012), as well as with microalbuminuria population, and in patients with T1DM (Ekstrand et al., 1998) and T2DM (Groop et al., 1993). Moreover, insulin signaling appears to be relevant for the adaptive endoplasmic reticulum stress response which, if not restored, can result in diabetic nephropathy (Madhusudhan et al., 2015).

1.2. Diabetic Kidney Disease

The combination of diabetes with kidney disease complications has the greatest death rates when compared to other microvascular complications (Afkarian et al., 2013). Diabetic kidney disease (DKD), also known as Diabetic Nephropathy (DN) occurs in 25% to 40% of patients with DM and is considered the leading cause of ESRD and the eventual need for kidney replacement therapy (Alicic et al., 2017). Traditionally, the clinical definition of DKD is a progressive deterioration in kidney function accompanied by chronic albuminuria with an albumin-to-creatinine ratio (uACR) greater than 30 mg/g and a subsequent decline in the estimated glomerular filtration rate (eGFR), eventually leading to dialysis (Coresh et al., 2019; Di Pino et al., 2021). Recent research has revealed a more nuanced understanding of DKD phenotypes with three distinct characterizations: albuminuria regression, non-proteinuria or non-albuminuric DKD, or rapid decline in GFR (Afkarian et al., 2016). The pathological alterations induced by hyperglycemia and hypertension can lead to damage to the tubulointerstitium and glomerulus, particularly in podocytes (Group, 2022). This diabetes-related microvascular complication is characterized by hyperfiltration and the expansion of the mesangial matrix, leading to kidney hypertrophy, glomerular basement membrane thickening, podocyte and glomerular injury, and tubular damage. Ultimately, these changes culminate in glomerulosclerosis and tubulointerstitial fibrosis (Oshima et al., 2021). The functional hallmarks of DKD could be classified into five categories and different trajectories (Fig 1.3).

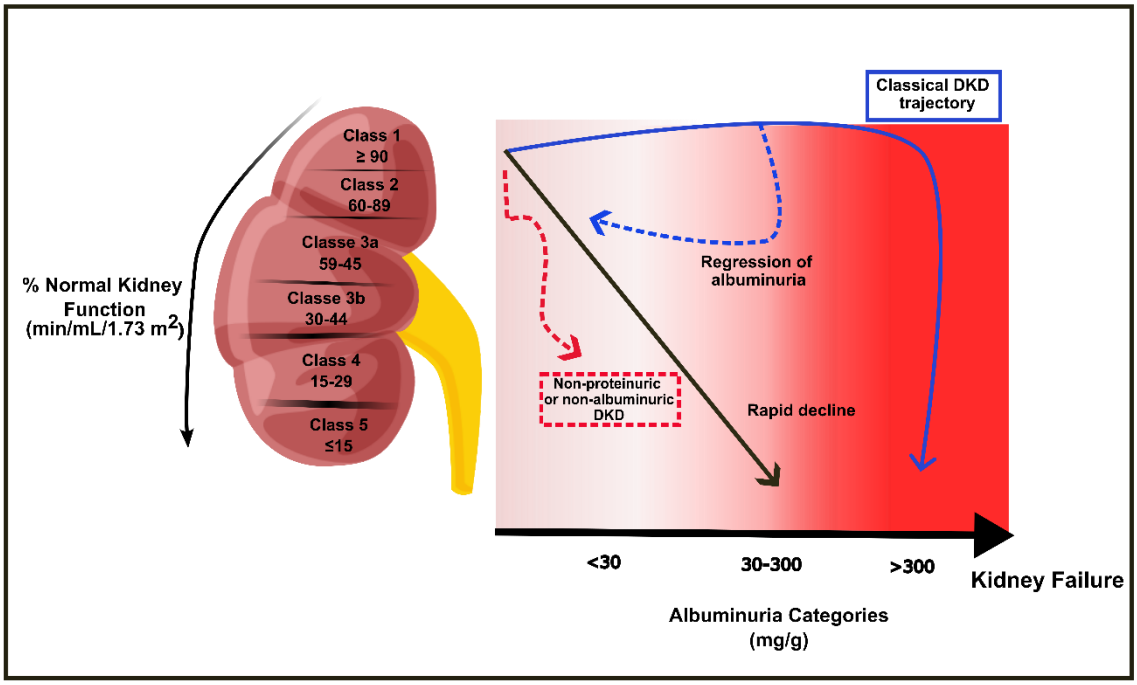

Fig.1.3- Different trajectories of diabetic kidney disease (DKD) development according to the decrease of glomerular filtration rate (GFR), divided into 5 categories and the increase of albuminuria levels, from normo, micro to macroalbuminuria. The classical DKD phenotype (represented in blue arrow) is characterized by a persistent increase of albuminuria followed by a progressive decline in GFR, where renal function is less than 15 min/mL/1.73 m^2, leading to renal failure. Moreover, another phenotype is possible to observe, where albuminuria levels can be reversed (represented as dashed blue arrow). This scenario can be observed due to pharmacological approaches used in patients, to control blood glucose and hypertension levels. On the other hand, people with diabetes may experience a rapid decline in GFR even without albuminuria (represented as a dark arrow). Lastly, another phenotype that deserves nowadays attention is represented by the patients with a rapid decline in GFR without reaching higher albuminuria excretion levels or even microalbuminuria, being referred as non-proteinuric or non-albuminuric DKD progressors (represented as dashed red arrow).

The earliest change (Class 1) involves thickening of the glomerular basement membrane (Dische, 1992). At this stage, patients typically exhibit normal albumin urine excretion rate and eGFR. Class 2 is characterized by a mesangial expansion exceeding 25 % of the glomerular tuft, while class 2b indicates severe expansion (Mauer et al., 1984). Consequently, the kidneys enlarge due to increased mesangial matrix and glomeruli hypertrophy, often accompanied by the onset of albuminuria. Class 3 (subdivided into 3a and 3b) denotes mesangial sclerosis due to mesangial expansion, and by biopsy it was observed nodular glomerulosclerosis, or Kimmelstein-Wilson nodules (Allen, 1941; Kimmelstiel & Wilson, 1936). Finally, class 4 is marked by a glomerular sclerosis affecting over 50% of glomeruli, often signifying ESRD, while class 5 denotes an GFR < 15 min/mL/1.73m^2 (Qian et al., 2008).

Emerging evidence suggests a non-linear progression of DKD. Indeed, Ekinci and collaborators, detected less glomerular damage in non-albuminuric patients (NA-DKD) compared to micro- or macroalbuminuric patients (A-DKD) (Ekinci et al., 2013). Additionally, NA-DKD patients tend to exhibit less typical glomerular damage but more advanced tubulointerstitial and vascular lesions compared to those with albuminuria (Jia et al., 2022). Consequently, particularly in this phenotype, DKD presents as diabetic tubulopathy. Roughly 90% of the cortex is composed of renal tubular cells. Since reabsorption is their main task, it is well known that it uses a lot of energy during the active reabsorption process (Bhargava & Schnellmann, 2017). Regardless of kidney health or DKD status, mitochondria are the main organelle in charge of producing energy in the kidney, being highly vulnerable to damage (Sharma, 2017). Mitochondrial dysfunction in diabetic tubulopathy is primarily linked to several pathological mechanisms that arise due to diabetes, involving energy imbalances, oxidative stress (OS), impaired dynamics, biogenesis deficits and renal tubular damage (Yao et al., 2022).

These findings challenge the traditional emphasis on glomerular injury as the primary driver and a decision-making factor for the progression of DKD. Recent research and theoretical discussions suggest that tubulopathy, particularly proximal tubule lesions, is a major contributor to DKD development and progression, leading some researchers to propose the term “diabetic tubulopathy” (Gilbert, 2017; Tang & Lai, 2012; Tang et al., 2011).

1.2.1. Biochemical overview of the main causes of DKD and pharmaceutical approaches to counteract the advance of the disease

DKD is a progressive and irreversible disease whose pathogenesis has been linked to changes in the structure and function of renal cells in response to metabolic stress induced by an excessive intake of glucose. This metabolic stress is caused by the activation of particular metabolic pathways that are connected to inflammation and redox imbalance (Turkmen, 2017). Thus, the four main factors that drive the progression DKD are hyperglycemia, hypertension, inflammation and OS, which are all interconnected and considered a consequence of one into another.

Pathophysiology of DKD induced by hyperglycemia

DKD is the primary consequence of long-term hyperglycemia, which is also the primary clinical symptom of DM. It is caused by two mainly mechanisms: autoimmune abnormality-induced dysfunction and apoptosis of pancreatic beta cells (T1DM) and/or overstimulation of insulin synthesis and secretion in the presence of IR, which is linked to obesity and overweight (T2DM) (Kwon & Pessin, 2013). The development of renal damage may be largely attributed to increase production of advanced glycation end products (AGEs), which can be caused by hyperglycemia, and inadequate clearance of these uremic toxins due to decreased eGFR, contributing to oxidative damage, hypoxia and thus to renal hypertrophy (Arsov et al., 2014) (Fig 1.4). The increase of endogenous pool of AGEs alters the structure and function of different proteins leading to glomerulosclerosis, as well as a significant accumulation of extracellular matrix proteins, contributing to the fibrosis process (Y. S. Kanwar et al., 2011).

The development of DKD from hyperglycemia is promoted by several mechanisms including the hexosamine, polyol, and protein kinase C (PKC). Regarding polyol pathway, the increase in the NADPH-dependent conversion of glucose by aldose reductase into sorbitol is one of the effects of the cells' increased absorption of glucose. Nicotinamide adenine dinucleotide (NAD) subsequently converts sorbitol into fructose (Aghadavod et al., 2016; Reis et al., 2008). An essential component for the antioxidant glutathione (GSH) renewal is NADPH. Therefore, the reduced availability of NADPH impacts the antioxidant defense, leading to redox impairment, considering enhanced aldose reductase activity (Blacker & Duchen, 2016). The increase in O_2^- inhibits the activity of glyceraldehyde 3-phosphate dehydrogenase (GADPH), which in turns inhibits glycolysis and activates alternative signaling pathways. Indeed, PKC is activated by diacylglycerol (DAG), which is produced in greater amounts as the NADH/NAD^+ ratio rises (Blacker & Duchen, 2016). An end-product of the pathway, namely fructose, has recently been linked to indicators of kidney impairment function (Toth-Manikowski & Atta, 2015). In renal cells, PKC stimulates the induction and the activation of endothelial nitric oxide synthase (eNOS), being this observed especially in diabetic kidney in the first stages of DKD (Geraldes & King, 2010). These pathways are further mediated or stimulated by many growth factors, such as insulin-like growth factor-1, platelet-derived growth factor, vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), and angiotensin II (Ang II) (Brownlee et al., 1984; Lin et al., 2018; Sharma et al., 2017; Warren et al., 2019). Prostanglandin E1 levels, Ang II activity and VEGF are all elevated in response to increasing NO. This leads to vascular endothelial dysfunction, increased permeability, glomerular hyperfiltration, and albuminuria (Noh & King, 2007). Tetrahydrobiopterin (BH4), an eNOS cofactor, is reduced in chronic diabetes

due to persistent hyperglycemia, which also causes a proportional decrease in NO synthesis in vascular endothelium, that contributes to vasoconstriction and systemic hypertension (Yashpal S. Kanwar et al., 2011).

To maintain glucose homeostasis in renal cells, its uptake occurs in non-insulin dependent, using GLUT1 and GLUT2 as glucose transporters. These glucose carriers stop to regulate the entry of glucose, as in normal conditions, leading to glucotoxicity. This scenario involves the increase of expression of these transporters, which results in increased glucose entry into renal cells. This is the case for the sodium- glucose co-transporters (SGLT)1 and 2, which are responsible for tubular reabsorption, and the high-affinity glucose transporter GLUT2, which is stimulated by hyperglycemia (Lagranha et al., 2007; Machado et al., 2006). This means that higher glucose reabsorption in the PT cells leads ultimately to hyperfiltration in DM patients (Vallon, 2011). Furthermore, the increase of stimulation of glucose oxidation pathways in non-insulin-dependent cells leads to activation of alternative pathways, and increase of the production of reactive oxygen species (ROS) and nitrogen species (NOS) (Donate-Correa et al., 2015). SGLT2, which is localized in the proximal tubule (Nespoux & Vallon, 2018), arises as essential on the glucose homeostasis, since it reabsorbs 90% of the filtered load of glucose (Wright, 2001). SGLT2 inhibitors, such as empagliflozin, canagliflozin and dapagliflozin, are widely used in the clinic for the treatment of hyperglycemia in T2DM (Nespoux & Vallon, 2020). They are able to lower plasma glucose concentrations during fasting and after meals by 25% (Merovci et al., 2014). They also lower hemoglobin glycated A1c (HbA1c) by around 1% in those with T2DM with a HbA1c of 8.0-8.5% (DeFronzo et al., 2017). In the kidneys, SGLT2 inhibitors are able to lower the renal threshold for glucose excretion from approximately 10 mmol/l (~180 mg/dl) to approximately 2.2 mmol/l (~40 mg/dl) (DeFronzo et al., 2013). The resultant glucosuria improves beta-cell activity and insulin sensitivity by lowering the mean plasma glucose concentration and reducing the glucotoxicity (Al Jobori et al., 2018). Thus, by reducing plasma glucose levels, SGLT2 inhibitors reverse these adverse intracellular metabolic effects. The unregulated glucose diverts to non-glycolytic pathways that promote kidney glomerular alterations as well as mitochondrial dysfunction, OS and inflammation (Yki-Järvinen & McClain, 2015). Thus, SGLT2 inhibitors, by reducing plasma glucose levels, are able to decrease lipid content, thereby promoting a decrease in the hyperfiltration and intraglomerular capillary hypertension, which lowers albuminuria and the need for oxygen in tubular reabsorption (Vallon & Verma, 2021).

The hexosamine pathway is also stimulated by hyperglycemia. This pathway promotes the conversion of fructose-6-phosphate, and the synthesis of uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) as end-product, which is O-glycosylated by the O-

GlcNAc transferase into N-Acetylglucosamine (O-GlcNAc) (Aghadavod et al., 2016; Reis et al., 2008).

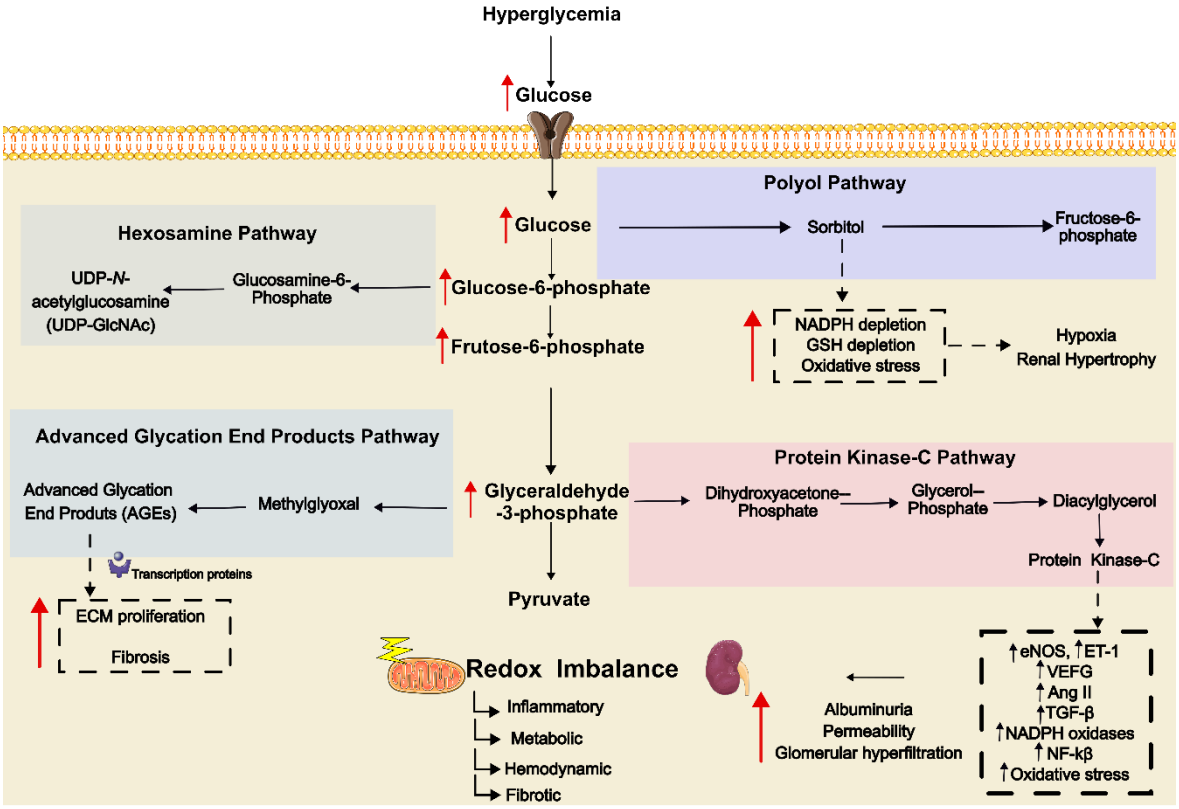

Fig.1.4- Alternative pathways due to a hyperglycemic condition that may lead to diabetic kidney disease (DKD) complications. Polyol pathway lowers the ratio of NADPH/$NADP^+$ which will result in a depletion of glutathione levels, a crucial antioxidant defense, leading to the increase in oxidative stress (OS), which in the kidney, leads to hypoxia and renal hypertrophy. Hexosamine pathway contributes to the modification of proteins related to extracellular matrix (ECM), resulting in the increase of fibrosis process. Advances Glycation End products (AGEs) formation due to a hyperglycemic state, are associated with the increase of extracellular matrix proteins. The activation of Protein Kinase-C (PKC) pathway is correlated with the decrease of endothelial nitric oxide synthase (eNOS), an increase of Prostanglandin E1 levels (ET-1), Angiotensin II (Ang II) activity and vascular endothelial growth factor (VEGF), contributing to the increase of transforming growth factor beta (TGF-β), nuclear factor kappa light chain of activated B cells (NF-κβ) and OS. All that contributes to the increase of albuminuria levels, as well as hyperpermeability, resulting in an increase of glomerular hyperfiltration.

Pathophysiology of DKD can also promote hypertension

The pathogenesis of DKD includes glomerular hypertension. For patients with DKD, the American Diabetes Association (ADA) proposed a higher target of 140/80 mm Hg (Armstrong, 2014; Passarella et al., 2018) while the American Heart Association and American College of Cardiology jointly published the 2017 guidelines, that indicate a lower target of 130/80 mm Hg (Whelton et al., 2018). Despite the benefit of hypertension

management, there is some specific issues that have not been fully clarified in DKD patients. The Kidney Disease/ Improving Global Outcomes (KDIGO) recommends blood pressure <140/90 mm Hg for patients with CKD without significant albuminuria and <130/80 mm Hg for patients with CKD and significant albuminuria ("KDIGO 2021 Clinical Practice Guideline for the Management of Blood Pressure in Chronic Kidney Disease," 2021). About one-third of T2DM patients have hypertension (Gross et al., 2005), which may cause or exacerbate diabetic kidney damage (Chawla et al., 2010). The complicated and multifaceted process of hypertension in DKD includes improper sodium regulation through an increase in its renal tubular reabsorption, aberrant activation of the sympathetic nervous system and renin-angiotensin-aldosterone system (RAAS), endothelial cell failure, and elevated OS. These mechanisms enhance extracellular volume and mediate vasoconstriction, which raises blood pressure (Maric-Bilkan, 2013; Van Buren & Toto, 2011). Moreover, compared to those with hypertension, normotensive patients with DKD exhibit a slower rate of illness progression (Patney et al., 2015). A meta-analysis with the major blood pressure lowering trial demonstrated that in 15 out of 17 studies, intensive lowering blood pressure was effective in reducing the number of patients at the end point of kidney disease and it was also beneficial in patients with diabetes, in which the progression of albuminuria was delayed (Lv et al., 2012). Thus, it has been suggested that patients with DKD should prioritize treating their hypertension using multifactorial risk factor reduction techniques, which should involve medication, lifestyle modification, and patient education to enhance the patients' ability to control these intricate risk factors on their own. The most commonly acknowledged hemodynamic element in the development of DKD is RAAS, and research has demonstrated that blocking it can slow the disease's progression (Márquez et al., 2015).

Renal cells produce Ang II in a higher rate in response to mechanical stress on the arterial wall caused by hypertension, hyperglycemia, inflammation, and ROS (Fig 1.5). This phenomenon also plays a role in the hyperactivation of the RASS (Chawla et al., 2010; Márquez et al., 2015), which in turn leads to systematic and renal vascular vasoconstriction, renal reabsorption of salt, and rises in pressure, intraglomerular pressure, and renal injury (Padda et al., 2015). Thus, the use of angiotensin-converting enzyme (ACE) inhibitors or Ang II receptor blockers (ARBs) for RAAS inhibition to lower albuminuria, cardiovascular, and the progression of DKD has been proposed (Lewis et al., 2001). These drugs can improve the systemic blood pressure as well as intraglomerular pressure, leading to the reduction of albuminuria in DKD (Persson et al., 2016). One example is a randomized, double-blind study performed with losartan in patients with T2DM and nephropathy. The aim was to determine whether losartan alone or in combination with conventional

antihypertensive therapy had any effect on the end stage renal diseases, or death. A total of 1513 patients were included in the study where 751 patients received losartan while 762 patients received a placebo. Losartan was able to reduce the incidence of a doubling of the serum creatinine concentration and ESRD. The level of proteinuria declined also by 35% with losartan in comparison with patients taking the placebo (Brenner et al., 2001). Furthermore, the combination of ACE-I and ARBs resulted in a greater decrease in proteinuria than monotherapy with either type of agent (Kunz et al., 2008). However, the combined therapy increased the risk of hyperkalemia and AKI, but there is an overall trend toward a lower risk in the combination therapy group when compared to the monotherapy group in patients that were in ESRD (Fried et al., 2013). Hyperkalemia is the most common electrolyte disturbance in patients with kidney disease, particularly in patients with diabetes that are under the medication of RAAS inhibitors. Due to the association of the increased hyperkalemia and the high risk of CKD in T2DM patients, the more recent guidelines, namely 2023 European Society of Hypertension, suggest the use of ACE-I and or ARBs, with the possibility to add SGLT2i for patients with diabetes and to non-diabetic CKD if eGFR is at least 20 mL/min/1.73 m^2 (Mancia et al., 2023). In addition, in the treatment of resistant hypertension, steroidal mineralocorticoid antagonists (MRAs) have been used in the clinic, such as spironolactone or eplerenone (non-selective and selective MRA, respectively), providing an alternative avenue for RAAS intervention. Aldosterone exerts its classical action of sodium absorption and potassium excretion by binding to MR at the distal nephron (Good, 2007). Two main mechanisms have been proposed for the kidney protective effects: a hemodynamic impact and an action on tissue inflammation and fibrosis (Sarafidis et al., 2023) since in DKD, an overexpression of MR leads to an exacerbation of inflammatory process and fibrosis. This pharmaceutical approach appears to be effective in reducing albuminuria alone or on top of a RAAS blocker in patients with CKD, diabetic or not (Group, 2023).

Contribution of inflammation to DKD development

Apart from the damage caused by blood pressure and glucose, the complexity of the diabetic environment upregulates other hemodynamic and metabolic pathways that in turn activate pro-inflammatory and profibrotic pathways, causing complications such as DKD (Su Woong Jung, 2021). Although DKD is typically characterized as a non-inflammatory glomerular disease, research using genome-wide transcriptome analysis repeatedly showed that inflammatory signaling pathways are strongly present (Woroniecka et al., 2011). Targeting inflammation to stop the progression of DKD has gained popularity with

the identification of the molecular pathways linking systemic and local inflammation to the pathology of T2DM and DKD (Saulnier et al., 2014). In response to kidney cell injury or stress, damage-associated molecular patterns (DAMPs) and cell adhesion molecules (CAMs) promote the activation of intracellular signaling pathways related to the inflammation process (Sourris & Forbes, 2009). The ongoing activation of innate immune damage sensors, kidney endothelial cells, mesangial cells and podocyte produce multiple inflammatory cytokines, chemokines and adhesion molecules. This will lead to the recruitment of macrophages (Nguyen et al., 2006), which result in the activation of a cascade of inflammatory responses, being renal ischemia as well as ECM and fibrosis a result of the activation of the inflammatory process (Wada & Makino, 2016). Inflammation and fibrosis indicators have been found in epidemiologic research to be independent indicators of the advancement of CKD in both patients with T1DM and T2DM. Furthermore, in non-albuminuric patients, tubular damage is linked to the presence of inflammatory markers in CKD (Fraser et al., 2014). Kidney cells (glomerular endothelial cells, mesangial, podocytes and tubular epithelial cells) express different cytokines, including the interleukin family, tumor necrosis factor-α (TNF-α), chemokines, and multiple adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) (Ijpelaar, 2019). From follow-up cohort studies, Niewczas and collaborators, demonstrated that in patients with T1DM and T2DM, there were elevated levels of kidney risk inflammatory signature proteins, being identified 17 novel circulating proteins enriched for TNF receptor superfamily members that were strongly correlated with a 10-year risk of ESRD, but also with early and advances stages of DKD (Niewczas et al., 2019). Moreover, these kidney risk inflammatory signatures of proteins on GFR decline are pointed to occur through albuminuria-independent pathways. In addition to that, activation of TNF-1α has also a strong correlation with diabetes and cardiovascular outcomes (Fernández-Juárez et al., 2017). The interleukin (IL)-1, IL-6 and IL-18 have been also implicated in the development of DKD. Through the production of prostaglandin E and phospholipase A2, IL-1 enhances the permeability of endothelial cells and causes glomerular filtration. IL-6 is involved in the stimulation of podocyte hypotrophy, albuminuria, GBM thickness, and neutrophil infiltration of the tubulointerstitium. Apoptosis, interferon-y release, and adhesion molecule expression are all induced by IL-18. IL-18 is severely correlated to increased levels of albuminuria in patients with diabetes (Alicic et al., 2018). The inflammation process as well as the above mechanisms highlighted (hyperglycemia and hypertension) cause the development of OS reactions and mitochondrial dysfunction. Traditionally, OS results from an imbalance between oxidants and antioxidants and causes oxidative damage to tissues (Kao et al., 2010).

Oxidative stress and DKD progression

Hyperglycemia, as aforementioned, due to the activation of other glucose oxidation pathways, induces mitochondrial overload and leads to the overproduction of ROS, which will further destroy glomerular podocytes and promote their apoptosis (Forbes et al., 2008). Under pathological conditions, the equilibrium between ROS production and detoxification by the antioxidant defense system is disrupted, resulting in system imbalance and the development of OS (Di Meo & Venditti, 2020). OS has been implicated as a key pathogenic factor in a wide range of diseases, including those affecting the kidneys (P. C. Braga et al., 2022). A key factor in the etiology of DKD is elevated ROS induced by hyperglycemia. The polyol, AGE and NADPH oxidase (NOX) are the primary contributors of ROS in diabetes (Krishan & Chakkarwar, 2011). In addition to NAPH oxidases, ROS can be produced through lipoxygenase, uncoupled nitric oxide synthase, xanthine oxidase and mitochondrial respiratory chain dysfunction (Y. Zhang et al., 2020). Indeed, the initiation and progression of DKD is suggested to be associated to mitochondrial dysfunction and development of OS, where higher amount of OS is able to disrupt some signaling pathways important for kidney function such as Pi3K/Akt, TGFβ-1/ p38 mitogen-aviated protein kinases (p38-MAPK) and nuclear factor kappa B (NF-kB), that ultimately leads to kidney damage (Ma et al., 2023). Tubulointerstitial fibrosis and proteinuria can arise from direct injury to mesangial cells, endothelial cells, and podocytes caused by OS (Duni et al., 2019; Khazim et al., 2013). This can occur because, in comparison to other nephron components, the glomerulus is more vulnerable to oxidative damage (Krishan & Chakkarwar, 2011). The importance of the mitochondria is highlighted in recent published works. For instance, in C57BL/6J mice in an early stage of DKD with no changes in albuminuria, it was observed a mitochondrial fragmentation and a decrease in ATP content in PT cells (Sun et al., 2019). Regarding antioxidant defense, in rats with DKD, it was observed a decrease in specific enzymes such as GSH, Superoxide dismutase (SOD) and total antioxidant status (Hussain Lodhi et al., 2021). Additionally, glomerular endothelial cells became dysfunctional, partly due to increased levels of mitochondrial super-oxides that are responsible for the increase in DNA damage (Casalena et al., 2020). In male Wistar rats, diabetes was induced through streptozotocin to evaluate the effect of oil vegetable oil blend (VOB) in DKD progression, which is an oil associated with antioxidant and anti-inflammatory properties. For that, the animals received VOB for 56 days. After the treatment it was observed a significant increase in glucose levels, blood urea nitrogen (BUN), N-acetyl-β-D-glucosaminidase (NAG), and proteinuria levels with a concomitant decrease in GFR. Furthermore, the diabetic group had higher levels of oxidative damage indicators, including 8-hydroxy-2′-deoxyguanosine (8-OHdG), TGF-β1, GSH, and malonaldehyde (MDA), along with decreased levels of

antioxidant enzymes, SOD and glutathione reductase (GR). Histological alterations were also observed in the diabetic group, that showed interstitial fibrosis and glomerulosclerosis. When VOB was administered, anomalies in tubular damage and renal dysfunction indicators were considerably reduced (Anwar et al., 2022). The convergence of these pathogenic factors, resulting in renal impairment, points mitochondria as a critical central point of control in DKD initiation and progression. Nevertheless, the absence of validated mitochondrial biomarkers for early-stage DKD detection remains a significant challenge that can have a great value for clinical practice.

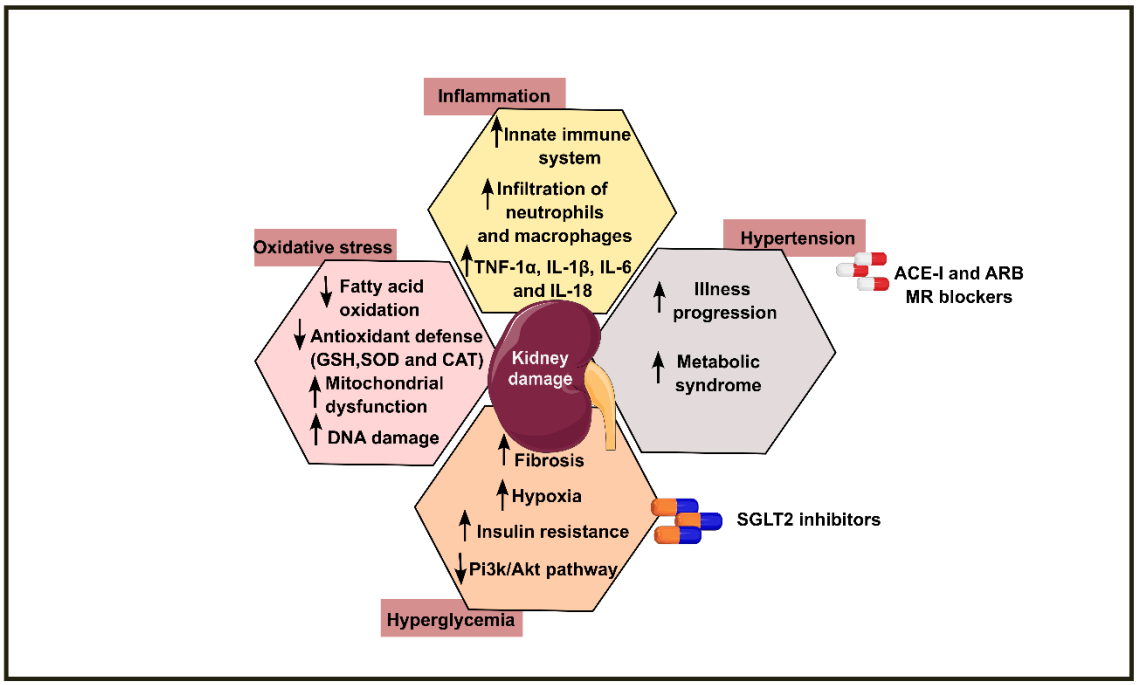

Fig.1.5- Key drivers of DKD progression. The main 4 causes are inflammation, hyperglycemia, hypertension and oxidative stress. They are all interconnected and are a cause-effect of each other. Indeed, an increase of arterial tension is one of the main contributors to kidney disease progression and is associated with metabolic syndrome. Hypertension can be regulated by the administration of angiotensin-converting enzyme inhibitor (ACE-I) and angiotensin II receptor-blockers (ARB), as well as mineralocorticoid receptor (MR) blockers. Hyperglycemia is another risk factor that contributes to the vulnerability of the organ, by contributing to a metabolic dysregulation that induces lower sensitivity to insulin leading to a downregulation of downstream signaling pathways such as PI3K/AKT pathway, as well as hypoxia and the development of fibrosis. Hyperglycemia also enhances sodium and glucose reabsorption in the proximal tubules via sodium-glucose cotransporter 2 (SGLT2). As more sodium is reabsorbed, less reaches the macula densa, disrupting tubuloglomerular feedback. and increasing glomerular filtration. To avoid the effects of hyperglycemia, SGLT2 inhibitors are widely used in the clinical practice. Nevertheless, hyperglycemia is connected to the increase of inflammation, by an increase of the innate immune system, leading to an increase of the infiltration of neutrophils and macrophages, as well as an increase in the expression of cytokines, such as tumor necrosis factor 1α (TNF-1α), Interleukin-1β (IL-1β), 6 (IL-6) and 18 (IL-18). Upon this dysregulation, oxidative stress also plays a key role in the progression of kidney damage, as expressed by a lower fatty acid oxidation as well as a decrease in antioxidant defense, by the decrease of Glutathione (GSH), Superoxide Oxidase Dismutase (SOD)

and Catalase (CAT) enzyme activities, being that reflected in a higher mitochondrial mechanism's dysfunction and higher DNA damage.

1.2.2.Non-albuminuric patients- the major challenge in the clinic

As already mentioned, patients with a history of DM are at increased risk for DKD (Levey et al., 2011; MacIsaac et al., 2004; Vistisen et al., 2019), and beyond the classical clinical course of the disease, a cluster of patients with diabetes that progress with normal levels of albuminuria, the NA-DKD patients, have gained clinical relevance because clinicians do not have risk urinary biomarkers. In patients with diabetes, GFR and uACR are not in steady states, often fluctuating due to several factors including blood pressure control and the use of RAAS antagonists. This is exemplified by the research of Klimontov and collaborators, where they demonstrated that approximately 9.3-71.1% of patients with eGFR <60 mL/min/1.73 m^2 have normal albuminuria levels (Klimontov & Korbut, 2019). In addition to that, others reported that normal levels of albuminuria but reduced eGFR were present in 8.3% of patients with diabetes and 63.3% of those who had an eGFR decline (Jia et al., 2022), highlighting that eGFR can be preserved, but the renal complications are already occurring. Furthermore, after the development of macroalbuminuria, patients with diabetes were considered to experience a rapid decline in GFR, but individuals without albuminuria were supposed to experience it infrequently. However, during the last decade, the studies are indicating the contrary. In a cohort of 286 patients with T1DM and normal albuminuria, 10% exhibited a progressive eGFR decline (≥ 3.3.%/year) prior to the onset of microalbuminuria (Krolewski et al., 2014). Therefore, the prevalence of DKD has not being reduced in the past decades and there is an opposing trend in its two primary markers: an increasing prevalence of low eGFR and a decreasing prevalence of albuminuria (Shi et al., 2022). This data demonstrated that both albuminuria and eGFR have limited efficacy as prognostic tools in DKD. In fact, individuals with microalbuminuria can either regress to normoalbuminuria or progress to macroalbuminuria (MacIsaac et al., 2014). Also, eGFR is not a totally reliable indicator of the extent of kidney injury, since when eGFR reaches the threshold of 60 mL/min/1.73 m^2, about 60% of the nephrons have already been lost, demonstrating that eGFR has limited interest as an early biomarker to follow in NA-DKD patients. This is also demonstrated by another study, where the national evaluation of the frequency of Renal Impairment Coexisting with non-insulin-dependent diabetes mellitus survey of primary care, in patients with T2DM, found that 55% of the patients with eGFR under 60mL/min/1.73 m^2 were persistently non-albuminuric (Thomas et al., 2009).

The increased use of RAAS blockers and their combinations with new classes of hypoglycemic agents, such as SGLT2 inhibitors and glucagon-like peptide 1 receptor

agonists (GLP1-RAs), may be one of the current causes of the increased prevalence of patients with non-albuminuric CKD and NA-DKD (D'Marco et al., 2022). Zeeuw D and collaborators indeed suggested that the continued modulation of RAAS, which lowers albuminuria by changing renal hemodynamics, may account for the increasing prevalence of non-albuminuric phenotype (De Zeeuw et al., 2004). That study demonstrated on a large scale, for the first time, in a prospective manner that the degree of albuminuria reduction is linearly related to the subsequent renal protection in T2DM. Another important conclusion taken by this study was the fact that the correlation between albuminuria and renal risk is maintained even after albuminuria lowering therapy. In addition to this, the use of additional antihypertensive drugs was well- balanced amongst the different albuminuria subgroups and the albuminuria response groups. These groups did show slight differences in responses to other risk markers such as blood pressure and cholesterol, but the significance of these changes did not explain the observed “antialbuminuric effect.” Further on, Loutradis C. and collaborators summarized all the data concerning RAAS blockers and the renoprotective effects against CKD patients, and they conclude that there are few randomized trials that explicitly address whether RAAS blockers should be started in patients with advanced CKD. Due to the increase lack of participants with normoalbuminuria in existing trials, many clinicians are not persuaded of the benefits of RAAS blockade in these patients (Loutradis et al., 2021), highlighting once again that there is an urgent need to find other pharmaceutical solutions to this cluster of patients. Other factors that can be associated to NA-DKD phenotype are female sex, hypertension, active smoking and the absence of diabetic retinopathy (Jia et al., 2022; Viazzi et al., 2019). Moreover, patients with renal impairment and albuminuria (31%) were more prone to develop retinopathy (Afghahi et al., 2013). Indeed, the renal insufficiency and cardiovascular events (RIACE) cohort study, demonstrated that NA-DKD patients were frequently female and non-smokers and had lower HbA1c, but did not have longer diabetes duration compared with patients with albuminuria. Interestingly, the regression analysis confirmed that non-albuminuric renal impairment phenotype was not associated with HbA1c and even less correlated with retinopathy and hypertension than albuminuria, either alone or associated with reduced eGFR. Curiously female sex presented a higher correlation with normoalbuminuric renal impairment while male sex was correlated with higher levels of albuminuria (Penno et al., 2011).

Morphological studies uncovered several distinctions between A-DKD and NA-DKD. In comparison to patients with normal uACR and eGFR >90 mL/min/1.73 m^2, it was shown that female patients with non-albuminuric T1DM, presenting an eGFR <90 mL/min/1.73 m^2, had a more prominent glomerular involvement, estimated by an increased width of the

glomerular basement membrane and the volume of the mesangial matrix (Caramori et al., 2003). Moreover, renal structural changes are more heterogeneous in T2DM, while tubulointerstitial abnormalities, arteriolosclerosis, and global fibrosis of some glomeruli may outweigh the common glomerulopathy symptoms (Fioretto et al., 2008). It is not well known how NA-DKD manifests, but some renal biopsy findings demonstrated that the typical alteration at glomerular levels are directly related with the increased levels of albuminuria, as observed by Ekinci et al., (Ekinci et al., 2013). Concurrently, there were more frequently observed interstitial and vascular alterations in patients with NA-DKD, which probably reflects the increased contribution from aging, hypertension, and arteriosclerosis (Ekinci et al., 2013) but may also be a clue to investigate other pathophysiologic tracks related with inflammation and hypoxia in tubular cell, as highly metabolic demanding site. As we acknowledge that NA-DKD has become a prevalent pattern, several crucial clinical issues need to be done. Finding NA-DKD diagnostic markers and factors that may indicate the disease's progression is crucial from a practical standpoint. Considering the findings of morphological assessments, tubular and interstitial involvement indicators may be helpful in the diagnosis of NA-DKD. Indeed, despite the still frail clinical evidence, other biomarkers are used in the clinical setting to follow the decrease of kidney function, more specifically to identify tubular damage. For instance, in clinical practice, liver fatty acid-binding protein (L-FABP), neutrophil gelatinase-associated lipocalin (NGAL), urinary immunoglobulin G and M, and kidney injury molecule-1 (KIM-1) are also used as primary biomarkers associated with DKD (Papadopoulou-Marketou et al., 2017). A study with 22 patients with T1DM and 20 patients with T2DM who all exhibit signs of DKD, suggests that urinary immunoglobulins may be useful for DKD risk stratification. Patients with T2DM showed higher urine excretion of IgG and IgM along with albuminuria (Bakoush et al., 2002). KIM-1 is a transmembrane glycoprotein expressed on the apical membrane of renal, and patients with tubular injury, present higher plasma levels of this protein. A study of patients with T1DM found that plasma KIM-1 levels above 97 pg/mL were a positive predictor of ESKD risk, independent of HbA1c, albumin excretion rate and eGFR outcomes (Sabbisetti et al., 2014). Additionally, plasma KIM-1 independently predicted both early eGFR decrease and progression to CKD in T1DM patients with normal eGFR and no macroalbuminuria, regardless of systolic blood pressure, Hb1Ac, albumin excretion rate, eGFR and TNF-α (Nowak et al., 2016). It has also been demonstrated that the progression of DKD is related to urinary L-FABP levels. High urine levels of L-FABP in patients with T1DM, regardless of albuminuria levels or other known risk factors, were predictive of all-cause mortality and the onset/ progression of DKD (Nielsen et al., 2010). A similar trend was observed in patients with T2DM, with notably higher levels of this protein in non-albuminuric patients (Kamijo-Ikemori et al., 2011).

When discussing tubular damage, the involvement of the mitochondria is inherent to the development of the disease. Mitochondrial dysfunction in DKD is primarily linked to several pathological mechanisms that arise due to diabetes. Renal oxygen consumption increases due to the elevated blood sugar concentration, causing hyperperfusion and hyperfiltration in the glomeruli due to the increase in glucose concentration. This will impact the renal hemodynamics, which contributes to a metabolic reprogramming, where mitochondria-related metabolism is highly affected. Studies that aim to explore the pathogenesis of NA-DKD at the metabolic level are limited. To properly comprehend the underlying mechanism and create suited treatments that can successfully mitigate the effects of mitochondrial dysfunction in DKD, more investigation is required. Recently, proteomic and metabolomic analyses emerged as promising tools for early-stage detection of DKD, particularly in NA-DKD patients, where knowledge is limited (Pereira et al., 2022). Understanding the role of mitochondria metabolism in this context can pave the way to create opportunities to personalized detection methods and therapeutic interventions for NA-DKD patients.

1.3. Kidney bioenergetics

The kidneys have a high density of mitochondria and thus a high consumption of oxygen to support the energy-intensive process for urine production (Forbes, 2020). This oxygen is metabolized within the mitochondria by oxidative phosphorylation (OXPHOS), through the electron transport chain (ETC), to produce ATP (Pebay-Peyroula et al., 2003). In a very simple way, a wide range of fuels (glucose, fatty acids, and amino acids) are converted into molecules of ATP. In this process, oxygen acts as an electron acceptor in highly regulated reactions carried out by protein complexes I–IV. The protons are pumped into the intermembrane space as electrons move through complexes I, II, and IV to ATP synthase to drive the conversion of ADP to ATP (Pebay-Peyroula et al., 2003). Additionally, the Krebs (TCA) cycle plays a pivotal role in mitochondrial energy production (Forbes, 2016). TCA is responsible for the production of the following coenzymes in the mitochondrial matrix, which englobes three molecules of NADH, and one molecule of flavin adenine dinucleotide ($FADH_2$), which is converted from pyruvate after the glycolysis pathway (Ralto et al., 2020). The Na^+-K^+-ATPase is powered by mitochondria to create ion gradients across the cellular membrane (Soltoff, 1986). In the kidney, active transport is essential for ion reabsorption in the proximal tubule, loop of Henle, distal tubule, and collecting duct (Soltoff, 1986). On the other hand, glomerular filtration is a passive process that driven by hydrostatic pressure in the glomeruli (Holechek, 2003). Since proximal tubules reabsorb 80% of the filtrate—which

includes glucose, ions, and nutrients—that passes through the glomerulus, they require more active transport mechanisms than other types of renal cells (Bhargava & Schnellmann, 2017). OXPHOS machinery can also be used for hormone and protein synthesis and glomerular filtration, including contraction of mesangial cells and podocytes (ZiMian Wang et al., 2010). The fuel preferences are different and tend to mirror ATP demands at these sites. Different regions of the kidney, more specifically along the nephron (Fig 1.6) carry out distinct physiological functions with varying energy requirements, contributing to a diverse metabolic profile within the organ.

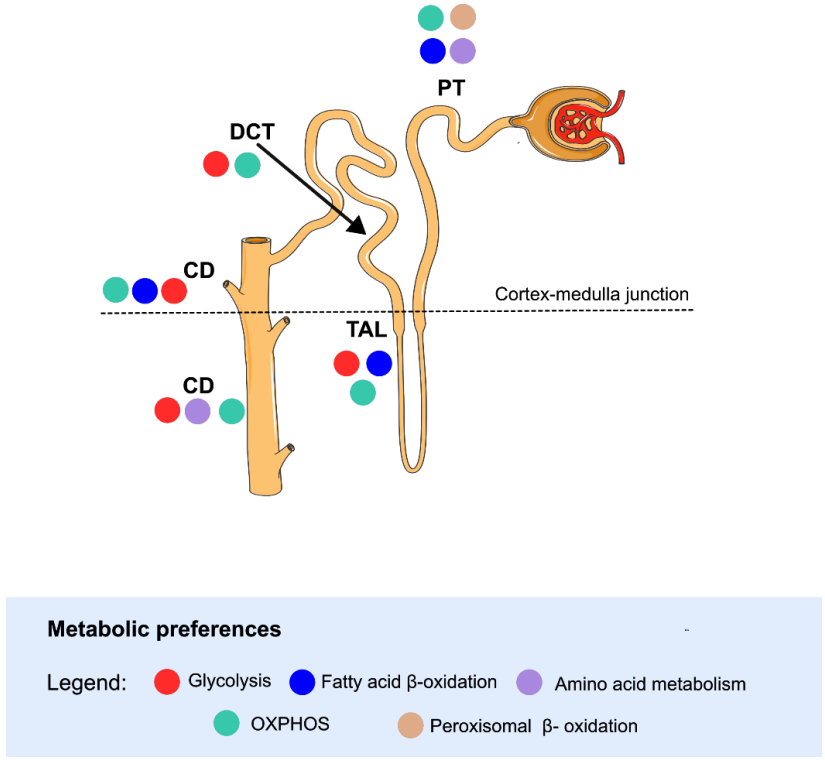

Fig.1.6- Overview of the metabolic pathways throughout the nephron segment. Colored circles indicate the main energy metabolic pathways used for the different areas. Abbreviations: PT- proximal tubules; DCT- distal convoluted tubule; CD- Collecting duct; TAL- Thick ascending limb of Henle´s loop.

Mitochondrial abundance is particularly high in cortical segments, including proximal and distal convoluted tubules, serving as the primary sites for fatty acid oxidation (FAO). Free fatty acid (FFA) oxidation is another fuel that can be used in kidney cells, particularly in the tubulointerstitial compartment (excluding the glomeruli). Oxidation of palmitic acid produces 106 molecules of ATP per molecule of substrate, being more efficient than glycolysis (Rich, 2003). The accumulation of FFA in AKI and DKD exhibit abnormalities in lipid metabolism, increasing the formation of lipid droplets inside the cells (Simon & Hertig, 2015). PT cells need ATP to produce glucose via gluconeogenesis (Krebs & Yoshida, 1963) (Fig 1.7), in which glucose is synthesized from precursors such as lactate, glutamate, and glycerol (Meyer et al., 2003).

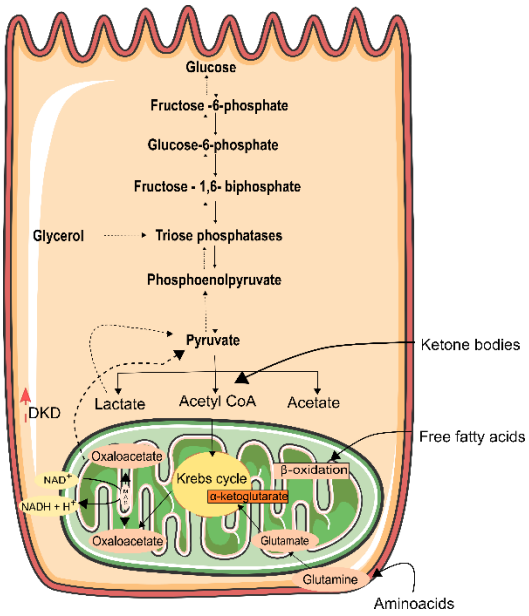

Fig.1.7-Schematic overview of the major metabolic pathways in renal epithelial cells. In renal cells, the uptake of free fatty acids, ketone bodies, glucose, and glutamine lead to increased oxygen consumption and ATP production, through ATP synthase. Glucose, in distal tubules in the healthy kidney, is metabolized to glucose-6-phosphate which is converted into fructose-1,6-biphosphate. Then, fructose-1,6-biphosphate is cleaved into two phosphorylated three-carbon compounds (glyceraldehyde 3-phosphate and dihydroxyacetone phosphate), known as triose phosphatases. Afterward, glyceraldehyde 3-phosphate is converted into phosphoenolpyruvate and finally into pyruvate. Free fatty acid oxidation is a more efficient substrate to use than glucose in terms of energy production; however, in diabetic nephropathy disease, some complications occur, i.e., increasing lipid droplets. Within the cortex, ketones bodies are also a substrate used for ATP production. Glycerol becomes an important gluconeogenic precursor in diabetes. It is broken into glucose-6-phosphate and then transformed into pyruvate and continues to follow the same path as glucose metabolization. Gluconeogenesis (up dashed arrows), which occurs in proximal tubules cells, needs ATP to produce glucose, which can be synthesized from lactate, and glycerol. Glutamine is degraded and converted to glutamate, which can be transaminated via glutamate-oxaloacetate-transaminase or glutamate-alanine-transaminase, yielding α-ketoglutarate, an intermediate of Krebs cycle. Pyruvate results from lactate, enters into the mitochondria, and is converted to oxaloacetate. Oxaloacetate, in the cytoplasm of the mitochondria, can be reduced to malate and be exported out and/ or in the cytoplasm. Here, through malate aspartate shuttle (MAS), first, malate is oxidized to oxaloacetate and then converted to phosphoenolpyruvate and subsequently converted to fructose-6-phosphate. After a phosphatase, fructose-6-phosphate is dephosphorylated, which results in glucose-6-phosphate, which then is converted into the release of glucose.

Glutamine is the most common amino acid in the human body and in the kidney makes a higher contribution to gluconeogenesis than does alanine (Stumvoll et al., 1998). Renal gluconeogenesis, in healthy individuals, represents nearly 60% of endogenous glucose release during the post-prandial period, while this proportion is half reduced in a patient with T2DM (Gerich et al., 2001; Meyer et al., 2003). In diabetic kidneys, these values are exacerbated via stimulation of the malate aspartate mitochondrial shuttle (Friedrichs, 1975),

increasing the generation of NADH, $FADH_2$, and ATP, and ultimately increasing renal gluconeogenesis (Stumvoll et al., 1999). Moreover, glutaminase, the enzyme responsible for converting glutamine into glutamate (Guder et al., 1986), is expressed at high concentrations in the tubular compartment of the kidney (Forbes & Thorburn, 2018). Nonetheless, in the diabetic kidney, the impairment of autophagy increases the use of less favorable fuel sources. For instance, glucose is used by PT cells and fatty acids are used by glomerular epithelial cells, leading to glucotoxicity and lipotoxicity, respectively (Ding & Choi, 2015). As happens with FFA, ketone bodies require higher amounts of oxygen to produce ATP (Guder et al., 1986). Notwithstanding, ketone bodies are an important fuel for OXPHOS within the kidney cortex (Harris et al., 1981). However, little is known about their contribution to the kidney. The use of ketone bodies along the nephron is reflected in the mitochondrial density, which is high in this site (Uchida & Endou, 1988). Moreover, increased ketogenesis also occurs in glomerular cells in diabetes. However, the rate of intake of ketones in the kidney is not altered in diabetes (Ikeda et al., 1993), and so ketone bodies may be an endogenous process, with no relation to diabetes per se, but with renal injury only. However, more studies are needed, particularly regarding the contribution of these metabolites and what is the consequence of their increase in mitochondrial dysfunction in the kidney. On the other hand, glomerular cells, that include podocytes, mesangial cells and glomerular endothelial cells, depend mainly of glucose for fuel (Abe et al., 2010; Bhargava & Schnellmann, 2017; Harzandi et al., 2021). Furthermore, podocytes have a moderate density of mitochondria that engage in both OXPHOS and glycolysis, while the collecting ducts in the renal medulla have fewer content of mitochondria and typically rely more on glycolysis (Pagliarini et al., 2008; Z. Wang et al., 2010). Podocytes can be damaged due to FFA accumulation, which can sense disruption of the glomerular filtration barrier, through the free passage of FFA (Fornoni et al., 2014; Kampe et al., 2014). In rodents, high circulation of FFA revealed susceptibility to renal injury (Chung et al., 2015). Lipid-lowering therapies are considered to have protective effects concerning kidney fibrosis (Kang et al., 2015). Nevertheless, these mechanisms are controversial once they can be considered to be pathogenic or compensatory responses to renal injury (Ferrannini et al., 2016; Rosca et al., 2012; Stadler et al., 2015).

Mitochondria have a crucial role in normal kidney function, to maintain the metabolic homeostasis. It is suggested that metabolic shift in renal cells can be caused by diabetes. Although several possible etiologies that could trigger this process have been identified, the underlying mechanisms are not entirely known. The relationship between mitochondria dysfunction and development of kidney disease has been recently highlighted and shown that a metabolic shift to anaerobic glycolysis to produce energy is present when mitochondria function is suppressed (Brinkkoetter et al., 2019). Furthermore, it was also

found that metabolic flux mediated by the TCA cycle was increased in diabetic kidney cortex, which was not corresponding to neither oxygen consumption nor ATP production increment (Brinkkoetter et al., 2019). Moreover, the excessive metabolic byproducts, such as citrate, succinate, fumarate and malate are caused by the decrease of mitochondria activity. These byproducts accumulation are toxic to mitochondria, resulting in inhibition of the ATP synthase (X. Fu et al., 2015) and decreased mitochondrial membrane potential (Karlstaedt et al., 2016). In addition, in the presence of persistent hyperglycemia, it has been demonstrated that the pyruvate dehydrogenase multienzyme complex, a crucial regulator connecting glycolysis to TCA cycle, by catalyzing pyruvate to acetyl coenzyme A (CoA) irreversibly in mitochondria, is hyperphosphorylated and inhibited. This results in diabetic kidney damage (Jeoung et al., 2006; Rardin et al., 2009). The pathological development of DKD is a long process, where the metabolic reprogramming can have different outcomes. To meet energy demands every kidney cell undergoes metabolic reprogramming, especially in the early phases of DKD.

1.3.1. Metabolic reprogramming in tubular cells

The pathogenic mechanisms behind DKD are complex and poorly understood. Metabolic issues that can lead to a variety of alterations in energy metabolism are what define diabetes. The kidney, as referred above, is one of the organs that uses the most oxygen in the body, and thus they need to maintain energy homeostasis. Changes in metabolism can cause malfunctions in cells or cause death. One theory suggests that exist a metabolic reprogramming, a switch from mitochondrial OXPHOS to glycolysis and its offshoots- plays a crucial role in the development and progression of DKD. The metabolic process is malleable and can adapt to changes in the surrounding environment. The “Warburg effect” or metabolic reprogramming, was first observed by Warburg in 1958 when he described that tumor cells could produce ATP through glycolysis in environments well oxygenated (Warburg et al., 1927). Technological developments over the past few decades have made it possible to comprehend energy metabolism better. Research on metabolic reprogramming´s critical environment in the emergence of CKDs, including renal fibrosis and autosomal dominant polycystic kidney disease, has grown in number (Pagliarini & Podrini, 2021; Zhu et al., 2021). In an effort to better understand DKD causes, there has been a growing amount of attention recently focused in the metabolic changes that drive the transition from mitochondrial OXPHOS to glycolysis and it main branches in the different kidney sections (Laustsen et al., 2013).

High baseline energy levels are necessary for the health of renal tubular epithelial cells. In PT cells, the demand of energy is high to sustain the activity of ATP-dependent pumps (e.g Na^+, K^+ and ATPase). In the kidney, about 40-70 % of the oxygen consumption is required to fuel those pumps and drive the reabsorption and secretion of water, solutes, and waste products (Bhargava & Schnellmann, 2017; Rolfe & Brown, 1997). Therefore, in diabetes, renal tubule epithelial cells are more susceptible to abnormalities in metabolism. Under typical circumstances, PT cells mostly rely on FAO to generate significant amounts of ATP to meet their physiological needs. The main pathways where fatty acids are transported into the cells are mediated by transmembrane protein CD36 and fatty acid-binding proteins (FABPs) (Glatz & Luiken, 2018; Pepino et al., 2014). After entering the cells, long chain fatty acids (LCFAs) can be converted into LFCA-CoAs under the catalysis of acyl-CoA synthetase (ACS). Fatty acids are then transported into the mitochondria, through carnitine shuttles involving carnitine palmitoyl transferase-1 (CPT1) and CPT2 catalyze FAO. Acetyl-CoA is created by this process and goes on to participate in the TCA cycle, which gives the kidney energy. Furthermore, the FAO-produced acetyl-CoA in mitochondria can be expelled via carnitine acetyltransferase (CACT) and resynthesized into new fatty acids via acetyl-CoA carboxylase. Using fatty acids as an energy source, brings different advantages. One is that the energy metabolism does not interfere with the glucose reabsorption in the PT cells, and thus glucose remains available for medullary tubular segments that face low availability, which allows the generation of glucose by gluconeogenesis. In fasting conditions, renal gluconeogenesis is essential for the maintenance of systemic glucose homeostasis. Moreover, in the PT cells, glutamine can be considered as an alternative energy source, which can provide the basis for gluconeogenesis and ammoniagenesis (Conjard et al., 2001).

In the context of DKD disease, tubular epithelial cells´ energy metabolic pattern is changed, and metabolic reprogramming takes place to produce energy and combat OS. Renal PT epithelial cells undergo a metabolic transition from FAO to glycolysis when exposed to elevated glucose levels (Hou et al., 2024; Singh, 2023). Several studies demonstrated that due to enhanced glycolysis, there was an elevated expression of glycolytic enzymes in diabetes, which further induce epithelial-mesenchymal transition (EMT) and exacerbate renal fibrosis (Li et al., 2020; Storch & Corsico, 2008; Yin et al., 2018). In addition to this, several metabolites accumulate in the TCA cycle due to decreased mitochondrial mechanisms, such as fumarate, which is also associated with EMT (Sciacovelli et al., 2016). TCA cycle is tightly regulated, with the intermediates of the cycle. The regulatory enzymes of the TCA cycle are activated under the conditions of high demands of ATP, which in turn impairs mitochondrial respiration, where the accumulation of NADH slows down the cycle (Martínez-Reyes & Chandel, 2020). Unconsumed FFAs are converted to triglycerides and

then into lipid droplets (Donkor et al., 2009), which trigger further lipotoxicity by inducing inflammation, OS, endoplasmatic reticulum stress, and so on and ultimately to cell death and renal fibrosis (Wang et al., 2014).

Collectively, this causes mitochondrial dysfunction, which in turn causes tubulopathy to develop and worsen kidney function (Hansell et al., 2013; Vallon, 2011).

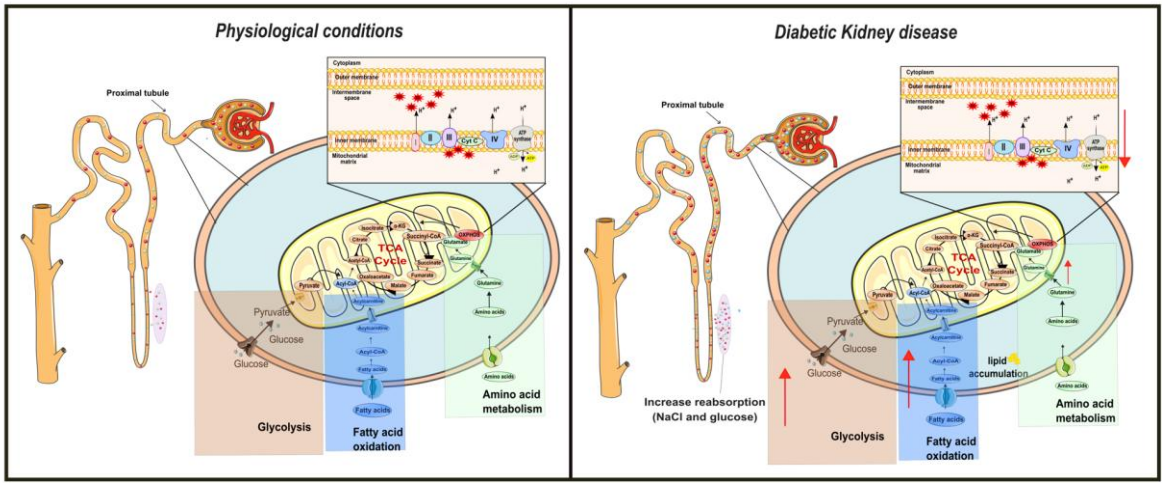

Fig.1.8 – Overview of the main metabolic pathways at the proximal tubule (PT) cells level in normal (left panel) physiological condition and in diabetic kidney disease (right panel). In normal conditions fatty acids are the preferable source of energy used. Fatty acid degradation occurs into acetyl coenzyme A, which enters the Krebs (TCA) cycle and progresses to oxidative phosphorylation (OXPHOS). Other energy sources may be used, such as glutamine (through gluconeogenesis). In stress conditions, glycolysis, represented in brown, increases. Here glucose is taken up to the cells and transformed into pyruvate, which then enters in the mitochondria and is converted into CoA, following the TCA cycle. Furthermore, in early stage of DKD, the PT cells are exposed to hypoxia due to hyper reabsorption of salts and glucose. Fatty acids metabolism is also increased, however there is an imbalance leading to the accumulation of lipids instead of its full oxidation. An alternative to fatty acid metabolism is the oxidation of amino acids, such as glutamine. Glutamine is transferred into the mitochondria and is converted into glutamate and α-ketoglutarate (α-KG), which fuels the TCA cycle. In DKD, an increase of TCA intermediates is observed, generating an impairment of OXPHOS. In the OXPHOS is observed an increased production of reactive oxidative species, resulting in a decrease of ATP production.

1.3.2. Metabolic reprogramming in podocytes

Podocytes are highly specialized cells that contribute to building the glomerular filtration barrier. They have intricate structures named focal adhesion complexes, slit diaphragms and interdigiting foot processes. To maintain their typical activity and intricate cellular structure, podocytes require a continuous energy source that includes glycolysis and mitochondrial OXPHOS (Imasawa et al., 2017). This fact occurs because podocyte bioenergetic status seems to depend on their differentiation stage. Indeed, aerobic glycolysis is the main source of energy before differentiation, while OXPHOS is predominant after, with the concomitant stimulation of mitochondrial function (Imasawa et al., 2017; Yuan et al., 2020). In differential podocytes, the metabolism also switches to glycolysis. It is though that the compensatory increase in glycolysis is believed to provide sufficient energy to meet the energy demand. However, in the setting of cells stress, such as hyperglycemia, the compensatory mechanism is not enough, leading to podocyte injury, caused by a decreased expression of functional proteins, namely podocin (Imasawa et al., 2017). In metabolic reprogramming, the increased flow of side branches of glycolysis is one of the main routes of podocyte damage. Rather than producing pyruvate, glucose enters into side branches, to form toxic metabolites such as sorbitol, methylglyoxal and DAG (Qi et al., 2018).

1.3.3.Glomerular mesangial and endothelial cells reprogramming

Specialized pericytes called glomerular mesangial cells (MCs) surround the glomerular capillaries inside the renal corpuscle. They are responsible for synthesizing the mesangial matrix, control glomerular hemodynamics by contracting the cells, being crucial in the regulation of blood flow and glomerular filtration rate by adjusting capillary diameter, and release a variety of cytokines (Ebefors et al., 2022).

Chronic hyperglycemia induces a loss of metabolic switching flexibility in MSc. This impaired response to an acute high glucose load results in bioenergetic deficits within these cells (Czajka et al., 2015). Moreover, the prolonged exposure to high glucose levels, more particularly an exacerbation of polyol pathway results in the loss of contractility followed by MCs swelling (Wakisaka et al., 2021), which are though to induce glomerular hyperfiltration. The dysregulation of this pathway depletes NADPH, leading to AGEs binding to the receptor on cell surfaces, causing OS, ROS generation and the activation of PKC and ERK1/2 signaling pathways. These events ultimately promote the secretion of TGF-β, the proliferation of mesangial cells, and the synthesis of extracellular matrix, all hallmarks of renal fibrosis (Huang & Zhao, 2020).

An essential part of kidney's filtration mechanisms is the glomerulus, a capillary ball housed in a tiny kidney capsule. The glomerular endothelial cells (GECs) are the first line of defense, contributing to the formation of the glomerular filtration barrier. A dysfunction in these cells was pointed as a key point to the onset and development of DKD (J. Fu et al., 2015; Maestroni & Zerbini, 2018; Shi & Vanhoutte, 2017). Despite having access to oxygen, GECs produce ATP largely by glycolysis rather than mitochondrial OXPHOS (Eelen et al., 2018). However, due to its ability to control OS and maintain Ca^{2+} homeostasis, mitochondrial respiration continues to be crucial for preserving the structural and functional integrity of GECs (Salnikova et al., 2021; Yamamoto et al., 2018; Yu et al., 2017). Again, an increase in glycolysis will lead to the increase of OS. This is observed, for instance, through the increase of O-linked β-N-acetylglucosamine (O-GlcNac) modification of eNOS in DKD and subsequent ROS production (Du et al., 2001). Additionally, the metabolism of the side branch pathway is further boosted by the excessive mitochondrial superoxide generation in defective mitochondria (Clyne, 2021).

1.4. The role of mitochondria quality control in renal tubular cells

Mitochondria are semi-autonomous organelles with an autotrophic system and mitochondrial turnover is a mechanism adopted to assure mitochondrial quality. Moreover, to increase the production of metabolic enzymes and increase the mitochondrial metabolic capacity, the nuclear and mitochondrial genomes must be coordinated. The mtDNA is a double-stranded circular molecule that is roughly 16 kb in size and encodes 37 genes, comprising 13 structural subunits of the mitochondrial respiratory chain (Jornayvaz & Shulman, 2010).To preserve the stability of the internal structure, they go through a dynamic process of fusion and fragmentation called mitochondrial dynamics. In this process, dysfunctional mitochondria are selectively eliminated and replaced through an increase in the number of mitochondria. Bioenergetic demand changes can influence mitochondrial morphology by regulating the activity of protein mitochondrial dynamics via metabolic pathway-related enzymes and signaling pathways, including mTOR and deacetylase sirtuins (Cha et al., 2021; Morita et al., 2017), which will influence the activity of both fusion and fission proteins. Furthermore, mitochondrial biogenesis can be influenced by multiple external factors, such as exercise, caloric restriction, and OS (Jornayvaz & Shulman, 2010). The peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) protein family is responsible for activating mtDNA transcription. It is also the key regulator of mitochondrial biogenesis in this family, being foremost expressed in proximal tubules

(Puigserver et al., 1998). Unsurprisingly, the dysregulation of mitochondrial biogenesis has been associated with kidney diseases. Nuclear respiratory factor 1 (NRF1) and nuclear respiratory factor 2 (NRF2) are transcriptional coactivators of PGC-1α that promote mitochondrial biogenesis, and are responsible for OXPHOS control, FAO, and the synthesis of NAD^+, which is a key metabolic coenzyme/co-substrate involved in cellular energy, that confers renal protection (Gureev et al., 2019; Lynch et al., 2018; Tran et al., 2016). PGC-1α coordinately upregulates the enzymes that synthesize NAD *de novo* from amino acids, however in AKI there is an attenuation of this *de novo* pathway. These results are supported by data obtained in PGC-1α knockout mice following IRI. Tran and collaborators demonstrated that PGC-1α knockout mice experienced substantial fat storage, a local deficit of the NAD precursor niacinamide, and an inability to restore normal renal function (Tran et al., 2016). Furthermore, in sepsis associated AKI, there is a suppression of PGC-1α which negatively affects the recruitment of NRF1 and NRF2, leading to an impairment of OXPHOS (Parikh et al., 2015; Tran et al., 2011) (Fig 1.9). According to a different study, PGC-1α protein levels rise and PGC-1α mRNA expression decreases on the third and tenth days following unilateral ureteral obstruction, and these changes are strongly linked to fibrosis (Hickey et al., 2011). Furthermore, the family of NAD^+- dependent deacetylases known as sirtuins (SIRT1-7) can regulate mitochondrial biogenesis and play a key role in kidney disease. It has been shown that SIRT1, a NAD-dependent deacetylase, can control PGC-1α expression and activity. When a SIRT1 activator (SRT1720) was administered to patients with IRI-AKI, the kidneys' expression of PGC-1α was restored. This enhanced mitochondrial biogenesis, evidenced by an increase in mitochondrial mass and ATP levels, ultimately led to improved renal function (Funk & Schnellmann, 2013). Besides, another activator of mitochondrial biogenesis can be addressed to recover renal function after an AKI episode (Jesinkey et al., 2014). Another example is formoterol, which is a potent agonist of β2-adrenoreceptor, that can induce mitochondria biogenesis by increasing mtDNA copy numbers, oxygen consumption rate, and PGC-1α expression in renal proximal tubular cells (Wills et al., 2012). SIRT3 is present in the mitochondrial matrix, and is responsible for maintaining OXPHOS efficiency (Haigis et al., 2012), mtDNA integrity (Osman et al., 2011), as well as mitochondrial biogenesis and fission (Morigi et al., 2015). In sepsis and cisplatin-induced AKI, SIRT3 overexpression has been shown to have a protective effect (Wang et al., 2018; Zhao et al., 2016). Moreover, in cisplatin induced AKI, a downregulation of PGC-1α was able to decrease the free FAO genes (like carnitine O-palmitoyltransferase and medium chain-specific acyl-CoA dehydrogenase), resulting in a decreased mitochondrial FAO. Thus, this impairment was reported as linked to ATP depletion in AKI (Jang et al., 2020). There are not many studies, but the ones found in the literature highlight a very important role for mitochondrial quality control and biogenesis in AKI.

1.4.1. Mitochondria dynamics in the kidney

Mitochondria, as dynamic organelles, possess the ability to regulate their morphology, orchestrating the formation of a tubular network through coordinated fusion and fission events. An excess of mitochondria fission leads to mitochondrial fragmentation, whereas when there is an enhanced mitochondrial fusion, it results in mitochondrial hypertubulation (Fig 1.9.). A precise balance between mitochondrial fusion and fission are crucial to maintain mitochondrial fitness (Galvan et al., 2017). This balance is primarily mediated by GTPases belonging to the dynamin family. Mitochondrial fission preserves the genetic, biochemical, and functional integrity of mitochondria. It is a process that allows the division of one mitochondrion in two daughter mitochondria, through dynamin-related protein 1 (Drp1). During the mitochondrial fission, Drp1 is recruited and triggers the recruitment of dynamin 2 to perform the membrane scission (Hu et al., 2017). On the other hand, the number and distribution of the mitochondria are regulated by mitochondrial fission (Roushandeh et al., 2019). This process is also responsible for removing the damaged mitochondria through mitophagy. Mechanistically, mitochondrial fusion is caused by mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2), which contribute to the outer mitochondrial membrane, while optic atrophy 1 (Opa1) contributes to the inner membrane fusion (Chandhok et al., 2018).

It is important to highlight that the changes in the expression of these proteins and the disruption of the balance between fission and fusion can impact the integrity and functionality of mitochondria, which have been highly associated with the development of kidney disease. Several studies have documented a correlation between AKI and an early-stage rise in mitochondrial fission following renal damage (Nowak et al., 2011; Zhan et al., 2013). In fact, an impaired regulation of Drp1 results in increased mitochondrial fission and subsequent release of cytochrome *c*, contributing to kidney organ damage (Galvan et al., 2017). Mitochondrial fragmentation results from a reduction in Mfn and Opa1, fusion regulators, whereas the protein Drp1, which controls mitochondrial fission, is quickly activated. To revert this event, Yan and collaborators demonstrated in a murine model that modulation of Mfn2 through microRNAs successfully prevented the disturbance of mitochondrial dynamics and the death of tubular cells in ischemic AKI (Yan et al., 2020). In agreement, Perry and collaborators, in an IR-induced mouse model, demonstrated that Drp1 causes mitochondrial dysfunction and increases maladaptive repair pathways, resulting in fibrosis (Perry et al., 2018). Additionally, in a rodent model with IRI in which was modulated the expression of Drp1 through an inhibitor, mdivi-1, there was an attenuation in mitochondrial fragmentation, cytochrome c release, caspase activation, and apoptosis (Brooks & Dong, 2007). Subsequently, mdivi-1 treatment showed a renoprotective effect on

mitochondrial function in sepsis-induced AKI. This was evident through an elevation in ATP production, a decrease in mitochondrial fragmentation, and a decrease of the inflammasome mediated pyroptosis of renal tubular epithelial cells caused by NOD-like receptor pyrin domain-3 (Liu et al., 2020). The NOD-like receptor is closely related to renal inflammation and fibrosis. These studies were further consolidated with research demonstrating that mitochondrial dynamics are critical to mediate AKI and the progression of fibrosis (Ludwig-Portugall et al., 2016).

Mitophagy is a sort of selective autophagy that targets and destroys damaged or redundant mitochondria. This process prevents cell death from mitochondrial OS and the release of pro-apoptotic proteins. Through the removal of damaged mitochondria, mitophagy protects the kidney from IRI. Renal function, tissue damage, and apoptosis are more severe in proximal tubules with a deletion of autophagy gene-related 7 (Atg7), a mitophagy-related protein, in comparison to the wild type mice (Jiang et al., 2012). Furthermore, renal tubular cells have an increase in autophagosomes in septicemia patients' kidney tissues, which is in contrast with hydropic mitochondria with cristae destruction. This pattern shows that damaged mitochondria are removed via mitophagy (Takasu et al., 2013). Further research revealed that p53 binds to Parkin protein, leading to an impairment of its translocation in mouse heart, thereby inhibiting mitophagy (Hoshino et al., 2013). Notably, the activation of p53 has been documented in AKI, with suggestions that the p53-mediated inhibition of mitophagy might play a role in the pathophysiology of AKI (Ishimoto & Inagi, 2016). Furthermore, Gibbs and collaborators, discovered that when serotonin [5-hydroxytryptamine (5-HT)]1F receptor, which is a physiological regulator of mitochondrial biogenesis, when deleted, the renal damage is exacerbated leading to worse recovery during kidney disease (Gibbs et al., 2018). Regarding Mfn2, Gall and collaborators, demonstrated that a knockdown of this gene in cultured primary PT epithelial cells resulted in increased apoptosis under metabolic stress (Gall et al., 2012). However, in contrast to this, the same authors later demonstrated that a conditional knockdown of Mfn*2* in mice proximal tubule could accelerate renal recovery in IRI- induced AKI, by increasing epithelial cell proliferation, rather than apoptosis (Gall et al., 2015). All of these reports demonstrated, as reviewed by Braga and collaborators (Braga PC. et al., 2022), that mitochondrial homeostasis is critical to the maintenance of normal kidney function. Thus, mitochondria dynamics represent possible mediators in kidney disease development and can be modulated to counteract or develop effective therapies to this disease.

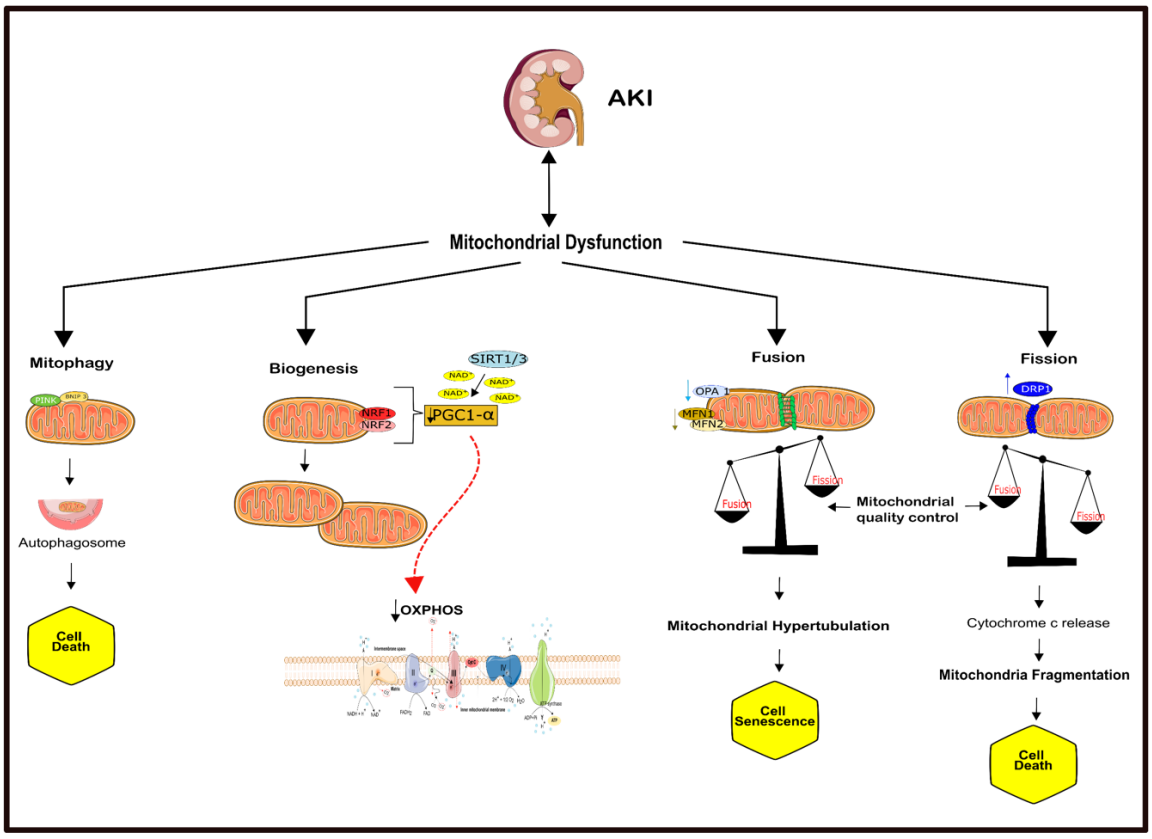

Fig.1.9- Schematic illustration of the pathological processes of mitochondrial dysfunction including biogenesis, fusion, fission, and mitophagy in AKI. Balance of fission and fusion processes is necessary to maintain the normal mitochondria quality control. In fusion, there is a dysregulation in the GTPases, where OPA1, MFN1, and MFN2 have a decreased activity. Excessive fusion is associated with mitochondrial hypertubulation, which leads to cell senescence. Biogenesis is also affected in AKI. There is a downregulation of PGC-1α which is related with the downregulation of the SIRT1 and 3, that will result in a downregulation of NRF1 and NRF2, culminating in a reduced activity of oxidative phosphorylation. Fission event is also altered in AKI, where it can be observed an upregulation of DRP1, that results in an increase of cytochrome C release, and further in mitochondrial fragmentation. Mitophagy protects kidney from IRI by removing damaged mitochondria. The two main mechanisms of mitophagy are the PTEN-induced putative kinase 1 (PINK1) and E3 ubiquitin ligase Parkin-dependent pathway and Bcl2/adenovirus E1B 19-kDa protein interacting protein 3 (BNIP3). An imbalance in mitochondrial control can also occur by excessive of fission over than fusion, leading the mitochondria to death. Abbreviations: AKI, acute kidney injury; IRI, ischemia–reperfusion injury; OPA1, optic atrophy 1; MFN1, mitofusin 1; MFN2, mitofusin 2; NRF1, nuclear respiratory factor 1; NRF2, nuclear respiratory 2; SIRT1/3, sirtuin 1 and 3; NAD+, nicotinamide adenine dinucleotide; PGC-1α, PPArgama-coactivator-1 alpha; and DRP1, dynamin-related protein 1.

1.4.2. Oxidative phosphorylation impairment in kidney disease

Electron transfer at complexes I and III has been suggested as the principal source of ROS overproduction (Ray et al., 2012) (Fig 1.10). In addition, several studies have been conducted to evaluate mitochondrial dysfunction as a potentiator and contributor to kidney

diseases (Ishimoto & Inagi, 2015). Indeed, Emma and collaborators reported a decrease in ATP production related with an increase in ROS production, a release of cytochrome C, and a disruption of mitochondrial cristae (Emma et al., 2016). Histologically, a loss of cristae structure is also observed in AKI, leading to the dissipation of the mitochondrial membrane potential that consequently halted ATP production (Emma et al., 2016). As expected, renal mitochondrial and ATP production are altered by diabetes. Curiously, early in diabetes, ATP generation is increased, yet it decreases as the disease progresses. Several studies already demonstrated that renal disease can have an origin in deficiencies in OXPHOS as a result of mutations in Coenzyme Q10 biosynthesis monooxygenase 6 , cytochrome C oxidase 10 (Valnot et al., 2000), Pdss2 (Peng et al., 2008), and p-53-controlled ribonucleotide reductase (Bourdon et al., 2007), in human and animal models. To have a deeper knowledge of the degree that ETC is affected, the evaluation of defects in the activity of the mitochondrial complexes, individually, may contribute to understanding how mitochondrial dysfunction and renal impairment are possibly correlated. Gene mutations that result in complex I deficiency are the most common oxidative phosphorylation disorders in humans (Tucker et al., 2011). Forbes and collaborators studied mice with a knockdown of the complex I gene, Ndufs6. Heterozygous mice, with an approximately 40% impairment of complex I activity in the kidney, had a kidney disease that precedes cardiac abnormalities, whereas homozygous mice died of cardiomyopathy and advanced CKD (Forbes et al., 2013). In line with these studies, complex I dysfunction was already identified in humans with diabetes. Here, it was observed a reduction of approximately 50% in complex I renal cortical activity (Hall & Unwin, 2006). Complex III deficiencies, associated with increased ROS production, were also detected in the diabetic kidney (Rosca et al., 2005). It is not a new theme of debate that mitochondria network changes, including bioenergetic alterations, are implicated in DKD. Coughlan and collaborators, in a study of diabetic rat mitochondria, at four weeks of age, reported that these rats revealed a decrease in ATP content and mitochondrial fragmentation in the kidney, within PT cells. By 8 weeks of age, they detected a generation of glomerular damage, due to an increase of mitochondrial capacity. Later, worsened effects were observed. At 16 weeks of age, there was tubular damage and mitochondrial uncoupling. This study unveils that changes in mitochondrial bioenergetics and dynamics may precede the development of renal lesion in diabetes and not the other way around (Melinda T. Coughlan et al., 2016). To complement this idea, a study in nondiabetic mice demonstrated that increasing kidney metabolism is not sufficient to induce kidney damage as it is in the presence of hyperglycemia and hypertension (Vives-Bauza et al., 2010). Furthermore, complex I deficiency is also associated with an increased mitochondrial superoxide production in skin fibroblast mitochondria. Skin fibroblasts, obtained from young individuals with DKD, also have mitochondrial dysfunction (Caramori

et al., 2015). In mitochondrial ATP generation, the electron can leak from the respiratory chain and form superoxide, through the binding with oxygen (Coughlan et al., 2009). Indeed, Complex I and Complex III are the main sites of superoxide production in renal mitochondria. Complex I generated superoxide is released to the mitochondrial matrix, while complex III generated superoxide is released to the matrix and intermembrane space (HAN et al., 2001; St-Pierre et al., 2002). Coenzyme Q10 (CoQ10) plays a crucial role in the mitochondria respiratory chain and protects against damage from ROS (Diomedi-Camassei et al., 2007; Ozaltin, 2014; Quinzii et al., 2010). CoQ10 deficiencies have been correlated with severe renal pathologies, including glomerular and tubular interstitial disease (Heeringa et al., 2011; López et al., 2006; Salviati et al., 2005). Mutation in genes related to the biosynthesis of CoQ10, such as PDSS2, COQ2, COQ6, COQ8, and COQ9, have been related to the genetic nephrotic syndrome in humans (Heeringa et al., 2011; López et al., 2006; Quinzii et al., 2010). Additionally, disturbed regulatory miRNAs, such as miR21, were reported in a CKD patient, which carried an impaired complex IV activity (Granata et al., 2009; McClelland et al., 2015). These studies highlight that mitochondria are intimately related to renal diseases.

1.4.3. Oxidative stress in kidney disease

OS plays a critical role in CKD progression, by inducing glomerular and tubular damage, that is indirectly associated with inflammation, hypertension, and/or endothelial dysfunction (Ling & Kuo, 2018). ROS disturb regulatory mechanisms of the kidney, such as RAAS, tubular glomerular feedback, and myogenic reflex in the arteriole. Therefore, the kidney fails to compensate for water-electrolyte and acid-base imbalances, resulting in an additional increase of OS (Podkowińska & Formanowicz, 2020). Morphological abnormalities and loss of function in mitochondria, through swelling and fragmentation, results in ROS production such as O_2^- and hydrogen peroxide (H_2O_2), leading to OS and inflammation, mechanisms that trigger the progression of renal fibrosis (Fig. 1.10) (Feng et al., 2018; Zhou et al., 2012). Moreover, OS is predominantly induced by excessive cytoplasmic and mitochondrial ROS emission (Duann & Lin, 2017).

Sources of ROS formation include the mitochondrial ETC, catecholamine oxidation, metabolism of arachidonic acid, endothelial cells (xanthine oxidase reaction), and inflammatory cells (myeloperoxidase and NADPH oxidase) (Ling & Kuo, 2018). NADPH oxidase 4 (NOX4) is the most important isoform, located in renal fibroblasts, renal tubules, glomerular mesangial cells, and podocytes in the kidney and in the membrane of mainly endothelial cells and fibroblast in the vasculature (Daenen et al., 2019). The enhanced Nox4

expression leads to an increase in ROS levels. These ROS reduced cytochrome C oxidase activity can cause mitochondrial dysfunction, by inhibiting mitochondrial OXPHOS (Quoilin et al., 2014). Several kidney cells change when OS imbalance occurs. As a consequence of the metabolic reprogramming, high glucose induces intracellular ROS in proximal tubular epithelial cells and in glomerular mesangial by AGEs and cytokines (Lee et al., 2003). It has been demonstrated, for instance, that when hexokinase abundance in hyperglycemia reaches the saturation level, the polyol pathway can metabolize up to 33% of this glucose (Chung et al., 2003). Additionally, activated sub-pathways, serving as non-mitochondrial sources of ROS and RNS, further amplify the production of these free radicals (Singh et al., 2022).

ROS production in response to several stimulus, activates the NADH/NADPH oxidase system in renal cells. NAD^+ is essential not only for the harvesting of energy in the form of ATP from different fuels, but also to protect the kidney tubule against acute stressor. NAD^+ augmentation may alleviate AKI triggered by IRI, systemic inflammation, and toxic injury. Indeed, data from experimental models and clinical studies have pointed to NAD^+ homeostasis as a key factor in kidney health (Ralto et al., 2020). Metabolic analysis of mouse urine samples identified an increase in quinolinic acid following IRI. Quinolinic acid serves as a precursor for NAD^+ biosynthesis. Accumulation of this acid in the urine suggested a reduction in the levels of the enzyme that uses QPRT substrate. Mehr and collaborators suggested that reduced QPRT expression increased AKI susceptibility in mice (Poyan Mehr et al., 2018). Moreover, in another study, quinolinic acid and tryptophan were measured in urine samples obtained from 215 patients within the first 24 hours of intensive care unit admission. Fifty-one patients developed AKI and the increased urinary quinolinic acid: the tryptophan ratio was independently associated with an increased risk of AKI, which raises the need for renal replacement therapy and adverse outcomes (Ralto et al., 2020; Zheng et al., 2019).

1.4.4. Antioxidant defense in the kidney

The imbalance between the generation of ROS levels and local antioxidant capacity is a biomarker of mitochondrial dysfunction in DKD (M. T. Coughlan et al., 2016). To overcome the excessive production of ROS, mitochondria have an antioxidant system (Fig 1.10). Indeed, homeostasis is maintained through a complex set of antioxidant mechanisms that prevent oxidative stress-induced injury. The physiologic formation of ROS is detoxified by endogenous antioxidants, which are classified into two groups: enzymatic and non-enzymatic. Enzymatic antioxidants include SOD, catalase (CAT), glutathione peroxidase

(GPx), glutathione S-transferase (GST), and GR, while non-enzymatic pathways include ETC, glucose autoxidation, AGEs, GSH, and others (Ducasa et al., 2019). Additionally, exogenous antioxidants obtained from daily food intake can be hydrophilic (Ascorbate/vitamin C and flavonoids) or lipophilic (α-tocopherol/vitamin E and carotenoids), being classified as nonenzymatic antioxidants (Forman et al., 2014).

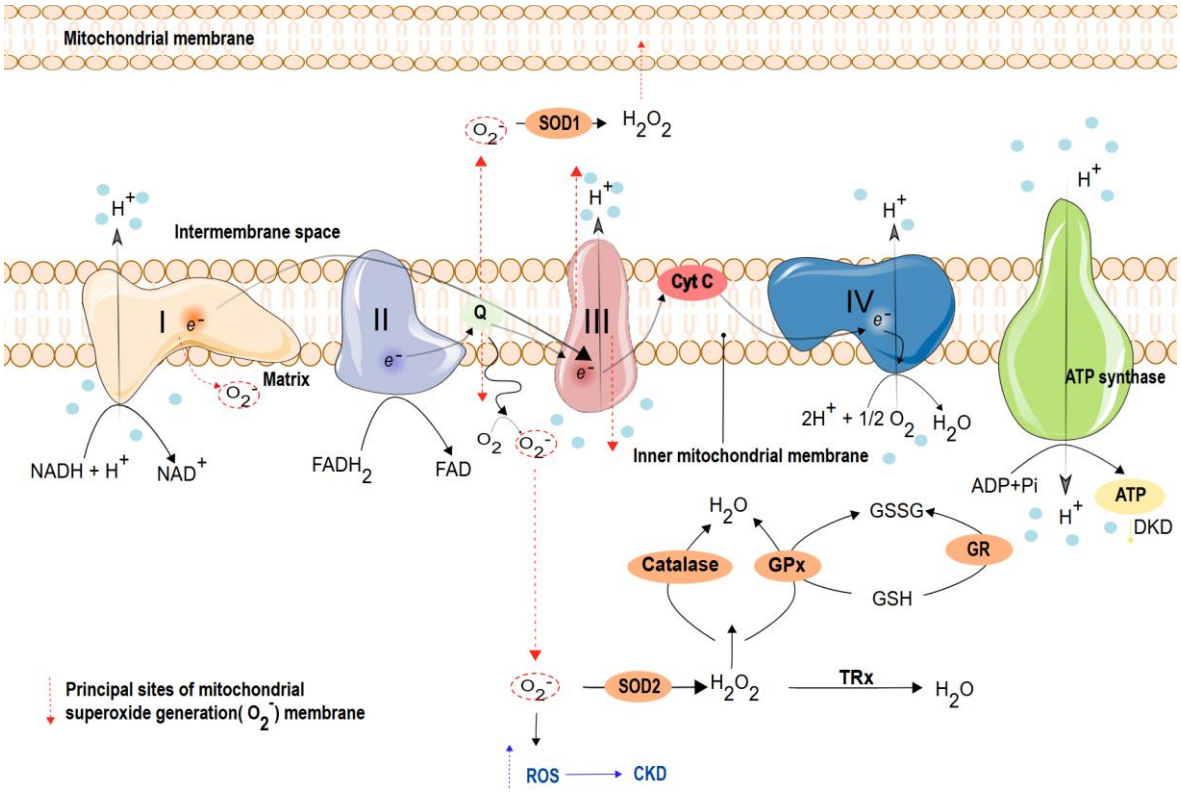

Fig.1.10- Mitochondrial oxidative phosphorylation and the mechanism of the main antioxidant enzymes present in the kidney. Reducing equivalents (NADH, $FADH_2$) produced in the Krebs cycle donate electrons to the electron transport system at complex I and complex II. These electrons are subsequently transferred to other electron carriers, including coenzyme Q, complex III, cytochrome C (Cyt C), and complex IV. In complex IV, the oxygen is reduced into water. The donation of electrons provides the energy to pump protons (H^+), which is responsible for the establishment of the electrochemical proton gradient at the inner mitochondrial membrane. This phenomenon generates the proton motive force that forces the protons back inside the matrix at ATP synthase to regenerate ATP from ADP and Pi. In diabetic kidney disease (DKD), the production of ATP is reduced. In physiological conditions, some part of the O_2 is converted into reactive oxygen species (ROS), where complexes I and III are considered the main sites of ROS production. In the kidney, when the electron transport chain and mitochondria functioning are impaired, there is an imbalance between ROS production and antioxidant defenses, which may be a potentiator and contributor of kidney-related diseases such as chronic kidney disease (CKD), and DKD. The main antioxidant enzymes are superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase (GR). SOD1 in the intermembrane space and SOD2 are released to the matrix. Levels of H_2O_2 are reduced to water through the cooperation between the antioxidant enzymes schematically represented in the scheme.

Under physiological conditions, mitochondria have an efficient antioxidant system to cope with ROS (Stowe & Camara, 2009), which includes, SOD that reduces O_2^- in H_2O_2 faster than the rate of O_2^- production (Bhargava & Schnellmann, 2017). Increasing levels of H_2O_2 lead to GPx activation which reduces H_2O_2 into water (Ribas et al., 2014). Increased levels of ROS have multiple consequences. It can cause breaks in mitochondrial DNA, which leads to mutations in the next generation, and it can also trigger apoptosis (through cytochrome C) (Ruiz et al., 2013). In an OS situation, NRF2 can boost an answer to ROS overproduction (Ruiz et al., 2013). The association of Nrf2 with kidney disease has been reported in some studies. Mice with DKD, Nrf2−/− showed increased ROS levels and higher oxidative DNA damage. Along to this, increased TGF-β1 and ECM accumulation were found. These aspects were all decreased when Nrf2 was activated, which significantly prevented the progression of AKI to CKD transition (Jiang et al., 2010). Nrf2 can activate transcription of heme oxygenase 1 (HO-1), and ferritin that can mitigate AKI and renal injury, on glomerular endothelium, cortical peritubular capillaries, and interstitial leukocytes (Abo El Gheit & Emam, 2016).

Other factors that contribute to OS are activation of NADPH oxidases, uncoupled eNOS and mitochondrial dysfunction together with decreases in antioxidant defenses, such as a decreased expression of antioxidant enzymes and intracellular GSH content. The glutathione redox cycle is the critical antioxidant mechanism found in several intracellular organelles, such as mitochondria (Ribas et al., 2014). Mitochondrial glutathione (mGSH) can be oxidized to glutathione disulfide (GSSG) by superoxide anions (Ribas et al., 2014). In addition, GSSG can be reversed again in mGSH, through glutathione reductase, which requires NADPH from the pentose phosphate pathway. To maintain the oxidative balance in the cells, the cell must contain low levels of GSSG and high levels of GSH (Daenen et al., 2019). Furthermore, severe OS can overcome the ability of the cell to reduce GSSG to GSH, resulting in the accumulation of GSSG (Minich & Brown, 2019). Hence, the ratio of GSH and GSSG is a marker of OS (Bajic et al., 2019). Furthermore, mutual maintenance of ascorbate and GSH can occur *in vivo*, since ascorbate can maintain intracellular GSH (Meister, 1994). Thus, these mechanisms are considered pivotal processes for preserving mitochondrial functions. An increasing number of studies demonstrated that enhancement of antioxidant enzyme activity resulted in a protective effect in animal models of AKI (Dennis & Witting, 2017). Administration of SOD or CAT can diminish ROS in proximal tubule injury after hypoxia *in vitro* (Paller et al., 1991). In rats, SOD was able to improve renal function and reduce tissue injury and cortical mitochondrial lipids peroxidation (Paller et al., 1984). Long-term treatment with a SOD mimetic (MnTMPyP) alleviated oxidation parameters in kidney fibrosis ischemic. MnTMPyP blocked O_2^- and peroxynitrite formation, and reversed functional kidney deficits, suggesting that antioxidant intervention is beneficial, and halting

ROS formation can alleviate microvascular failure and renal injury (Kim et al., 2009). Another similar study is in early-diabetes, where hypertensive rats were administrated with tempol, another SOD mimetic, corrected the oxidative imbalance and improved oxidative stress-induced renal injury, decreasing albuminuria and fibrosis (Quiroz et al., 2009). Decreases in renal SOD are accompanied by mitochondrial biogenesis and less mitochondrial glucose oxidation in diabetic mice (Dugan et al., 2013). Mitochondrial biogenesis can occur as a cellular response to OS (LEE et al., 2000). Regarding the progression of DKD, the contribution of mitochondrial superoxide production is not clear. However, some data led to the theory that superoxide production can be an indicator of mitochondrial healthy activity (Sharma, 2015).

1.5.The potential role of mitochondria in discriminating diabetic kidney disease

As discussed, the development of DKD is more related to tubular damage than glomerular damage. The primary function of the PT cells is the reabsorption process requiring a large amount of energy (Bhargava & Schnellmann, 2017). Variations in glomerular filtration are balanced by changes in tubular reabsorption, which maintains the fractional reabsorption of fluid and solutes (sodium and chloride) in the proximal tubules though constant glomerular-tubular balance. Glomerular filtration is regulated by tubulo-glomerular feedback. Normally, elevated sodium, chloride, and potassium delivery to the distal nephron activates the transporter in the macula densa cells. This results in adenosine release and depolarization, which in turn activates the afferent arteriole's adenosine receptor A1, causing vasoconstriction and a decrease in glomerular filtration (Vallon, 2011). In diabetes, the increase in solute reabsorption will increase glomerular filtration. This effort will impact mitochondrial function, becoming vulnerable and less functional, leading to the reduction of ATP production, inflammation and/ or renal epithelial cell death (Honda et al., 2019). In primary mitochondrial diseases, different kidney related diseases are included, such as DKD, tubulointerstitial nephropathy and glomerular disease (Bakis et al., 2023). Mitochondria can have a critical role in the onset of DKD, since metabolic alterations are reported to precede the development of albuminuria and renal histology hints of the progression of the disease (M. T. Coughlan et al., 2016). Furthermore, throughout the course of diabetes, OXPHOS measured as oxygen consumption rates, has a bimodal

pattern. In the early stages of diabetes, oxygen consumption rates are higher in the renal cortex and PT cells, but later there is a decrease as albuminuria progresses (Friederich et al., 2008). These findings provide credence to the theory that DKD develops concurrently with renal changes, with mitochondrial dysfunction serving as a pathogenic mechanism, and in a more specific way in non-albuminuric situation. Moreover, the metabolic reprogramming that the different sections of the kidney suffer due to effects of hyperglycemia in DKD relies on the proper function of the mitochondria, since the main alterations relied on the intermediates of TCA cycle and the proper FAO. In fact, the dysregulation of lipid transport and metabolism, that result into lipid accumulation in the kidney, could be observed in biopsies samples of patients with DKD by tubular and glomerular lipid deposits (Herman-Edelstein et al., 2014). This was observed in kidney samples obtained from kidney biopsies of patients with DKD (n=34) and healthy kidneys (n=12) from the pathological archives of the Pathology at Rabin medical Center. In the DKD group, renal biopsy was performed according to the clinical recommendations, that included the degrees of sclerosis, thickening and fibrosis. The higher lipid accumulation was observed in DKD kidney biopsies when compared to the control group and was associated to a dysregulation in genes related with lipid metabolism, and to a decrease of fatty acid β-oxidation pathways, where PPAR-α, carnitine palmitoyltransferase 1, acyl-CoA oxidase, and L-FABP were downregulated. The increase of lipid accumulation leads to mitochondrial OS lipotoxicity (Mohandes et al., 2023). However, despite the increasing flux through β-oxidation and glycolytic pathways, OXPHOS and mitochondrial metabolism appear to be uncoupled, as evidenced by the lack of ATP production. Moreover, it has already been described that other metabolic alterations occur, such as the influence of the TCA cycle on the pathophysiology of DKD (Limonte et al., 2022). Collectively, this causes mitochondrial dysfunction, which in turn causes tubulopathy to develop and worsen kidney function (Hansell et al., 2013; Vallon, 2011). Thus, impaired mitochondria play an important role in the tubulopathy of DKD, particularly in the early stages of kidney injury, before microalbuminuria appears, being metabolites derived from mitochondria essential to evaluate different metabolic signatures, especially among the NA-DKD phenotype. Thus, we may speculate that mitochondrial-derived metabolites may serve as valuable biomarkers for identifying metabolic signatures associated to individuals with NA-DKD. Therefore, there is a pressing need to investigate the underlying pathological mechanisms linking mitochondrial dysfunction and the progression of DKD. This holds the potential to uncover crucial mechanisms that could pave the way for the development of innovative therapeutic strategies aimed at preserving or enhancing mitochondrial function, ultimately preventing or delaying the progression of DKD.

1.5.1. Mitochondria as contributors to the understanding of non-albuminuric DKD patients

Metabolomic fingerprints of patients have been growing over the years. However, there are not many studies regarding only the comparison of the metabolome between NA-DKD and A-DKD patients. Reis-Pereira P and collaborators, in a revision manuscript highlighted different studies with the most dysregulated metabolites in DKD disease in patients with T1DM or T2DM with kidney dysfunction, in different stages of the progression of the disease. The authors were able to identify a total of 36 studies, subdivided regarding the possible biomarkers related with aminoacids metabolism (12), lipid (11), TCA derivate metabolites (8) and related with nucleic acids (5). However, it was observed limited studies discriminating the possible biomarker involved in patients with NA-DKD (Pereira et al., 2022). This highlights the need to search for new possible fingerprints in this specific phenotype of DKD. Some metabolites are already highlighted to have a possible role in the discrimination of patients with DKD, with and without normal albuminuria levels, such as some branched-chain amino acids (Hallan et al., 2023; Zhu et al., 2022), TCA intermediates (Hallan et al., 2023; Li et al., 2013; Liu et al., 2021) some metabolites related with fatty acid metabolism, butyrate metabolism (Kwan et al., 2020), linoleic metabolism (Liu et al., 2021; H. Zhang et al., 2020), arginine and proline metabolism (Liu et al., 2021), aspartate and asparagine metabolism (Trifonova et al., 2022).

As previously explained, albuminuria is not a reliable biomarker, and in DKD, especially in a non-albuminuric situation, it is important to look closely at eGFR rates, since DKD is mainly characterized by hyperfiltration, leading to tubular damage. In this sense, some studies, that follow the decrease of eGFR, came up with some extra information (Zhou et al., 2021) where again, the metabolites that appeared to be dysregulated in non-albuminuric scenario are the ones related with tyrosine and glutamine metabolites, which will impact TCA cycle (Kwon et al., 2023). Due to the lack of information it is also important to analyze the studies performed in T1DM (Tofte et al., 2020; Trifonova et al., 2022; van der Kloet et al., 2012), as well as studies related with non-proteinuric patients (Peng et al., 2022). Considering these diverse scenarios, summarized in Table 1.1, it is crucial to obtain more precise information regarding NA-DKD patients. Although not every study specifically addresses the non-albuminuric condition, there is strong evidence suggesting that mitochondria play a vital role and may represent a powerful tool in identifying novel biomarkers for this DKD phenotype.

Table 1.1. List of different studies that identified possible metabolic alterations that can have a role in diabetic kidney disease progression, especially in non-albuminuric conditions.

Authors	Study design	Population	Applied Method	Biological sample	Metabolites altered	Main outcomes/findings
(Niewczas et al., 2019**)**	Prospective study (8-12 years of follow-up)	T2DM that develop ESRD (N=40) T2DM without ESRD (N=40)	Untargeted LC-MS	Serum	↑Leucine ↑Valine ↑Phenylacetyl glutamine ↑p-cresol	Risk of ESRD
(Hallan et al., 2023**)**	Prospective study	Controls (N=60) vs. NA-DKD (N=14) vs. A- DKD patients (N=26)	Targeted GC-MS	Urine	↓short-chained acylcarnitines ↓ long-chained fatty acids ↓ Isoleucine, leucine and valine ↑ TCA intermediates (citric acid, 2-ketoglutaric acid and succinic acid)	DKD progression
(Zhu et al., 2022**)**	Case control study	Healthy patients (N=30) vs. T2DM (N=30) vs. DKD (N=30) patients	Untargeted LC-MS	Serum	↓ L-valine and betaine	eGFR decline
(Zhou et al., 2021**)**	Case control study	Healthy control (N=122) vs. T2DM (N=101) vs. DKD (=101)	Untargeted UPCL-MS/MS	Serum	Stage 1-2 CKD: ↑histidine ↓valine	eGFR decline
(Pena et al., 2014**)**	Prospective study	Healthy patients (N=21) vs. NA-DKD (N=24) and A-DKD (N=21)	Untargeted FIA-MS/MS	Urine	↓tyrosine, hexose and glutamine	Early DKD stages

(van der Kloet et al., 2012**)**	prospective study (5.5. years)	T1DM NA-DKD (N=26) vs. A-DKD (N=26)	Untargeted GS-MS and LC-MS	Urine	↑hippuric acid ↓substitute carnitine and S (3-oxododecanoyl) cysteamine	DKD incidence
(Han et al., 2011**)**	Case control study	Healthy patients (N=30) vs. T2DM (N=30) and DKD patients (N=52)	Targeted NPLC-TOF-MS	Serum	↑ NEFAs in macroalbuminuric patients	DKD incidence
(Klein et al., 2014**)**	Prospective study (14-20 years follow-up)	T1DM (normoalbuminuria= 291; microalbuminuria =141 and macroalbuminuria =65	Untargeted UPCL-MS	Serum	↑ ceramide long-chain species in macroalbuminuric patients vs. normalbuminuric (C22:1, C24 and C24:1)	Early DKD stages
(Mogos et al., 2023**)**	Cross sectional study	T2DM NA-DKD (N=20) Micro albuminuric DKD patients (N=20) and macroalbuminuric patients (N=20)	UPLC-QTOF-ESI*MS	Urine and serum	↑ serum tryptophan ↓ serum taurine ↑ tiglyglycine ↓serum phenylalanine, tyrosine and methoxytryptophan ↑ urine uric, aspartic and O-phosphothreonine ↑ methoxytryptophan	Early DKD stages
(Li et al., 2013**)**	Case control study	**Animal model (N=80)** 6 weeks (non-albuminuric), 8,10,12,14 and 16 weeks	GS-MS/MS	Serum and urine	↓ serum TCA intermediates (fumarate, citrate, α-ketoglutarate, malate and succinate) ↑ urine malate, glutarate anderythronate	Early DKD stages
(Kwan et al., 2020**)**	Cross sectional	DKD patients NA-DKD (N=294), microalbuminuric patients (N= 275) and macro albuminuric (N=421)	Untargeted QTOF-ESI*MS	Urine	↑3-hydroxyisobutyrate	eGFR decline

(Tofte et al., 2020**)**	Prospective study	T1DM (N=637) Normo-to macroalbuminuric transition	Untargeted Multiple-MS	Serum	↑3,4-dihydroxybutanoic acid	eGFR
(Liu et al., 2021**)**	Prospective study	DKD (N=1513) (early and late stage)	Untargeted GS-MS	Serum	↑ glycerol-3-galactoside	eGFR decline
(Feng et al., 2020**)**	Case control study	T2DM (N=271) vs. NA-DKD and A-DKD patients (N=116)	Untargeted UPLC-MS/MS	urine	↓ Linoleic acid and ϒ- Linoleic acid ↑ L-Malic acid ↑-proline, L-erythro-4-Hydroxyglutamatea and Spermidine	DKD progression
(Peng et al., 2022**)**	Case control study	Healthy controls (N=30) T2DM (N=30) vs. DKD divided in moderate proteinuria (N=12) Heavy proteinuria (N=17)	Untargeted LC-MS/MS	Serum	↓ Asn-Met-Cys-Ser, Asn-Cys-Pro-Pro and Isorhamnetin 3-(3”,6”-di-p-coiumaryglucoside	DKD progression
(Trifonova et al., 2022**)**	Case control study	T1DM vs. DKD Early and late DKD	DIMS	serum	↓-cysteine, 4-guanidonobutanamide and N2-succinyl-L-ornithine ↓ citrulline, thiosulfate, thiocysteine and 3-sulfinylpyruvic acid, ↓ phosphoglycolic acid and 2-oxo-3-hydroxy-4-phosphobutanoic acid	DKD progression
(Zhang et al., 2020**)**	Case control study	NA-DKD (N=20) vs. A-DKD (N=24)	UPLC-MS/MS	serum	↑Linolelaidic Acid ↑L-dihydroortic Acid	DKD progression

(Kwon et al., 2023**)**	Prospective study (4.5 years follow-up)	Healthy patients (N=26) vs. DKD patients to progress to ESRD (N=208)	Targeted NMR	Urine	↑ N-N-dimethylglycine	Risk of ESRD

Abbreviations: DKD- diabetic kidney disease; NA-DKD- non-albuminuric diabetic kidney disease; ESRD-end stage renal disease; eGFR-estimated glomerular filtration rate; LC-MS- liquid chromatography mass spectrometry; GC-MS- gas chromatography mass spectrometry; UPLC-MS- ultra performance liquid chromatography mass spectrometry; FIA-MS/MS- flow injection analysis-tandem mass spectrometry; NPLC-TOF-MS - Normal phase liquid chromatography, time-of-flight mass spectrometry; QTOF-ESI-MS- quadrupole time-of-flight mass spectrometry chromatography electrospray ionization tandem mass spectrometry.; DIMS- Direct injection mass spectrometry; NMR- nuclear magnetic resonance; UPLC-QTOF -ESI-MS- quadrupole time-of-flight mass spectrometry , ultra- performance liquid chromatography electrospray ionization tandem mass spectrometry.

Thus, a mitochondria-centric view can be an asset to developing more specialized diagnosis methods in DKD progression, especially at the onset of the disease. The possibility of the discovery of a possible biomarker at this stage of the disease, especially if it is found in urine, will give to the patient a less invasive way to diagnose the disease than the typical biopsy that must be performed to correctly diagnose NA-DKD. Understanding how this relationship varies across the classical and non-classical DKD should help to clarify the pathophysiological mechanism that underlie the disease progression in each phenotype and ultimately will facilitate the development of targeted diagnostic tools and therapeutic interventions specifically tailored for NA-DKD, without the need of the usage typical cocktails of pills, which will significantly improve patient outcomes, and potentially delay or prevent the progression to overt DKD, i.e. the need for dialysis or even transplant and its associated complications.

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Chapter 2

Aims

2. Aims

Kidney diseases are currently one of the most challenging health and societal issue worldwide. Furthermore, diabetic kidney disease (DKD) is one of the most common microvascular complications of diabetes mellitus (DM). Most of the patients with DKD eventually advance to end stage renal disease (ESRD) once the condition has progressed. The ESRD risks persist despite numerous novel therapy approaches aimed to address the pathophysiological cause of DKD.

The pathological mechanisms that contribute to the development of DKD are complex, with multiple factors involved. Notably, DM is characterized by metabolic abnormalities, such as hyperglycemia and hyperlipidemia, that cause deleterious effects on kidney function. The main role of the kidney is to filter the blood to remove accumulated toxins along with concentrating the urine to avoid dehydration. To do that it needs a large amount of energy. In fact, kidney is one of the most energy-consuming organs, to maintain homeostasis. Along the tubule, the filtered fluid moves along the tubule, while the blood vessel reabsorbs almost all the water, along with minerals and nutrients. To support these functions, the energy generation in the proximal tubules relies on oxidative phosphorylation. An aberration in energy metabolism can lead to cellular dysfunction or even death. Structural changes of renal cells occur in response to the multiple insults induced by excessive glucose inflow and the imbalance of oxidative stress and antioxidant capacity. It has been already reported that mitochondrial metabolic reprogramming influences the pathophysiological progression of DKD. Alteration in kidney metabolism, more particularly the shift from mitochondrial oxidative phosphorylation to glycolysis and its side branches, is thought to play a critical role in the development and progression of DKD that can culminate in renal fibrosis and kidney insufficiency. Therefore, understanding the mitochondria function and the management of its quality control in certain time points of the disease progression, especially at the onset of the disease, has a great importance.

In the clinic, albuminuria and the estimation of glomerular filtration rate (eGFR) are the primary diagnostic biomarkers currently used for DKD diagnosis. However, a substantial proportion of patients with DKD are non-albuminuric (NA-DKD) and eGFR is the only available biomarker in this cluster of patients. Nonetheless, even in this phenotype, eGFR loss is considered a non-specific marker of DKD. NA-DKD patients are a concern in the clinic since the detection of loss of kidney function is possible to obtain only in an advanced stage of the decrease of renal function. Despite several pharmaceutical approaches being widely accepted to improve renal function, there is an urgent need to find a more accurate method to diagnose DKD in patients, especially in those that are non-albuminuric, which are diagnosed through a biopsy, a very invasive procedure.

Despite the limited studies available, it is undeniable that mitochondrial (dys)function has a key role in DKD onset and progression. Research consistently shows that mitochondrial quality control mechanisms, combined with metabolic approach techniques, can provide a better understanding of this phenotype, with a simple sample of urine, becoming the process to achieve the diagnostic of DKD in a much less invasive manner. This understanding can aid in the development of targeted diagnostic methods and therapies to be implemented in clinical practice. In this work we aimed to understand how hyperglycemia impact the metabolic reprograming and how mitochondria cooperate to overcome this insult. Furthermore, we also aim to evaluate how mitochondria dynamics are affected upon a change in a diet, since obesity is also a great contributor to the advancement of DKD. Finally, we aim to understand, how urine metabolome changes among DKD group, with a particular emphasis to the cluster of patients that are non-albuminuric, to find a panel of possible biomarkers to diagnose DKD. In detail, the following specific goals were outlined to guide this pursuit:

- **Study the role of glucose on the metabolic profile and mitochondrial activity of epithelial human kidney cells with induced insulin resistance.** It is already known that hyperglycemia causes renal dysfunction with an impact of the mitochondrial function. Moreover, it is also a fact that insulin resistance, which can be caused by lipid toxicity is a problem in the development of DKD, since insulin resistance itself, without being associated with diabetes, has been directly associates with the development of kidney function loss. However, the effects from a mild to a hyperglycemic condition, associated or not with insulin resistance are not fully understood. Thus, we aimed to evaluate the effect of different concentrations of glucose associated or not with insulin resistance induced by lipotoxicity and understand the occurring metabolic reprogramming and how it impacts the mitochondria bioenergetics.

- **Unveil the role of mitochondrial proteins in kidney disease progression among different dietary habits.** High-fat diets (HFD) are prevalent and have been linked to non-communicable diseases, including chronic kidney injury. Although it is known that HFD can lead to glomerulopathy and damage to the proximal convoluted tubule, the mechanisms underlying obesity-related kidney impairment remain unclear. Research indicates that DKD is influenced by multiple cellular stress responses triggered by metabolic alterations and changes in energy metabolism. However, the exact pathophysiological mechanisms leading to obesity-related

kidney impairment, particularly after dietary correction, are still not fully understood. Our goal was to investigate how HFD consumption alters the kidney proteome and whether dietary reversal in adulthood can mitigate the harmful effects of early-obesity in the kidney, with a particular focus on mitochondrial quality control.

- **Evaluate the urine metabolome profile between non-albuminuric and albuminuric DKD patients in comparison to health individuals.** Hyperglycemia, dyslipidemia, obesity, hypertension and smoking are the major risk factors for DKD spectrum. Moreover, the typical biomarkers used, especially albuminuria are not fully reliable in the clinic, since the patients with DKD but that do not present elevated levels of albuminuria are increasing, leading to the advance of the disease in a silent way. Thus, there is a need for the identification of other biomarkers beyond proteinuria. Over the last years a large amount of serum/ urine molecules has been proposed as candidate biomarkers for DKD. It is undeniable that mitochondria must have a critical role in the different stages of DKD disease, since extensive research has consistently demonstrated a correlation between mitochondrial quality control mechanisms and DKD. Thus, employing metabolic profiling techniques can provide valuable insights into the pathophysiology of DKD. A mitochondria-centric view can be an asset to develop more specialized diagnosis methods in DKD progression, especially at the onset of the disease. Our aim with this work was to find a possible candidate for biomarkers in DKD patients, more particularly in the non-albuminuric population, through untargeted metabolomic approach, and detect, if possible, in a urine sample, a way less invasive to obtain a more accurate diagnose than by a typical biopsy.

Overall, this work aimed to unravel the possible biomarkers that are associated with the mitochondria function, that could be an indicator of the possible mechanisms that are involved in DKD, beyond the glomerular filtration rate.

Chapter 3

Practical concepts- Determination of Glycolysis flux by extracellular flux measurements

This chapter was adapted from the published work:

Bernardino RL, **Braga PC**, Oliveira PF, Alves MG (2024). Chapter 19 - Determination of glycolysis flux by extracellular flux measurements. In: Ferreira R, Oliveira PF, Nogueira-Ferreira R, eds. *Glycolysis: Academic Press*; 2024: 443-54. https://doi.org/10.1016/B978-0-323-91704-9.00004-5

Abstract

Glycolysis is a metabolic pathway that breaks down glucose into different products and generates energy. The final product of glycolysis is lactate in anaerobic conditions and pyruvate in aerobic conditions. These reactions can change the composition of the medium surrounding the cells and consequently variations in pH. An alteration of this pathway could indicate a change in cellular differentiation, proliferation, or survival. Glycolysis measurement by some biochemical assays provide low resolution that do not offer a comprehensive picture of metabolic pathways utilization, and usually elucidate only one parameter at a time. With the Seahorse XF analyzer, these limitations are overcomed. Both glycolysis and oxidative phosphorylation pathways can be measured simultaneously. Moreover, the users do not need to use multiple instruments, hazardous chemicals and compounds, a high number of cells or tissues, or spend much time analyzing the data. Furthermore, the technique is very versatile since it is possible to analyze these parameters in adherent or suspended cells and/or in tissues. The glycolysis stress test kit allows us to examine different parameters, such as glycolysis, glycolytic capacity, glycolytic reserve, and non-glycolytic acidification, that is translated in alterations of extracellular acidification rate (ECAR) of the medium, giving a more complete overview of the behavior of the cells or tissues in a specific situation. So, the evaluation of the metabolic profile of the cells and tissues through the Seahorse XF analyzer offers numerous advantages relative to other methods. The flexibility and ability to perform real-time measurements by this technique can often allow one to deduce pathway engagement.

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3.1. Introduction

Energy-producing metabolic pathways mainly include glycolysis and mitochondrial oxidative phosphorylation that are used to support cellular functions, such as cell proliferation (Vander Heiden et al., 2009). Glycolysis is a series of 10 reactions that converts one molecule of glucose into two molecules of pyruvate, with the concomitant production of a small amount of adenosine triphosphate (ATP) (TeSlaa & Teitell, 2014). The first step of glycolysis involves the transfer of a phosphate from the ATP to glucose, resulting in glucose-6-phosphate, by the action of hexokinase (Harper & Harris, 2001). Regulation of a pathway is achieved by modulation of the rates of reactions that control the overall flux through the pathway. For example, hexokinase activity is regulated by product inhibition. If flux through

the glycolytic pathway is diminished, the concentration of glucose 6-phosphate increases, blocking the hexokinase active site by the product saturation (Harper & Harris, 2001). Indeed, managing hexokinase activity is considered to be a possible way to evaluate the glycolytic function of a cell, once this enzyme is considered as a control point of the pathway, as we will describe later.

Furthermore, glycolysis can supply biosynthetic intermediates for cell development. Glucose 6-phosphate can also be oxidized via the pentose phosphate pathway to carbon dioxide and ribulose 5-phosphate, in which carbon flux generated will contribute to nucleotide biosynthesis that is essential for DNA replication and RNA transcription (Locasale, 2013).

In cells that use aerobic respiration as the primary energy source, oxidative phosphorylation is the energetic preferential pathway. Pyruvate can be used by mitochondria to produce substrates for oxidative phosphorylation, producing 34 molecules of ATP per one molecule of glucose (Nolfi-Donegan et al., 2020), through ATP synthetase complex. Glycolysis can be used as an emergency backup for energy production or as the preparation step before oxidative phosphorylation (OXPHOS), being markedly less efficient in ATP production when compared to OXPHOS (Koppenol et al., 2011).

Furthermore, pyruvate has different fates, such as the production of lactate (Jin & Zhou, 2019; Matschinsky & Wilson, 2019). Pyruvate can be converted into lactate in the cytosol by lactate dehydrogenase with oxidation of NADH to NAD^+. The Seahorse Analyzer can be used to measure cellular responses to modulators of glycolysis and oxidative phosphorylation. Seahorse technology uses fluorescent probes sensitive to oxygen and protons to determine oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), respectively (Yang et al., 2017). The conversion of glucose to pyruvate, and subsequently lactic acid, results in the production and extrusion of protons into the extracellular medium, resulting in the acidification of the medium, which can be evaluated through the ECAR (Perez et al., 2010; Wu et al., 2007).

ECAR is a measure of lactic acid production, generated by anaerobic glycolysis, in a general way representing the extracellular glucose that is taken up by the cell and converted into acidic end products, such as lactate, translating medium pH as an indirect measurement of the glycolytic pathway (Newell et al., 1993). With Seahorse XF Glycolysis Stress Test it is possible to evaluate the different parameters, such as glycolysis, glycolytic capacity, glycolytic reserve, and non-glycolytic acidification. For that, different modulators that are injected sequentially in cells or tissues are used, and it is possible to control the time of injection in real time. First, glucose is injected, to supply substrate for glycolysis and then oligomycin is injected, an inhibitor of ATP synthase in the mitochondria, shifting the burden of ATP supply entirely in glycolysis. Finally, 2-deoxyglucose (2-DG) is injected, which blocks

the hexokinase and consequently the glucose-6-phosphate production (Chen & Guéron, 1992) (Fig 3.1).

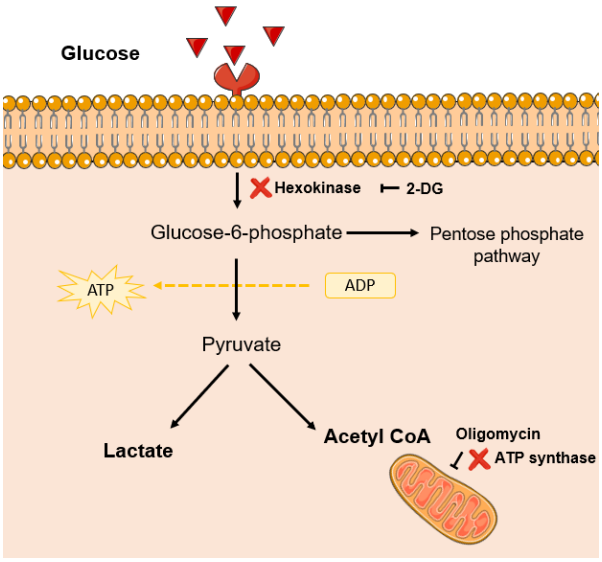

Fig.3.1- Glycolysis Stress Test Modulators of Glycolysis. Glucose feeds glycolysis. Oligomycin inhibits oxidative phosphorylation of the electron transport chain through ATP synthase inhibition, therefore preventing aerobic ATP generation and forcing cells to increase glycolytic flux. 2-deoxyglucose (2-DG) is used as a competitive inhibitor of glucose, resulting in a decrease in the glycolysis pathway**.**

3.2. Materials, equipment and reagents

- Seahorse XFe24 Analyzer
- Agilent Seahorse XF Glycolysis Stress Test Kit
- XF24 Cell Culture Microplates
- XFe24 Sensor Cartridge
- Seahorse XF Calibrant Solution
- Seahorse XF 200 mM glutamine solution
- Seahorse XF DMEM medium, pH 7.4
- Cell culture medium
- Phosphate-buffered saline (PBS)

Alternatives: Seahorse XFe96 Analyzer (for this equipment it is necessary to adjust the volumes of all components)

3.3. Protocols

Day before experiment

Task 1: Seed cells and prepare XFe24 sensor cartridge

a. For adherent cells, trypsinize the cell culture until it detaches from the culture flask.
b. Add culture medium with serum to inhibit trypsin action, break up cell clumps with a pipette to create a cellular suspension, and count cells (e.g., automatic counter, hemacytometer).
c. Resuspend the desired number of cells in the culture medium to the volume of 100 µl per well.

Note: The number of the cells seeded in culture microplates need to be adjusted to the cell type.

d. Pipette 100 µl of the cell suspension into each well of the XF24 Cell Culture Microplates.
e. Incubate the XF24 Cell Culture Microplates with cells seeded at 37°C, 5% CO_2 to allow cells to adhere for 1-6 hours. Add additional 400 µl of culture media and allow cells grow overnight in incubator.

Note: The cell plate will likely need to be prepared one or many days in advance, depending on cell proliferation and number of seeded cells.

Note: The optimization of cell number is required. Before the experiment, different numbers of cells per well should be seeded to assess growth and the desired confluence. Experimental cell density should be optimized to obtain ECAR value of at least 20 mpH/min, which corresponds to the lowest detection range of the machine.

f. The XFe24 sensor cartridge needs to be hydrated overnight to obtain stable results. Add Seahorse XF Calibrant Solution to each well of XFe24 well microplate. The cartridge contains fluorophores associated with O_2 and H^+ for each well.
g. Incubate the XFe24 sensor cartridge with 1 mL of Seahorse XF Calibrant Solution to hydration at 37°C without CO_2 for at least 6 hours.

Note: Make sure that the calibrant solution level is enough to maintain the sensor submerged. Lower the sensory cartridge back onto the utility plate gently and avoid creating air bubbles.

h. Turn on the Seahorse XFe24 Analyzer equipment before the assay so that it reaches the correct temperature before the assay.

Day of the experiment:

Task 1. Preparation of glycolysis stress assay medium and cell plate

a. The Seahorse XF DMEM medium supplemented with 2 mM L-glutamine need be warm until 37ºC and adjusted pH to 7.4.

Note: 2 mM L-glutamine is the recommended concentration, but the composition of the medium may be different depending on the cell type or culture conditions.

b. Removal of the cell culture medium from each well of the plate where the cells are seeded and wash all wells with saline solution (PBS) heated to 37°C.

Note: Washing should be done carefully so as not to disrupt the cell monolayer.

c. Add 500 µl of glycolysis stress assay medium to each well and incubate the plate for 1 hour at 37°C in an incubator without CO_2.

Note: CO_2 can alter the pH of the medium and consequently alter ECAR measurements.

d. After 1-hour, the glycolysis stress assay medium needs to be replaced by a new fresh medium (37°C).

Task 2. Preparation of inhibitors

a. The inhibitors will be added through the injection ports in the cartridge (Fig 3.2), and they should be at a concentration of 10x higher than desired final concentration. First, it is necessary to prepare the stock solution with a glycolysis stress assay medium and follow the dilutions shown in Table 3.1.

Note: Vortex for approximately 1 minute to ensure full resuspension of compounds.

Note: If you are performing a standard assay, i.e., with no additional injections, load the compounds into the appropriate ports (glucose at Port A; oligomycin at Port B and 2-DG at Port C). However, if you inject an additional compound before glucose, use port A for the desired compound and then load glucose at Port B, Oligomycin at Port C, and 2-DG at Port D.

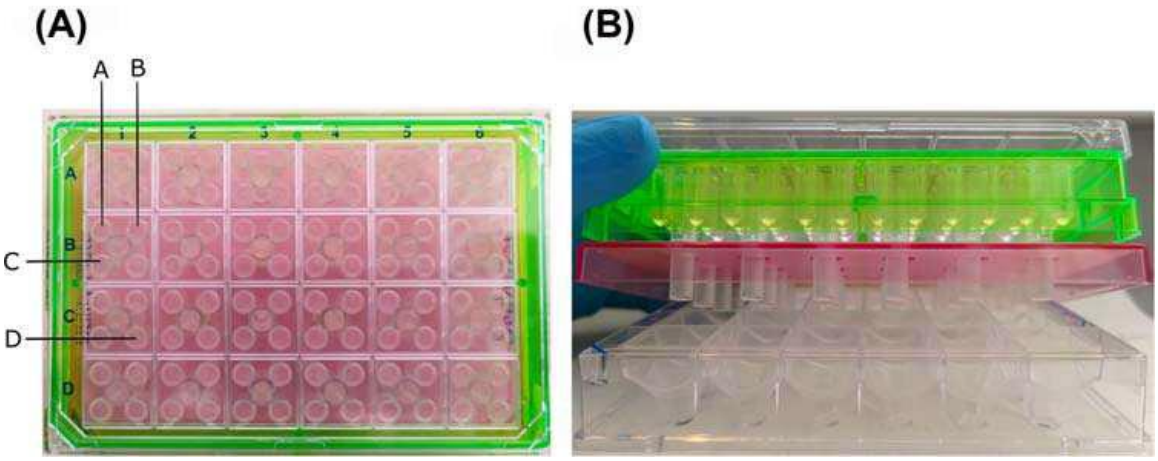

Fig. 3.2- Seahorse cartridge layout. (A) Ports of the cartridge: A, B, C and D. (B). Cartridge constitution: Utility plate, pink plate, cartridge and lid**.**

Table 3.1. Preparations of stock and work solutions for loading sensor cartridge ports.

Compound	Volume of assay medium (AM)	Stock solution Concentration (SS)	Work Solution (10x)	Volume to add to the port (µL)	Final well concentration
Glucose Port A	3000 µL	100 mM	3000 µL (SS)+ 0 µL (AM)	56	10 mM
Oligomycin Port B	720 µL	100 µM	300 µL (SS)+ 2700 µL (AM)	69	1.0 µM
2-DG Port C	3000 µL	500 mM	3000 µL (SS) + 0 µL (AM)	69	50 mM

Note: The protocol outlined is for the 24-well format of the instrument. Volumes will need to be adjusted if another format is used. Work solution concentration can need to be adjusted to the cell and tissue type.

b. After cartridge loading, place the utility plate with the loaded sensor cartridge on the Seahorse XFe24 Analyzer to perform calibration. It will take approximately 20 minutes.

Note: Before placing the cartridge, remove the cover and the pink plate that is between the utility plate and the cartridge (Fig 3.2).

Task 3. Preparation of protocol

a. Open the Program Wave and select Glycolysis stress test on the Templates window and set up the program.

b. At the plate map tab, click “add group” and select corresponding wells, in which each group has a different color associated (Fig 3.3).

Note: Each sample should be measured in triplicate.

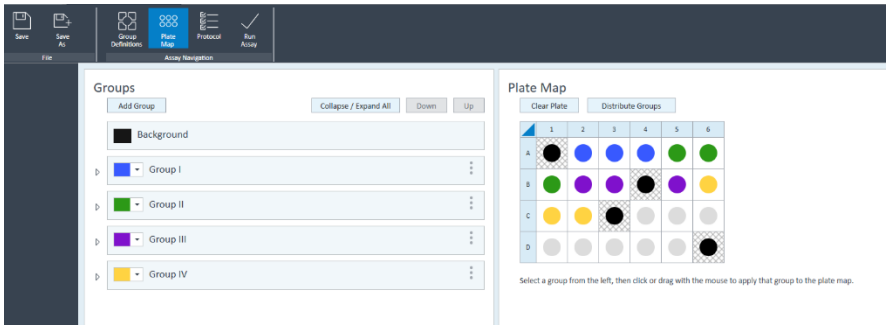

Fig. 3.3 - Seahorse plate layout. Triplicated of different experimental groups.

c. At the Protocol tab, the parameters are as follows: 3 cycles - injection of Port A, 3 cycles - injection Port B, 3 cycles - injection port C.

Note: All protocol parameters can be modulated, like the number of cycles between each injection. These parameters need to be adjusted related to cells or tissue in analysis.

d. After cartridge calibration, the utility plate is replaced by a cell or tissue culture plate.

Task 4. Results analysis

a. Through the results obtained by graphs provided by Seahorse XF Glycolysis Test Report Generator (Fig 3.4), we can export all values to an excel or prism file to calculate the principal parameters:

- **Glycolysis =** ECAR after addition of glucose – ECAR after 2 -DG injection
- **Glycolytic capacity =** ECAR after addition of oligomycin – ECAR after 2- DG injection
- **Glycolytic reserve =** ECAR after addition of oligomycin- ECAR after addition of glucose
- **NonGlycolytic acidification=** ECAR after 2-DG injection.

Note: The results should be normalized to cell number, protein concentration or other methods.

3.4. Analysis and statistics

The glycolysis stress test begins with a baseline measurement of the ECAR in the cells with a glucose free-medium (Fig 3.4). At this point, cells are at their basal minimum, a stage that is considered as non-glycolytic acidification. This acidification corresponds to CO_2 generated in the TCA cycle, being converted to HCO_3^- and H^+. The injection of glucose activates glycolysis. This process is characterized by the conversion of glucose to pyruvate that can be converted into lactate, acetyl-CoA, or acetate. The catabolism of one glucose to two lactate generates two H^+, which presents an increase in extracellular acidification. The cells are then challenged with the injection of oligomycin, which shuts down the generation of ATP through OXPHOS. Cells respond to this decrease in ATP production by driving the cells to use glycolysis to its maximum capacity, resulting in an increase in ECAR levels, being characterized as glycolytic capacity. Depending on cellular models, glycolytic capacity can be a predictor of drug sensitivity, like in tumors (Issaq et al., 2014) and dendritic cells (Malinarich et al., 2015). It can also be associated with cell damage, where for

instance, a decline in glycolytic capacity is observed in a heart failure model (Phillips et al., 2010). Furthermore, a decrease in glycolytic capacity can be linked with cellular reprogramming and differentiation (Chung et al., 2010; Folmes et al., 2011).

Notwithstanding, in a cell with high glycolytic capacity, the glycolytic rate in the absence of OXPHOS may fully meet the whole of the cell´s current ATP demand without being maximal. To evaluate if the cells are operating at their maximum glycolytic level or not, the cellular ATP demand should be increased experimentally, where a lack of glycolytic response will be demonstrated that it was running at its full capacity. However, if an increase in glycolytic acidification is observed, it will demonstrate that it was not (Mookerjee et al., 2016).

Finally, the experiment is ended with the injection of 2-DG, which returns ECAR to its non-glycolytic level. With this injection, it is possible to determine the glycolytic reserve, which indicates the capacity of a cell to respond to an energetic demand, as well as how close the glycolytic function is to the cell's theoretical maximum (Fig 3.4).

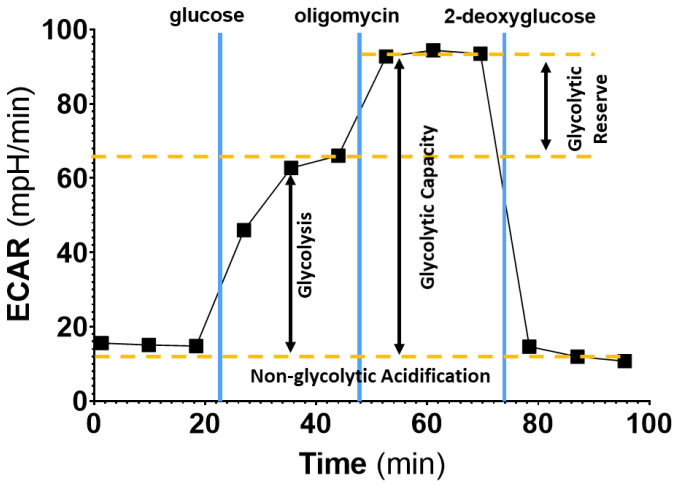

Fig. 3.4 - Schematic representation of the Extracellular Acidification Rate (ECAR) during glycolysis extracellular flux assay, after injection of different modulators through time. The different parameters can be measured by the ECAR values as a function of time, where Glycolysis is the difference between ECAR after addition of glucose and ECAR after 2-deoxyglucose (2-DG) treatment; Glycolytic Capacity is the difference between ECAR after addition of oligomycin and ECAR after 2- DG treatment; Glycolytic Reserve is the difference between ECAR after Oligomycin injection and ECAR after addition of glucose and Non- glycolytic Acidification corresponds to ECAR value after 2- DG treatment.

3.5. Related techniques

For an accurate data interpretation, it is important to do a normalization of the results, to minimize inconsistency and variations from well to well. First of all, it is important that, after

the experiment on Seahorse Analytics, to remove as soon as possible, the plate with the cells to avoid the detachment of cells and gently remove the medium.

To normalize the data, several approaches can be useful. One possible way to normalize is through cell number density. However, it is not the most reliable method of normalizing the data, if the proliferation is affected throughout the duration of the experiment.

Still, regarding the cell number, another approach is to proceed to DNA quantification. This technique is predicated on the hypothesis that the content of DNA is linearly proportional to the number of cells. The content of nuclear DNA can be easily measured in a standard plate reader by using commercial reagent kits (Angelini et al., 2022).

It is possible to normalize by evaluation of cellular density through colorimetric sulforhodamine B (SRB) assay (Martins et al., 2019). Briefly, cells are fixed in 1% acetic acid in methanol for 1 hour at -20ºC. Next, cells are stained with 0.05% (w/v) SRB dissolved in 1% acetic acid for 1 hour at 37ºC. Afterward, unbound SRB are removed through washing with 1% acetic acid solution. SRB bound to cell proteins is extracted afterward, with 10 mM Tris solution (pH 10) in a shaker for 10 minutes at room temperature. The optical density of the resulting media is determined at 490 nm.

Furthermore, to correct the mitochondrial content of the sample, OCR can be normalized through citrate synthase activity (Klein Gunnewiek et al., 2020). In brief, the background citrate synthase activity is recorded in the absence of its substrate acetyl-CoA, followed by its addition. In practice, after completion of OCR measurement, the seahorse medium must be replaced by a buffer solution composed with Triton X-100 and Tris-HCl, and the plate must be stored at -80ºC. After thawing the plate, it is added in each well a solution with Acetyl-CoA, 5,5′-dithiobis- (2-nitrobenzoic acid) (DTNB), oxaloacetate, and Triton X-100. The background reduction of DTNB is measured spectrometrically at 412 nm and 37ºC for 10 min, collecting points at every minute. The citrate activity is determined by the background DTNB conversion rate, using an extinction coefficient of 13800 $M^{-1}cm^{-1}$, and expressed as nmol/min/ mg protein (Monteiro-Cardoso et al., 2015). Apart from normalization through mitochondrial enzymatic activities, mitochondrial protein content is also an option. To do that, expression of some mitochondrial proteins such as CTP1B, TIMs, and TOMs can be quantified. Nevertheless, with so few cells per well, it might be challenging to determine the mitochondrial content (Yépez et al., 2018).

The most accurate method to measure for the normalization of the results is the cellular or tissue lysate protein quantification. Total protein can be obtained using RIPA buffer. After that, total protein concentration can be determined by routine methods in the laboratory.

3.6. Advantages and disadvantages

The advantages and disadvantages of the use of Seahorse Analytic to evaluate the glycolytic capacity are summarized in table 3.2. In sum, it is important to highlight that effectively this approach is faster and has a high resolution and high sensitivity; however, the costs are high. So, it is important to evaluate if this approach is the one that can provide the necessary information to obtain a more accurate result.

Table 3.2. Advantages and disadvantages of evaluating the glycolytic capacity through seahorse analytics.

Advantages	Disadvantages
Evaluation of OCR and ECAR simultaneously in the same cells.	The cost is high.
Evaluation of different samples in different conditions at the same cell culture plate.	Optimization of the compounds is needed.
Very small quantities of cells are necessary (as few as 1% of cells required by other methods).	Only four injectable compounds are possible to be tested in a single assay.
Most assays can be completed in 1.5-2 hours.	Injectable compounds may potentially interfere with sensor fluorescence or the plastic plate and produce misleading data.
High resolution and sensitive equipment.	Complementary techniques are necessary to normalize the data.
Background correction wells.	

3.7. Alternative methods and procedures

The Seahorse extracellular flux (XF) analyzer is a powerful tool for measuring glycolysis and OXPHOS simultaneously in the same cells. However, enzymatic approaches are possible to mimic what happens in seahorse equipment. One example is the kit Glycolysis Stress Test Companion Assay, which has the same rationale of the glycolysis stress test kit of Seahorse. The principal reagents, like in glycolysis stress kit, are oligomycin, 2-DG, and glucose oxidase, which have positive control of the assay, and glucose-free respiration buffer. The results are read in a microplate reader, with controlled temperature and fluorescence intensity at 360 ± 40 nm.

Furthermore, there are other approaches, which can indirectly be used to measure the glycolytic rate of a cell. One possible method is measuring the activity of rate-specific glycolytic enzymes. Indeed, specific enzymes are considered key points in the glycolysis pathway, in which their activities control the maximum possible flux. The activity of

hexokinase, phosphofructokinase, and pyruvate kinase, the main enzymes studied, can be evaluated. It is usually done through the conversion of NAD^+ to NADH or $NADP^+$ and NADPH by spectrophotometric methods (Vander Heiden et al., 2009).

Metabolite measurements is also a possibility to study glycolytic flux. Unlabelled or ^{13}C labelled metabolites can be quantified by nuclear magnetic resonance spectrometry. Indeed, ^{13}C- labelled glucose provides glycolytic flux data by quantifying ^{13}C-labeled glucose in downstream metabolites. Even though labelled substrates are expensive and sometimes challenging to detect, the identification of ^{13}C-glucose-derived carbons in glycolytic intermediates is robust, and therefore, considered a good method for evaluating glycolysis (Gkiouli et al., 2019).

3.8. Troubleshooting and optimization

During the experiment, several problems can appear, which are more or less easy to solve. Before the experiment, it is highly recommended that the cells and or tissue be at confluency over 80% to obtain a good signal. Nonetheless, some issues can occur during the injection of the different modulators, which are described in table 3.3., as well as some solutions that need to be taken into count to solve the troubleshooting.

Table 3.3. Problems that can occur during the seahorse analytics experiment and the possible solution.

Problem	Solution
Variability between wells	Test the number of cells to be seeded per well and the time it takes to double, to avoid excess confluence and cell detachment that can lead to variation in the readings.
OCR levels are <40 pmol O_2/min	Increase the seeded cell number.
Minimal response to oligomycin	Cells growing in nutrient-rich media such as Optimem barely use mitochondria to produce ATP. Therefore, incubate the cells in DMEM with a reduced glucose concentration (or galactose-based medium) to promote the use of mitochondria for ATP production
Post-assay normalization by cell count does not yield a reliable result	Use an alternative cell counting method before seeding, such as trypan blue. This may yield improved accuracy when analyzing sensitive cell lines that are more prone to cell death.

Detachment of permeabilized cells from the microplate	Several changes can be made to improve cell adhesion: 1- Increase cell seeding density; 2- Grow cells for longer (2 days rather than 1); 3- Consider a cell coating (polylysine, gelatin solution, collagen, Cell-Tak, Matrigel, or others)
Intact cells cannot be accessible to the full dose of drugs.	It will be needed to permeabilize the cell membrane. Digitonin can be a solution to permeabilize the cells.

3.9. Authors contribution

Patrícia C. Braga: Writing-original draft, methodology, formal analysis, data curation, investigation and writing-review; Raquel L. Bernardino: conceptualization, methodology and writing-review and editing; Pedro F. Oliveira: conceptualization, methodology, writing-review and editing, project administration and funding acquisition. Marco G. Alves: conceptualization, methodology, writing-review and editing, project administration and funding acquisition.

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Chapter 4

The progression from mild to severe hyperglycemia coupled with lipotoxicity causes mitochondrial dysfunction and alters the metabolic secretome of epithelial kidney cells

This chapter was adapted from the published work:

Braga PC, Bernardino RL, Guerra-Carvalho B, Carrageta DF, Oliveira PF, Rodrigues AS, Alves MG (2023). The progression from mild to severe hyperglycemia coupled with insulin resistance causes mitochondrial dysfunction and alters the metabolic secretome of epithelial kidney cells, 113744. Experimental Cell Research. https://doi.org/10.1016/j.yexcr.2023.11374

Abstract

Diabetic kidney disease (DKD) and insulin resistance (IR) in kidney cells are considered main causes for end-stage renal failure. However, it is unclear how IR affects early stages of the disease. Here, we investigate the impact of mild (11 mM) and severe (22 mM) hyperglycemia, with and without induced IR, on cellular metabolism and mitochondrial bioenergetics in a human kidney cell line (HK-2). IR in HK-2 cells was induced with palmitic acid and cellular cytotoxicity was studied. We evaluated the impact of mild and severe hyperglycemia with and without IR on the metabolic secretome of the cells, their live-cell mitochondria function, mitochondrial membrane potential, and mitochondrial complex activities. Furthermore, we measured fatty acid oxidation and lipid accumulation. Cells cultured under mild hyperglycemic conditions exhibited increased mitochondrial bioenergetic parameters, such as basal respiration, ATP-linked production, maximal respiration capacity, and spare respiration capacity. However, these parameters decreased when cells were cultured under higher glucose concentrations when IR was induced. Our data suggests that progression from mild to severe hyperglycemia induces a metabolic shift, where gluconeogenic amino acids play a crucial role in supplying the energy requirements of HK-2. To our knowledge, this is the first study to evaluate the progression from mild to severe hyperglycemia allied to IR in human kidney cells. This work highlights that this progression leads to mitochondrial dysfunction and alters the metabolic profile of kidney cells. These results identify possible targets for early intervention in DKD.

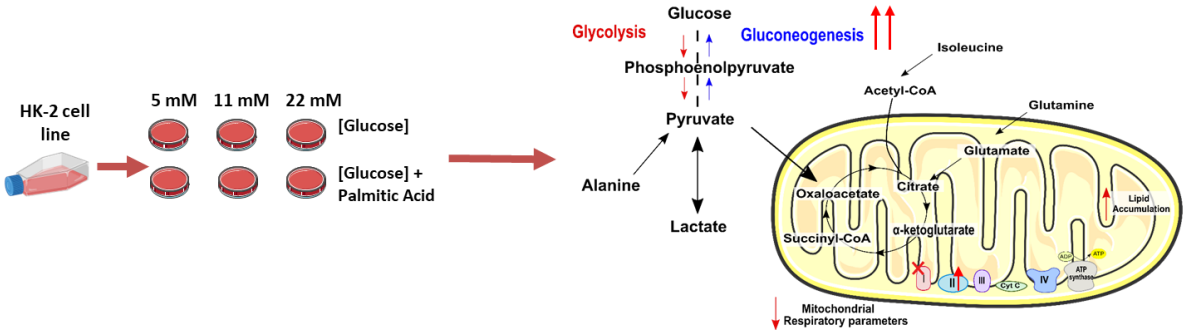

Fig. 4 – Graphical abstract. In this work, we provide evidence of the impact of mild and severe hyperglycemia with and without palmitic acid (PA), to induce lipotoxicity on mitochondria physiology and bioenergetics. The progression from mild to severe hyperglycemia, especially allied with IR-induced by PA was able to diminish mitochondrial respiratory function and a mitochondrial rearrangement towards an increase in gluconeogenic metabolites.

4.1. Introduction

Diabetic Kidney disease (DKD) is considered a major cause of morbidity and mortality in patients with diabetes and is associated with the leading cause of chronic and end-stage renal disease (Fox et al., 2012). Elevated plasma glucose levels can trigger cellular dysfunctions, leading to changes in energy utilization and mitochondrial dysfunction, both of which play a critical role in disease initiation (Kanwar et al., 2011). The kidney is an energetically demanding organ that requires large amounts of energy in the form of ATP due to the re-absorption process (Clark & Parikh, 2020). In the renal cortex, proximal tubules depend on the efficiency of oxidative phosphorylation (OXPHOS) to produce ATP, while glomerular cells have a lower oxidative capacity. Since proximal renal tubular cells have high mitochondrial content they are particularly susceptible to metabolic and mitochondrial injury from hyperglycemia (Bhargava & Schnellmann, 2017). The role of mitochondrial (dys)function in kidney physiology remains largely unknown, although it has been suggested that the detection of mitochondrial dysfunction may promote the development of personalized therapies or the identification of specific proteins to improve renal function (as reviewed by Braga PC and collaborators (Braga PC et al., 2022). Furthermore, after approximately 15 years following a diabetes diagnosis, nearly 40% of patients do not develop albuminuria, a commonly used biomarker for renal disease, which suggests that the disease can progress silently and albuminuria may no longer be a reliable biomarker (Alicic et al., 2017). In addition, chronic hyperglycemia is associated with excessive inflammation, which accelerates insulin resistance (IR), a hallmark that contributes to renal injury (Navarro-González & Mora-Fernández, 2008). Indeed, IR is present in non-diabetic patients already in the early stages of chronic kidney disease (CKD), being more prevalent as the disease progresses (Shinohara et al., 2002; Thomas et al., 2015). IR is a pathophysiologic state where there is a decline in the response to the normal actions of the hormone insulin, leading to high levels of blood glucose (Thomas et al., 2015). Several clinical trials have shown that IR can be considered as an independent risk factor in the development of CKD (Becker et al., 2005; Kosmas et al., 2018; Tsai et al., 2016). However, the exact mechanism by which IR affects kidney disease, even in non-diabetic patients, remains poorly understood (Liao et al., 2012). Elevated circulating free fatty acids (FFA) and triglycerides are typical symptoms of dyslipidemia in diabetic patients. Indeed, one of the main causes of IR, including in the kidney, is lipotoxicity caused by high FFA levels (Herman-Edelstein et al., 2014; Yang et al., 2017). FFA and their metabolites interfere with insulin signaling by inhibiting glucose uptake and glycogen synthesis (Herman-Edelstein et al., 2014; Yang et al., 2017). FFA and their metabolites interfere with insulin signaling by

inhibiting glucose uptake and glycogen synthesis (Chabowski et al., 2013). Palmitate is the most abundant saturated fatty acid present in human serum and elevated levels in the bloodstream have been described to promote lipid accumulation, IR, oxidative stress as well as cell death (Kakimoto et al., 2020). Nonetheless, its impact in DKD is still not well comprehended, especially in combination with IR. Studies investigating mitochondrial bioenergetics and its association with the progression of DKD are scarce. To our knowledge, the ones that exist only explore the differences between physiological and diabetic states (Bernardo-Bermejo et al., 2019; Wei et al., 2019). Herein, we explore the impact of mild to severe hyperglycemia as well as IR under normo and different hyperglycemic *in vitro* conditions in renal epithelial cells' metabolic and mitochondrial bioenergetics profiles.

4.2. Materials and methods

4.2.1. Cell line culture

Human kidney cell line 2 (HK-2), an immortalized proximal tubule epithelial cell line from the adult human kidney, was purchased by ATCC (Manassas, VA, USA), and was cultured in DMEM:F12 medium (1:1, pH = 7.4 with a final concentration of glucose of 7.7 mM) (Sigma -Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS, Sigma), 50 mg/mL gentamicin, 1% Penicillin-Streptomycin (Pen-Strep), 1% amphotericin B, 15 mM HEPES and 14 mM $NaHCO_3$. The cells were incubated at 37◦C with a 5% (v/v) CO_2 humified atmosphere.

4.2.2. Experimental Groups

HK-2 cells were grown until reaching a confluence of 80–90%. Then, the culture medium was replaced by DMEM:F12 medium with Insulin Transferrin-Selenium supplement (ITS, in mg/mL: insulin 10, transferrin 5.5 and selenium 0.0067), pH 7.4. To assess the effects of glucose and IR, cells were treated for 24 h with varying concentrations of glucose (5 mM, 11 mM, and 22 mM) to mimic physiological, mild, and severe hyperglycemic conditions, respectively. In addition to these 3 experimental groups, we also evaluated the effect of different glucose concentrations (in mM: 5, 11 and 22) combined with IR. To induce IR, 250 μM of palmitic acid (PA) was used as described by Ge and collaborators (Ge et al., 2019) . The preparation of palmitate-BSA conjugated was adapted from Qin and collaborators (Qin et al., 2009). After 24 h, extracellular media was collected for further analysis by proton nuclear magnetic resonance (^{1}H-NMR) and the cells were detached with trypsin-

ethylenediamine tetraacetic acid (EDTA) solution. After detachment, the cells were counted with a Neubauer chamber and stored at − 20 ◦C for protein, RNA and mitochondria isolation. Moreover, to assess insulin sensitivity and stimulation, we added two additional groups in our study. The first group was treated with 5 mM of glucose, while the second group was treated with 5 mM of glucose along with PA. Both groups were subjected to a 100 nM insulin stimulation for a duration of 3 h. Subsequently, the cells were collected for analysis using the slot blot technique.

4.2.3. Western blot and slot blot

Cell lysates were homogenized using RIPA buffer (PBS 1×, 1% NP–40, 0.5% Sodium deoxycholate and 0.1% SDS 10%), supplemented with 1% of protease inhibitor mixture (AEBSF, Aprotinin, Bestatin, E− 64, Leupeptin and Pepstatin A), 100 mM of sodium orthovanadate and 100 mM of phenylmethylsulphonyl fluoride (PMSF). Then the final supernatants were obtained by centrifugation at 14 000 g for 20 min at 4 ◦C. Protein concentration was calculated using BCA protein assay kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. Total protein (20 µg) from HK-2 cells was prepared with electrophoresis sample buffer (0.125 M Tris–HCl pH = 6.8; 4% SDS; 20% glycerol, 10% 2-mercaptoethanol), fractioned on a SDS-PAGE system with TGX™ Stain-Free Acrylamide gel (Bio-Rad), 90 V gel for 90 min, at room temperature and transferred to polyvinylidene difluoride membranes in a Trans-Blot® Turbo™ transfer System (Bio-Rad; 1.3 A, up to 25 V, 10 min). Total protein was measured using fluorescence, which eliminates the need for a housekeeping protein control that is often found to be erroneous used in the literature, as explained by Maloy and colleagues (Maloy et al., 2022). The membranes were blocked in a Tris-buffered saline solution with 0.05% Tween 20 (TBS-T) containing 5% nonfat dry milk. Thereafter, membranes were incubated with the primary antibodies, anti-p-IRS1(Ser1001) (#2385, Cell Signalling, dilution 1:2500) and anti-PI3 kinase, p85 (#06–195, Millipore, dilution 1:2500), diluted in TBS-T containing 1% nonfat dry milk, overnight, at 4 ◦C. Afterward, membranes were incubated with the respective peroxidase-conjugated secondary antibody (anti-rabbit, A3687, Sigma, 1:5000), for 90 min, at room temperature. The immunoreactive bands were observed using a WesternBright™ ECL Chemiluminescent HRP Substrate (Bio-RAD) and then exposed to ChemiDoc™ MP Imaging system (Bio-Rad, California, USA). Each band was quantified using BIO-PROFIL Bio-1D Software from Quantity One (VilberLourmat, Marne-la-Vall´ee, France). The value of band density was normalized with the value of total protein and expressed as fold variation to the control group (5 mM). To evaluate insulin sensitivity/stimulation, slot blot

technique was performed. The serine residues of p-IRS1 were evaluated as described by Oliveira PF and collaborators (Oliveira et al., 2015).

4.2.4. RNA extraction, cDNA synthesis and quantitative real-time PCR (qPCR)

The total RNA extraction of HK-2 cells was performed using Total RNA isolation kit (NZYTech), following the manufacturer's instructions. Total RNA was reversed transcribed with M-MuLV Reverse Transcriptase (NZYTech), resulting in cDNA synthesis (1 µg/µL) according to the manufacturer's instructions. To analyze Insulin receptor (IRS), mRNA expression was performed by qPCR assay. In brief, to each sample containing 0.07 µg of cDNA, 0.64 µL of forward and reverse primer (10 µM), and 8.0 µL of Speedy NZYtaq 2× Green Master Mix (NZYthech). The volume was adjusted with RNase-free water until 16 µL. The protocol for qPCR was the following: 2 min at 95 ◦C for polymerase activation, 5 s at 95 ◦C for DNA denaturation and, 30 s at 72 ◦C for the extension. The melting curve was determined by an additional step: 1 min at 95 ◦C, 30 s at 55 ◦C, and 30 s at 95 ◦C. qPCR reactions were carried in duplicate, and the optical density was assessed by a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The primer sequences, the annealing temperatures, and the number of cycles required for the exponential phase of amplification in qPCR are listed in Table 4.1. All conditions were optimized for each set of primers. The quantitative analyses were calculated according to the $2^{-\Delta\Delta Ct}$ expression formula (Pfaffl, 2001).

Table 4.1. Primer sequences and qPCR conditions used to assess mRNA abundance in human kidney cells. Human Insulin Receptor (IRS) and Beta- 2 microglobulin (hβ2M). hβ2M was used as a housekeeping gene. Ann. T(ºC): Annealing temperature. C: Number of cycles.

Gene (Accession number)	Sequence (5´-3´)	Ann. T (˚C)	C
hIRS (NM_001185098.1)	FWD: CTGCATCAGAAGAGGCCATCA RVS: CCCCACATGCTTCACGAGC	63	30
hβ2m (NM_004048.4)	FWD: AGATGAGTATGCCTGCCGTG RVS: TCATCCAATCCAAATGCGGC	58	35

4.2.5. Cell viability using LDH and MTT assays

HK-2 cells were cultured in a 48-well plate at a density of 4×10^4 cells per well and incubated overnight. After that, cells were treated with different glucose concentrations (in mM: 5, 11 and 22) with or without PA for 24 h. Then, LDH release was determined by measuring the

extracellular activity using a commercial assay kit following the manufacturer's instructions (LDH Citox Assay Kit, Biolegend, San Diego, CA, USA). In brief, 100 µL of extracellular media were collected into a 96 well plate, to which were added 100 µL of working solution. LDH assay substrate was added to all samples in a dark environment and this mixture was incubated at 37 ◦C for 15 min. After that time, a 50 µL stop solution was added to stop the enzymatic activity and absorbance was read at 490 nm using the BioTek Synergy HT (BioTek, Winooski, VT, USA) microplate reader. Subsequently, the metabolic toxicity of PA was assessed through the MTT colorimetric assay (van Meerloo et al., 2011). Cells were washed with PBS, and incubated with 3-(4,5-dimethylthiazole-2-yl)-2, 5-biphenyl tetrazolium bromide (MTT, Merk Millipore, Burlington, MA, EUA), in a final concentration of 5 mg/mL, for 2 h, in a dark environment. Then, the medium was removed, and it was added DMSO to each well, to dissolve the formazan crystals formed inside the cells and 100 µL of each well was transferred to a 96-well plate culture and a blank was made with DMSO. The absorbance was read at the wavelengths of 570 nm and 650 nm using the BioTek Synergy HT (BioTek, Winooski, VT, USA) microplate reader. Metabolic viability was calculated by the difference between 570 nm and 650 nm.

4.2.6. Nuclear magnetic resonance (^{1}H-NMR) spectroscopy

^{1}H-NMR spectra of the extracellular media collected were acquired using a Brucker Avance 600 MHz spectrometer equipped with a 5 mm QXI probe with a z-gradient (Bruker Biospin, Karlshruhe, Germany), at 14.1 T and 25 ◦C, using standard methods (Jarak et al., 2018). Sodium fumarate was used as internal reference (singlet, 6.50 ppm.), in a final concentration of 2 mM, to quantify the following metabolites present in solution (chemical shift: ppm): lactate (quartet, 4.10), alanine (doublet, 1.45), glutamate (multiplet, 2.34), isoleucine (dublet, 0.93), pyruvate (singlet, 2.35), glutamine (multiplet, 2.44) and H1-α glucose (doublet, 5.22) were determined when present in medium. The spectra were manually phased, and baseline corrected. NUTS-ProTM NMR software (Acorn NMR, Inc, Fremont, CA, USA) was used to integrate the chosen metabolite peaks. Metabolite consumption or release (production) was determined for each condition by the difference between pre and post treatment. The concentration of the metabolites is expressed as µmol/10^6 cells.

4.2.7. Mitochondrial respiration

Mitochondrial oxygen consumption (OCR) of cultured HK-2 cells, was measured using Seahorse XFe24 Analyzer (Agilent Technologies), following the manufacturer's instructions. Two days before the analysis, 6×10^4 cells were seeded in each well of the 24-well culture plate. On the next day, the cells were treated with conditions of the different experimental groups. On the day of the experiment, the injections ports were loaded. For standard mitochondrial oxygen consumption assessment, it was used Mito Stress Test Kit, and the injections ports were filled with the following compounds: oligomycin (an inhibitor of oxidative phosphorylation), fluorocarbonylcyanide phenylhydrazone (FCCP, a mitochondrial uncoupler), and rotenone/antimycin A (inhibitors of complex I and III, respectively). We used sequential injections of oligomycin (1.5 μM), FCCP (1 μM) and rotenone/antimycin A (0.5 μM). Concerning cell plate, on the day of the experiment, cells were washed with PBS and the medium culture was replaced with assay medium, supplemented with Pyruvate (10 mM) and Glutamine (20 mM) (DMEM medium, pH = 7.4, Agilent). To have access to LCFA, we used Etomoxir to inhibit this pathway. We further used Mito Stress Test Kit, and sequential injections of Etomoxir (4 μM), injections of oligomycin (1.5 μM), FCCP (1 μM) and rotenone/antimycin A (0.5 μM). This assay was performed under conditions of saturating palmitate and L-carnitine by adding them to each well at the time of reading, avoiding the end of substrate. Between each injection, three measurements of each OCR were recorded. All recordings were 8 min apart and the running time was nearly 100–120 min. Test analysis provided measurements of basal respiration, ATP production, maximal respiration, spare or reserve capacity, proton leak and nonmitochondrial respiration. OCR values were normalized to protein concentrations, using a standard BCA assay.

4.2.8. JC-1 assay for mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was evaluated using the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; T3168, Invitrogen™, Carlsbad, CA, USA). To sum up, 1.5×10^4 cells were seeded in a 96-well plate culture, with a black plate and clear bottom (Corning Incorporated costar®). When a 60–70% cell confluence was reached, cells were treated with different concentrations of glucose and/or with PA for 24 h. After treatment, cells were washed with PBS and incubated in culture medium with JC-1 (2 μM), at 37 ◦C for 30 min, in a dark environment. The media with JC-1 was then replaced by fresh media without JC-1. As a positive control, cells were incubated with 20% DMSO to decrease the MMP. The fluorescence of the JC-1 monomers

(ex 485/530 nm; excitation/emission) and JC-1 aggregates (535/590 nm; excitation/emission) were assessed by a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). The ratio between JC-1 aggregates/monomers was calculated and used as an MMP marker and the results were expressed as fold variation to the control group (5 mM). JC-1 aggregates/monomers fluorescence was observed in a Nikon Eclipse Ci-plus microscope equipped with DS-Fi3 microscope camera, a CI-FL-2 Epi-Fluorescence Attachment, and pE300 Lite Multi Band Power Supply (Nikon, Shinagawa, Tokyo, Japan). Nuclei were stained with Hoechst 33 342 (H3570, Thermo Scientific, Waltham, MA, USA). Pictures were captured with a 200 × magnification and a DAPI filter (395/425 nm; excitation/emission) was used for observing the nuclei, FITC filter (480/510 nm; excitation/emission) for JC-1 monomer, and G-2A filter (510/575 nm; excitation/emission) for JC-1 aggregates. All pictures were captured with the same exposition time and gain. The pictures obtained were processed using the NISElements Br 4.60.00 software (Nikon, Shinagawa, Tokyo, Japan).

4.2.9. Mitochondrial isolation and mitochondrial complexes I and II activities

HK-2 cells were homogenized in ice-cold extraction buffer (in mM: 130 sucrose, 50 KCl, 5 KH_2PO^4, 5 HEPES, pH = 7.4) using a glass-Teflon Potter Elvejhem (Kimble, Millville, NJ), and then centrifuged at 1000 g for 10 min, at 4 ◦C. The resulting supernatant was then centrifuged at 12 000 g for 15 min, at 4 ◦C to obtain a mitochondria-free cytosolic fraction and a mitochondria-enriched fraction, as described by Monteiro Cardoso and collaborators (Vera F. Monteiro-Cardoso et al., 2015). The pellet obtained corresponded to the mitochondria-enriched fraction, which was resuspended in a washing buffer (250 mM sucrose, 5 mM HEPES, pH = 7.2), and centrifuged at 12 000 g, for 15 min at 4 ◦C. The final protein content of this fraction was determined by the BCA method. Activities of the mitochondrial complexes were assessed at 37 ◦C, in a 96-well plate, according to well-established protocols (V. F. Monteiro-Cardoso et al., 2015). Complex I (CI) activity was determined following NADH oxidation. The reaction occurred in a potassium phosphate buffer (25 mM KH_2PO_4, 5 mM $MgCl_2$, pH = 7.5), supplemented with 1 mM KCN, 193.8 µM decylbiquinone,4 µM antimycin A, 10 µM rotenone (or an equal volume of buffer) and the volume corresponding to 5 µg of mitochondrial protein. The reaction was triggered by the addition of NADH (100 µM), and fluorescence at 450 mM (excitation: 366 nm) was measured every 30 s for 15 min. CI activity was determined by the difference between NADH oxidation rate in rotenone-inhibited wells and noninhibited wells (nmol $NADH.min^{-1}$ .mg $protein^{-1}$). Complex II (CII) activity was evaluated by monitoring the reduction of 2,6-

dichlorophenolindo-phenol (DCPIP) at 600 nM (ϵ = 20.7 mM^{-1} cm^{-1}) in a potassium buffer (25 mM KH_2PO_4, pH = 7.5), supplemented with 250 µM DCPIP, 1 mM KCN, 193.8 µM decylbiquinone, 10 µM rotenone, 4 µM antimycin A, 162.5 µM and the volume corresponding to 5 µg of the mitochondrial enriched fraction. The negative control wells were additionally supplemented with 5 mM oxaloacetate (CII inhibitor). The reaction was initiated by adding 2 mM succinate and absorbance was measured every 30 s for 20 min. CII activity was expressed as nanomoles of DCPIP per minute per milligram protein (nmol DCPIP.min^{-1}.mg $protein^{-1}$). Citrate synthase (CS) activity was assessed spectrophotometrically, by monitoring the reduction of DTNB. The reaction buffer consisted of 200 mM Tris-HCl buffer (pH = 8.0), 0.02% (m/v) Triton X-100, which was supplemented with 0.4 mM DTNB, 2 mM oxaloacetate, and the corresponding volume of 5 µg of protein. The reaction was triggered by the addition of acetyl-CoA (122 µM), and the absorbance at 412 nm was recorded at 30 s for 10 min. CS activity was calculated by DTNB (ε = 13.6 $mM^{-1}cm^{-1}$) reduction rate, determined in the linear range of the plot and expressed as nanomoles per minute per milligram of protein (nmol.min^{-1}.mg $protein^{-1}$). The activity of mitochondrial redox chain complexes were normalized with CS activity.

4.2.10. Oil Red O staining

To assess intracellular lipid accumulation, cells were stained with Oil Red O staining (ORO) (Chen et al., 2019). Briefly, 5 × 10^4 cells were seeded in 24-well plate. After the treatment, cells were fixed with 4% formalin for 1 h at room temperature. Then, after rinsing twice with PBS supplemented with Ca^{2+} and Mg^{2+}, cells were incubated for 5 min with 60% isopropanol. Then, cells were stained with freshly diluted (in a final concentration of 0.02%) ORO working solution at room temperature for 20 min. After that, cells were washed firstly with 40% of isopropanol and then with PBS. For the quantification of lipid accumulation, it was added to the stained samples, 200 µL of 100% isopropanol to each well, and the plates were slowly agitated at room temperature for 10 min to dissolve the ORO staining. Thereafter, 100 µL of the samples of each well were transferred to a 96-well plate, and the absorbance was measured at 510 nm using a BioTek Synergy HT (BioTek, Winooski, VT, USA) microplate reader. The results are expressed as fold variation to the control group (5 mM).

4.2.11. Statistical analysis

Analysis was performed using GraphPad 8 (GraphPad Software, Inc). The distribution of the data was tested, and groups were compared using two-way ANOVA with Bonferroni's multiple comparisons tests. Statistical significance was considered at $p < 0.05$ and data are presented as mean ± SEM (N = 3 to N = 8; N refers to distinct passages of the HK-2 cell line, where each condition that was performed in cell culture plates were done in triplicates).

4.3. Results

4.3.1. Palmitic acid (PA) induces IR and downregulates insulin signaling pathway in HK-2 cells

To investigate the molecular mechanisms underlying palmitic acid (PA)-induced IR in HK-2 cells, we assessed insulin signaling in both nonexposed and PA-exposed cells. Specifically, we first examined the expression of the insulin receptor. As demonstrated in Fig. 4.1 (Panel A), HK-2 cells of all experimental groups expressed the insulin receptor (INSR). Activation of INSR by insulin typically leads to the recruitment and phosphorylation of insulin receptor substrate proteins (p-IRS), which then activates the PI3K cascade. Our results demonstrated that, upon INSR activation, the levels of p-IRS in HK-2 were lowered in the cells of the groups treated with PA (Fig. 4.1, Panel B). Between the groups of HK-2 exposed only with glucose, there was a decrease in the p-IRS1 protein expression after these cells were cultured in severe hyperglycemia condition (22 mM; 0.46 ± 0.12, fold variation to control) in comparison to the mild hyperglycemia condition (11 mM; 1.59 ± 0.34, fold variation to control). Moreover, we observed a similar decrease in p-IRS1 levels in cells treated with 11 mM glucose along with PA treatment (0.51 ± 0.15, fold variation to control) and in those exposed to glucose in physiological conditions (5 mM) with PA (0.34 ± 0.01, fold variation to control). PI3K levels were also affected in the cells of the groups treated with PA (Fig. 4.1, Panel C). However, this decrease was more pronounced when the cells were treated with severe hyperglycemic conditions and PA (0.58 ± 0.07, fold variation to control) compared with those from the groups treated with increasing concentrations of glucose, although with no statistical differences.

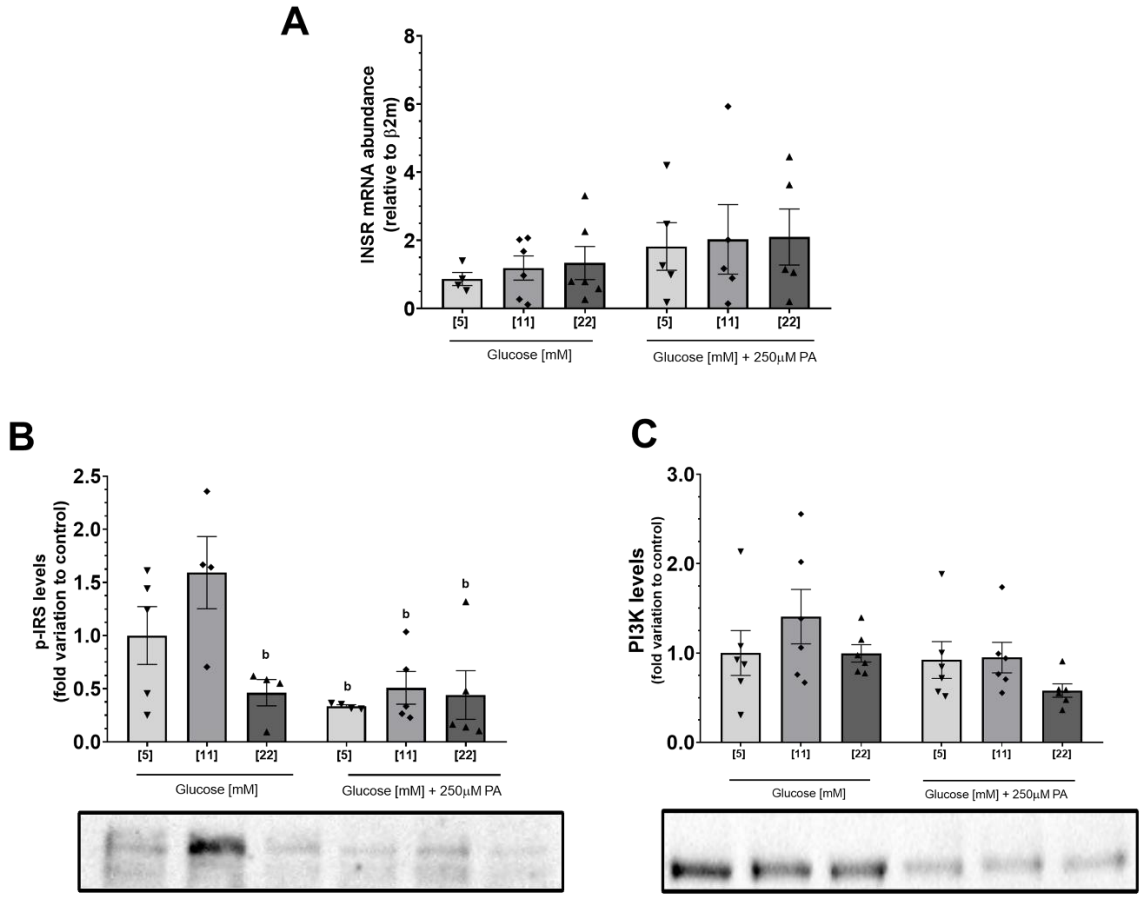

Fig. 4.1 - Insulin pathway inhibition in human kidney cells by exposure to palmitic acid (PA). Quantification of mRNA abundance of Insulin Receptor (INSR) (Panel A), protein levels of p-IRS1 (Panel B) and PI3K (Panel C) in human kidney cells (HK-2), with and without treatment with PA. Results were tested by two-way ANOVA with Bonferroni's multicomparison test. The results are expressed as mean ± standard error of the mean (SEM) (N = 6, in triplicates each experimental group). Significantly different results (P< 0.05) are indicated as: b-relative to 11 mM. Fold variation to the control represented as 5 mM of Glucose.

4.3.2. PA-induced IR in HK-2 cells induces an increase in LDH release and a decrease in the cells metabolic viability

To evaluate the effect of PA-induced IR on cell membrane integrity in HK-2 cells, we performed the LDH assay. When the cells were cultured with PA, all glucose concentrations tested induced an increase in LDH release in comparison to the control group (1.00 ± 0.11, fold variation to the control) (Fig. 4.2, Panel A). Furthermore, regarding the experimental groups where the cells were cultured with PA, the highest concentration tested of glucose led to a decrease in LDH release (2.53 ± 0.19, fold variation to the control) in comparison

to the lowest concentration of glucose tested (3.00 ± 0.04, fold variation to the control). In addition, in PA-induced IR condition, when the cells were exposed to 11 mM of glucose there was an increase in LDH release (3.79 ± 0.33, fold variation to control) in comparison to the cells only treated with 11 mM of glucose (1.07 ± 0.03, fold variation to the control group). The same results were obtained when we compared the cells treated with 22 mM of glucose and PA (2.54 ± 0.20, fold variation to control) and with only 22 mM of glucose (1.37 ± 0.18, fold variation to the control). When we assessed the metabolic viability, by using the MTT assay (Fig. 4.2, Panel B), all the concentrations of glucose tested in combination with PA had a decrease in metabolic viability in comparison to the control group (1.00 ± 0.09, fold variation to the control). Furthermore, when the cells were exposed to a mild hyperglycemia condition and PA (11 mM) there was a significant decrease of the metabolic viability (0.33 ± 0.01, fold variation to the control) in comparison to that of cells treated only with 5 mM of glucose, 11 mM of glucose (0.73 ± 0.05, fold variation to the control) and 22 mM of glucose (0.82 ± 0.04, fold variation to the control). The same results were obtained when the cells were treated with 5 mM of glucose with PA, which also presented a decrease in metabolic viability (0.48 ± 0.05, fold variation to the control). Furthermore, the group treated with 22 mM of glucose and PA (0.56 ± 0.03, fold variation to the control) presented a decrease in metabolic viability compared with the control group (5 mM of glucose) and with the cells treated with only 22 mM of glucose.

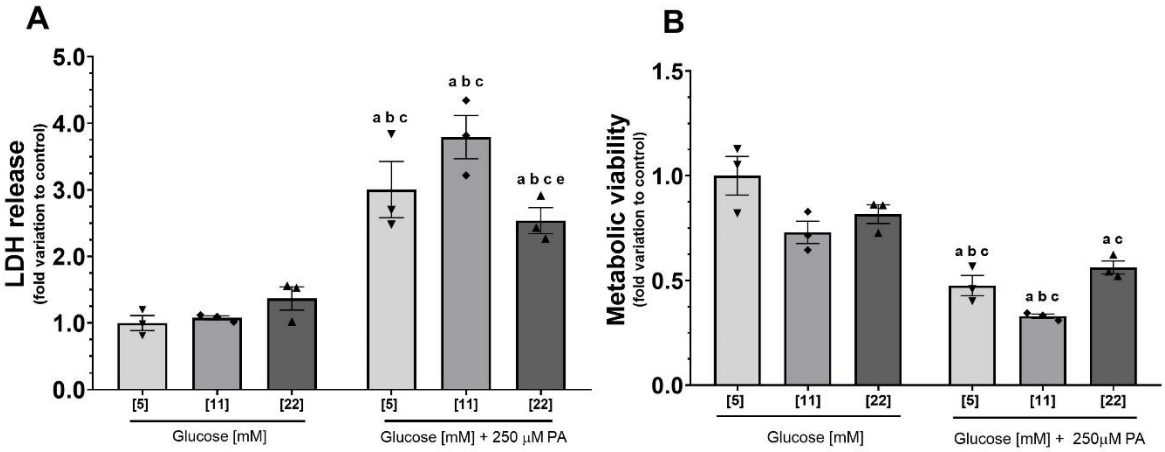

Fig. 4.2 – Evaluation of Lactate Dehydrogenase (LDH) release (Panel A), and metabolic viability (Panel B) of HK-2 cells treated with different glucose concentrations (5 mM, 11 mM and 22 mM) without and in the presence of palmitic acid (PA). Results were tested by two-way ANOVA with Bonferroni's multicomparison test. The results are expressed as mean ± standard error of the mean (SEM) (N = 3, in triplicates each experimental group). Significantly different results ($P < 0.05$) are indicated as: a-relative to 5 mM Glucose, b-relative to 11 mM Glucose, c-relative to 22 mM Glucose and e− relative to 11 mM + PA. Fold variation to the control represented as 5 mM of glucose.

4.3.3. The combination of PA-induced IR with mild to severe hyperglycemia alters the metabolic profile HK-2 cells

In HK-2 cells, glucose consumption was increased when the cells were treated with increasing concentrations of this hexose and when IR was induced by PA (Fig. 4.3, Panel A). Concerning only the treatment with glucose, cells in mild hyperglycemia demonstrated an increase (32.51 ± 3.18 μmol/10^6 cells) in glucose consumption in comparison to those cultured under normoglycemia (18.07 ± 2.05 μmol/10^6 cells). In addition, this increase was also observed in PA-induced IR conditions, in cells treated with mild (43.89 ± 5.04 μmol/10^6 cells) to severe hyperglycemic conditions (44.12 ± 3.79 μmol/10^6 cells). Furthermore, the higher increase was observed between matched groups of glucose (22 mM), where the highest glucose consumption was observed when the cells were cultured with PA (44.12 ± 3.79 μmol/10^6 cells vs. 29.31 ± 5.47 μmol/10^6 cells). Kidney cells utilize glutamine as an important source to meet their metabolic demands, and this amino acid also plays a crucial role in maintaining acid-base homeostasis. Among all our experimental conditions, glutamine consumption in these cells (Fig. 4.3, Panel B) was higher when the cells were treated with 11 mM of glucose, with PA induced IR, followed by the cells treated with the same concentration of glucose but without PA (8.62 ± 1.56 μmol/10^6 cells and 5.44 ± 0.89 μmol/10^6 cells, respectively), particularly in comparison to that of cells under normoglycemia, with or without PA-induced IR (3.41 ± 0.77 μmol/10^6 cells and 1.96 ± 0.33 μmol/10^6 cells, respectively). The cells also presented an increased consumption of glutamine when cultured also under severe hyperglycemic condition after PA-induced IR (5.89 ± 0.61 μmol/10^6 cells). Similar results were obtained when we evaluated pyruvate consumption by HK-2 cells (Fig. 4.3, Panel C). The highest consumption of pyruvate was observed in cells cultured in mild hyperglycemia with or without PA-induced IR (2.09 ± 0.40 μmol/10^6 cells and 1.80 ± 0.32 μmol/10^6 cells, respectively), particularly in comparison to the normoglycemia with or without PA-induced IR (0.35 ± 0.04 μmol/10^6 cells and 0.31 ± 0.03 μmol/10^6 cells, respectively). Regarding the groups treated only with different concentrations of glucose, a decrease in pyruvate consumption was observed when the cells were cultured under severe hyperglycemia (0.90 ± 0.18 μmol/10^6 cells) in comparison to the mild hyperglycemic condition (1.80 ± 0.33 μmol/10^6 cells). Furthermore, comparing only the PA-induced IR groups, the severe hyperglycemic condition caused an increase in pyruvate consumption (1.15 ± 0.23 μmol/10^6 cells) in comparison to the cells under mild hyperglycemic condition. Moreover, isoleucine consumption was also altered in our experimental design (Fig. 4.3, Panel D). The highest consumption of isoleucine was observed when the cells were treated with 11 mM of glucose, without and with PA-induced IR (1.47 ± 0.40 μmol/10^6 cells and 1.57 ± 0.30 μmol/10^6 cells, respectively). Lactate is

considered a major precursor of gluconeogenesis including in the kidney. We observed increased production of lactate (Fig. 4.3, Panel E) when the cells were treated with increasing concentrations of glucose (11 mM and 22 mM) and even higher after PA-induced IR, however with no statistical significance among the groups. Alanine is also an amino acid that contributes to gluconeogenesis. Its production was increased in a hyperglycemic condition plus PA (2.51 ± 0.26 μmol/10^6 cells) in comparison to the normoglycemic and mild hyperglycemic condition without PA (1.59 ± 0.09 μmol/10^6 cells, 1.54 ± 0.18 μmol/10^6 cells, respectively) (Fig. 4.3, Panel F). Glutamate production was also affected after PA-induced IR (Fig. 4.3, Panel G). In fact, this effect was noticed in the cells cultured under physiological condition with PA-induced IR (3.62 ± 0.53 μmol/10^6 cells), when compared to cells without PA-induced IR (0.71 ± 0.11 μmol/10^6 cells), and this increase was more noticed in the higher concentrations of glucose (11 mM: 0.96 ± 0.12 μmol/10^6 cells; 11 mM with PA-induced IR: 3.41 ± 0.62 μmol/10^6 cells; 22 mM: 1.89 ± 0.26 μmol/10^6 cells; 22 mM with PA-induced IR: 3.88 ± 0.52 μmol/10^6 cells).

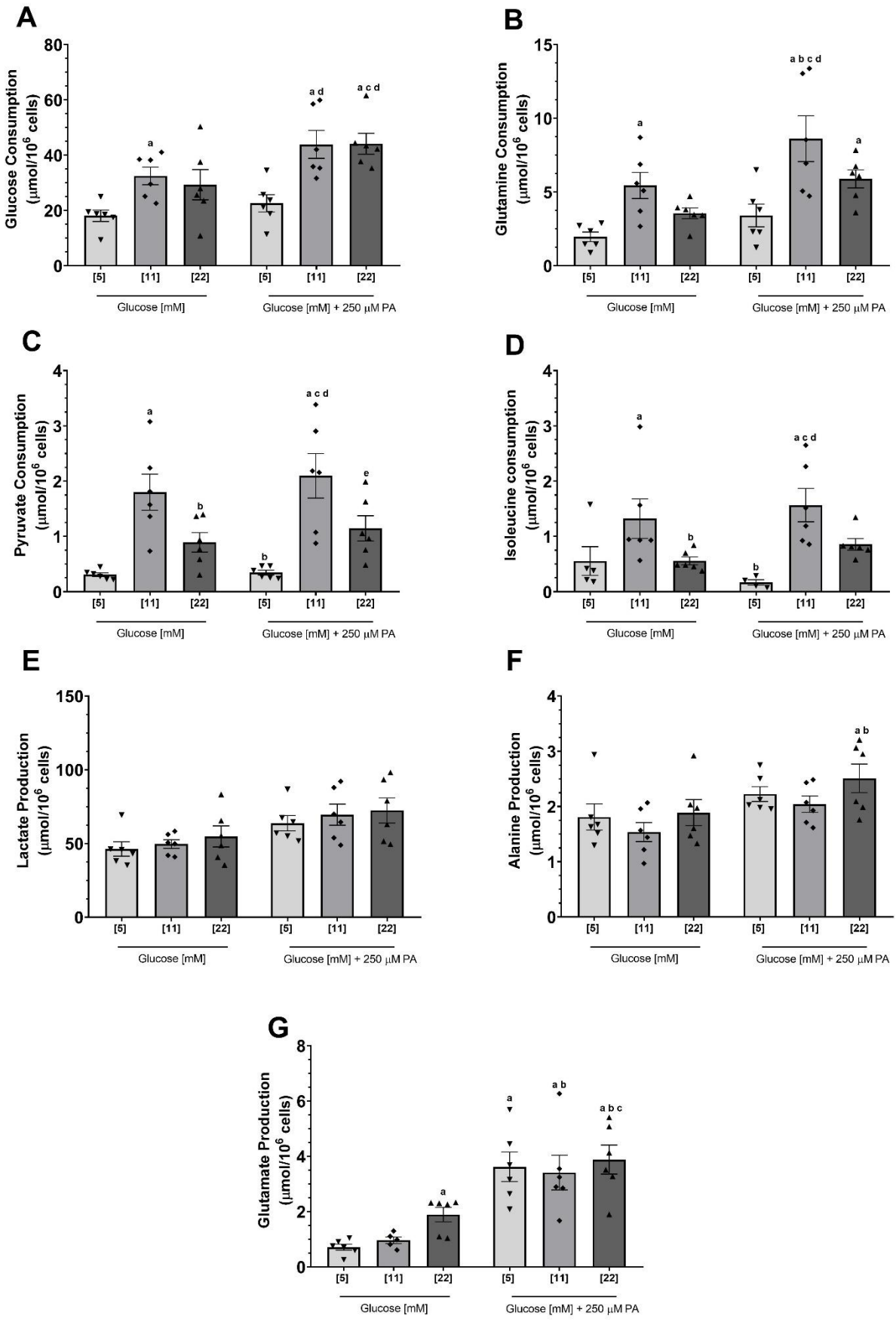
A
Glucose Consumption (µmol/10^6 cells)
B
Glutamine Consumption (µmol/10^6 cells)
C
Pyruvate Consumption (µmol/10^6 cells)
D
Isoleucine consumption (µmol/10^6 cells)
E
Lactate Production (µmol/10^6 cells)
F
Alanine Production (µmol/10^6 cells)
G
Glutamate Production (µmol/10^6 cells)
[5]
[11]
[22]
Glucose [mM]
Glucose [mM] + 250 µM PA

Fig. 4.3 – Evaluation of the consumption and production of metabolites by HK-2 cells treated with different glucose concentrations (5 mM, 11 mM and 22 mM) without or with palmitic acid (PA)-induced IR. Evaluation of glucose (Panel A), glutamine (Panel B), pyruvate (Panel C), and isoleucine (panel D) consumption after 24 h of treatment. Quantification of lactate (Panel E), alanine (Panel F) and glutamate (Panel G) production. Results were tested by two-way ANOVA with Bonferroni's multicomparison test. The results are expressed as mean ± standard error of the mean (SEM) (N = 6, each N corresponds to the medium collected of one Petri dish per each experimental condition). Significantly different results (P< 0.05) are indicated as: a-relative to 5 mM Glucose, b-relative to 11 mM, c-relative to 22 mM Glucose, d-relative to 5 mM + PA and e− relative to 11 mM + PA.

4.3.4. Mitochondrial performance of HK-2 cells is affected by PA-induced IR in a glucose-dependent manner

The data above demonstrated that PA was able to affect HK-2 cells metabolic pathways to obtain energy. Therefore, we examined whether this condition could affect the cellular energy production by mitochondria. To examine oxygen consumption rates (OCR) and bioenergetic parameters in all experimental groups, we used the Seahorse XFe24 analyzer in a live cellular environment (Fig. 4.4). OCR including basal respiration, ATP production, maximal respiration, spare reserve capacity, proton leak and non-mitochondrial respiration were evaluated using specific modulators. HK-2 cells showed decreased basal respiration after PA-induced IR in a glucose-dependent manner (Fig. 4.4, Panel A). Concerning the groups only treated with glucose, the cells cultured in 11 mM of glucose (1.38 ± 0.15 µmol/min/µg protein) had higher basal respiration in comparison to those cultured in 5 mM (0.82 ± 0.06 µmol/min/µg protein). Comparing the groups treated with 22 mM of glucose, the minimal rate of basal respiration was observed when the cells were treated with PA, (0.63 ± 0.08 µmol/min/µg protein vs. 1.08 ± 0.14 µmol/min/µg protein). Following the injection of oligomycin, the electron flow through electron transport chain (ETC) decreases due to inhibition of ATP synthase (Fig. 4.4, Panel B). The ATP production by HK-2 cells associated to respiration was significantly higher in the groups treated with 11 mM (0.80 ± 0.11 µmol/min/µg protein) and 22 mM (0.75 ± 0.07 µmol/min/µg protein) of glucose in comparison to the normoglycemia group (5 mM; 0.41 ± 0.06 µmol/min/µg protein). Moreover, there was also an increase between glucose-matched groups in ATP production when the cells were treated with 5 mM of glucose with PA-induced IR (0.75 ± 0.12 µmol/min/µg protein). It was also observed a decrease in ATP production parameter when cells were treated with 22 mM of glucose and PA in comparison to the matched group, however without statistical significance (p=0.074). Injection of FCCP, a mitochondrial uncoupler, caused an increase in OCR and allowed the measurement of the maximal mitochondrial respiration (Fig. 4.4, Panel C) and spare respiratory capacity (Fig. 4.4, Panel

D). The maximal respiration was higher in the cells treated with only 11 mM of glucose (3.00 ± 0.67 µmol/min/µg protein) than in the cells cultured with only 5 mM of glucose (1.78 ± 0.17 µmol/min/µg protein). Additionally, in PA-induced IR condition, there was a decrease in the mild hyperglycemia condition (1.79 ± 0.12 µmol/min/µg protein). The lowest value on maximal respiration OCR was observed when the cells were treated with 22 mM of glucose plus PA (1.32 ± 0.07 µmol/min/µg protein), when compared with the matched group and with the group where the cells were treated with 11 mM of glucose and PA. The same tendency was observed in the spare respiration capacity (Fig. 4.4, Panel D), since the cells treated with 11 mM of glucose (2.00 ± 0.50 µmol/ min/µg protein) demonstrated an increase in comparison to the cells cultured with only 5 mM of glucose (0.98 ± 0.18 µmol/min/µg protein), but this parameter decreased when compared to the matched group of glucose treatment (0.79 ± 0.08 µmol/min/µg protein). The lowest value was observed when PA-induced IR was present in the concentration of 22 mM of glucose (0.68 ± 0.06 µmol/min/µg protein) when compared to the cells treated only with 22 mM of glucose (1.47 ± 0.19 µmol/min/µg protein; p =0.052). We also measured the energy dissipation as proton leak (Fig. 4.4, Panel E) and we found that only cells from the groups where IR was induced presented a decrease in proton leak. More specifically, we observed a significant decrease in the cells cultured with a concentration of 11 mM of glucose (0.31 ± 0.06 µmol/min/µg protein) when compared to those from the group treated with the same concentration of glucose but without PA-induced IR (0.48 ± 0.03 µmol/ min/µg protein). No alterations were observed in non-mitochondrial respiration (Fig. 4.4, Panel F), except for the cells grown under the concentration of 11 mM of glucose plus PA (1.34 ± 0.51 µmol/min/µg protein), that presented a slight increase in comparison to the cells grown only with 5 mM of glucose (1.17 ± 0.63 µmol/min/µg protein), although with no statistical significance.

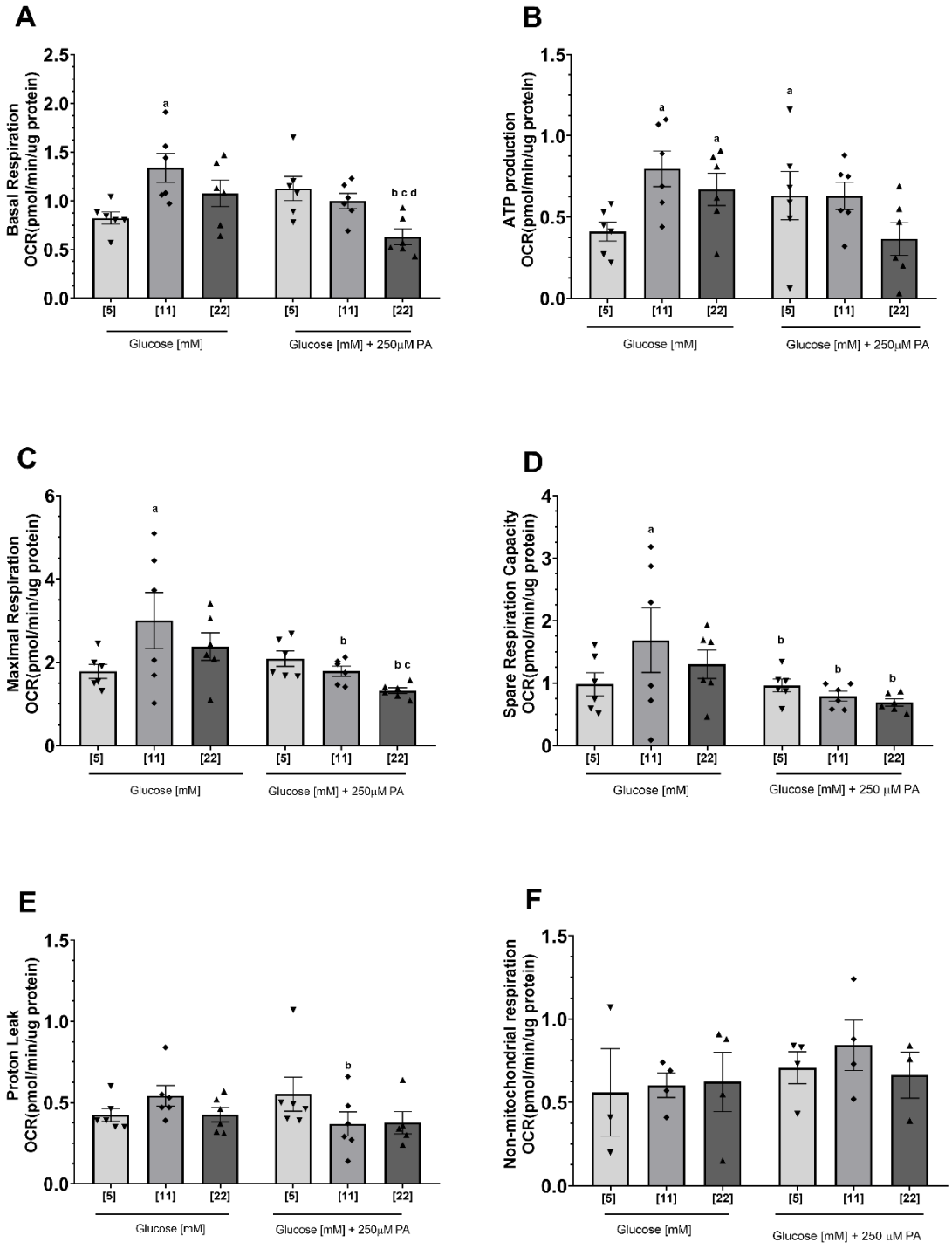

Fig. 4.4– Mitochondrial profile of HK-2 cells treated with different concentrations of glucose and/or IR induced with palmitic acid (PA). Oxygen consumption rates are reported in Basal Respiration (Panel A), ATP Production (Panel B), Maximal Respiration (Panel C), Spare Respiration Capacity (Panel D), Proton Leak (Panel E) and Non-mitochondrial Respiration (Panel F). Results were tested by two-way ANOVA with Bonferroni's multicomparison test. The results are expressed as mean ± standard error of the mean (SEM) (N = 6, in triplicates each experimental group). Significantly different results (P<0.05) are indicated as: a-relative to 5 mM Glucose, b-relative to 11 mM, c-relative to 22 mM Glucose and d-relative to 5 mM + PA.

4.3.5. PA-induced IR in HK-2 cells reduces mitochondrial membrane potential and disrupts complex I activity

As previously described, PA-induced IR was able to reduce the mitochondria functional parameters. Thus, we evaluated the possible mechanism that could mediate those alterations. Mitochondria Membrane potential (MMP) is an indicator of mitochondrial functionality and can be used as a measure of ETC activity. Our results demonstrated that, when IR was induced by PA, there was a decrease in the MMP of HK-2 cells (Fig. 4.5, Panel A and Panel B), particularly in the physiological glycemic concentration and mild hyperglycemic condition (0.87 ± 0.04, fold variation to the control and 0.97 ± 0.03, fold variation to the control, respectively). Additionally, the cells treated with 11 mM of glucose plus PA have a decreased MMP in comparison to the cells treated with only 11 mM of glucose (1.14 ± 0.03, fold variation to the control). Furthermore, still in PA-treated groups, the hyperglycemic condition demonstrated an increase of MMP (1.06 ± 0.04, fold variation to the control) when compared to the cells treated with 5 mM of glucose (0.87 ± 0.04, fold variation to the control). The complexes of ETC maintain the MMP, which is essential for ATP production. Our results demonstrated that PA-induced IR allied to severe hyperglycemia had an impact on the activity of specific complexes of ETC (Fig. 4.5, Panel C and D). The activity of CI in HK-2 cells after PA-induced IR decreased, particularly in severe hyperglycemia condition ($2.41 \times 10^5 \pm 1.13 \times 10^5$ NADH oxidized/min/mg protein) in comparison to the matched group ($1.31 \times 10^6 \pm 1.96 \times 10^5$ NADH oxidized/min/mg protein). Furthermore, it was also observed a decrease in cells treated with normoglycemia and mild hyperglycemia condition ($2.83 \times 10^5 \pm 1.44 \times 10^5$ NADH oxidized/min/mg protein, and $4.40 \times 10^5 \pm 7.35 \times 10^4$ NADH oxidized/min/mg protein, 5 mM + PA and 11 mM + PA respectively). Moreover, regarding the groups treated with different concentrations of glucose, cells treated with 22 mM presented a higher CI activity in comparison to those in physiological condition ($1.31 \times 10^6 \pm 1.44 \times 10^5$ NADH oxidized/min/mg protein (Fig. 4.5, Panel C). The activity of mitochondrial complex II (CII) (Fig. 4.5, Panel D), was also evaluated, and similar results were obtained in the groups treated with glucose, where the cells treated in severe hyperglycemia conditions demonstrated an increase in CII activity in comparison to the normoglycemia condition (0.81 ± 0.14 DCPIP reduced/min/mg protein vs. 0.12 ± 0.01 DCPIP reduced/min/mg protein, respectively). Moreover, the highest increase of CII activity was observed when cells were cultured in the mild hyperglycemic condition (1.23 ± 0.45 DCPIP reduced/min/mg protein) when compared to those in normoglycemic condition with PA (0.22 ± 0.14 DCPIP reduced/min/mg protein) or without PA, and with the matched group (0.19 ± 0.06 DCPIP reduced/ min/mg protein).

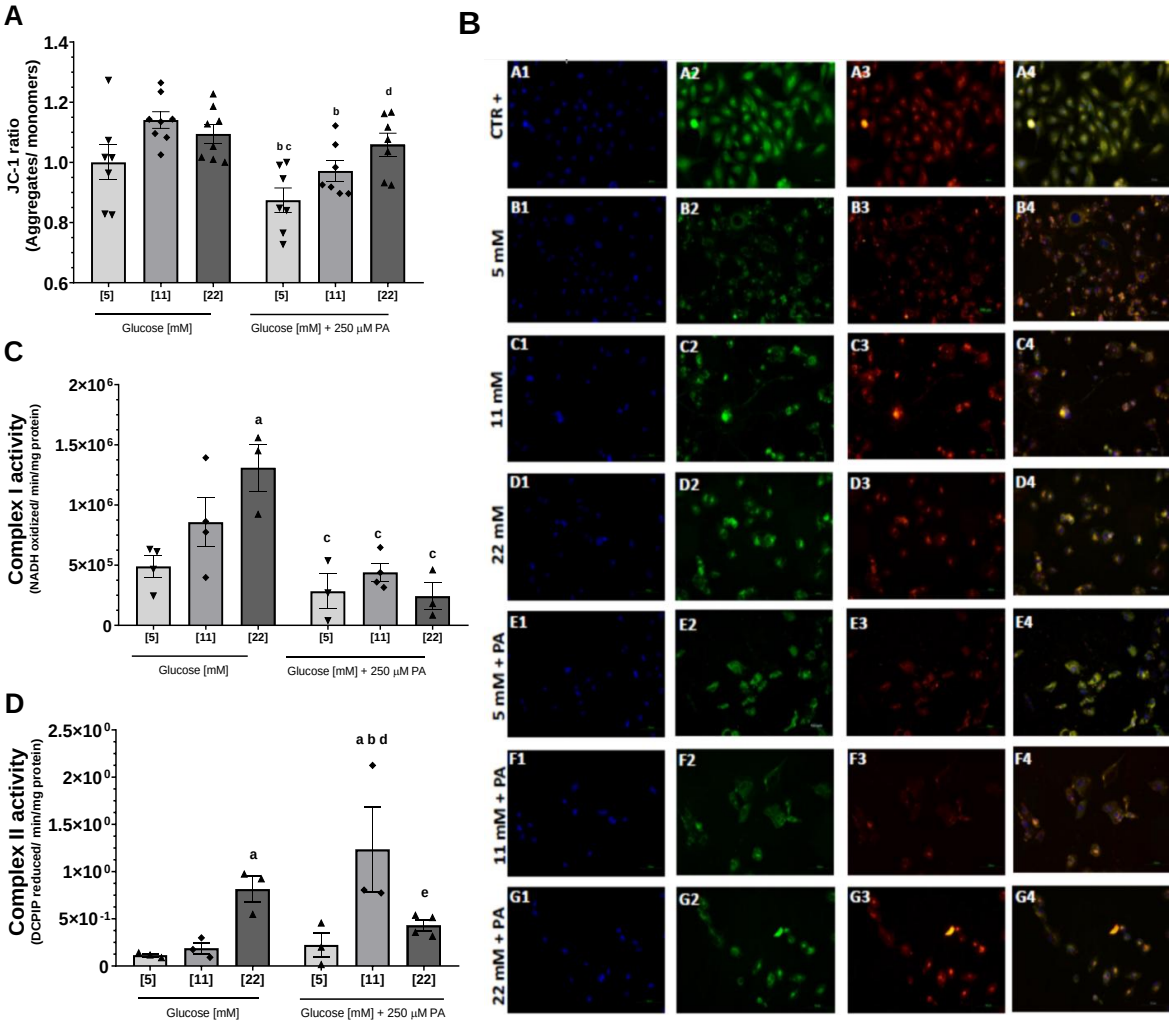

Fig. 4.5 – Evaluation of mitochondrial membrane polarization and effect of increasing concentration of glucose and/or IR in HK-2 cells through JC-1 assay (Panel A). Analysis of JC-1 J-aggregates (red) or J-monomers (green) in HK-2 cells, by fluorescence microscopy (Panel B). In A1, B1, C1, D1, E1, F1 and G1 are represented nuclei (DAPI filter), in A2, B2, C2, D2, E2, F2 and G2 are represented monomers (green, FITC filter), in A3, B3, C3, D3, E3, F3, G3 and H3 are represented JC-1 J-aggregates (red, G-2A filter) and in A4, B4, C4, D4, E4, G4 and H4 are represented the overlap between JC-1 aggregates and monomers, with a scale bar of 100 μm. Panel C represents the activity of the complex I and panel D represents the complex II of the electron transport chain in mitochondria. Results were tested by two-way ANOVA with Bonferroni's multicomparison test and expressed as mean ± standard error of the mean (SEM) (N = 8 for JC-1 assay, and N = 3-4 for mitochondrial complexes, in triplicates for each experimental group). Significantly different results ($P< 0.05$) are indicated as: a-relative to 5 mM Glucose, b-relative to 11 mM and c-relative to 22 mM Glucose, d- relative to 5 mM + PA and e− relative to 11 mM + PA. Abbreviations: CTR + - positive control of the experiment of JC-1 (20% of DMSO).

4.3.6. PA-induced IR in HK-2 cells does not promote lipid oxidation but it leads to the accumulation of lipids in the cells under mild to hyperglycemic conditions

Kidney cells can utilize not only conventional amino acids, but also lipids, specifically fatty acids, as a source of energy, particularly in the tubulointerstitial compartment. However, despite their ability to use lipids as a fuel source, these cells can also accumulate them. Our results demonstrated that the cells in PA-induced IR conditions, independently of the concentration of glucose tested, increased lipid accumulation (Fig. 4.6, Panel A) in comparison to the same concentration without IR induced (5 mM: 2.04 ± 0.25 vs. 1.00 ± 0.32,11 mM: 2.13 ± 0.28 vs. 0.89 ± 0.07 and 22 mM: 1.97 ± 0.25 vs. 0.92 ± 0.11, respectively). Thus, we next intended to investigate in our experimental groups subjected to PA-induced IR, if FFA were being used to obtain energy, through the seahorse Mito Stress kit and Etomoxir (FFA oxidation inhibitor) (Fig. 4.6, Panel B). Our results demonstrate that FFA are not the preferential substrate to produce ATP when cells are treated with 5 mM of glucose (0.84 ± 0.11 µmol/min/µg protein), in comparison to the same concentration when the pathway is inhibited (2.64 ± 0.63 µmol/ min/µg protein) since the production of ATP was higher. No differences were observed in the matched group, when lipid oxidation pathway was inhibited. It was only observed a decrease in mild (1.18 ± 0.53 µmol/ min/µg protein) and severe hyperglycemic (0.98 ± 0.16 µmol/min/µg protein) condition, when FFA oxidation was inhibited.

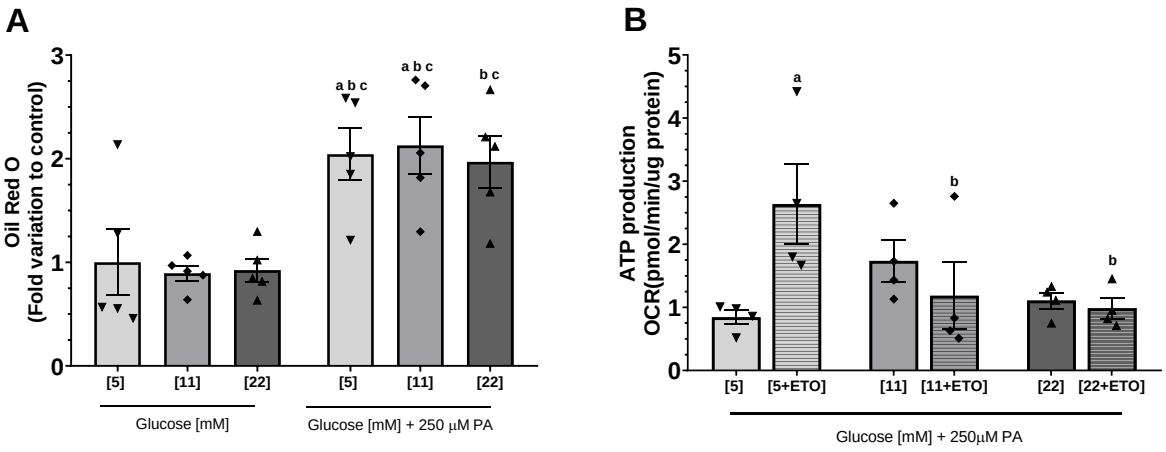

Fig. 4.6 – Lipid accumulation through Oil Red O staining (Panel A), in HK-2 cells with or without PA-induced IR in increasing glucose concentrations (5 mM, 11 mM and 22 mM), and ATP production in increasing glucose concentrations (5 mM, 11 mM and 22 mM) with IR induced with palmitic acid (PA), with or without free fatty acid (FFA) oxidation inhibition, through Etomoxir (ETO) (Panel B). Results were tested by two-way ANOVA with Bonferroni's multicomparison test and are expressed as mean ± standard error of the mean (SEM) (N = 5 and N = 4, panel A and B respectively, in triplicates each experimental group). Significantly different results ($P < 0.05$) are indicated in Panel A: a-relative to 5 mM glucose, b-relative to 11 mM glucose and c-

relative to 22 mM Glucose. Fold variation presented as 5 mM of Glucose. Panel B as: a-relative to 5 mM + PA, b-relative to 5 mM + PA + ETO.

4.4. Discussion

DKD is a significant but under-recognized contributor to the global burden of diabetes (Johansen et al., 2022). Furthermore, impaired insulin sensitivity is strongly associated with alterations in renal cellular metabolism, which can lead to kidney damage (Ismail et al., 1999). It is generally accepted that IR is related with CKD or complications of CKD can cause IR; however, the development of IR is not restricted to DKD or specific kidney disease (Groop et al., 2005). Nonetheless, IR is reported as a predictor of DKD, since patients without diabetes can present IR and still develop CKD (Kurella et al., 2005). Thus, the exact impact of IR on kidney disease pathophysiology remains a topic of debate requiring further investigation. Our experimental approach aimed to uncover the metabolic and mitochondrial mechanisms underlying kidney cell performance under different glucose stimuli, with and without PA-induced IR. In addition, IR may inhibit β-oxidation of fatty acids (Lu et al., 2016), resulting in the accumulation of FFA (such as PA), being reported in Diabetes Mellitus (DM) or DKD. As described by Ge and collaborators (Ge et al., 2019), PA induced IR through a downregulation on insulin signaling pathway in HK-2 cells. Our findings provide compelling evidence that IR was induced through the downregulation of the insulin substrate (p-IRS1) and subsequent inhibition of downstream signaling, specifically affecting the expression level of PI3K. Furthermore, the impact of palmitate on insulin sensitivity was evident in our supplementary data (see Figure A1 in Annex section), where insulin stimulation at basal glucose concentration revealed a reduction in p-IRS1 expression, highlighting palmitate's role in inducing IR. In addition to the aforementioned signaling pathway, it is worth noting that IR has been associated with an increase in the content of branched-chain amino acids (L. Wang et al., 2019). This enhancement is associated with the activation of the mTOR/S6K1 kinase signaling pathway and subsequent phosphorylation of serine on IRS-1 residues, which further cause IR (Newgard et al., 2009). Our results indicate changes in cell membrane integrity, as observed through LDH release, and metabolic viability, suggesting the induction of lipid toxicity. These findings are highly associated with DKD progression and IR. Therefore, the PA-induced IR model used in this study appears to be an excellent approach to achieve our research goals since our results demonstrated an increase of specific amino acids, that are correlated with the downstream of p-IRS1 signaling pathway. Our next goal was to investigate the metabolic and mitochondrial changes that may occur. Previous studies have shown that in diabetes, high glucose levels

due to nutrient overload can affect nutrient transport, sensing, and metabolism in proximal tubular cells (Hinden et al., 2018; Kogot-Levin et al., 2020). Our results indicate that under mild to severe hyperglycemic conditions, glucose uptake increases when IR is induced. Previous studies have demonstrated that downregulation of insulin signaling leads to decreased glucose uptake (Liao et al., 2012). However, in the kidney, particularly in the proximal tubules (from which HK-2 cells are derived), glucose reabsorption is mediated through glucose transporters that are not sensitive to insulin (Liu et al., 2019; Rahmoune et al., 2005). Furthermore, Mueckler and collaborators demonstrated that in diabetic patients, the expression of these glucose transporters were increased (Mueckler & Thorens, 2013). Interestingly, our results pointed that the uptake of glucose increased in mild glycemic condition and even higher when PA was used, being this effect dissipated in a severe hyperglycemic condition. The kidney is a metabolically complex organ, with proximal tubular (PT) cells tuned to gluconeogenesis rather than glycolysis (Wirthensohn & Guder, 1986), where the main renal gluconeogenic substrates are lactate (60%), glutamine (20%), glycerol (10%) or alanine (5%) (Alsahli & Gerich, 2017; Garibotto et al., 2010). Renal gluconeogenesis makes a greater contribution during starvation conditions, where it is estimated to be responsible for 40–45% of total glucose production (Soty et al., 2017). Our findings show that glutamine consumption is significantly increased under mild hyperglycemia conditions, with an even higher increase observed when IR is induced. Glutamine is one of the most abundant amino acids in the human body, and in the kidney, it is estimated to contribute more to gluconeogenesis than alanine (Stumvoll et al., 1998). Our results showed that, in our experimental design, HK-2 cells secrete alanine instead of consuming it, particularly when IR is induced and under the stimuli of the highest glucose concentration. In 1996, Fouque and colleagues concluded that the human kidney-cortex tubule could adapt its metabolism to the available carbon source. Following incubation with increased amounts of alanine, a higher contribution of this amino acid to the production of not only glucose but also lactate and glutamate was observed. These findings suggest that the metabolism of alanine may be adjusted in response to the metabolic needs of the kidney for anabolic and catabolic purposes (Fouque et al., 1996). In our study, the experimental conditions maintained constant and elevated levels of glucose, which prevented HK-2 cells from metabolizing alanine and instead resulted in its production and export to the extracellular environment. The increased alanine production observed in cells under IR condition may be related to altered consumption of glucose and glutamine. Indeed, it was reported elevated plasma concentrations of alanine in insulin-resistant states (Ikeda, 2022) suggesting that PT cells might contribute to that effect, leading to the overall exacerbation of the IR status. Glutamate can act as a respiratory substrate in the oxidative deamination direction of glutamate dehydrogenase (GDH), which also generates α-ketoglutarate for the

Krebs cycle. In a cross-sectional study, Ogawa and colleagues found that individuals with diabetes had increased urinary excretion of glutamate, while the blood glutamate concentration decreased, and the reabsorption of glutamate declined (Ogawa et al., 2018). They also proposed that the depletion of glutamate in PT cells is compensated by an increase in glutamine levels from the blood, which is consistent with our results in IR conditions, showing that lower levels of glutamate are accompanied by increased glutamine consumption. Furthermore, we observed increased consumption of isoleucine in the presence of mild hyperglycemia, with or without induced IR. A recent follow-up study of diabetic patients with DKD found that higher baseline levels of isoleucine were associated with an increased risk of rapid decline in glomerular filtration rate, and was identified as an independent risk factor for development of DKD (Zhu et al., 2022). Our data also suggest that isoleucine may play a role in mild hyperglycemia, independently of IR. Indeed, as DKD progresses, there is a noticeable disruption in the metabolism of certain amino acids. Among these, branched-chain amino acids (BCAAs) such as isoleucine play a crucial role in glucose metabolism. An insightful study by Wang and collaborators explored the metabolic changes in plasma samples from healthy volunteers, patients with diabetes, and patients with DKD, aiming to identify potential biomarkers for DKD. Their study revealed a significant increase in the levels of valine, leucine, and isoleucine in the plasma of diabetic patients compared to the control group. However, interestingly, these BCAAs did not exhibit the same increase in patients with DKD (L. Wang et al., 2019). This observation underscores the potential utility of amino acids as biomarkers for diabetes in the body. Moreover, the distinctive trend of these amino acids being upregulated in diabetes and downregulated in DKD could serve as an early warning sign for the onset and progression of DKD. These findings hold promise for better understanding the metabolic changes associated with diabetes and DKD could pave the way for the development of targeted therapies and early detection strategies to manage and treat DKD effectively. Additionally, lactate can be converted to pyruvate and utilized in gluconeogenesis after entering the mitochondria. *In vivo* and *in vitro* tracer studies of early renal changes associated with DM have reported an increase in glycolysis-driven lactate production or pyruvate-to-lactate conversion (Wang et al., 2022; Z. Wang et al., 2019). Our experimental findings indicate that lactate production remains relatively stable under the given experimental conditions. However, we observed a noteworthy increase in pyruvate consumption, particularly in mild hyperglycemic conditions, irrespective of PA treatment. This intriguing observation suggests that the excess pyruvate generated could potentially be converted into lactate. Notably, lactate serves as one of the renal gluconeogenic carbon sources for renal metabolism. These results shed light on the intricate metabolic pathways and adaptations that occur in response to hyperglycemic conditions, providing valuable insights into the renal

metabolism's dynamic nature. The conversion of pyruvate into lactate may have implications for glucose homeostasis in the kidneys, and further investigation into these mechanisms could enhance our understanding of renal physiology and potential therapeutic approaches for managing hyperglycemic-related disorders. In addition, these results may also reflect an impaired mitochondrial function in DKD (Hansell et al., 2013). That is expected since the kidney is an organ that demands high amount of energy in the form of ATP to fuel membrane pumps that are needed for active transepithelial transport (Soltoff, 1986). Since the ATP is mainly provided through OXPHOS, kidney function depends on mitochondria, being highly susceptible to damage, especially in DKD (Forbes & Thorburn, 2018). Taking in consideration the metabolic dysfunction detected after the different glucose stimuli and PA-induced IR, we speculated that cellular respiratory chain (dys)function in the kidney could be a good indicator for disease progression in response of glucose. Thus, we assessed the bioenergetic profile of HK-2 cells to explore the mechanisms by which different glucose stimuli and/or IR can affect cellular respiration. Our results showed that exposure to hyperglycemia negatively impacted the bioenergetic profile of the cells, with greater susceptibility observed when IR was induced. In the early stages of DKD, renal ATP demand is raised (Mogensen et al., 1983), but with the progression of the disease ATP synthesis (Coughlan et al., 2016), glucose oxidation (Dugan et al., 2013), and FAO (Kang et al., 2015) start to decline. Our findings suggest that mild hyperglycemia, which mimics the onset of the disease, enhances all mitochondrial bioenergetic parameters, including basal respiration, ATP production, maximal respiration, and spare respiratory capacity. However, these parameters decrease when cells are cultured in higher concentrations of glucose and when IR is induced. This is consistent with studies that reported changes in mitochondrial activity after exposure to high concentrations of glucose. It has been shown that exposure to high glucose concentrations can result in a decreased mitochondrial respiration, an increased ROS production, and ultimately mitochondrial dysfunction (Czajka & Malik, 2016; Schiffer & Friederich-Persson, 2017; Valdés et al., 2021). Czajka and collaborators demonstrated concurrent results, where, after 4 and 8 days of exposure to 25 mM of glucose, HK-2 cells presented a decrease in mitochondrial oxygen uptake (Czajka & Malik, 2016). Curiously, they also demonstrated that decreased mitochondrial respiratory capacity coincides with increased glycolysis in HK-2 cells. Our results also support that the increased consumption of glucose and pyruvate and the subsequent increase in lactate production can be a compensatory mechanism when mitochondrial activity is diminished. To further understand the decline in mitochondrial respiratory capacity observed, we evaluated individual activity of CI and CII of the ETC. Our results showed that both complexes increased their activity in severe hyperglycemic condition, and the activity was decreased when IR was induced. This damage was more pronounced in CI, especially in

cells exposed to the severe hyperglycemic condition, which led to a depolarization of MMP, when compared to the matched group. This finding is in agreement with a study by Forbes and collaborators, which showed that knockdown of CI in mice was associated with the progression of CKD (Forbes et al., 2013). Contrary to the previously mentioned results, an interesting finding was the observed increase in the activity of CII when cells were exposed to a mild concentration of glucose in combination with PA. This observation aligns with the simultaneous increase in gluconeogenic amino acids under the same condition, implying an overall boost in metabolic activity. However, despite this metabolic upregulation, it appears that the increase was insufficient to elevate the overall energy status. Our data strongly indicate that the decline in mitochondrial respiration capacity could serve as a crucial indicator of DKD progression. These findings suggest that mitochondrial performance plays a pivotal role in the context of DKD. Targeting the mitochondrial machinery, especially focusing on CI functionality, emerges as a promising therapeutic strategy. By addressing these specific mitochondrial components, we might uncover potential avenues to manage and mitigate the progression of DKD (Panov et al., 2022). Understanding the link between mitochondrial dysfunction and DKD could offer crucial insights into the disease's underlying mechanisms. Further studies in this area are warranted to explore the full therapeutic potential of modulating mitochondrial function in DKD. Finally, our experimental findings provide compelling evidence that FAO is not the primary energy pathway utilized by HK-2 cells in response to glucose stimuli under our specific experimental conditions. However, inhibiting FAO during induced IR in a hyperglycemic environment resulted in several notable effects. Firstly, there was a significant decrease in ATP-linked production, suggesting the importance of FAO as a potential compensatory energy source during IR. Additionally, the inhibition of FAO led to the accumulation of fatty acids as lipid droplets within the cells. These observations align with previous research, which has shown that enzymes and regulators of FAO are reduced in PT cells of patients with DKD and in animal models of DKD. This reduced FAO capacity contributes to intracellular lipid accumulation, mitochondrial dysfunction, and the development of tubulointerstitial fibrosis—important hallmarks of kidney disease progression (Forbes & Thorburn, 2018; Stadler et al., 2015; Weinberg, 2006). These findings shed light on the intricate metabolic alterations that occur in response to IR and hyperglycemia in the context of DKD. Understanding the role of FAO and its impact on cellular lipid metabolism and mitochondrial function is crucial for identifying potential therapeutic targets to intervene in the progression of DKD. Further research in this area could help the development of novel treatment strategies aimed at preserving renal function and ameliorating kidney disease in diabetic patients.

4.5. Conclusion

The current study provides important insights into the pathogenesis of kidney disease progression, particularly DKD, and its association with hyperglycemia and IR. Our findings demonstrate that these conditions have a negative impact on the bioenergetic profile of kidney cells, leading to a metabolic shift that is characterized by the altered metabolism of gluconeogenic amino acids. Our findings reveal a notable increase in multiple metabolic and bioenergetic parameters under mild hyperglycemic conditions, whereas these effects were not observed in the severe hyperglycemic condition. This intriguing observation implies that hyperglycemia might offer a critical window of opportunity to identify potential biomarkers in the early stages of the disease. The distinct response of metabolic and bioenergetic parameters in mild hyperglycemia indicates the dynamic nature of cellular adaptations during the early phases of the disease process. Identifying reliable biomarkers during the early stages of DKD is of paramount importance as it allows for timely diagnosis and targeted therapeutic interventions. Our research contributes valuable evidence to the potential significance of hyperglycemia as a pivotal factor in the search for biomarkers that can aid in the detection and management of DKD at its onset. Further exploration of these metabolic changes and their correlation with disease progression could provide essential insights into the underlying mechanisms of DKD.

4.6. Authors contribution

Patricia C. Braga: Writing-original draft, methodology, formal analysis, data curation, investigation and writing-review; Raquel L. Bernardino: conceptualization, methodology and writing-review and editing; Barbara Guerra-Carvalho: formal analysis and writing review; David.F.Carrageta: formal analysis and writing review. Pedro.F.Oliveira: conceptualization, methodology, writing-review and editing, project administration and funding acquisition; Anabela.S.Rodrigues: conceptualization, methodology, writing-review and editing funding acquision and project administration. Marco.G.Alves: conceptualization, methodology, writing-review and editing, project administration and funding acquisition.

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Chapter 5

High fat diet reversion in mice is not able to mitigate the effects of early obesity in kidney mitochondria quality control mechanisms

This chapter was adapted from the published work:

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Abstract

High fat diet (HFD) induces glomerulopathy and proximal tubule injury. The precise pathophysiological mechanisms triggering obesity-related kidney impairment remain elusive, especially after dietary correction. Male C57BL6/J mice (n=15) were divided in: control group (CTR) fed with standard chow; a group fed with HFD for 200 days; and a group fed with HFD for 60 days and then with standard chow (HFDt). Biometric data and whole-body metabolism were assessed. Expression of genes associated with mitochondrial dynamics, mitochondrial complexes and antioxidant defenses were analyzed. Kidney homogenates enriched in mitochondria were prepared and characterized by mass spectrometry-based proteomics. Kidney tissue of mice fed with HFD exhibited reduced PGC-1α expression, supported by an imbalance between fusion (increased MFN1 and decreased OPA1) and fission (decreased FIS1 and DRP1) processes. The activity of mitochondrial complex I (CI) increased, while activity of complex II (CII) decreased in the kidney after HFD and HFDt. Antioxidant defense Manganese Superoxide dismutase (MnSOD) was decreased in the kidney of HFD, while Glutathione reductase (GR) increased, with both activities being restored upon dietary reversion. Proteomic analysis showed alterations in proteins associated with glutathione and glycine metabolism, fatty acid oxidation (FAO), and peroxisomal function. HFD negatively impacted kidney glutathione related proteins (Gsta3 and Gsr) however dietary correction reverted this condition. Acsm3 protein was downregulated in kidney after HFD and upregulated after dietary correction. Some machinery is shared by mitochondria and peroxisomes, with their network being crucial particularly under stress conditions. A HFD impaired kidney FAO in peroxisomes, as evidenced by downregulation of Pecr after HFD and HFDt. Dietary correction after early-obesity mitigates the systemic metabolic dysfunction and can attenuate mitochondria dysfunction but is unable to completely restore mitochondria dynamics and bioenergetics. The results highlight the integrity of mitochondrial network as a main point for targeted therapeutic strategies aimed at preventing the progression of kidney disease.

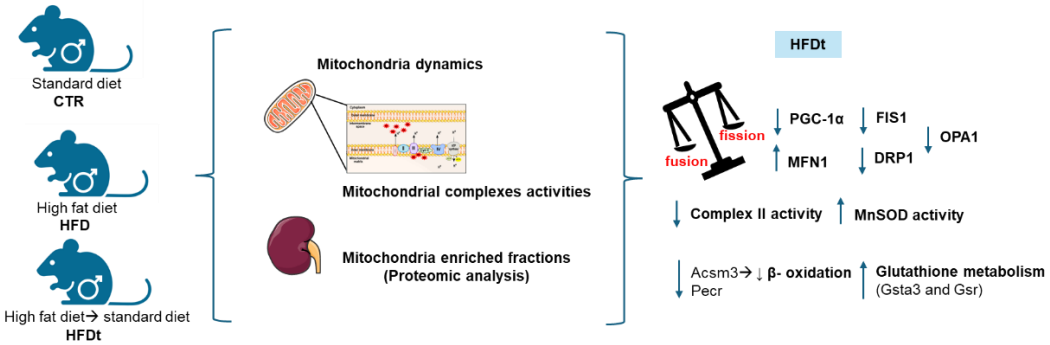

Fig. 5 -Graphical abstract. In this work, we provide evidence that reversion of the diet in mice fed with a high fat diet was not enough to restore mitochondrial dynamics and function. In more detail, it was observed an imbalance of kidney mitochondria fusion and fission, and antioxidant capacity in the kidney after HFD. Furthermore, the proteome analysis of the kidney revealed that while glutathione-related protein levels were restored, proteins involved in β-oxidation (such as Acsm3) remained downregulated, even after the dietary intervention. Overall, this work demonstrated that the reversion of the diet is not enough to restore mitochondria pathophysiology in the kidney.

5.1. Introduction

High-fat diets (HFD) are widespread and have been associated with non-communicable diseases, including chronic kidney injury. While it is established that an HFD can trigger glomerulopathy and proximal convoluted tubule injury (Szeto et al., 2016), the mechanisms initiating obesity-related kidney impairment remain elusive. Evidence suggests that diabetic kidney disease (DKD) is shaped by several cellular stress responses induced by metabolic changes and alterations in energy metabolism (Sakashita et al., 2021). In DKD, the renal cortex, shifts towards an accumulation of glucose and metabolites involved in the tricarboxylic acid (TCA) cycle (Hasegawa et al., 2020), highlighting that DKD progression is related with mitochondrial dysfunction (Sharma, 2017). Insulin resistance (IR) redirects the renal metabolic profile from glucose-mediated aerobic metabolism to fatty acid β-oxidation (FAO) and amino acid metabolism (Mori et al., 2021). Metabolic reprogramming, a phenomenon intricately linked to kidney diseases, influences the optimal functioning of mitochondria, that can trigger a cascade of signaling pathways, prompted by oxidative stress (Ahmad et al., 2021), leading to fibrosis and glomeruli/podocyte damage. Thus, mitochondrial quality plays a crucial role in renal tubular cell stress caused by HFD consumption (Sun et al., 2020). Mitochondria are dynamic organelles that continually undergo fusion and fission in response to metabolic and environmental stresses (Youle & van der Bliek, 2012). Given the kidney's high energetic demand, it has been proposed that mitochondrial dysfunction caused by HFD consumption contributes to kidney diseases.

Maintaining mitochondrial integrity requires a balance between fission and fusion (Hu et al., 2017). An imbalance in mitochondria dynamics can lead to disruptions in kidney function (Narongkiatikhun et al., 2022). Notably, metabolic changes that occur in kidney profile when transitioning from a HFD to a healthy/normal diet remain unknown. Mitochondria biogenesis, bioenergetics and related oxidative stress contribution to early stages of kidney dysfunction induced by an HFD remain understudied. Exploring by proteomic analyses the intricacies of these interactions deepen our comprehension of kidney-related diseases and may unveil promising markers for targeted interventions to mitigate adverse effects on mitochondria. Therefore, proteomic markers are gaining increasing recognition (Provenzano et al., 2021), particularly their association with mitochondrial (dys)function. We aim to elucidate how early life HFD feeding alters the proteome of kidney as well as how this relates to mitochondrial function. Moreover, we aim to unveil if the reversion of the diet after an early life HFD can mitigate the deleterious effects in the kidney later in adulthood with focus on mitochondrial quality control. Overall, we aim to understand the impact of a HFD in mitochondrial function in the kidney, and how this can be correlated to the onset of kidney related diseases and the possible mitigating effect of dietary correction.

5.2. Materials and methods

5.2.1. Animal Model

Mus musculus C57BL6/J (n=15), after weaning (21-23 days), were randomly divided into three groups, control (CTR, n=5), high-fat diet (HFD, n=5) and transient HFD (HFDt, n=5). All male mice originated from normoponderal males and females and were uniformly exposed to random in-utero stimuli. Mice in the CTR group were fed with a standard chow diet (#F4031, BioServ, USA) containing 61.6 % carbohydrates, 20.5 % protein, and 7.2 % fat. Mice in the HFD group were fed with a diet rich in fats (#F3282, BioServ, USA) comprising 35.7 % carbohydrates, 20.5 % protein, and 36.0 % fat for 200 days following weaning. Mice in the HFDt group were initially placed on a HFD for 60 days after weaning, followed by a switch to a standard chow diet. Animals were killed by cervical dislocation after 200 days after weaning (Fig. 5.1), and tissues were collected for further analysis. Total body weight, water, and food intake were monitored weekly until euthanization.

Fig.5.1-Illustration of the experimental groups used in the present work. After weaning, mice generated from normoponderal males and females were weighed and randomly assigned to an experimental group: CTR – standard diet; HFD – high-fat diet; HFDt – transient high-fat diet during 60 days after weaning, and then, the diet was reverted to standard diet until the 200th day.

5.2.2. Glucose tolerance test and insulin resistance test

A week prior to euthanization the animals underwent an intraperitoneal Glucose Tolerance Test (ipGTT) and intraperitoneal Insulin Tolerance Test (ipITT) following the protocols outlined by the NIH Mouse Metabolic Phenotyping Center (MMPC) Consortium guidelines

(Ayala et al., 2010). In brief, the animals were subjected to overnight fasting (16-18 hours without access to food) before the tests. For the ipGTT, each animal received an intraperitoneal injection of 6 mL of 30% glucose solution per kg of body weight, and blood glucose levels were assessed at 30-minute intervals over a span of 2 hours. In the case of the ipITT, the animals were injected intraperitoneally with insulin (0.75 U per kg of body weight) as per the method described by McGuinness and collaborators (McGuinness et al., 2009).

5.2.3. Endocrine and metabolic function

Before euthanization (at 200 days after weaning), fasting glycemia levels were determined following an overnight fast of 8 hours. This was accomplished by utilizing a glucometer (One Touch Ultra Lifescan-Johnson, Milpitas, CA) to measure glucose levels in a small blood sample from the tail vein. Blood was then collected through cardiac puncture and centrifuged at 1500 g, 4°C, for 10 minutes to obtain serum. The assessment of glucose homeostasis was conducted based on HOMA2 scores (Levy et al., 1998)**.** These scores were computed using the HOMA2 calculator provided by the University of Oxford, UK.

5.2.4.Quantitative real-time PCR

To evaluate mitochondrial dynamics, it was evaluated the mRNA abundance of specific genes. In brief, RNA of kidneys was extracted by using NZY Total RNA Isolation Kit (#MB13402, NZYtech Lisbon, Portugal), according to the manufacturer´s instructions. The extracted RNA was quantified using a dedicated plate (BioTek Take3 Micro-Volume Plate) and measured with the BioTek Synergy HT microplate reader (BioTek, Winooski, VT, USA). Then, RNA was reversely transcribed using the NZY M-MuLV Reverse Transcriptase (#MB30801, NZYtech, Lisbon, Portugal). The resultant complementary DNA (cDNA) was used with primer sets designed to amplify the cDNA fragments of interest. Quantitative Real-Time PCR (qPCR) was performed to evaluate the abundance of mouse Fission 1 protein (FIS1), Dynamin-related protein (DRP1), Mitofusin 1 and 2 (MFN1 and MFN2), Mitochondrial optic atrophy-1 (OPA1), Peroxisome proliferator-activated receptor-gamma coactivator alpha (PGC-1α) and Beta-2 microglobulin (β2m). Specific primers were designed and are presented in **Table 5.1**. Amplification conditions: 2 min at 95 ºC, followed by 30 or 40 runs of a 3 steps cycles: 5 s at 95 ºC for DNA denaturation, 30 s with a specific temperature for each set of primers to perform the annealing, and 30 s at 72 ºC for the extension. The optical density was assessed by a CFX Connect TM Real-Time PCR

Detection System (Bio-Rad). Primers' efficiency was set to 90-110%. β2m transcript levels were used to normalize gene expression levels. Fold variation of gene expression levels were calculated following the model proposed by Pfaffl, using the formula $2^{-\Delta\Delta Ct}$ (Pfaffl, 2001).

Table 5.1. Primer sequences and qPCR conditions used to assess gene expression and mRNA abundance in mouse kidney. Mouse fission 1 protein (FIS1), mouse Dynamin related protein (DRP1), mouse Mitofusin 1 and 2 (MFN1 and MFN2), mouse mitochondrial optic atrophy-1 (OPA1) and mouse peroxisome proliferator-activated receptor-gamma coactivator alpha (PGC-1α) were studied. Mouse Beta-2 microglobulin (β2m) was used as a housekeeping control.

Gene	Sequence (5´-3´)	Annealing temperature	Cycles
mFIS1	**FWD:** TAAATGTGCGAGGGCTGTT	56	35
	RVS:TTATCAATCAGGCGTTCCAGC		
mDRP1	**FWD:**TAGCTATGGTGAACCGGTGG	56	35
	RVS: TTGCGGTTCCTTCAATCGTG		
mMFN1	**FWD:** CAATTGCAAGCTGTGTTC	56	35
	RVS:CTTCTGTGCATTTGTGGAACC		
mMFN2	**FWD**:TGTGCTTTTCAGGAGGAC	56	40
	RVS:TGTAGCCTAGCAAGGCCCG		
mOPA1	**FWD:**ACCCAAAGGAAAGGAACACG	57	40
	RVS:GCTATCCTCGGCAAAGTCGT		
mPGC1-α	**FWD:**TGTGTGTCAGAGTGGATTGGAG	56	35
	RVS:GCAGGCTCATTGTTGTACTGG		
mβ2m	**FWD:**ACGTAACACAGTTCCACCCG	58	35
	RVS: TCTCGATCCCAGTAGACGGT		

5.2.5. Isolation of mitochondria-enriched fraction

A portion of kidney from each animal was homogenized in 2 mL of ice-cold buffer (130 mM sucrose, 50 mM KCl, 5 mM KH_2PO_4, 5 mM HEPES, pH= 7.4) using a glass-Teflon Potter Elvejhem (Kimble, Millville, NJ) and centrifuged at 1000 *g* for 10 min, at 4 ºC. The resultant supernatant was fractionated by differential centrifugation (12 000 *g* for 15 min, 4ºC) to obtain a mitochondria-free cytosolic fraction and a mitochondria-enriched fraction, as described by Mendes et al., 2018 (Mendes et al., 2018). The mitochondria enriched fraction was resuspended in a washing buffer (250 mM sucrose, 5 mM HEPES, pH=7.2), and centrifuged at 12 000 *g*, for 15 min at 4ºC. The final protein content of this fraction was determined by the BCA method. The mitochondria-enriched fraction was used to assess

the activity of mitochondrial enzymes, while the mitochondria-free cytosolic fraction was used to evaluate the enzymes of the antioxidant defense system.

5.2.6. Determination of mitochondrial complexes activity

Activities of the OXPHOS complexes I, II, and IV as well as citrate synthase (CS), were assessed, at 37ºC, in a 96-well plate, by adaptation of previous protocols (Mendes et al., 2023). Complex I (CI) activity was assessed spectrofluorimetrically following NADH oxidation. The reaction occurred in a potassium phosphate buffer (25 mM KH_2PO_4, 5 mM $MgCl_2$, pH=7.5), supplemented with 1 mM KCN, 193.8 µM decylubiquinone, 4 µM antimycin A, 10 µM rotenone (or an equal volume of buffer) and the volume corresponding to 10 µg of mitochondrial protein. The reaction was triggered by the addition of NADH (100 µM), and fluorescence at 450 mM (excitation: 366 nm) was measured every 30 s for 15 min. CI activity was determined by the difference between the slopes of NADH oxidation rate in rotenone-inhibited wells and noninhibited wells ($_{nmol}$ NADHmin^{-1}.mg $_{protein}$).

Complex II (CII) activity was evaluated by monitoring the reduction of 2,6-dichlorophenolindophenol (DCPIP), which was used as an exogenous acceptor of electrons, at 600 nM (ε= 20.7 $mM^{-1}cm^{-1}$) in a potassium buffer (25 mM KH_2PO_4, pH=7.5), supplemented with 250 µM DCPIP, 1 mM KCN, 193.8 µM decylubiquinone, 10 µM rotenone, 4 µM antimycin A and the volume corresponding to 10 µg of the mitochondrial enriched fraction. The negative control wells were additionally supplemented with 5 mM oxaloacetate (competitive CII inhibitor). The reaction was initiated by adding 2 mM succinate and absorbance was measured every 30 s for 20 min. CII activity was determined by the difference between the slopes in the absence and presence of oxaloacetate and was expressed as nanomoles of DCPIP per minute per milligram protein (nmol $_{DCPIP}min^{-1}mg^{-1}$ $_{protein}$).

Complex IV (CIV) was evaluated by the oxidation of cytochrome c (CytC). CytC (50 mM) was freshly prepared by its reduction through the addition of saturated sodium dithionite solution to increase the absorbance of CytC solution in 0.6 units at 550 nm by its saturation of nitrogen gas. The CytC oxidation reaction was performed in a potassium buffer (25 mM KH_2PO_4, pH=7.5) supplemented with 10 µM rotenone, 8 µM antimycin A, 1 mM KCN (specific inhibitor of CIV) or the equal volume of buffer, and the volume corresponding to 10 µg of the mitochondrial enriched fraction. The reaction was initiated by adding 60 µL reduced CytC and the absorbance at 550 nm was recorded every 15 s for 5 min. CIV activity was calculated by CytC (ε= 29.5 $mM^{-1}cm^{-1}$) oxidation rate, corrected by the spontaneous oxidation rate obtained in KCN-inhibited wells and expressed as nanomoles of oxidized CytC per minute per milligram of protein ($_{nmol}$CytC $min^{-1}mg^{-1}_{protein}$).

CS activity was determined spectrophotometrically, by monitoring the reduction of 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) by CoASH. The reaction buffer consisted of 200 mM Tris-HCl buffer (pH=8.0), 0.02% (m/v) Triton X-100, which was supplemented with 0.4 mM DTNB, 2 mM oxaloacetate, and the corresponding volume of 10 µg of protein. The reaction was triggered by the addition of acetyl-CoA (122 µM), and the absorbance at 412 nm was recorded at 30 s for 10 min. CS activity was calculated by DTNB (ε= 13.6 $mM^{-1}cm^{-1}$) reduction rate, determined in the linear range of the plot and expressed as nanomoles per minute per milligram of protein ($_{nmol}min^{-1}mg^{-1}{}_{protein}$). The activity of mitochondrial redox chain complexes was normalized with CS activity.

5.2.7. Antioxidant enzymes activity

The activities of cytosolic antioxidant enzymes: Manganese- dependent superoxide dismutase (MnSOD), glutathione reductase (GR) and glutathione peroxidase (GPx) were evaluated using a microplate reader at 37ºC. MnSOD activity was indirectly evaluated based on competition for $O_2^{\bullet-}$, generated by xanthine/xanthine oxidase system (Mendes et al., 2023). The test was performed by mixing 25 µg of mitochondria free cytosolic fraction with potassium phosphate buffer (100 mM KH_2PO_4, 5 mM EDTA, pH=7.5, supplemented with 2 mM NBT, and 0.05 U xanthine oxidase (no. X1875-10U, Sigma-Aldrich/ Merck KGaA, Darmstadt, Germany). After addition of 10 µL hypoxanthine (2 mM), absorbance at 560 nm was measured every 20 s for 5 min. Results were expressed as units per minute per milligram of protein (U $min^{-1}mg^{-1}_{protein}$), where U is the enzyme activity that inhibited the reduction of NBT to blue.

Following NADPH oxidation associated with the conversion of GSSG to GSH, GR activity was measured spectrofluorimetrically using 25 µg of mitochondria free cytosolic fraction with potassium phosphate buffer (100 mM KH_2PO_4, 5 mM EDTA, pH=7.5), supplemented with 0.4 mM GSSG and 0.2 mM NADPH. NADPH oxidation was recorded by the fluorescence decrease at 450 nm (excitation: 360 nm) every 20 s for 5 min. NADPH oxidation not associated with GSSG reduction was assessed in assays carried out in the absence of GSSG. To convert the slopes of NADPH fluorescence decay promoted by GR activity, a calibration curve of NADPH fluorescence intensity as a function of concentration was prepared. Results were expressed in nanomoles per minute per milligram of protein ($_{nmol}$ NADPH $min^{-1}mg^{-1}_{protein}$).

GPx activity was evaluated by the rate of NADPH oxidation with H_2O_2 reduction. Assays were performed by using 25 µg of mitochondria free cytosolic fraction in potassium phosphate buffer (100 mM KH_2PO_4, 5 mM EDTA, pH=7.5), supplemented with 0.4 mM GSH and 1-3 U GSR (no. G3664-500 UN, Sigma-Aldrich/ Merck KGaA, Darmstadt, Germany).

The reaction started by the addition of 0.6 mM of H_2O_2, and fluorescence at 450 nm (excitation: 360 nM) was measured every 20s for 5 min. GP_X activity was determined, in the first step, by the difference between slopes of NADPH oxidation in the presence and absence of GSH, expressed in nmol NADPH per minute per milligram of protein ($_{nmol}$ NADPH $min^{-1}mg^{-1}_{protein}$).

5.2.8. Analysis of mitochondria enriched fractions by SDS- gel electrophoresis followed by liquid chromatography tandem mass spectrometry (GeLC-MS/MS)

A total of 50 µg of mitochondria enriched fraction proteins were separated using a 12.5 % SDS-PAGE, as previously described (Laemmli, 1970). After staining the gels with colloidal Coomassie Blue G250 and destained with 25 % methanol until an optimal contrast was achieved, the protein bands were cut off from the gels, in a total of 6 sections. Each gel sections were incubated with 25 mM ammonium bicarbonate (AMBIC)/ 50% acetonitrile (ACN) for 30 min, twice. Then, to break the cysteine residues, 5 mM dithiothreitol (DTT) was added, and after 45 min, it was added 55 mM iodoacetoamide to alkylate the cysteine residues for 30 min at room temperature and in the dark. Afterwards, the gel portion were washed with 25 mM AMBIC/50% ACN, and then each portion was incubated for 10 min with ACN 100%. Afterwards, the gel portion were digested with trypsin (enzyme-to-total protein ratio of 1:20) (Shevchenko et al., 2006). Next, the peptides were extracted from gel pieces with consecutive incubations with formic acid and lyophilized in a SpeedVac (SC210A; ThermoSavant, Milford, MA, USA). The tryptic peptides were resuspended in 1% formic acid (FA). Protein identification was performed using a nanoLC-MS/MS system consisting of an Ultimate 3000 liquid chromatography system connected online to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific). Approximately 500 ng of peptides were loaded onto a trap cartridge (Acclaim PepMap C18 100 Å, 5 mm × 300 µm i.d., Thermo Scientific) in a mobile phase, containing 0.1 % FA at a flow rate of 10 µL/min. After 3 minutes of loading, the trap column was switched to EASY spray columns with 50 cm and 75 µm inner diameter (ES803, PepMap RSLC, C18, 2 µm, Thermo Scientific) at 300 nL/min. Separation was performed by mixing A: 0.1 % FA and B: 80 % ACN using the following gradient: 5 min (2.5 % B to 10 % B), 120 min (10 % B to 30 % B), 20 min (30 % B to 50 % B), 5 min (50 % B to 99 % B) and 10 min (99 % B hold). The column was then equilibrated with 2.5% B for 17 min. Data acquisition was controlled by Xcalibur 4.0 and Tune 2.9 software (Thermo Scientific). The mass spectrometer was operated in data-dependent (dd) positive acquisition mode, alternating between a full scan (m/z 380–1580)

and subsequent HCD MS/MS of the 10 most intense peaks from the full scan (normalized collision energy of 27%). The ESI spray voltage was 1.9 kV.

Protein identification and label-free quantification (LFQ) was performed with MaxQuant (version 2.5.0.0) using the Andromeda search engine with default search settings, including a 1% false discovery rate at the PSM, peptide, and protein levels. The MS/MS spectra were searched against the UniprotKB/Swiss-Prot protein sequence database under *Mus musculus* (July 2023 version). A minimum number of two unique peptides or razor peptides was required for quantification. Further data analysis was performed using Perseus software (version 2.0.11.0) after loading the files from MaxQuant. Proteins with fewer than three valid values in at least one group were removed, and missing values were imputed from a normal distribution around the detection limit.

Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were applied to examine proteome dynamics between groups (the raw data were log-transformed for these analyses). Volcano plots were used to annotate proteins that were present in significantly different amounts between groups. These analyses were performed using the MetaboAnalyst web portal (version 5.0, www.metaboanalyst.ca, accessed in July 2023). Mass spectrometry proteomics data were deposit with ProteomeXchange Consortium through PRIDE partner repository with the dataset identifier PXD052773.

5.2.9. Statistical analysis

Different statistical methods were applied depending on the objective and the nature of the analyzed data. Shapiro test was used to test data normality. Whenever normality was followed, one way ANOVA with the Tukey's HDS post hoc test was applied as the default test. If the data did not follow normalization, a nonparametric Kruskal-Wallis test was performed, using the Mann-Whitney U test as a post hoc test.

5.3.Results

5.3.1. Normalization of biometric data and systemic body metabolism are achieved by correcting early obesity through dietary adjustments

Body weight was monitored (Table 5.2). After weaning, no differences were observed between the weight of the groups. By day 28th day, the CTR group started to present a lower weight in comparison to mice fed with HFD (CTR: 20.2 ± 0.6 g; HFD: 23.8 ± 0.5 g and HFDt: 25.9 ±0.9 g). The difference increased up at the 56th day, when mice fed with HFD (31.9 ± 0.9 g) and HFDt (34.6 ± 1.4 g) weighed more 40% than CTR mice (23.6 ± 0.5 g). At 60th day the diet from HFDt group was reversed to standard chow, but only on the 147th day, the mice from this group (32.5 ± 0.8 g) reached the mean values of mice from the CTR group (29.7 ± 0.4 g), being this tendency maintained until sacrifice (30.1 ± 0.8 g, 31.1 ± 1.3 g, CTR and HFDt, respectively). In contrast, the mice fed with HFD were 50% heavier than those in the other groups.

Those mice also exhibited higher glycaemia levels during ipGTT (Fig 5.2A) and during IPTT (Fig 5.2B) compared to HFDt group. They also presented a higher fasting glycaemia (104.5 ± 8.3 mg/dL) in comparison to mice of HFDt (68.6 ± 10.8 mg/dL) (Fig 5.2C). Moreover, concerning the fat mass (Fig 5.2D), HFD group (13.24 ± 0.95 %) presented more percentage of fat mass in comparison to the control (4.04 ± 0.39 %) and to HFDt group (4.27 ± 0.47 %).

After evaluating the biometric parameters, we explored the systemic metabolic homeostasis. We could detect those mice fed with HFD (310 ± 53) had β-cell function (HOMA2-%B) higher than CTR (84 ± 34). This effect was reversed when the HFD was changed to chow after early- obesity (137 ± 15) (Fig 5.3A). The mice fed with HFD (12 ± 4) exhibited a lower glucose sensitivity when compared to the mice from the CTR group (162 ± 67) (Fig 5.3B). Insulin resistance index was also calculated (Fig 5.3C) and was concurrent with the observations regarding β-cell function, i.e. mice in HFD group presented a higher index (11 ± 2) in comparison to mice from CTR group or those subjected to dietary correction (1.9 ± 0.4).

Table 5.*2*. Body weight of mice during the experimental work. The mice were fed with standard chow (CTRL), early-life high-fat diet (HFD) or subjected to diet correction after 60 days (HFDt). Total body weight evolution through all the experiment. Weight was evaluated individually every 7 days. Results were tested by repeated measures (RM) two away ANOVA with Tukey's multicomparison test. Results are expressed as mean ± SEM (N=5 for each group). $^{\#}$CTR vs HFD; $^{\varphi}$CTR vs HFDt; ‡HFD vs HFDt.

Days	Conditions (weight, g)		
	CTR	HFD	HFDt
0	8.6 ± 0.2	6.2 ± 0.1	7.8 ± 0.3
7	15.1 ± 0.4	12.8 ± 0.2	15.8 ± 0.6$^{\varphi}$
14	17.7 ± 0.5	19.1 ± 0.4	21.3 ± 0.7$^{\varphi}$
28	20.2 ± 0.6	23.8 ± 0.5$^{\#}$	25.8 ± 0.9$^{\varphi}$
49	23.6 ± 0.4	30.7 ± 0.9$^{\#}$	33.2 ± 1.2$^{\varphi}$
56	23.6 ± 0.5	31.9 ± 0.9$^{\#}$	34.6 ± 1.4$^{\varphi,\ddagger}$
140	27.8 ± 0.5	48.5 ± 0.6$^{\#}$	31.7 ± 0.6$^{\varphi,\ddagger}$
147	29.7 ± 0.4	49.2 ± 0.7$^{\#}$	32.5 ± 0.8‡
196	30.1 ± 0.8	47.3 ± 1.7$^{\#}$	31.1 ± 1.3‡

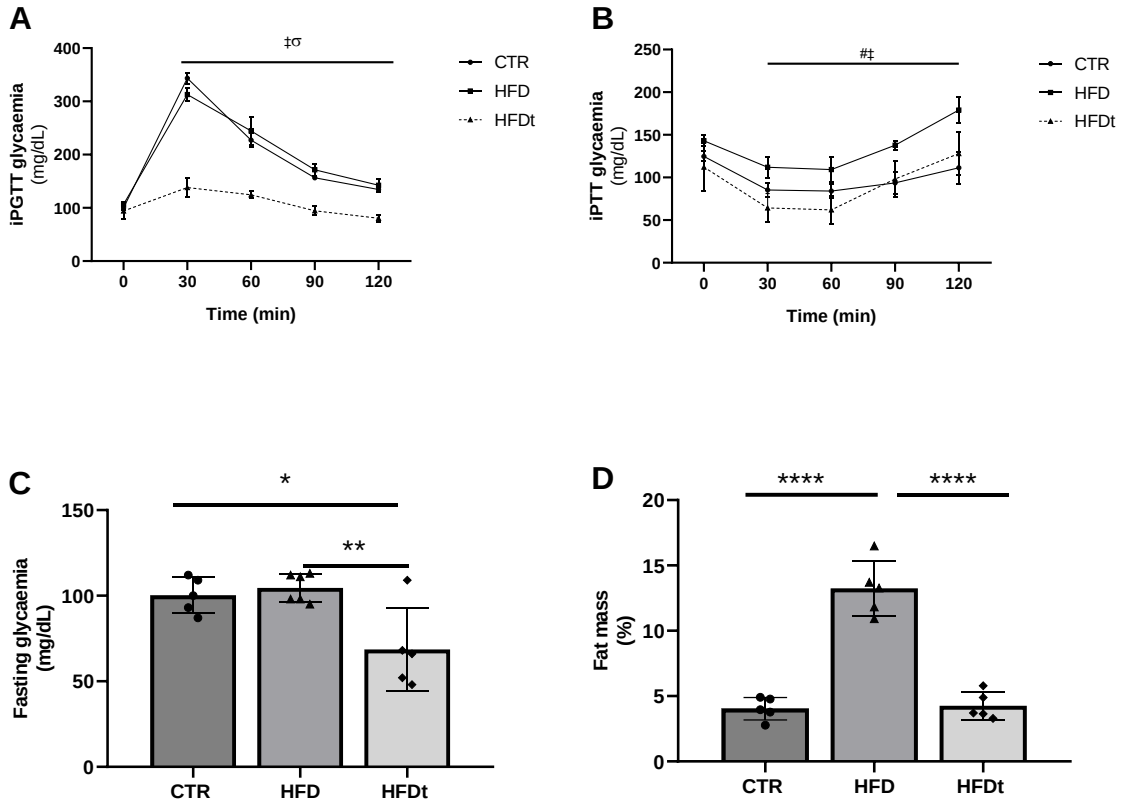

Fig.5.2- Metabolic characterization of mice in the experimental groups: fed with standard chow (CTR); early-life high fat diet (HFD) or to high-fat diet during 60 days and then standard chow until 200 days (HFDt). Results are expressed as mean ± SEM (N=5 for each group). (A) Mice glycaemia during the iPGTT-intraperitoneal glucose tolerance test and (B) during the ipITT-intraperitoneal insulin multicomparison test. (C) Fasting glucose before sacrifice and fat mass (%) (D). * P< 0.05, **P<0.005, ***P<0.0005 and **** P<0.0001. Data was compared using ordinary one-way ANOVA with Tukey´s multicomparison test. #CTR vs HFD; ºCTR vs HFDt; ‡HFD vs HFDt. * P< 0.05, **P<0.005,***P<0.0005 and ****P<0.0001.

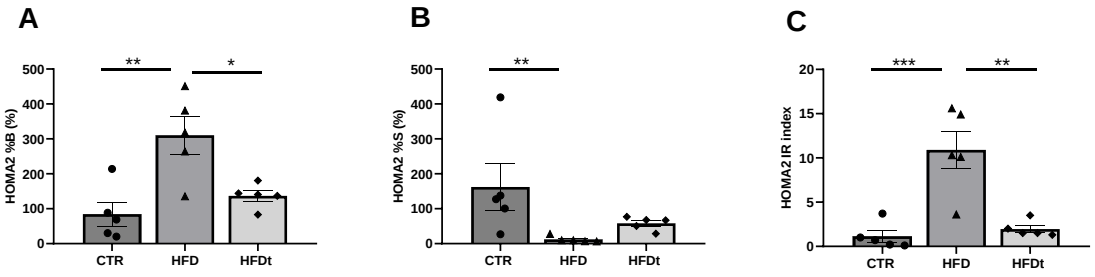

Fig.5.3- HOMA2 indexes of mice in the experimental groups: fed with standard chow (CTR); high fat diet (HFD) or high-fat diet during 60 days and then standard chow until 200 days (HFDt). Results are expressed as mean ± SEM (N=5 for each group). Fasting glycemia and insulin were used as inputs for the interactive HOMA2 calculator (University of Oxford, UK) and (A) represents the β-cell function (HOMA2 %B), (B) represents the insulin sensitivity (HOMA 2% S) and (C) HOMA 2 Insulin resistance (IR) index. Data was compared using ordinary one-way ANOVA with Tukey´s multicomparison test. #CTR vs HFD; ºCTR vs HFDt; ‡HFD vs HFDt. * P< 0.05, **P<0.005 and ***P<0.0005.

5.3.2. Dietary correction after early life HFD consumption is not able to mitigate the disruption of kidney mitochondrial dynamics

We also evaluated the impact of HFD consumption and diet correction on kidney mitochondrial dynamics. Our results show that PGC1-α relative expression decreased in the kidney of mice fed with HFD (0.20 ± 0.04-fold variation to control) and in mice fed with HFD early-life and then subjected to dietary correction (0.3 ± 0.1-fold variation to control), in comparison to CTR group (1 ± 0.2-fold variation to control) (Fig 5.4A). In addition, HFD had an impact on kidney mitochondrial fusion. Indeed, the levels of MFN1 in the kidney of HFD mice increased (2.3 ± 0.4-fold compared to control) and reverting the diet after early-obesity did not reduce these levels to those detected in the CTR group (Fig 5.4B). No alterations were observed in the relative expression of MFN2 in the kidney of all groups (Figure 5.4C). Mitochondrial fission is a regulated process that maintains the mitochondria and cell health. The levels of genes associated with fission (FIS1) decreased in the kidney of mice fed with HFD (0.4 ± 0.1-fold variation to control) and HFDt (0.6 ± 0.1-fold variation to control) groups in comparison to the levels detected in the kidney of control mice (Fig 5.4D). The relative expression of DRP1 levels were also decreased in the kidney of mice from HFDt (0.4 ± 0.1-fold variation to the control) in comparison to the CTR (Figure 5.4E). Finally, OPA1 is responsible for fusion in the inner membrane of the mitochondria. The kidney of mice from HFDt (0.2±0.02-fold variation to control), presented a decreased expression levels of OPA1 when compared with the levels detected in the kidney of mice from the HFD group and from CTR group (Figure 5.4F).

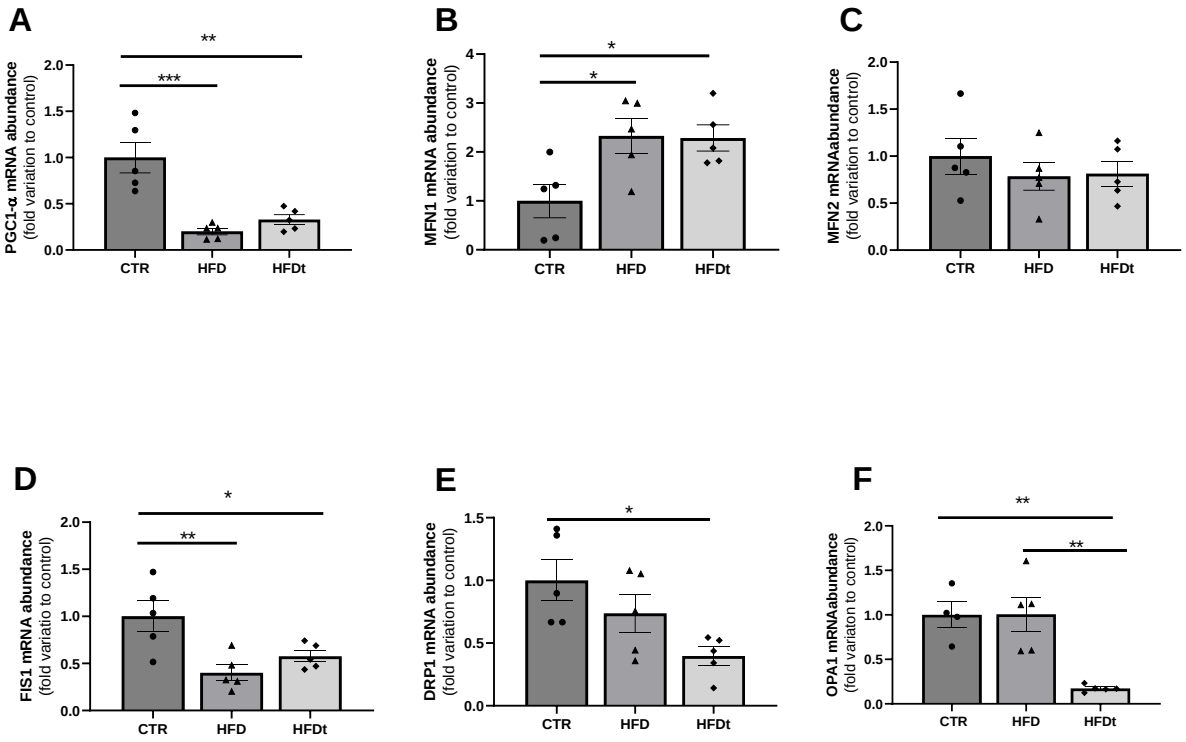

Fig. 5.4 – Quantification of mRNA abundance of genes involved in mitochondrial dynamics in kidney tissue of mice fed with standard chow (CTR); early-life high fat diet (HFD) or to high-fat diet for 60 days and then standard chow until 200 days (HFDt). (A) mRNA abundance of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), (B) mitofusin 1 (MFN1), (C) mitofusin 2 (MFN2), (D) mitochondrial fission 1 protein (FIS1), (E) dynamin-related protein (DRP1) and (F) mitochondrial optic atrophy 1 (OPA1). Results are expressed as mean ± SEM (N=5 for each group). Data were tested with one-way ANOVA with the Tukey's HDS correction of group comparison. Significance was considered when P<0.05. * P<0.05, ** P<0.005 and ***P<0.0005.

5.3.3. Dietary correction after early-life HFD consumption is not able to mitigate the effects in kidney mitochondrial and antioxidant activities

Mitochondrial proton circuit affects kidney functions. Thus, we investigated whether consumption of HFD or its reversal to normal diet affects electron transport chain complexes in kidney. Complex I (CI) activity was increased in the kidney of mice from HFD group (1666000 ± 65500 NADH oxidized per $min^{-1}mg^{-1}_{protein}$) in comparison to the CTR group (394000 ± 78100 NADH oxidized per $min^{-1}mg^{-1}_{protein}$) (Fig 5.5A), while complex II(CII) had a decreased activity in the kidney of mice from the HFDt (0.3 ± 0.1 DCPIP reduced $min^{-1}mg^{-1}_{protein}$) in comparison to the CTR group (Figure 5.5B). Complex IV (CIV) and Citrate Synthase (CS) activity (Fig 5.5C and D) did not present any differences in the kidney of mice from any of the groups.

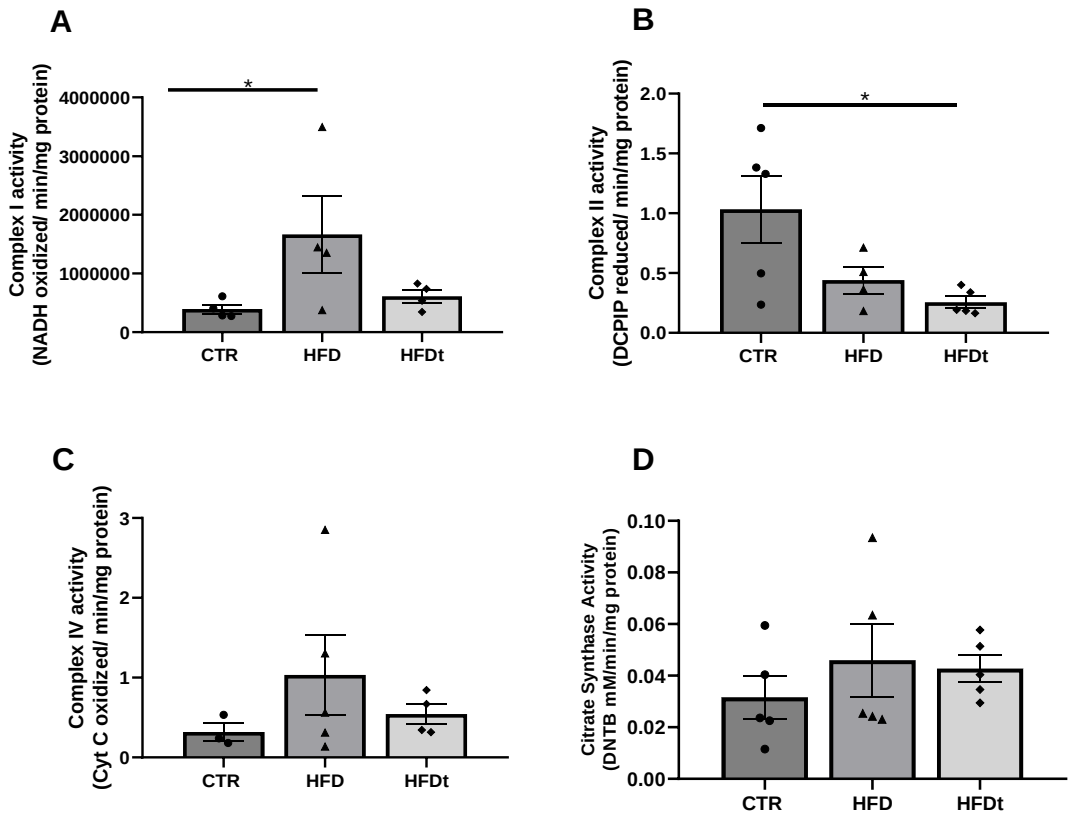

Fig. 5.5 - Mitochondrial complexes activity in kidney tissue of mice fed with standard chow (CTR); early-life high fat diet (HFD) or to high-fat diet for 60 days and then standard chow until 200 days (HFDt). Complex I was followed by the oxidization of NADH (A), complex II was evaluated through DCPIP reduction (B), while complex IV was evaluated by the oxidation of cytochrome C (C) Citrate synthase was also evaluated by the reduction of DTNB (D). Results are expressed as mean ± SEM (N=5 for each group). Data were tested with one-way ANOVA with the Tukey's HDS correction of group comparison. Significance was considered when *P<0.05.

Under normal physiological conditions, the equilibrium between reactive oxygen species (ROS) production and detoxification by the antioxidant defense system is tightly regulated. Since we observed a disruption in OXPHOS function, we evaluated the activity of antioxidant enzymes, particularly Manganese-dependent Superoxide Dismutase (MnSOD), Glutathione Reductase (GR), and Glutathione Peroxidase (GPx). MnSOD activity was increased in the kidney of mice subjected to diet reversion (43.1 ± 4.2 U $min^{-1}mg^{-1}_{protein}$) in comparison to the levels detected in the kidney of HFD-fed mice (12.1 ± 2.7 U $min^{-1}mg^{-1}_{protein}$) (Fig 5.6A). Furthermore, GR activity was increased in the kidney of mice fed with HFD (1.2 ± 0.2 $_{nmol}NADPHmin^{-1}mg^{-1}_{protein}$) when compared to the levels detected in control mice (0.4 ± 0.1 $_{nmol}NADPHmin^{-1}mg^{-1}_{protein}$) (Fig 5.6B). The dietary regime did not alter the activity of kidney GPx activity (Fig 5.6C), however its slight increase in mice fed with HFD.

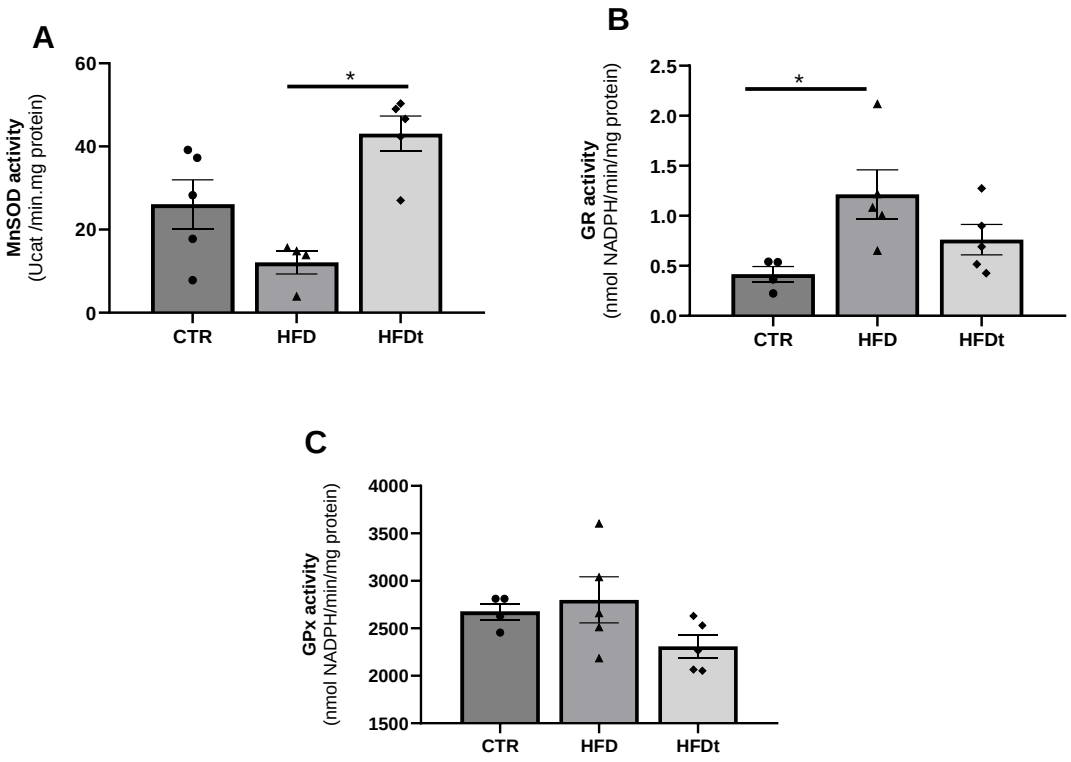

Fig. 5.6 - Activity of antioxidant defenses in kidney tissue of mice fed with standard chow (CTR); early-life high fat diet (HFD) or to high-fat diet for 60 days and then standard chow until 200 days (HFDt). Manganese-dependent Superoxide dismutase (MnSOD) (A), glutathione-disulfide reductase (GR) (B) and glutathione peroxidase (GPx) (C). Results are expressed as mean ± SEM (N=5 for each group). Data were tested with one-way ANOVA with the Tukey's HDS correction of group comparison. Significance was considered when *P<0.05.

5.3.4. Proteomic characterization of kidney mitochondrial enriched fractions

Taking in consideration the differences in mitochondrial dynamics and OXPHOS complexes activities in the kidney of mice on different diets, we used proteomics to identify the biological processes and protein targets in the kidney's enriched mitochondria fraction. A total of 1139 proteins were identified with at least two peptides and a false discovery rate of 1% (Supplementary table S1 in Annex section). Among these, 746 proteins were consistently detected in a minimum of 2 animals per group (Supplementary Fig A2.1, Annex section).

To observe group clustering, both unsupervised PCA and supervised PLS-DA were applied to proteins common to all group's (Fig 5.7A and B). Regarding PCA, notable intragroup variability is observed, particularly within the CTR group. The PLS-DA score plot indicated that kidney mitochondrial fractions from all groups were distinct, suggesting unique protein expression patterns that differentiate the three experimental groups. To identify proteins with statistically significant changes between the study, volcano-plot analyses were

performed. Quantitative analysis was performed to highlight the most abundant proteins using Log2 ratio of iBAQ values across groups, with only two missing values considered in each group **(**Supplementary Fig A2.2; tables A2.1, A2.2, A2.3. and A2.4 in Annex section). Selected proteins are highlighted in Table 5.3. The proteins were selected based on their significant p-values < 0.05, highlighting their higher impact across the various dietary regimens. The dysregulated proteins associated with dietary regimens predominantly represent biological functions related to FAO, glycine metabolism, glutathione and glycolytic processes and TCA metabolism. Less represented functions include mitochondria functioning, renal transporters related to proton transporters and other amino acid metabolic processes. Additionally, peroxisomal proteins are also dysregulated across the different groups. HFD effectively modulated the metabolic phenotype. Acyl-coenzyme A synthetase 3 (Acsm3) emerged as significantly altered, alongside with Acsm1 regulation, crucial in initiating FAO metabolism. Acsm1 (0.669- fold change) and Acsm3 (0.939-fold change) are downregulated in the kidney of HFD group, when compared with the CTR group. However, diet reversion was able to restore Acsm1 levels. Conversely, following early-obesity and diet reversion, Acsm3 was upregulated in the kidney when compared to the CTR (0.323-fold change) and with HFD group (1.319-fold change), suggesting altered FAO. Similar trend was observed with isovaleryl-CoA dehydrogenase (Ivd). Moreover, 3-ketoacyl-CoA thiolase (Acca2), a mitochondrial protein, and 3-ketoacyl-CoA thiolase (Accad1), a peroxisomal protein, were upregulated in enriched mitochondria fractions in the kidney of HFD-fed mice. Diet reversion after early-obesity was able to restore Accad1 but not Acca2, which remained upregulated (0.338-fold change). Other peroxisomal-associated proteins in the enriched mitochondrial fractions of kidney were also diet-sensitive. Specifically, peroxisomal trans-2-enoyl-CoA reductase (Pecr) protein was downregulated in the HFD group (0.432-fold change) when compared to the CTR group. Glycine metabolism, represented by several proteins (Keg1, Aldhl1, Dao and Glyat), was found to be downregulated in enriched mitochondria fractions of kidney from HFD-fed mice. Proteins involved in glycolysis, such as ATP-dependent dihydroxyacetone kinase (Dak), which catalyzes the phosphorylation of dihydroxyacetone and glyceraldehyde; and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were upregulated in kidney enriched mitochondria fractions from HFD-fed mice, suggesting glycolysis stimulation. However, these proteins may also function in different cellular compartments and have alternative roles (Fujisawa et al., 2009; Werelusz et al., 2024). Other processes related to amino acids metabolism were also downregulated in the enriched mitochondrial fractions of kidney from HFD-fed mice, including fumarylacetoacetate hydrolase domain-containing protein 2A (Fahd2a), methylmalonyl-CoA epimerase, Mut (succinate metabolic process), proline dehydrogenase 1, Prodh (Proline catabolic process), and proteins involved in glutathione

metabolism such as glutathione S-transferase A3, Gsta3, and glutathione hydrolase 1 heavy chain, Ggt1. Furthermore, proteins associated with kidney function were increased in the kidney enriched mitochondrial fraction of HFDt group, including Na(+)/H (+)exchange regulatory cofactor NHE-RF3 (Pdzk1), and Na(+)/H (+)exchange regulatory cofactor NHE-RF1(Slc93r1).

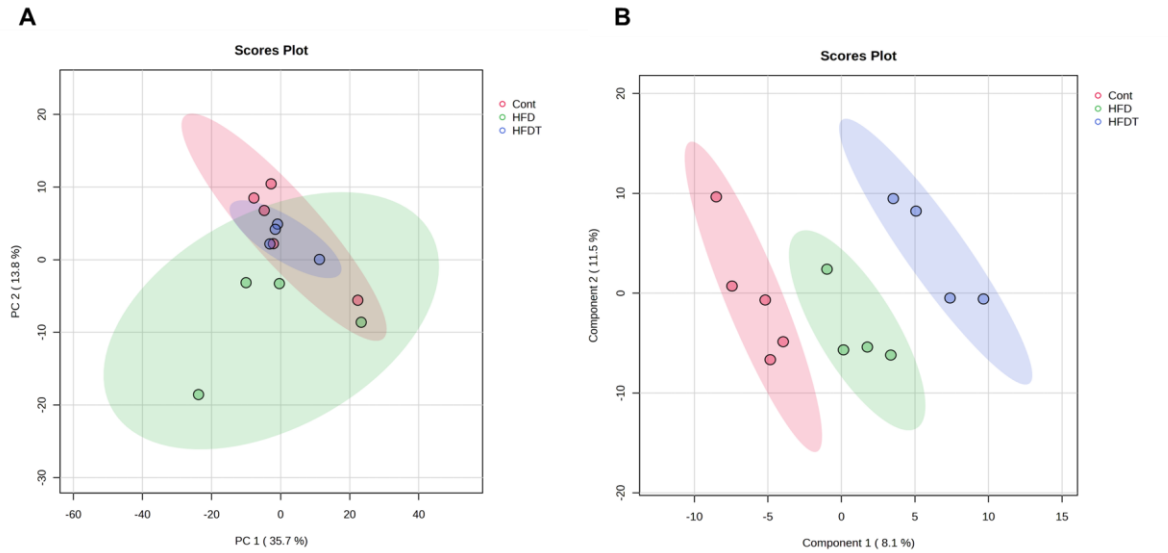

Fig. 5.7- (A) Principal component analysis (PCA) of the proteomics data of kidney mitochondrial enriched fractions of mice fed with standard chow (CTR); early-life high fat diet (HFD) or to high-fat diet during 60 days and then standard chow until 200 days (HFDt). (B) orthogonal partial least-square discriminant analysis (PLS-DA) score showing the differential metabolic features of the in the different experimental groups. Control group (CTR) is shown in pink, (HFD) high fat diet group in green and the HFDt group in blue. The ellipses represent 95 % confidence intervals for each experimental group.

Table 5.3. Biological function of the most significant consistently changed proteins in enriched mitochondrial fractions of kidney mice fed with a chow diet (CTR), with high-fat diet (HFD) and with diet reversion after 60 days of weaning (HFDt). Significance was considered for ($p<0.05$). The downregulated are represented with (-) and the upregulated proteins with positive values.

			Fold change (log2)			
Gene Name	**Protein Name**	**UniProt Accession**	**CTR vs. HFD**	**HFD vs. HFDt**	**CTR vs. HFDt**	**Biological process**
Acsm3	Acyl-coenzyme A synthetase mitochondrial	Q3UNX5	0.939	(-)1.319	-0.323	Fatty acid beta-oxidation
Acaa2	3-ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	(-)0.646	0.311	-0.338	Fatty acid beta-oxidation
Keg1	Glycine N-acyltransferase-like protein	Q9DCY0	0.448	(-)0.459	-	Glycine metabolic process
Q922Q1	Mitochondrial amidoxime	Q922Q1	0.351	-	0.366	Nitrate metabolic process

	reducing component 2					
Mep1a	Meprin A subunit alpha	P28825	0.634	(-)0.528	-	Dipeptidyl-peptidase activity
Acsm1	Acyl-coenzyme A synthetase 1, mitochondrial	Q91VA0	0.669	(-)0.425	-	Fatty acid beta-oxidation
Aldh1l1	Cytosolic 10-formyltetrahydrofolate dehydrogenase	Q8R0Y6	0.402	(-)0.353	-	Glycine biosynthetic process from serine
Gsta3	Glutathione S-transferase A3	P30115	0.587	(-)0.327	-	Glutathione metabolic process
Dak	ATP-dependent dihydroxyacetone kinase	Q8VC30	(-)0.650	0.528	-	Glycolytic process
Dao	D-amino-acid oxidase	P18894	0.280	(-)0.297	-	Glycine biosynthetic process from serine, peroxisomal protein import
Pter	Phosphotriesterase-related protein	Q60866	(-)0.356	0.307	-	Hydrolase activity, acting on ester bonds and endodeoxyribonuclease activity
Amacr	Alpha-methylacyl-CoA racemase	O09174	0.286	(-)0.349	-	Fatty beta-oxidation using acyl-CoA Oxidase / Protein import into peroxisome matrix
Ivd	Isovaleryl-CoA dehydrogenase, mitochondrial	Q9JHI5	0.395	(-)0.397	-	Fatty acid beta oxidation using acyl-CoA dehydrogenase
Cndp2	Cytosolic non-specific dipeptidase	Q9D1A2	0.318	(-)0.480	-	Carnosine metabolic process
Slc3a2	4F2 cell-surface antigen heavy chain	P10852	0.448	-	0.337	L-leucine import across plasma membrane; valine transport, Phenyalanine transport
Atp6v1h	V-type proton ATPase subunit H	Q8BVE3	0.266	(-)0.232	-	Vacuolar proton-transporting V-type ATPase
Aadat	kynurenine/alpha-aminoadipate aminotransferase, mitochondrial	Q3UNS8	0.389	(-)0.473	-	L-Kynurenine catabolic process
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	P16858	(-)0.482	-	-	Canonical glycolysis
Acly	ATP-citrate synthase	Q91V92	0.405	-	-	Citrate metabolic process

Pecr	Peroxisomal trans-2-enoyl-CoA reductase	Q99MZ7	0.432	(-)0.517	-	Fatty acid beta-oxidation using Acyl-CoA oxidase, protein import into peroxisome matrix
Prodh	Proline dehydrogenase 1, mitochondrial	Q9WU79	0.436	(-)0.462	-	Proline catabolic process to glutamate
Hk1	Hexokinase-1	P17710	0.342	-	-	Glucose 6-phosphate transport
Ckmt1	Creatine kinase U-type, mitochondrial	P30275	0.348	(-)0.252	-	Mitochondrial ATP/ADP transmembrane transport
Ehhadh	Enoyl-CoA hydratase/3,2-trans-enoyl-CoA isomerase	Q9DBM2	0.574	-	-	Fatty acid beta-oxidation using Acyl-CoA oxidase
Ces1d	Carboxylesterase 1D	Q8VCT4	(-)0.629	-	-	Monocarboxylic acid metabolic process
Pdzk1	Na(+)/H (+) exchange regulatory cofactor NHE-RF3	Q9JIL4	-	(-)0.669	(-)0.575	Positive regulation of inorganic anion transmembrane transport
Slc9a3r1	Na(+)/H (+) exchange regulatory cofactor NHE-RF1	P70441	-	(-)0.831	(-)0.879	Positive regulation of inorganic anion transmembrane transport
Pcx	Pyruvate carboxylase, mitochondrial	Q05920	-	0.294	-	Glycerol biosynthetic process from pyruvate
Hao2	Hydroxyacid oxidase 2	Q9NYQ2	-	0.481	-	Glyoxylate metabolic process
Slc22a12	Solute carrier family 22 member 12	Q8CFZ5	-	(-)0.872	-	Renal urate salt excretion
Lap3	Cytosol aminopeptidase	Q9CPY7	-	(-)0.153	-	Glutathione catabolic process
Acaa1b	3-ketoacyl-CoA thiolase B, peroxisomal	Q3TUU3	-	0.554	-	Fatty beta-oxidation using Acyl-CoA oxidase
Shmt2	Serine hydroxymethyltransferase, mitochondrial	Q9CZN7	-	(-)0.292	-	Histidine biosynthetic process
Ndufa5	ADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5	Q9CPP6	-	0.267	-	Mitochondrial respiratory chain complex I assembly
Aldh2	Aldehyde dehydrogenase, mitochondrial	P47738	-	(-)0.282	-	Monoamine oxidase activity

Mut	Methylmalonyl-CoA epimerase, mitochondrial	P16332	-	(-)0.404	-	Succinate metabolic process
Gsr	Glutathione reductase, mitochondrial	P47791	-	(-)0.312	-	Glutathione biosynthetic process
Pabpc1	Polyadenylate-binding protein 1	P29341	-	(-)0.370	(-)0.370	Positive regulation of cytoplasmic translation
Hibadh	3-hydroxyisobutyrate dehydrogenase, mitochondrial	Q99L13	-	0.199	-	Valine catabolic process
Ggt1	Glutathione hydrolase 1 heavy chain	Q60928	-	(-)0.654	-	Glutathione biosynthetic process
Glyat	Glycine N-acyltransferase; Mitochondrial acyltransferase	Q91XE0	-	(-)0.465	-	Benzoyl-CoA metabolic process
Hspa9	Stress-70 protein, mitochondrial	P38647	-	(-)0.174	-	Protein import into mitochondrial matrix
Fahd2a	Fumarylacetoacetate hydrolase domain-containing protein 2A	Q3TC72	-	(-)0.292	-	Tyrosine catabolic process
Eef1a1	Elongation factor 1-alpha 1	P10126	-	0.321	(-)0.468	Cytoplasmic translation
Atp5a1	ATP synthase subunit alpha, mitochondrial	Q03265	-	0.216	-	Proton motive force-driven mitochondrial ATP synthesis
Pck1	Phosphoenolpyruvate carboxykinase, cytosolic	Q9Z2V4	-	0.424	-	Citrate metabolic process
Acox3	Peroxisomal acyl-coenzyme A oxidase 3	Q9EPL9	-	-	(-)0.379	Fatty-acid beta-oxidation
Fgg	Fibrinogen gamma chain	Q8VCM7	-	-	(-)0.603	Blood coagulation
Acy3	N-acyl-aromatic-L-amino acid amidohydrolase	Q91XE4	-	-	0.325	Aminoacylase activity

5.4. Discussion

The impact of HFD on the progression of kidney disease and the potential mitigating effects of dietary correction remain unknown. Our results demonstrate that HFD-fed mice gained weight and presented dysregulated ipGTT and ipTT, as previously reported (Crisóstomo et al., 2019). Dietary intervention after early-obesity was able to reestablish the insulin resistance index and systemic glucose homeostasis. However, the deleterious effects on kidney mitochondrial quality control mechanisms and associated proteins that primarily affect FAO caused by HFD consumption are not fully recovered. Analysis of enriched mitochondria kidney extracts revealed multiple changes in proteins involved in different mitochondrial-related pathways. One notable alteration was the decrease of kidney glycine metabolism in HFD-fed mice, which was reverted with dietary correction, as evidenced by D-amino-acid oxidase (Dao) protein. Dao is primarily located in the kidney and catalyzes the oxidation of D-serine, producing ammonia (Koga et al., 2017). Approximately 10% of renal ammonia production originates from the conversion of glycine to serine in the proximal tubule, a process involved in the synthesis of gluconeogenic precursors (Brosnan, 1987). DAO's breakdown of D-serine may cause kidney toxicity through production of hydrogen peroxide and ROS (Krug et al., 2007), thereby disrupting kidney metabolism. Proteins involved in FAO also showed distinct patterns. Acsm3 plays a crucial role in activating fatty acid metabolism (Cazarin & Altman, 2024). Our findings suggest a trend towards upregulation of Acsm3 expression in enriched mitochondria fractions from kidneys' of HFDt group when compared to the other groups. This upregulation of Acsm3 appears to be more associated with kidney function rather than diabetes onset. In a rat model characterized by hypertriglyceridemia, obesity, and IR, liver transcriptomics showed that Acsm3 expression was suppressed, implicating Acsm3 downregulation in fat accumulation and hypertriglyceridemia (Junková et al., 2021). Acaa2, responsible for catalyzing the final step of the mitochondrial β-oxidation pathway, was also found to be altered. Our proteomic analysis revealed that it was upregulated after early-life HFD consumption, suggesting that HFD, even temporarily, has enduring effects on kidney FAO. Under these stress conditions, peroxisomal proteins were also altered, with Pecr being downregulated in response to HFD group. A proteomic analysis on glomeruli and kidney cortex of mice with early DKD showed a downregulation of peroxisomal proteins, Pecr and Enoyl-CoA hydratase/3,2-trans-enoyl-CoA isomerase (Ehhadh) at early stages. Furthermore, low levels of EHHADH mRNA were detected in human DKD patients (Gholaminejad et al., 2021). These findings underscore the potential impact of dietary intervention in restoring the crosstalk between mitochondria and peroxisomes (Tserga et al., 2022). Mitochondria and peroxisomes closely interact to regulate cellular metabolism. Chain-shortened acyl-CoAs are synthesized through the

oxidation of very long FFA within peroxisomes, yet their complete oxidation occurs in mitochondria. The downregulation of peroxisome-related lysates of HFD-fed mice highlights the disruption of this peroxisome-mitochondria interplay. Mitochondria and peroxisomes share essential components of membrane fission machinery, such as FIS1 (Koch et al., 2005) and DRP1 (Koch et al., 2003; Lee et al., 2004) and collaborate in anti-viral signaling and defense mechanisms (Demarquoy & Le Borgne, 2015). In stressful conditions, dysregulation induces peroxisomal fragmentation and mitochondrial elongation. Previous research suggests that mitochondrial fragmentation resulting from peroxisome depletion is mediated by the translocation of Drp1 from peroxisomes to mitochondria (Tanaka et al., 2019). Drp1 is identified as a mediator of mitochondrial fragmentation and subsequent cytochrome-c release in cases of peroxisomes deficiency (Lee et al., 2004). As these organelles share the same machinery, dysfunction in one can impact the other. A HFD increases lipids availability, leading to a higher peroxisomes' biogenesis (Piao et al., 2019). However, peroxisome dysfunction can cause lipid accumulation and mitochondrial dysfunction through mechanisms such as OS, OXPHOS dysfunction and mitochondrial depolarization (Lismont et al., 2015; Waterham et al., 2016). In our results, this was reflected in mitochondria dynamics. PGC-1α, the master regulator of mitochondrial biogenesis (Gureev et al., 2019), was decreased in both kidney fractions of HFD-fed and HFDt-fed mice. Prem P and collaborators, showed that in rats subjected to a HFD, kidney had lower mitochondria copy numbers and diminished gene expression of PGC-1α (Prem & Kurian, 2021). This underscores impaired mitochondrial biogenesis, consequently affecting the modulation of fission/fusion due to dietary regimens. We observed an increase in mitochondrial fusion in kidney of HFD-fed mice with dietary correction after early-life obesity unable to revert those effects. However, the expression of OPA1, which mediates inner membrane fusion, was decreased after early-obesity and dietary correction. Additionally, fission in the kidney is compromised in HFD-fed and HFDt-fed mice as both FIS1 and DRP1 expression decreased. Navarro A and colleagues established an obese mouse model, where after 14 weeks of a HFD, there was an increase in renal Drp1 protein expression, but no alteration in renal Mfn1/2. These findings suggested increased mitochondrial fragmentation and damage due to oxidative and endoplasmic reticulum-stress following HFD consumption (Sánchez-Navarro et al., 2021). Our data suggests that dietary correction after early-obesity is unable to fully reverse the damage caused by consumption of HFD in mitochondria-peroxisome crosstalk. Additionally, a protective role for FIS1 in DKD has been suggested (Kato et al., 2021). In cases of acute renal injury, overexpression of FIS1 can lead to mitochondrial fragmentation, nephrotoxicity, cell death, pyroptosis, and mitochondrial division (Yi et al., 2022). *Fis1* gene expression is lower in diabetic mice and decreased in mouse glomerular mesangial cells exposed to high glucose levels (Kato et al.,

2021). Since FAO impairment is linked to mitochondrial (dys)function (Chen et al., 2021), we evaluated the activities of mitochondrial complexes after different dietary regimens. CI activity was increased in kidney HFD-fed mice, while kidney CII activity was only affected after early-obesity and diet correction. Additionally, MnSOD activity was decreased in the kidney of HFD-fed mice but diet correction after early-obesity was able to reestablish MnSOD activity. CI is a well-known site of ROS production in the mitochondria. Furthermore, an *in vivo* model showed that HFD induces higher levels of MnSOD in the kidney (Sun et al., 2020), suggesting a response to increased ROS production. These effects can be attenuated when CI is inhibited, decreasing H_2O_2 release and mitochondrial membrane potential. This led to the hypothesis that early OS-related changes, but not mitochondrial bioenergetic dysfunction per se, contribute to the onset and development of obesity-linked DKD (Ruggiero et al., 2011). Indeed, in a rat model of diet-induced obesity, Yu H. and colleagues observed a reduction of CII activity, similar to our results, after eight weeks of HFD (Yu et al., 2018). HFD aggravated mitochondrial damage, causing dysregulation of TCA and OXPHOS. Our results showed a decrease in CII activity, which may be compensated by increased CI activity, contributing to an increase in ROS production, and impairment of mitochondria (Yu et al., 2018). This, in turn, induces mitochondrial dysfunction beyond impaired ATP generation. Notably, mutations affecting TCA enzymes are implicated in predisposing kidney tubular epithelium to transformation (Sciacovelli et al., 2020). When TCA is inhibited, total glutathione (GSH) levels increase, indicating a disturbance in redox homeostasis (Ryan et al., 2021; Sreekumar et al., 2021). Additional enzymes with antioxidant activity that support the intracellular balance of GSH include GR and glutathione peroxidase (Sies, 1999). Although the redox status and tissue GSH concentrations are genetically regulated, it is unknown how food and genetic background interactions impact GSH homeostasis. Disruption in GSH metabolism turns organelles more vulnerable to stress-induced damage, culminating in tissue remodeling, and to the onset renal disorders (Dong et al., 2023). GR activity is reported to be downregulated in erythrocyte, liver, heart and kidney in rats fed with HFD for 10 weeks, when compared to control group (Muthulakshmi & Saravanan, 2013). Also, in albino rats, where after 16 weeks of an HFD it was observed a lower GR activity in kidney extracts (Noeman et al., 2011). This is contradictory to our results, where we observed that GR activity was increased in HFD-fed mice, whereas no alterations were noted in HFDt-fed mice. Similar to our results, others demonstrated that the ratio of GSH/GSSG expression levels in HFD (10 weeks) are higher in renal tissue in AKR mice, a strain susceptible to development of hypertension and metabolic syndrome, when compared to B6 and D2 mice (Norris et al., 2016). Moreover, enzymatic activity of GR from enriched fraction of kidney tissue was increased in Sprague-Dawley rats in a HFD condition (6 months), when

compared to rats fed with a control diet (Fejerčáková et al., 2012). Dysregulated GSH metabolism results in unbalanced redox and excessive production of ROS, which will trigger off mitochondria-mediated intrinsic cell apoptosis and lipid peroxidation-related cell ferroptosis (Dong et al., 2023). This further aligns with our proteomic analysis, which revealed upregulation of proteins associated with glutathione metabolism (Ggt1), and upregulation of Gsta3 in HFDt-fed mice suggesting an imbalance of GSH/GSSG.

In conclusion, our findings demonstrate that dietary correction after childhood obesity mitigates the systemic metabolic dysfunction and can attenuate kidney mitochondria dysfunction but is unable to completely restore mitochondria dynamics and bioenergetics. The results highlight the integrity of mitochondrial network as a main point for targeted therapeutic strategies aimed at preventing the progression of kidney disease.

5.5. Authors contribution

Patrícia C. Braga: Conceptualization, Data curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Validation, Visualization, Writing-original draft and Writing-review & editing; Rui Vitorino: Formal analysis, Methodology, Software, Validation, Visualization and writing-review & editing, Rita Ferreira: Formal analysis, Methodology, Software, Validation, Visualization and writing-review & editing; Mariana Marques: data curation and investigation; Pedro F. Oliveira: Conceptualization, Project administration, Resources, Supervision, Validation and Writing-review & editing; Anabela S. Rodrigues: Conceptualization, Methodology, Project administration, Supervision and Writing-review & editing; Marco G. Alves: Conceptualization, Funding Acquisition, Methodology, Project Administration, Resources, Supervision, Validation, Visualization and Writing-review & editing.

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Chapter 6

Metabolic profile of urine of albuminuric and non-albuminuric nephropathic diabetic patients suggests TCA-cycle related biomarkers

This chapter was adapted from the unpublished work:

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Abstract

Diabetic kidney disease (DKD) has emerged as the most prevalent and life-threatening consequence for patients with diabetes. It is characterized by an increase in albuminuria levels, however, ~25% of DKD population is non-albuminuric, creating a grey zone for diagnosis. We aimed to investigate possible metabolic biomarkers in the urine of healthy and DKD patients with and without albuminuria (A-DKD and NA-DKD, respectively).

Patients were selected and divided into healthy (n=23) and DKD (n=17) groups. DKD patients were further split into NA-DKD (n=5) and A-DKD (n=12). Urine was analyzed using a non-targeted proton nuclear magnetic resonance(^{1}H-NMR) metabolomics approach to search specific metabolic.

It was possible to establish a distinct metabolic profile between healthy and DKD groups. DKD patients exhibited lower levels of citrate, hypoxanthine, formate, isobutyrate, glycine, phenylacetate, dimethylamine, and valine in urine samples, and higher levels of trans-aconitate, glycolate, and taurine. Moreover, citrate presented the strongest negative correlation with albuminuria (r=-0.65, p<0.0001), followed by hypoxanthine (r=-0.49, p=0.004), isobutyrate (r= -0.45, p=0.004) and formate (r=-0.41, p=0.009). On the other hand, glycolate (r=0.46, p=0.003), taurine (r=0.47, p=0.007), and trans-aconitate (r=0.41, p=0.05) were positively correlated with albuminuria. When stratifying patients with DKD, glycine and alanine were down and upregulated, respectively in NA-DKD when compared to A-DKD patients. Additionally, receiver operating characteristic (ROC) suggested that citrate, hypoxanthine, and taurine are the best predictors for discriminating between non-albuminuric and albuminuric patients in kidney disease progression.

Urinary metabolites related to Krebs cycle and purine metabolism can potentially serve as a marker to individualize therapeutic choices in DKD, especially for non-albuminuric phenotype.

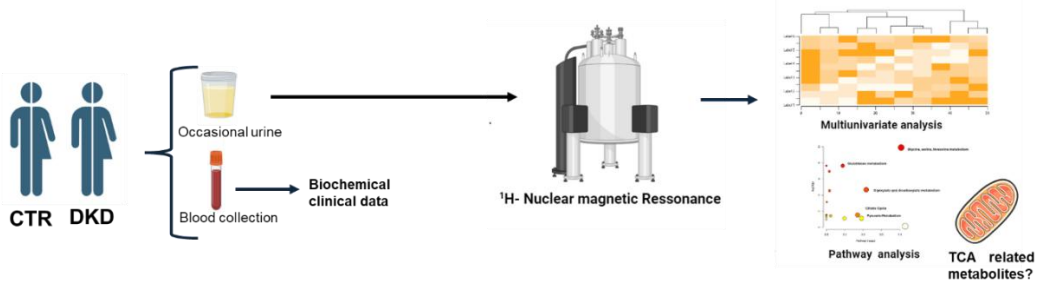

Fig.6 – Graphical abstract. Our work provides evidence that urinary metabolome from healthy patients is different from patients with diabetic kidney disease (DKD) particularly in what concerns metabolites related with mitochondria function, related with Krebs (TCA) cycle and those with antioxidant defense. They are important in the characterization of patients with DKD, especially in non-albuminuric patients.

6.1. Introduction

Diabetic kidney disease (DKD) is a major complication in diabetic patients emerging as a serious clinical and economic burden. It has a high morbidity and death rate, being the main cause of end-stage renal disease (ESRD) (Han et al., 2017). Notably, about 40% of individuals with type 2 diabetes mellitus (T2DM) and 30% of patients with type 1 diabetes mellitus (T1DM) eventually develop DKD (Alicic et al., 2017). In clinical practice, the urinary albumin to creatinine ratio (uACR) >30 mg/g is the major DKD diagnostic criterion, being albuminuria considered the single best predictor of kidney function loss. However, it is reported that 25% of patients with T2DM have little or no albuminuria, but present renal structure alterations, characteristic of DKD (Qi et al., 2017).

To diagnose DKD, the estimated glomerular filtration rate (eGFR) must gradually decline to less than 60 mL/min/1.73 m^2 (Afkarian et al., 2016). Based on epidemiological data, albuminuria may be reversible whereas eGFR can continue to decline until ESRD. The histological features can also support the DKD diagnosis, but renal biopsy is an invasive method (Stiles et al., 2000) with risks, only done in atypical clinical presentations. Serum creatinine and serum cystatin C are used as markers of the eGFR, however, serum creatinine is also influenced by muscle mass and daily protein intake, whereas cystatin C is linked to body weight and disorders associated with obesity and inflammation (Lin et al., 2016). In addition, in the early stages of DKD, glomerular hyperfiltration will result in a higher eGFR (Hou et al., 2024). In conclusion, traditional metrics are not capable of accurately predicting which patients will develop DKD. Thus, more sensitive and specific targets are critically needed for early diagnosis and assessment of the nature and progression of DKD.

The progression of DKD is associated with several metabolic alterations, due to the reprogramming that cells must experience to overcome the exposure to a hyperglycemic environment. In the context of DKD, proximal tubular epithelial cells undergo a metabolic transition from fatty acid oxidation to glycolysis when exposed to elevated glucose levels (Hou et al., 2024; Singh, 2023). Several studies reported an elevated expression of glycolytic enzymes due to enhanced glycolysis in diabetes, which further induces epithelial-mesenchymal transition and exacerbates renal fibrosis (Li et al., 2020; Storch & Corsico, 2008; Yin et al., 2018). Moreover, several metabolites from the Krebs (TCA) cycle accumulate because of reduced mitochondrial function (Sciacovelli et al., 2016), while unmetabolized fatty acids are transformed into triglycerides, then stored as lipid droplets (Donkor et al., 2009), leading to glomerulosclerosis and tubulointerstitial injury (Bobulescu, 2010). Thus, it is crucial to evaluate metabolic alterations.

Metabolomics, a cutting-edge technology, identifies and quantifies metabolites, which are the end products of metabolic reactions. As a result, it can more accurately and promptly

reflect metabolic changes occurring in both healthy and pathological conditions (Shao et al., 2020; Zhu et al., 2022). Moreover, some progresses were already made regarding the study of the possible metabolites altered in DKD that could be found in different biofluids (plasma and urine), as demonstrated by Pereira R and collaborators (Pereira et al., 2022). Among biofluids, urine is easily obtainable and considered a non-invasive sample, making it a viable alternative to biopsy procedures. In this study, we utilized untargeted metabolomics, through proton nuclear magnetic resonance (^{1}H-NMR), to analyze the urine metabolite levels of patients diagnosed with DKD and healthy individuals who were admitted for living kidney donation. We aim to identify metabolic patterns and potential biomarkers of DKD, particularly in non-albuminuric conditions.

6.2. Materials and methods

6.2.1. Study design of the participants

A total of 40 participants, being 17 diagnosed with DKD (eGFR <60 ml/min/1.73 m^2) and 23 healthy subjects (CTR group, presenting glycated hemoglobin (HbA1c) < 6.5 %, eGFR >60 ml/min/1.73 m^2 and without albuminuria), were recruited at the Santo Antonio Hospital, Porto, Portugal. According to Kidney Disease Improving Global Outcomes (KDIGO) guidelines, patients with DKD were classified into two categories based on their albumin levels: those with normal albumin levels (uACR ≤30 mg/g) and those with elevated albumin levels (uACR>30 mg/g) (Awan et al., 2023) as represented in Fig 6.1. All the selected patients were informed of the purpose of the study and the methodology that was going to be used. The privacy and confidentiality of the participants were protected during all the process. The research was performed according with the Santo António University Hospital ethics committee (119-DEFI/122-CE and 178-DEFI/179-CE), and in accordance with the Declaration of Helsinki. The inclusion criteria for patients with DKD were: (1) age above 18 years; (2) diagnosed with T2DM for more than 5 years and (3) not suffering from any other clinically diagnosed severe disease. The inclusion criteria for healthy controls were as follows: (1) age above 18 years; (2) patients recruited for living donor kidney transplantation which were submitted to a standard evaluation according to international guidelines that discarded any significant chronic disease (Lentine et al., 2017).

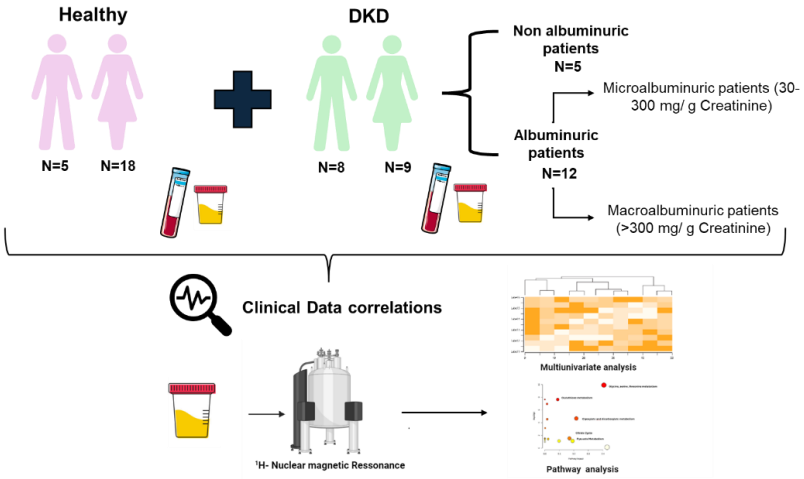

Fig. 6. 1 - Illustration of the experimental design of the work. Occasional urine and blood sample were collected in healthy individuals and in diabetic kidney disease (DKD) patients. For this study 23 (5 men and 18 women) healthy individuals and 17 (8 men and 9 women) patients with DKD were selected. DKD were then split into non (5 patients*; NA-DKD*) and albuminuric (12 patients*; A-DKD)*. Urine and blood were collected. With urine, untargeted ^{1}H-NMR was performed as well as clinical data correlations.

6.2.2. Clinical data acquisition

Data concerning age, gender, weight, BMI, diabetes status, complete blood count, serum creatinine, serum urea, sodium, potassium, chloride, uric acid levels, fasting glucose, glycated hemoglobin, total serum proteins, lipid profile (total cholesterol, Low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL), cholesterol, triglycerides), urinalysis, and measurements of albuminuria, proteinuria and uACR, collected on an occasional urine collection, for every participant in this study. We used the CKD epidemiology Collaboration (CKD-EPI) creatinine equation to calculate eGFR (Levey et al., 2009). To avoid dietary influence, biofluids samples were collected in the morning, after overnight fasting and stored at -80 ºC until lyophilization.

6.2.3. NMR Spectroscopy

The metabolites present in the urine samples were assessed through ^{1}H-NMR and spectra analysis. One dimensional ^{1}H-NMR spectra were acquired on a 500 MHz Bruker Avance III HD spectrometer equipped with a 5mm TXI probe (Bruker Biospin, Karlshruhe, Germany) at 298 K. A zgpr pulse sequence was used to suppress the water, and 32 scans were acquired for each sample. Protein was precipitated from urine samples before ^{1}H-NMR analysis. For that, 30 mg of lyophilized urine was diluted in 105 µL of an ice-cold methanol/water solution (1:2 v/v ratio) and incubated at -20 ºC for 30 min. After that, the samples were centrifuged at 13 000 × g for 30 min. The supernatant was collected and

subsequently dried in a SpeedVac concentrator. Dried samples were resuspended in 225 µL of sodium fumarate in 0.2 M phosphate buffer (pH 7.0) in D_2O solution ([fumarate]= 2mM). Samples were analyzed using 3-mm NMR tubes (Norell, Landisville, NJ, USA) with the NOR5X3INSB optimizer inserts (Norell, Landisville, NJ, USA). Sodium fumarate was used as an internal reference for chemical shifts and metabolite quantification. The quantified metabolites are described in Table 6.1. The spectra were manually phased, and baseline corrected. To integrate the chosen metabolite peaks, NUTS-Pro TM NMR software (Acorn NMR, Inc., Fremont, CA, USA) was used. The concentration of the metabolites is expressed as nmol/mL/ mg of urine.

Table 6. 1. Resonance assignment in the ^{1}H-NMR spectra of urine sample with the listening of all the metabolites identified.

Metabolite	**δ ^{1}H ppm (multiplicity)**
Acetate	1.90 (s)
Alanine	1.48 (d); 3.77 (q)
Benzoate	7.86 (d), 7.54 (t), 7.47 (dd)
Betaine	3.26 (s), 3.90 (s)
Citrate	2.51 (d); 2.67 (d)
Creatinine	3.01 (s); 3.91 (s)
Dimethylamine	2.50 (s)
Formate	8.46 (s)
Glucose	5.22 (d), 4.66 (d)
Glycine	3.54 (s)
Glycolate	3.93 (s)
Hippurate	7.83 (dd);7.63 (tt); 7.54 (m); 3.96 (d)
Hydroxyacetone	2.27 (s); 4.40 (s)
Hypoxanthine	8.19 (s), 8.21 (s)
Isobutyrate	1.05 (td); 1.11 (dt); 1.11 (dd); 1.13 (dd); 2.37 (m); 3.70 (s)
Lactate	1.31 (d); 4.10 (q)
Phenylacetate	3.52 (s); 7.29 (s); 7.37 (s)
Pyruvate	2.46 (s)
sarcosine	2.73 (s); 3.60 (s)
Succinate	2.40 (s)
Taurine	3.26 (t), 3.43 (t)
Trans-aconitate	3.40 (s); 5.85 (s); 6.33 (s)
Trimethylamine	2.89 (s)

Valine	0.97 (d); 1.02 (d); 2.25 (m); 3.59 (d)

6.2.4. Metabolic data analysis

Data normalization was performed using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/) statistical analysis module. Data were normalized by sum, log-transformed, and auto-scaled before univariate and multivariate analyses, and the missing values were replaced by LoD 1/5 of the minimum positive value for their corresponding variable. For multivariate statistical analysis, unsupervised principal component analysis (PCA) and partial least squares-based discriminant analysis (PLS-DA) were performed to find potential clusters in the groups. Differential metabolites were screened through variable importance in projection (VIP). VIP>2 and FDR-adjusted p-value <0.05 were considered differentially expressed metabolites (DEMs). Likewise, variables with Student's *t*-test p<0.05 and univariate analysis |fold change|>1.0 were selected as DEMs. For pathway analysis, we used Enrichment Analysis - Over Representation Analysis in Metaboanalyst 6.0, which evaluates groups of functionality-related metabolites according to the Small Molecule Pathway Database version 2 to observe whether they occur more often than expected (Fischer exact Test).

6.2.5. Statistical analysis

The Shapiro-Wilk test was used to determine the normality of the clinical and experimental data. The clinical data are expressed as the mean ± standard deviation (SD). Patient baseline characteristics were compared using chi-squared test, two-sided Student's *t*-test and One-way Anova, depending on the variables type and the number of groups to be compared. With GraphPad 8.0 software, Pearson´s correlation was conducted to describe the correlation between the differentially expressed metabolites and the main kidney disease outcomes. Receiver operating characteristic (ROC) curve analysis was performed on the differential metabolites between healthy and DKD patients with uACR, <30 mg/g > 30 mg/, respectively. The corresponding area under the ROC curve (AUC), cutoff value, sensitivity, specificity, and accuracy were conducted to evaluate the predictive performances of the key metabolites for DKD. The accuracy was calculated using the equation: (A x sensitivity + B x specificity)/(A+B), where A is the participant number of the corresponding disease group, and B is the participant number of the corresponding control group. A p-value lower than 0.05 was considered statistically significant.

6.3. Results

6.3.1. Clinical characteristics of the patients

The clinical characteristics of the patients in the healthy and DKD groups are summarized in Table 6.2. There was a homogenous distribution between the groups regarding the sex, and no differences were observed between the healthy controls and DKD patients. All other clinical data were significantly different between the two groups. BMI was higher in DKD patients compared to healthy controls (29.0 ± 3.0 vs. 24.7 ± 3.4 kg/m², p=0.005), and DKD patients were older (73.4 ± 9.5 vs 51.8 ± 8.6 years for DKD and CTR patients, respectively, p<0.0001), as clinically expected. Additionally, the metabolic profile in DKD fits the disease status. Patients with DKD had higher levels of HbA1c (7.1 ± 1.2 % for DKD vs. 5.3 ± 0.4 % for CTR, p<0.0001) and lower HDL-cholesterol (39 ± 14 mmol/L for DKD vs. 66 ± 24 mmol/L for CTR, p=0.0004), while cystatin C (2.8 ± 1.0 mg/L for DKD vs. 0.8 ± 0.1 mg/ L for CTR, p<0.0001), serum creatinine (2.3 ± 1.3 vs. 0.70 ± 0.14 mg/dL) and triglycerides (191 ± 115 mmol/L vs. 89 ± 29 mmol/L p<0.0001) levels were increased in patients with DKD, when compared to healthy individuals. Regarding kidney function, DKD patients had increased total proteinuria (0.8 ± 1.2 vs. 0.1 ± 0.06 g/g creatinine, for DKD and CTR, respectively, p=0.0002), higher levels of albuminuria (527 ± 960 vs. 8.9 ± 11.1 mg/g creatinine, for DKD and CTR, p<0.0001), and lower eGFR (33 ± 19 vs. 79 ± 21 mL/min/1.73 m², for DKD and CTR, p<0.0001).

Table 6. 2. Clinical and biochemical parameters of Healthy Patients (CTR) and Patients with Diabetic Kidney Disease (DKD).

	CTR	**DKD**	**p-value**
BMI (kg/m²)	24.7 ± 3.4	29.0 ± 3.2	0.005
Age	51.8 ± 8.6	73.4 ± 9.8	<0.0001
HbA1c(%)	5.3 ± 0.4	7.1 ± 1.2	<0.0001
Female	18	9	0.17
Male	5	8	
Total cholesterol (mmol/L)	188 ± 34	146 ± 44	0.004
Triglycerides (mmol/L)	89 ± 29	191 ± 115	<0.0001
HDL-cholesterol (mmol/L)	66 ± 25	39 ± 14	0.0004
Cystatin C (mg/L)	0.8 ±0.1	2.8 ± 1.0	<0.0001
SerCreatinine (mg/dL)	0.70 ± 0.14	2.3 ± 1.3	<0.0001
Albumin (mg/g creatinine)	1.08 ± 0.13	1.4 ± 0.2	<0.0001
Urine			
Creatinine (mg/dL)	116.61 ± 56.20	110 ± 200	0.004
Proteinuria (g/L)	0.13 ± 0.16	0.5 ± 0.8	0.11

Total proteins (g/g creatinine)	0.1 ± 0.06	0.8 ± 1.2	0.0002
Albuminuria (mg/g creatinine) (uACR)	8.9 ± 11.1	507 ±965	<0.0001
eGFR (mL/min/1.73 m^2)	100 ± 13	34 ± 20	<0.0001

CTR- *healthy group*; DKD- *diabetic kidney disease*; BMI- *Body mass index*; HbA1c- *glycated hemoglobin* 1Ac; HDL- *high density lipoprotein*, Ser- *Serum*; eGFR- *estimated glomerular filtration rate*.

6.3.2. Urine metabolic profile of the included patients

Principal component analysis (PCA) was used to provide qualitative information on the observed data. Partial Least Square- Discriminant Analysis (PLS-DA) was used to assess class separation and to identify the main metabolites involved to the class discrimination (Fig 6.2, A and B). Exploratory unsupervised method PCA demonstrated a clustering between the two groups although a small overlap between the 95% confidence bands for healthy and DKD individuals is observed, being this last population more diverse in intragroup (Fig 6.2A). Supervised method PLS-DA scores scatter plot, which was used to maximize the difference between the groups, demonstrated a clear separation between both groups, which suggests that DKD affects the urine metabolome, exhibiting a different phenotype in comparison to healthy individuals, the control group. which can be used to discriminate between patients with DKD and healthy individuals (Fig 6.2 B).

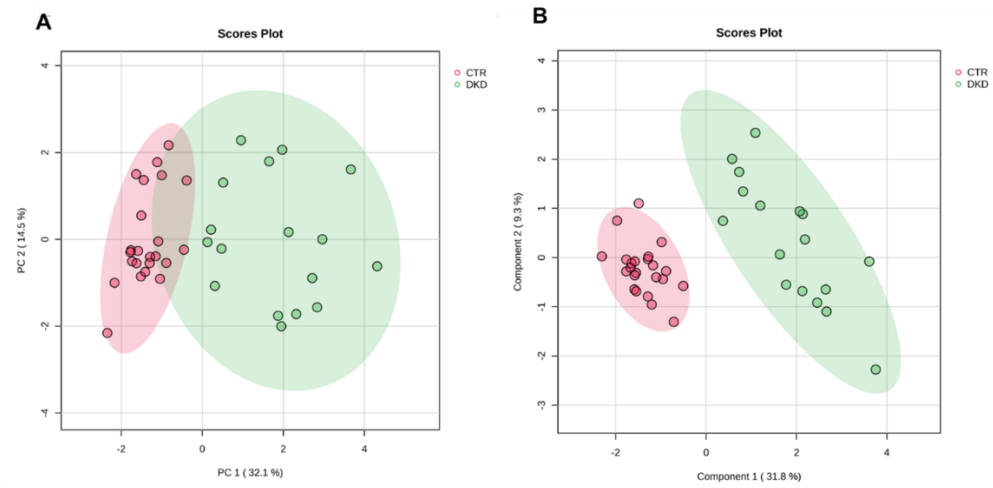

Fig. 6. 2- Principal Component Analysis (PCA, Panel A) and Partial least square discriminant analysis (PLS-DA, Panel B) of the main metabolites quantified in the ^{1}H-NMR spectra of the urine samples of healthy patients (represented in red, CTR) and patients diagnosed with diabetic kidney disease (Represented in green, DKD).

6.3.3. Dysregulated metabolites in DKD patients´urine

DKD patients urine samples presented significantly higher levels of glucose ($p<0.0001$), trans-aconitate ($p<0.0001$), taurine ($p<0.0001$) and glycolate ($p=0.0004$) and lower levels of citrate ($p<0.0001$), formate ($p<0.0001$), hypoxanthine ($p<0.0001$), isobutyrate ($p<0.0001$), glycine ($p=0.0003$), phenylacetate ($p=0.003$), dimethylamine ($p=0.01$), valine ($p=0.01$) and creatinine ($p<0.0001$) when compared to healthy individuals. These alterations are highlighted in Table 6.3. Furthermore, to understand the metabolic pathways that were affected in DKD, we used MetaboAnalyst 6.0 to perform pathway enrichment and topological analysis of the metabolites identified in urine. We screened out 24 metabolic pathways (Fig 6.3). According to the -log(P) value and the impact score (supplementary table A3.1), the top 5 metabolic pathways responsible for group clustering included glucose metabolism (that includes glucose), glyoxylate and dicarboxylate metabolism (glycolate), purine metabolism (that includes hypoxanthine). Those pathways presented a higher impact and lower p-values. In addition, glutathione metabolism (glycine) and citrate cycle (which are related to metabolites like citrate and trans-aconitate and the aminoacids) were also detected as altered but had lower impact but also lower p-values.

Table 6.3. Dysregulated metabolites in Diabetic Kidney disease (DKD) patients in comparison to healthy patients (CTR).

Metabolites	**CTR vs. DKD** (Fold Change)	**p-value**
Glucose	-6.94	6.59e-14
Citrate	2.54	8.15e-08
Hypoxanthine	1.84	5.60e-08
Trans-aconitate	-3.59	4.81e-07
Creatinine	1.00	5.20e-06
Formate	2.09	5.27e-05
Isobutyrate	1.57	2.98e-05
Glycine	1.55	0.0003
Glycolate	-1.15	0.0004

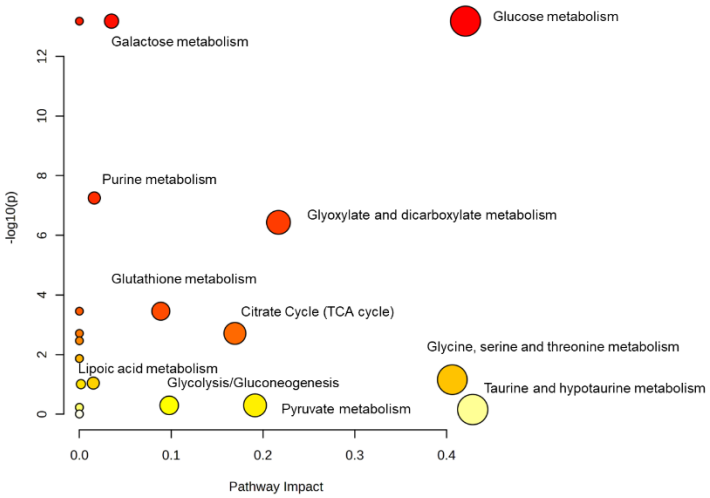

Fig. 6.3- Pathway analysis of the dysregulated metabolites detected in the urine of healthy and patients with DKD. Size and node colors are indicators of the impact and p value, and darker nodes correspond to pathway that are more impacted with lower p-values**.**

6.3.4. Correlation of urine metabolites levels with albuminuria and eGFR

To assess a possible association between the metabolites quantified in the urine and the main outcomes of kidney function present in the urine, we performed Pearson´s correlation between the levels of the metabolites identified in urine and the albuminuria and eGFR, as demonstrated in Fig 6.4 and Fig 6.5 Notably, the metabolites identified by the analysis through ^{1}H-NMR presented different tendencies of correlation. The levels of isobutyrate, formate, citrate, creatinine, and hypoxanthine in the urine were negatively correlated with the levels of albuminuria, while glycolate, taurine, succinate, and trans-aconitate levels in urine were positively correlated with albuminuria. More particularly, citrate presented the strongest negative correlation (r=-0.65, p<0.0001), followed by hypoxanthine (r= -0.49, p=0.004), isobutyrate (r= -0.45, p= 0.004), formate (r=-0.41, p=0.009) and creatinine (r=-0.37, p=0.02). On the other hand, glycolate (r=0.46, p= 0.003), taurine (r=0.47, p=0.007) and trans-aconitate (r=0.41, p=0.05) presented the strongest positive correlation with albuminuria. Regarding the correlations with eGFR (Fig 6.5), they are opposite to the correlations of albuminuria, i.e., the metabolites that are positively correlated with albuminuria are negatively correlated with eGFR and vice versa. In more detail citrate presented the strongest positive correlation (r=0.69, p<0.0001), followed by hypoxanthine (r= 0.45, p=0.008), creatinine (r= 0.44, p=0.005) and formate (r=0.35, p=0.03). On the other hand, trans-aconitate (r=-0.51, p=0.0007), taurine (r=-0.48, p=0.005) and glycolate (r=-0.41, p= 0.009), and were negatively correlated with eGFR. Furthermore, phenylacetate and succinate were positively correlated with eGFR (r=0.42, p=0.009, r=0.33, p=0.05,

respectively), while no such correlation was observed when the same metabolite was correlated with albuminuria.

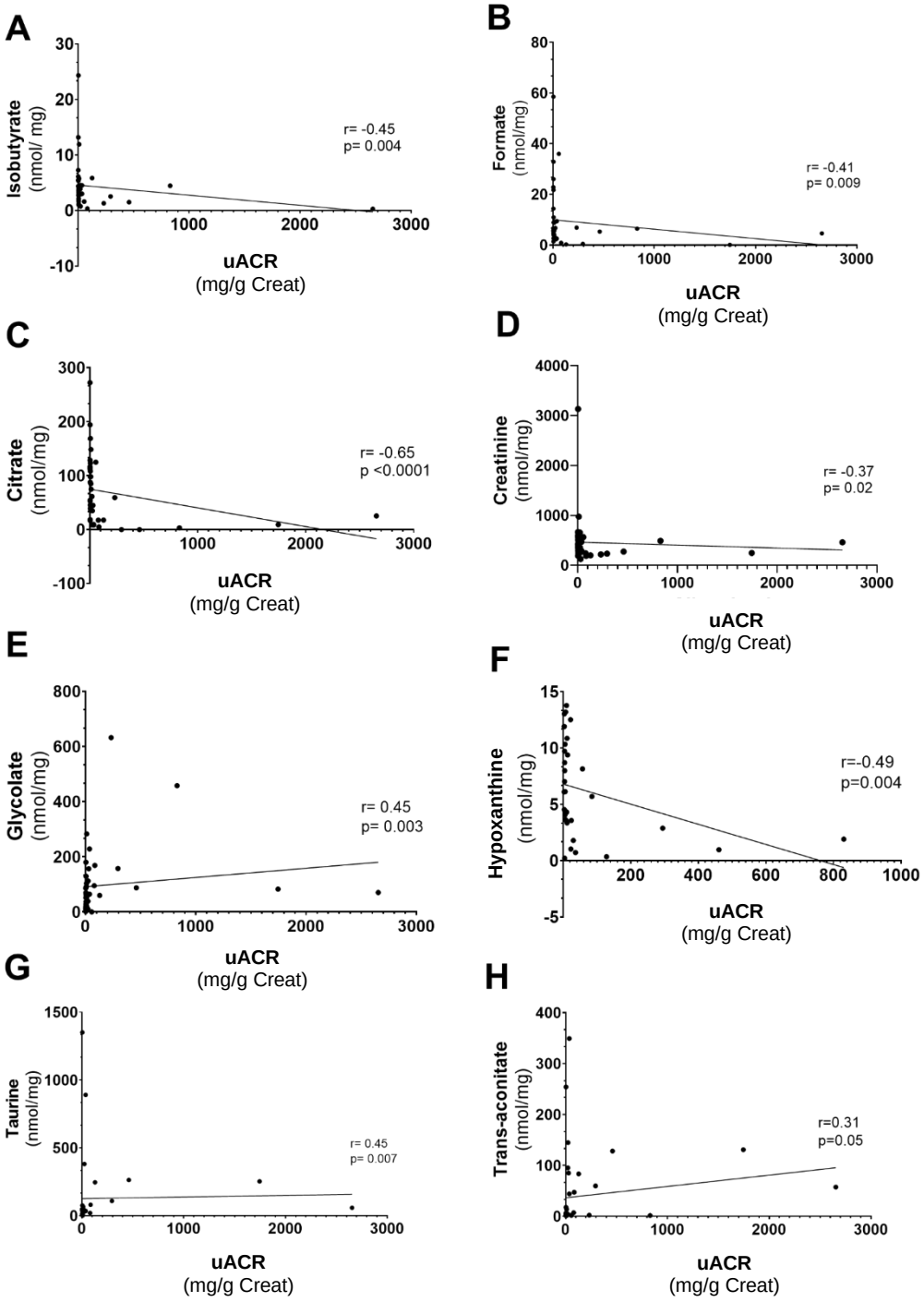

Fig.6. 4 - Pearson's correlation among the different metabolites quantified in the urine and albuminuria levels (uACR), with a p value <0.05. Isobutyrate (A), Formate (B), Citrate (C), Creatinine (D), Glycolate (E), Hypoxanthine (F), Taurine (G) and Trans-aconitate (H).

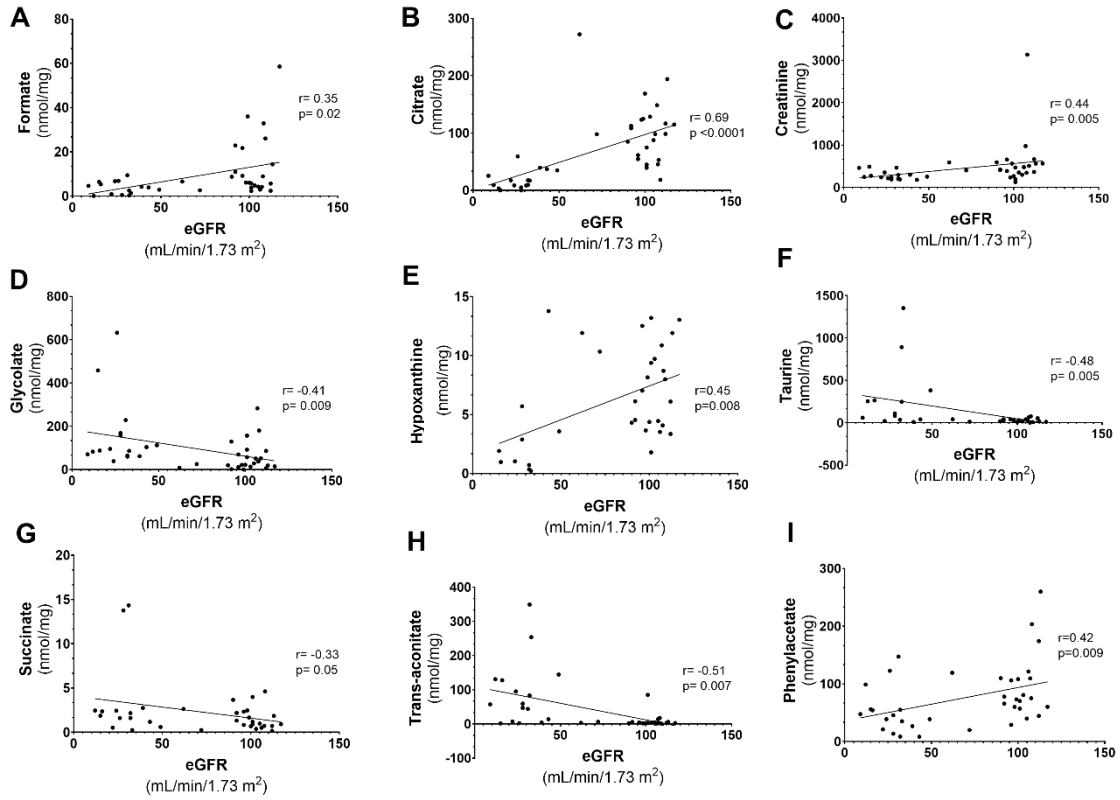

Fig.6. 5 -Pearson's correlation among the estimated glomerular filtration rate (eGFR) and the different metabolites detected in the urine, with a p value <0.05. Formate (A), Citrate (B), Creatinine (C), Glycolate (D), Hypoxanthine (E), Taurine (F), Succinate (G), Trans-aconitate (H) and Phenylacetate (I).

6.3.5. Urine metabolic profile of DKD patients with non-albuminuria progressor patients

According to the normal levels of albuminuria, we divided the patients into NA-DKD (<30mg/g) and A-DKD (>30 mg/g). PCA analysis (Fig 6.6A), suggested that NA-DKD patients and A-DKD patients presented overlapping metabolomes, however, in PLS-DA analysis it is noticeable a clustering trend between groups. When we applied PLS-DA, we could distinguish diverse urine metabolic profile in NA-DKD and A-DKD patients (Fig 6.6 B).

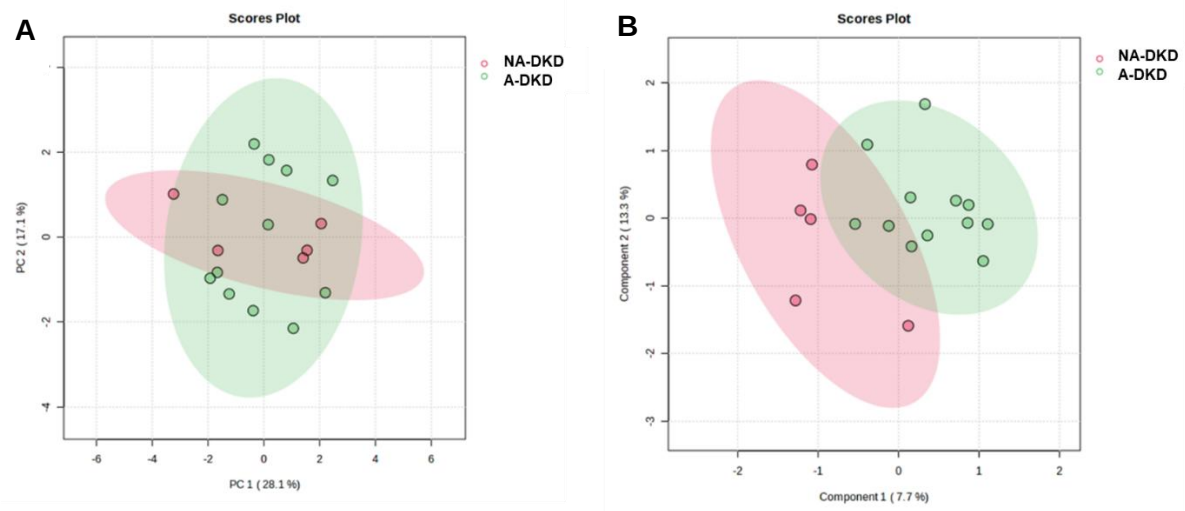

Fig. 6. 6 - Principal Component Analysis (PCA, Panel A) and Partial least square discriminant analysis (PLS-DA, Panel B) of the main metabolites identified in the urine, quantified by ^{1}H-NMR spectra of the urine samples of DKD patients that have uACR under 30 mg/g (NA-DKD, represented in red) and patients with uACR above 30 mg/g (A-DKD, represented in green).

The metabolites with the highest fold change are represented in Table 6.4. Glycine and pyruvate were downregulated in NA-DKD patients (1.32 and 1.88, respectively).

Notably hypoxanthine and citrate were the metabolites detected in the urine with the highest fold change between groups (2.60 and 1.43, respectively), being upregulated in NA-DKD patients when compared to A-DKD patients. Nonetheless, only glycine and alanine reached significant statistical differences between groups (p=0.02, and p=0.03, respectively).

Table 6.4. Dysregulated metabolites in Diabetic Kidney disease (DKD) patients divided in NA-DKD and A-DKD.

Metabolites	NA-DKD vs. A-DKD (Fold Change)	p-value
Citrate	1.43	0.44
Formate	1.32	0.32
Hypoxanthine	2.60	0.32
Glycine	-1.32	0.02
Trimethylamine	1.44	0.06
Pyruvate	-1.88	0.29
Alanine	1.20	0.03

A-DKD patients presented lower urinary alanine levels but higher levels of glycine, when compared to NA-DKD patients (Fig 6.7).

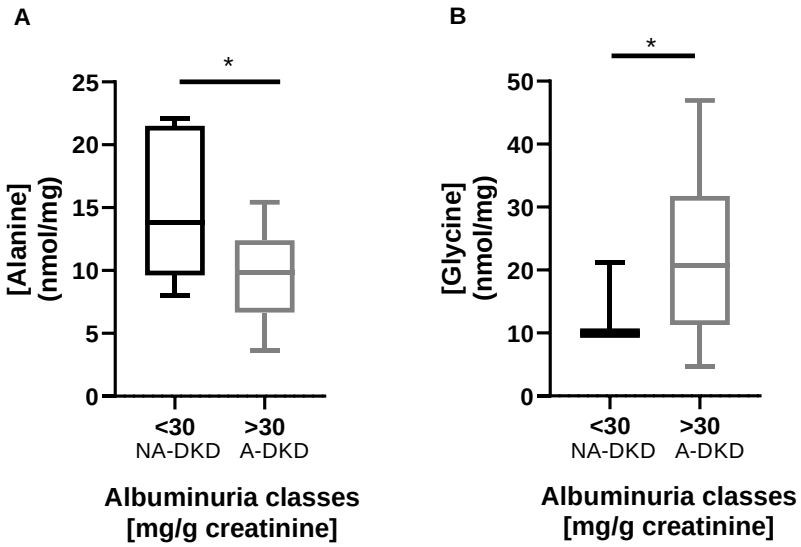

Fig.6. 7- Alanine (Panel A) and Glycine (Panel B) quantification in 24-urine sample of patients with DKD divided in non-albuminuric (NA-DKD) and albuminuric (A-DKD). * p<0.05.

Furthermore, to understand the metabolic pathways that may be affected in DKD, we used MetaboAnalyst 6.0 to perform pathway enrichment and topological analysis of the metabolites quantified in the urine samples. We screened out 23 metabolic pathways and according to the -log(P) value and the impact score, the top 6 metabolic pathways were selected (Fig 6.8), which included glutathione, glyoxylate and dicarboxylate metabolism, purine metabolism, citrate cycle, glycine, serine and threonine metabolism and taurine and hypotaurine metabolism.

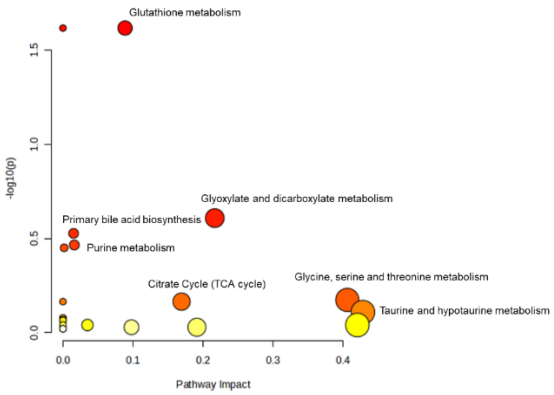

Fig.6. 8 - Pathway analysis of the dysregulated metabolites between non-albuminuric and albuminuric DKD patients. Size and node colors are indicators of the impact and p value, which darker node corresponds to pathway that are more impacted with lower p-values**.**

In addition, we performed a correlation analysis between the levels of the urine metabolites between NA-DKD and A-DKD (Table 6.5). Our results showed that urine alanine and lactate levels were strongly positively correlated with NA-DKD (0.95, 0.96, p=0.001, respectively),

Table 6. 5. Pearson's correlations of the metabolites quantified in urine samples of Diabetic Kidney disease (DKD) patients with normal levels of albuminuria (<30 mg/g, NA-DKD) and patients with DKD with higher levels of albuminuria (>30 mg/g, A-DKD).

	NA-DKD		A-DKD	
	r	**p-value**	**r**	**p-value**
Isobutyrate	0.001	0.99	-0.37	0.30
Formate	0.49	0.41	-0.06	0.86
Citrate	-0.22	0.72	-0.17	0.61
Creatinine	0.52	0.36	0.38	0.23
Glycolate	-0.03	0.96	-0.06	0.87
Alanine	0.95	0.01	0.29	0.35
Lactate	0.96	0.01	0.45	0.15
Valine	-0.19	0.81	0.09	0.81
Dimethylamine	0.63	0.25	0	>0.99
Pyruvate	0.34	0.66	0.43	0.30
Benzoate	0.20	0.80	0.13	0.70
Hippurate	0.37	0.53	0.03	0.92
Hypoxanthine	-0.20	0.80	0.14	0.78
Trimethylamine	0.66	0.23	0.22	0.49
Taurine	-0.26	0.74	0.1	0.81
Sarcosine	0.53	0.47	-0.55	0.08
Hydroxyacetone	0.42	0.48	-0.21	0.56
Glycine	-0.74	0.47	0.31	0.32
Succinate	0.06	0.92	0.30	0.41
Betaine	-0.26	0.67	-0.42	0.18
Trans-aconitate	0.002	0.99	-0.009	0.77
Phenylacetate	0.47	0.42	0.24	0.49
Glucose	-0.34	0.58	-0.51	0.11

6.3.6. Validation of the potential biomarkers to discriminate non-albuminuric vs. albuminuric patients

To test the possible relevance of urine metabolites as biomarkers for distinguishing between DKD patients with and without albuminuria, ROC analysis was performed. More in detail, the aim with this approach was to use urine metabolites to classify the patients based on whether they had albuminuria. Among all the urine metabolites evaluated in this study, only 6 had a statistical significance, p<0.05, as observed in Table 6.6.

Table 6. 6. Area under the curve (AUC) of ROC curves of the different metabolites in all patients in the study (non-albuminuric vs. albuminuric patients).

Metabolites	AUC	p-value	Cutoff	Sensitivity (y)(%)	Specificity (x) (%)	Accuracy (%)
Citrate	0.82	0.001	<30.13	76.92	80.77	79.13
Creatinine	0.72	0.03	<295.3	69.23	77.78	74.15
Glycolate	0.77	0.006	>86.99	61.54	77.78	70.88
Hypoxanthine	0.83	0.005	<3.12	75.00	92.31	86.71
Taurine	0.81	0.005	>56.25	77.78	84.00	81.48
Trans-Aconitate	0.72	0.03	>6.96	76.92	77.78	77.42

Urine citrate (AUC=0.82), hypoxanthine (AUC=0.83), and taurine (AUC= 0.81) levels demonstrated to have better AUC, which also had a higher percentage of sensitivity and specificity, suggesting that these differences in the metabolites may provide a way to discriminate the critical cut off to determine the disease progression (Fig 6.9). Furthermore, formate also revealed a possible potential to be an indicator, since p=0.07 and AUC= 0.69, as well as pyruvate (AUC=0.71, p=0.09) and isobutyrate (AUC= 0.68, p= 0.08) (represented in supplementary figure A3.1). The remaining metabolites quantified in urine did not present any statistical difference when compared the non-albuminuric and albuminuric patients (Supplementary Fig A3.2).

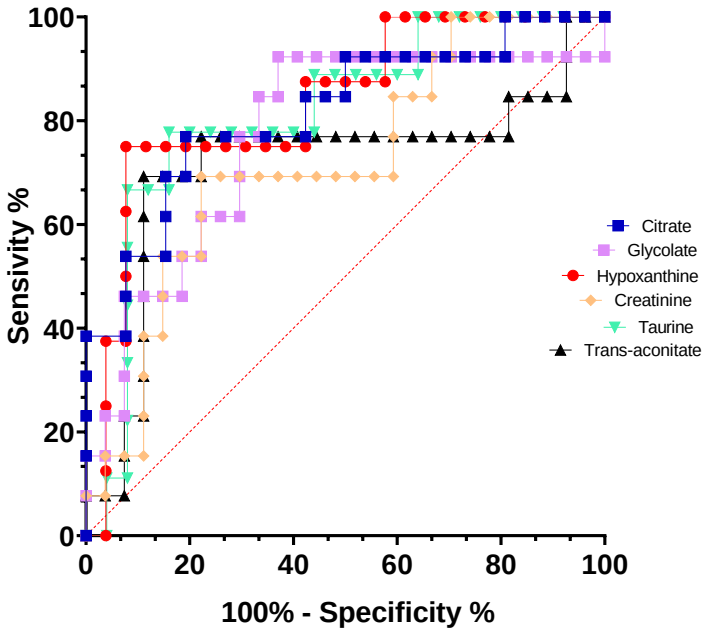

Fig.6.9- ROC curves of the main metabolites identified with p value ≤ 0.05, between patients considered non-albuminuric and albuminuric patients. Citrate ( ), Glycolate ( ), Hypoxanthine ( ), Creatinine ( ), Taurine ( ) and Trans-aconitate ( ).

6.4. Discussion

One of the major challenges in preventing and treating DKD is the lack of effective early detection strategies. The discovery of novel biomarkers for the beginning and progression of DKD is therefore urgently necessary. Urine metabolite concentrations are more variable than in blood which means that urine metabolites may provide information not available from blood tests (Schlosser et al., 2020). Therefore, urinary metabolomics is an attractive tool to study a broad spectrum of kidney disease, allied to the fact that the collection of the sample is non-invasive (Schlosser et al., 2020). We used ^{1}H-NMR metabolomics approach to characterize the urine profile of 23 metabolites that are easily and consistently detectable and can be used to distinguish patients with DKD from healthy patients. Our findings align with the typical DKD phenotype: patients were older, had elevated HbA1c, and had dyslipidemia (Guebre-Egziabher et al., 2013). Notably, traditional markers like serum creatinine were less sensitive (Cala et al., 2008) in predicant kidney function compared to cystatin C, which has been linked to lipid metabolism in DKD (Knopfholz et al., 2014).

In our study, we observed dysregulation in 13 of the detected metabolites, with most of these alterations associated with mitochondrial dysfunction. This is consistent with established research linking DKD progression to mitochondrial impairment (Yang et al., 2017), expressed by reduced levels of metabolites such as citrate, glycolate, and hypoxanthine in patients with DKD, although some distinct variations in urinary metabolomes were found (Mogos et al., 2023; Mutter et al., 2022; Zhu et al., 2022). This could be explained by the differences in patient populations, lifestyle factors such as diet, and disease stages. PCA results showed some overlap in the urine metabolome between healthy individuals and patients with DKD, so we performed PLS-DA which allowed us to verify a clear separation within the groups (healthy and DKD patients), indicating a distinct metabolic signature. Nevertheless, the studies showing which metabolites are involved in this mitochondrial dysfunction and the direct correlation between urine metabolite levels and DKD progression, especially in a non-albuminuric situation, are not fully clear. It has already been associated with a decrease of branched-chain amino acids levels, such as valine in patients with DKD when compared with diabetic patients without nephropathy (Esmati et al., 2021), which is concomitant with our results.

The reduction levels of citrate, a key TCA metabolite, suggests diminished mitochondrial function, which may contribute to decrease energy production in kidney cells (Hallan et al., 2017). Citrate's positive correlation with eGFR aligns with prior studies, supporting its protective role against tubulointerstitial injury and its potential for slowing DKD progression (Phisitkul et al., 2010).

Taurine has been documented in individuals with CKD either in serum and urine (Berg et al., 2013; Posada-Ayala et al., 2014) samples, particularly in the early-stages (Goek et al., 2013). This metabolite has been previously reported and may be linked to known osmoregulatory roles in kidney function, (Chesney et al., 2010), as well as with antioxidant properties (Schaffer et al., 2009), and prevention of oxidative stress and lipid peroxidation (Parvez et al., 2008). Lower levels of taurine in the plasma of patients with diabetes, in comparison with healthy subjects, was already reported (Ito et al., 2012). In our study we observed a higher taurine excretion in urine in DKD patients when compared to healthy patients, being that reflected in a negative correlation with eGFR, which suggests a reduced tubular reabsorption in DKD, reflecting early tubular damage. Another metabolite related with mitochondria in our study was glycolate. It is found to help in combat of oxidative stress by helping to restore the redox potential (Saulnier et al., 2018). Our results demonstrate that glycolate levels are upregulated in the urine of DKD patients with a correlation with eGFR and albuminuria. According to the disease stage, it was observed in patients with T1DM with a follow-up of a median of 9-years, the urine of progressor patients presented a decrease of citrate, glycine, glycolate, hypoxanthine, and trans-aconitate levels, among other metabolites, when compared to the non-progressor patients (Mutter et al., 2022). Moreover, alanine in urine was also nominally associated with the overall progression of the disease (Mutter et al., 2022).

Interestingly, our results demonstrated that alanine was present in higher levels in the urine in NA-DKD when compared to A-DKD patients. Moreover, the opposite effect was observed with glycine, i.e., higher levels in A-DKD patients. Glycine and alanine are involved in tubular damage (Nicholson et al., 1985), where glycine was already suggested to be related to ameliorate renal hemodynamics through an as-yet-undefined mechanism (Bądzyńska et al., 2014). Furthermore, it was also pointed to inhibit oxidative stress, as well as being a contributor to the synthesis of glutathione (Rochette et al., 2022). Further supporting the diagnostic potential of the altered metabolites, we performed a ROC analysis, with the possible biomarkers being identified citrate, hypoxanthine, and taurine as the most accurate metabolites for distinguishing between patients that have normal and higher levels of albuminuria. Indeed, the appearance of low levels of hypoxanthine in the urine of DKD patients is suggested to be associated with the decline of kidney function. Animal and human studies already demonstrated an association of hypoxanthine in plasma with the increased expression of key markers of inflammation, fibrosis (Leo et al., 2023), but also a pro-oxidant substrate in inducing oxidative stress (Knudsen & Thorgaard Andersen, 1985). Not less important, hypoxanthine can be also considered as a biomarker of hypoxia (Saugstad, 1977). In our results, hypoxanthine was less excreted in the urine of DKD patients, being positively correlated with eGFR and negatively correlated with albuminuria,

suggesting it may be more closely linked to hyperfiltration rather than proteinuria, and may be an earlier metabolic diagnostic tool. Indeed, in patients with ESRD, the pattern of serum metabolites was evaluated in patients receiving hemodialysis (HD) and peritoneal dialysis and it was demonstrated that hypoxanthine and inosine were present in HD patients, which is likely to represent more hypoxic and oxidative stress (Choi et al., 2011). Overall, we found that altered metabolites, especially in NA-DKD patients, can shed light towards new research for a more accurate diagnostic method. Most of the metabolites altered in the urine of DKD patients are related to mitochondria dysfunction, a process that may be mediating this disease, and serve as a marker to individualize therapeutic choices in DKD.

6.5. Conclusion

Our results provide a basis for the early pathophysiology DKD identification grounded on changes in urine levels of some metabolites and can guide future studies on the etiology, pathophysiology, and treatment of DKD. Specifically, it was noted that mitochondria- and antioxidant-related metabolites are highly altered in DKD patients, and this tendency is maintained in DKD patients with macroalbuminuria. In the NA-DKD population, certain metabolites, with a special emphasis on TCA metabolites and with associated antioxidant properties may serve as footprints of lesion progression. Despite these promising results, our study is limited by a relatively small sample size and the heterogenous use of different antidiabetic drug classes which may have influenced the urine metabolite levels. Future studies should focus on larger cohorts and longitudinal designs to validate these findings and further explore the diagnostic utility of urine metabolites in DKD, particularly in NA-DKD patients.

6.6. Authors contribution

Patrícia C. Braga: Writing-original draft, Methodology Validation, Software, Investigation, Formal Analysis and Data curation. Bárbara Guerra-Carvalho: Validation, Methodology, Investigation, Formal Analysis and Writing-review & editing. Manuela Almeida: Data curation, Methodology, Investigation and Writing-review & editing. Sofia S. Pereira: Validation, Formal Analysis and Writing-review & editing. Pedro F. Oliveira: Resources, Project administration, funding acquisition and Writing-review & editing. Marco G. Alves: Resources, Methodology, Project administration, funding acquisition and Writing-review &

editing. Anabela S. Rodrigues: Resources, Methodology, Project administration, funding acquisition and Writing-review & editing.

6.7.References

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Chapter 7

Conclusion

7. Conclusion

The global rise in diabetes prevalence is contributing to an increasing incidence of health complications, particularly DKD. Importantly, histological signs of DKD may appear even when standard glomerular function parameters remain unchanged, as seen in non-albuminuric DKD (NA-DKD) patients. These silent histological changes can progress over time, emphasizing the need for early and accurate biomarkers beyond conventional clinical markers. DKD is primarily driven by diabetes-induced metabolic dysregulation, which triggers a cascade of metabolic, hemodynamic, inflammatory, and fibrotic processes (Schlondorff, 2008). Due to the effects of hyperglycemia, the association of impaired insulin with renal cellular metabolism was already described as contributor for kidney damage (Ismail et al., 1999). Furthermore, the increase in the stimulation of glucose oxidation pathways in non-insulin-dependent cells leads to the activation of alternative pathways and increases ROS production (Donate-Correa et al., 2015). It is reported that IR can be a predictor of DKD since patients without diabetes can also be insulin-resistant (Kurella et al., 2005). However, the precise impact of IR on kidney pathophysiology is not yet understood and remains an area of interest. Thus, our first aim in the work was to evaluate the role of different glucose stimuli (from a normoglycemic, mild hyperglycemic to a severe hyperglycemic condition) with or without IR-induced through lipotoxicity (with administration of palmitic acid) in an immortalized human kidney (HK-2) cell line. We aimed to understand how metabolomic reprogramming would impact the mitochondria bioenergetics. We demonstrated a higher preponderance for gluconeogenic metabolites and the effects were noticed in a mild hyperglycemic condition and then ceased when the cells were in a severe hyperglycemia condition. This effect was also noticed when IR was induced through lipotoxicity, regardless of the glucose stimuli (5 mM, 11 mM, 22 mM). The glucose uptake occurs in non-insulin-dependent transporters, namely SGLT1 and 2, responsible for tubular reabsorption, along with the high-affinity glucose transporter GLUT2, which are overregulated in hyperglycemia (Lagranha et al., 2007; Machado et al., 2006), having this a higher contribution to hyperfiltration in patients with DM (Vallon, 2011). Moreover, the preponderance of gluconeogenic metabolites suggests that TCA cycle must be overloaded in these conditions, leading to a decrease in mitochondrial function (Sciacovelli et al., 2016). When ATP demands are high, the regulatory enzymes of the TCA cycle are triggered. This hinders mitochondrial respiration, where NADH accumulation slows down the TCA cycle (Martínez-Reyes & Chandel, 2020). In our results this was highlighted by the higher activity of complex II in mild hyperglycemic condition allied with IR, demonstrating a possible TCA disruption. We were also able to demonstrate that the exposure to hyperglycemia negatively impacted the bioenergetic profile of the cells, with greater susceptibility when IR was

induced. Notably, our findings align with evidence that in early DKD stages, kidney cells experience high ATP demand, but over time, ATP synthesis (Coughlan et al., 2016), glucose oxidation (Dugan et al., 2013), and FAO (Kang et al., 2015) decrease, resulting in increased ROS production, and ultimately mitochondrial dysfunction (Czajka & Malik, 2016; Schiffer & Friederich-Persson, 2017; Valdés et al., 2021). This underscores the vulnerability of renal tubule epithelial cells to metabolic imbalances, particularly as FAO is central to their energy metabolism. Under typical circumstances, PT cells mostly rely on FAO to generate significant amounts of ATP to meet their physiological needs. Our results demonstrated that in our cell model, lipid accumulation was occurring, especially in the groups where IR was induced, being the cells more dependent on FAO metabolism when exposed to higher concentrations of glucose. Understanding the role of FAO and its impact on cellular lipid metabolism and mitochondrial function is crucial for identifying potential therapeutic targets to intervene in the progression of DKD. By knowing the importance of FAO metabolism in kidney correct function, it is important to understand how the imbalance of FAO has an impact on mitochondria kidney function.

Obesity is a condition associated with IR and lipid accumulation, a common ground shared with T2DM (Gupta et al., 2012). Nowadays, diets high in fat (HFD) are prominent and have been linked to non-communicable diseases, such as chronic kidney injury. However, the exact processes causing obesity-related kidney impairment are yet unknown. Notably, metabolic changes that occur in the kidney profile when transitioning from an HFD to a healthy/normal diet remain unknown, as well as the effect that this metabolic reprogramming has on mitochondria function, after restoration of the diet. Thus, in the second work, we aimed to characterize the deleterious effects of HFD on kidney mice mitochondrial quality control mechanisms and associated proteins in the kidneys of mice. In this work, one of the biggest alterations detected was the proteins involved in FAO, in different critical points of the β-oxidation pathway, more particularly at the first (Acsm3) and final (Acca2) steps. These alterations suggested that HFD, that persisted even with the reversion of the diet, has enduring effects on kidney FAO. Under HFD stress condition, peroxisomal proteins were also altered, with Pecr being downregulated in the kidney in response to HFD. The proximal tubule of the kidney has the largest density of peroxisomes, indicating that peroxisomes and mitochondria may work together to mediate FAO (Gupta et al., 2012). Peroxisomes can oxidize very long-chain fatty acids (VLCFA); however, they are not able to produce ATP since they do not have respiratory chain enzymes. Moreover, these organelles share some machinery (Koch et al., 2003; Lee et al., 2004). Our findings underscore the potential impact of dietary intervention in restoring the crosstalk between mitochondria and peroxisomes (Tserga et al., 2022), which could result in lipid accumulation and mitochondrial dysfunction (Lismont et al., 2015; Waterham et al., 2016). Although there

are some studies on mitochondrial dysfunction and DKD, the molecular drivers of mitochondrial remodeling in DKD are poorly understood. Our results further demonstrated that after reverting the diet, mitochondrial dynamics could not be fully recovered, since PGC1-α expression in mice kidney was downregulated in comparison to the control, which affect the balance of fission (DRP1 and FIS1 mRNA expression) and fusion processes (MFN1 and OPA1 mRNA expression). In sum, this will result in mitochondria being more elongated and less functional, resulting in an impact at ETC, more particularly in complex I and II activities, yielding antioxidant defenses (MnSOD, Gpx and GR) disruption. An imbalance in GSH metabolism turns organelles more vulnerable to stress-induced damage (Dong et al., 2023). Overall, our results led to the hypothesis that early OS-related changes contribute to the onset and development of obesity-linked DKD. This point of view was already described by Ruggiero and collaborators (Ruggiero et al., 2011). Thus, we may suggest that mitochondrial-derived metabolites can have a role in identifying possible diagnosed biomarkers, clinically relevant as they express independently of current used biomarkers, such as albuminuria. After this translational overview (from 2D cell model to an animal model), our last challenge was to identify the possible diagnosed biomarkers that could be discriminative between a control population and patients that present DKD, either albuminuric or non-albuminuric. Thus, we aimed to identify through untargeted ^{1}H-NMR, the urinary metabolome phenotype of healthy patients vs. DKD patients. There are studies already showing that metabolites present in urine and or plasma are correlated with mitochondrial dysfunction in DKD, however in a non-albuminuric situation, the studies are not so clear (Pereira et al., 2022). We were able to identify 23 easily detectable metabolites in urine that can distinguish between patients with DKD and healthy individuals. Key metabolites (citrate, hypoxanthine, and taurine) are candidates to be potential biomarkers for DKD, achieving good discriminatory power in ROC analysis. Besides clinically relevant associations were documented as changes in specific metabolites (e.g., reductions in citrate and increases in taurine) correlate with kidney function indicators, shedding light on the metabolic disruptions in DKD.

Our data showed other metabolites that are related to the to the mitochondria function, more particularly with TCA cycle. This supports some assumptions made that postulated that a reduction of TCA activity, affects in a direct way, mitochondria function (Hallan et al., 2017; Sharma et al., 2013). Notably the study on urine metabolomics in DKD patients, along with TCA cycle related metabolites, we could also observe distinct metabolic signatures when comparing NA-DKD vs. A-DKD. Alanine and glycine presented alterations, where it was observed a downregulation and upregulation, respectively.

Alanine in urine was nominally associated with the overall progression of the disease (Mutter et al., 2022). With our work in HK-2 cells, we were able to identify possible alterations in alanine production by cell line exposed to hyperglycemia and IR and consolidated these results in humans, since alanine excretion was increased, particularly when DKD-associated mechanisms were present, as observed already in plasma of in IR patients (Ikeda, 2022) Regarding glycine, higher urinary levels were linked to ESRD in patients with macroalbuminuria, while lower levels were associated with the progression of albuminuria (Mutter et al., 2022). This is in agreement with our results, as we observed a decreased concentration of glycine in the urine of DKD patients when compared to healthy controls. This also aligns with our proteomic analysis, which revealed upregulation of proteins associated with glutathione metabolism, with an upregulation of Ggt1, and Gst3 in the kidney of HFDt-fed mice. The injuries provoked by HFD aggravate mitochondrial damage, causing dysregulation of TCA and OXPHOS, and thus, being reflected in the urine metabolome of DKD patients. In sum, our results provide evidence of the importance FAO in DKD and how its unbalance even with efforts to recover the metabolic reprogramming, due to diabetes and obesity, impacts the overall status of mitochondria. These works highlight that mitochondria can be considered a golden star, and it is worth continuing the research on this field to identify DKD, particularly at the onset of the disease. Mitochondria can be useful to uncover the basis for the early identification of DKD grounded on changes in urine levels of some metabolites. This allows understanding the impact of metabolic reprogramming to guide future studies on the etiology, pathophysiology, and treatment of DKD, beyond the clinical assessment to observe the decrease tubular kidney function.

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Chapter 8

Final Remarks

8. Final Remarks

This work provides important insights into the pathogenesis of DKD, more specifically in target mitochondria as a valuable tool for diagnosis and possible development of tailored therapies. With this work we could confirm that metabolic reprogramming plays a pivotal role in mechanisms associated to DKD, such as hyperglycemia and the high availability/ accumulation of fatty acids. Moreover, it was possible in these different scenarios to evidence metabolic signatures of albuminuric and non-albuminuric DKD. In our work, the ultimate goal was to be able to discriminate in urine the metabolome of DKD patients and find a panel of possible biomarkers that can be correlated with the advance of the disease, more particularly in the patients that present normal values of albuminuria, lacking clinically accessible biomarkers of progression. With this work we explored and confirmed that untargeted ^{1}H-NMR is useful to identify metabolites that are altered in urine. Urine metabolome, especially in kidney disease, has a valuable meaning and may discriminate risk in earlier stages of kidney disease.

Additionally, our investigation opened new tracks that escalate its clinical relevance particularly in diabetic patients that often suffer from obesity and a “fat kidney”. Since it is not completely understood, which metabolic reprogramming exists in patients that were candidates to bariatric surgery, and how the urine metabolome changes after such intervention it is now an opportune investigation for further knowledge on the best approach to these patients. Moreover, it has also a great value to understand which metabolic alterations occur, added to the adaptative glomerular hyperfiltration, in living kidney donors. By extending our metabolomic approach to these groups of disease, we gain a better understanding of different metabolomic patterns, in spite that hyperfiltration is transversal in all the tree scenarios, either diabetes, obesity or kidney donor status. Thus, preliminary results from nephrology group of UMIB already demonstrated that the urine metabolome from healthy individuals is completely distinct from patients that have DKD and patients and were subject to bariatric surgery (Fig 8.1).

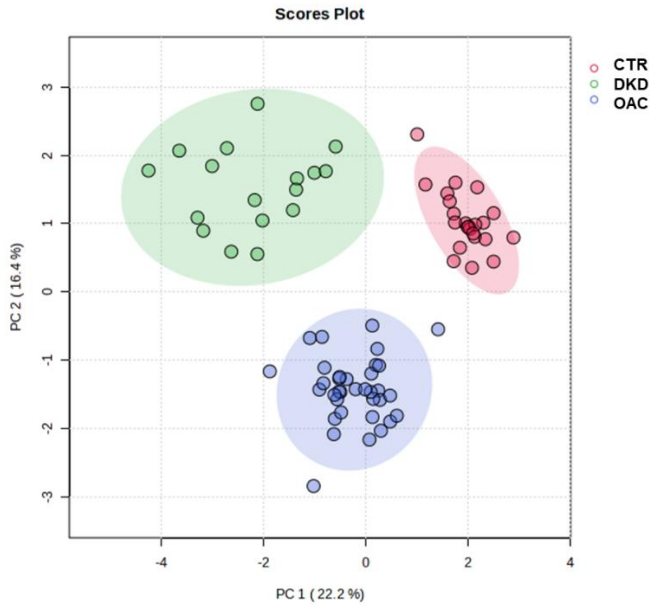


Fig 8.1- Principal Component Analysis (PCA) of the main metabolites quantified in the ^{1}H-NMR spectra of the urine samples of patients considered healthy and living kidney donors (CTR, represented in red), patients that are diagnosed with diabetic kidney disease (DKD, represented in green) and patients after 1 year of bariatric surgery (OAC, represented in blue).

Additionally, if we do a follow up of 6 months is also possible to discriminate the urinary metabolome from healthy patients before and after 6 months of kidney donation (Fig 8.2.)

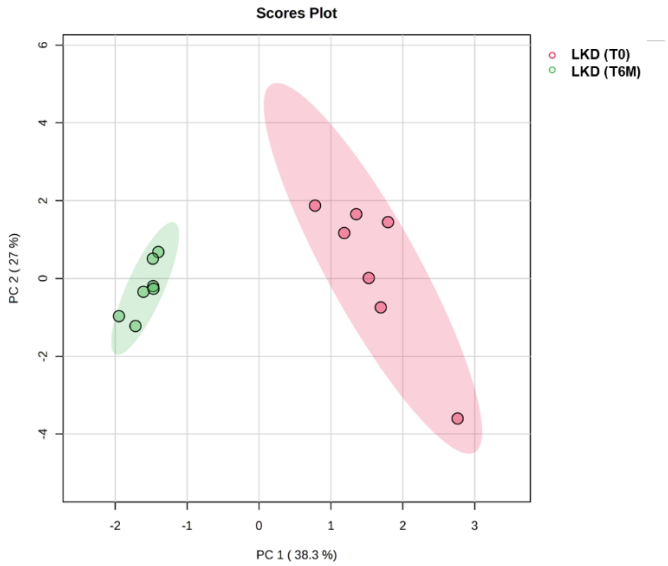


Fig 8.2 - Principal Component Analysis (PCA) of the main metabolites quantified in the ^{1}H-NMR spectra of the urine samples of patients considered healthy and living kidney donors (LKD) before kidney donation (LKD, T0, represented in red) and after 6 months of kidney donation (LKD, T6M, represented in green).

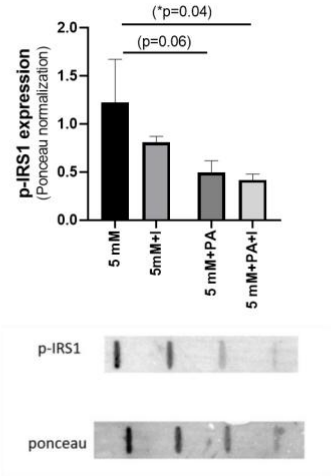
Taken together, these preliminary results highlight that metabolomics are an important tool to advance in the knowledge of kidney function and damage on top of renal hyperfiltration.

Having mitochondria as centric point of view could pave the track to new therapies in earlier stages of renal damage, avoiding renal deterioration.

Annexes

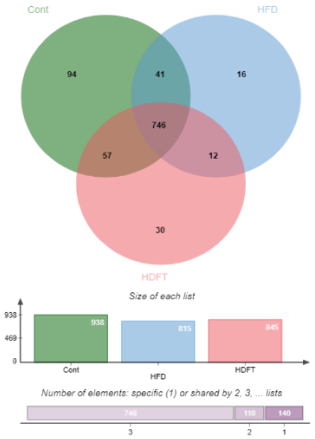
Annex A – Supplementary data

A1. Supplementary data from Chapter 4



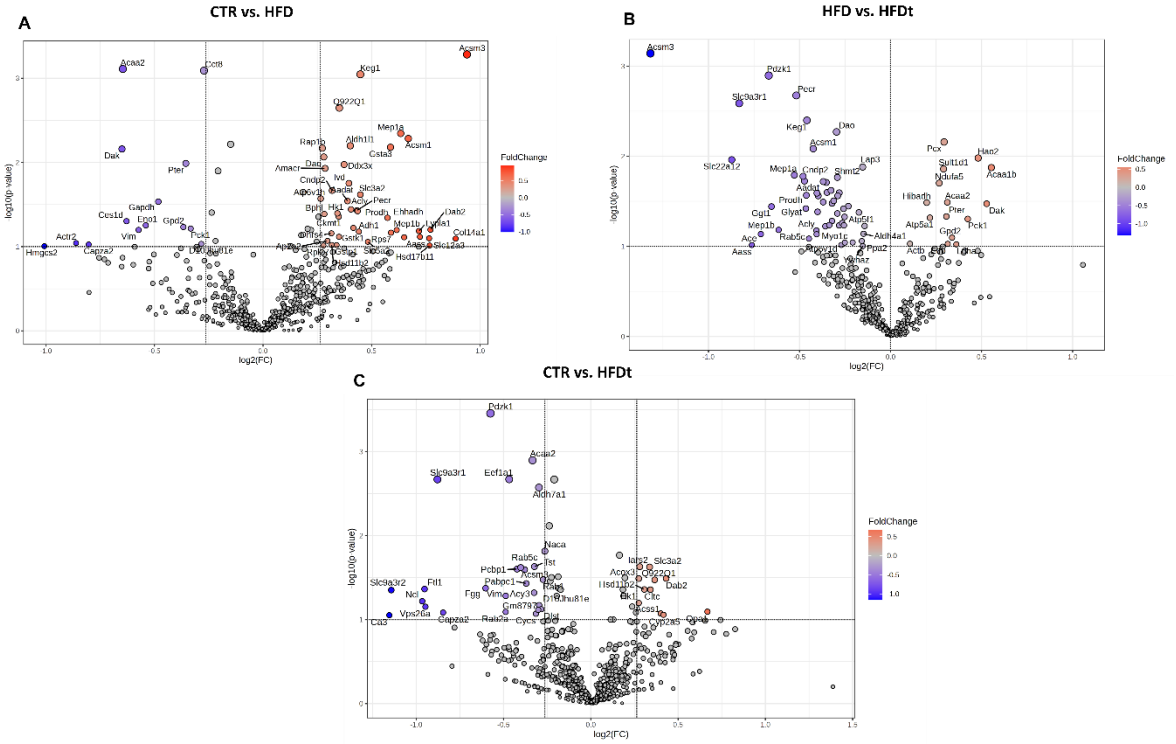
Supplementary Fig. A1. Expression of phosphorylated Insulin Receptor Substrate-1 (p-IRS1) in HK-2 cells subjected to various conditions. The cells were cultured in a medium containing 5 mM glucose, with or without the presence of palmitic acid (PA). Additionally, insulin stimulation (I) was applied at a concentration of 100 nM for 3 hours. The graph illustrates the quantitative assessment of p-IRS1 expression levels in response to these different treatments. The results are expressed as mean ± standard error of the mean (SEM) (N=3 at each experimental group). Significantly different results ($p<0.05$) are indicated as *.

A2. Supplementary data from Chapter 5



Supplementary Fig A 2.1. Venn diagram analysis of proteins reported in in kidney mitochondria enriched fraction of mice fed with standard chow (CTR); early-life high fat diet (HFD) or to high-fat diet for 60 days and then standard chow until 200 days (HFDt). It was possible to identify 938 proteins in the kidney fractions of mice from CTR group, 815 in the kidney fractions of mice from HFD group and 845 in the kidney fractions of mice from HFDt group. In the kidney mitochondria-enriched

fraction, 746 proteins were found to be common across all groups, regardless of their dietary regimen.



Supplementary Fig A 2.2- Volcano plot of kidney proteins from different groups in the study. The proteins with the largest (Manhattan) distance from the origin above thresholds are highlighted. (A) Red dots are thus proteins overexpressed in mice fed with chow diet (CTR group) and blue dots are thus proteins under-expressed, when compared to the mice group fed with high fat diet, (B) Red dots are thus proteins overexpressed in mice fed high fat diet and blue dots are thus proteins under-expressed, when compared to the mice from HFDt group and (C) Red dots are thus proteins overexpressed in mice fed with chow diet (CTR group) and blue dots are thus proteins under-expressed, when compared to the mice from HFDt group. |Fold change (log2)| > 1.5 and significance > 2 were set as thresholds.

Supplementary table A2.1. List of the total proteins identified in the MS analysis, in the present study. The proteins were detected in the kidney fraction enriched with mitochondria of mice fed with normal diet (CTR), with High-fat Diet (HFD) and, the group of was subjected to the reversion of the diet (HFDt)

NOTE: Due to the large size of this table, it is available in: https://docs.google.com/spreadsheets/d/14ks2bbr29aNbDI6R-n02tisa6JSLTGim/edit?usp=drive_link&ouid=112760375937649998246&rtpof=true&sd=true

Supplementary table A2.2. List of proteins identified in the MS analysis of kidney fraction enriched with mitochondria of mice from CTR vs. HFD groups, with corresponding fold change values,p-value and the most relevant biological processes of each protein (according to UniProt and Gene Ontology).

Gene Name	Protein Name	UniProt acession	Fold change	p-value	Biological Process (Gene Ontology)
Acsm3	Acyl-coenzyme A synthetase, mitochondrial	Q3UNX5	0.93937	0.00052166	Fatty acid beta-oxidation (GO:0006635)
Acaa2	3-ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	-0.64575	0.00077504	Fatty acid beta oxidation (GO:006635)
Cct8	T-complex protein 1 subunit theta	P42932	-0.27178	0.00081105	Chaperone mediated protein (GO:0061077)
Keg1	Glycine N-acyltransferase-like protein	Q9DCY0	0.44821	0.00089815	Glycine metabolic process (GO: 0006544)
Q922Q1	Mitochondrial amidoxime reducing component 2	Q922Q1	0.35148	0.0022535	Nitrate metabolic processo (GO:0042126)
Mep1a	Meprin A subunit alpha	P28825	0.63412	0.0045227	Dipeptidyl-peptidase activity (GO:0008239)
Acsm1	Acyl-coenzyme A synthetase 1,mitochondrial	Q91VA0	0.66927	0.0051979	Fatty acid beta oxidation (GO:006635)
Aldh1l1	Cytosolic 10-formyltetrahydrofolate dehydrogenase	Q8R0Y6	0.40197	0.0063377	Glycine biosynthetic process from serine (GO0019264)
Gsta3	Glutathione S-transferase A3	P30115	0.58708	0.0066059	Glutathione metabolic process (GO:0006749)

Rap1b	Ras-related protein Rap-1b	Q99JI6	0.27301	0.0067714	Rap protein signal transduction (GO0032486)
Dak	ATP-dependent dihydroxyacetone kinase	Q8VC30	-0.64969	0.006918	Glycolytic process (GO0061625)
Dao	D-amino-acid oxidase	P18894	0.27995	0.0086071	Glycine biosynthetic process from serine (GO0019264)
Pter	Phosphotriesterase-related protein	Q60866	-0.35559	0.010273	Hydrolase activity, acting on ester bonds and endodeoxyribonuclease activity
Ddx3x	ATP-dependent RNA helicase DDX3X	Q62167	0.37314	0.010592	NLRP3 inflammasome complex assembly (GO:0044546)
Amacr	Alpha-methylacyl-CoA racemase	O09174	0.28577	0.011762	Fatty beta-oxidation using acyl-Coa Oxidase (GO:0033540) / Protein import into peroxisome matrix (GO: 0016558)
Ivd	Isovaleryl-CoA dehydrogenase, mitochondrial	Q9JHI5	0.39543	0.017685	Fatty acid beta oxidation using acyl-CoA dehydrogenase (GO0033539)
Cndp2	Cytosolic non-specific dipeptidase	Q9D1A2	0.31797	0.021591	Carnosine metabolic process (GO: 0035498)
Slc3a2	4F2 cell-surface antigen heavy chain	P10852	0.44817	0.024102	L-leucine import across plasma membrane (GO:1903801)
Atp6v1h	V-type proton ATPase subunit H	Q8BVE3	0.26559	0.026902	Vacuolar proton-transporting V-type ATPase (GO:0030641)
Aadat	kynurenine/alpha-aminoadipate aminotransferase, mitochondrial	Q3UNS8	0.38923	0.028728	L-Kynurenine catabolic process (GO:0097053)

Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	P16858	-0.4821	0.029226	Canonical glycolysis (GO: 0061621)
Acly	ATP-citrate synthase	Q91V92	0.40525	0.036058	Citrate metabolic process (GO: 0006101)
Pecr	Peroxisomal trans-2-enoyl-CoA reductase	Q99MZ7	0.43258	0.037109	Fatty acid beta-oxidation using Acyl-CoA oxidase (GO:0033540)/ Protein import into peroxisome matrix (GO: 0016558)
Prodh	Proline dehydrogenase 1, mitochondrial	Q9WU79	0.43569	0.037852	Proline catabolic process to glutamate (GO: 0010133)
Hk1	Hexokinase-1	P17710	0.34243	0.040237	Glucose 6-phosphate transport (GO:0015760)
Bphl	Valacyclovir hydrolase	Q8R164	0.28195	0.041001	Methionyl-tRNA aminoacylation (GO: 0006431)
Ckmt1	Creatine kinase U-type, mitochondrial	P30275	0.34845	0.043975	Mitochondrial ATP/ADP transmembrane transport (GO1990544; 0140021)
Ehhadh	Enoyl-CoA hydratase/3,2-trans-enoyl-CoA isomerase	Q9DBM2	0.57351	0.045433	Carnitine metabolic process, CoA-linked (GO: 0019254)
Ces1d	Carboxylesterase 1D	Q8VCT4	-0.62863	0.049837	Monocarboxylic acid metabolic process (GO: 0032787)
Eno1	Enolase 1, alpha non-neuron	P17182	-0.54005	0.055839	Methylglyoxal biosynthetic process (GO: 0019242)
Gpd2	Glycerol-3-phosphate dehydrogenase, mitochondrial.	Q64521	-0.36689	0.05848	Glycerol catabolic process (GO: 0019563)

Gstk1	Glutathione S-transferase kappa 1	Q9DCM2	0.41608	0.059904	Glutathione metabolic process (GO:0006749)
Pck1	Phosphoenolpyruvate carboxykinase, cytosolic [GTP]	Q9Z2V4	-0.33321	0.060933	Citrate metabolic process (GO: 0006101)
Dab2	Disabled homolog 2	P98078	0.77127	0.0629	Postsynaptic neurotransmitter receptor internalization (GO0098884)
Vim	Vimentin;	P20152	-0.57313	0.063309	Epithelial to mesenchymal tansition involved in endocardial cushion formation (GO: 0003198)
Mep1b	Meprin A subunit beta	Q61847	0.61566	0.063476	Metallodipeptidase activity (GO 0008238)
Lypla1	Acyl-protein thioesterase 1	P97823	0.71816	0.065124	Phosphatidylserine acyl-chain remodeling (GO:0036150)
Adh1	Alcohol dehydrogenase 1	P00329	0.44142	0.066284	Fructose catabolic process (GO: 0006001)
Rps7	0S ribosomal protein S7	P62082	0.58948	0.068292	cytoplasmatic translation (GO: 0002181)
Dhrs4	Dehydrogenase/reductase SDR family member 4	Q99LB2	0.3148	0.069516	9-cis-retinoic acid biosynthetic process (GO:0042904)
Atp5f1	ATP synthase F(0) complex subunit B1, mitochondrial	Q9CQQ7	0.34992	0.076258	Proton motive force-driven mitochondrial ATP synthesis (GO: 0042776)
Aass	Alpha-aminoadipic semialdehyde synthase, mitochondrial	Q99K67	0.72267	0.076461	Glutamine family amino acid biosynthetic process (GO: 0009084)

Cyp2a5	Cytochrome P450 2A5	P20852	0.64996	0.077313	Dibenzo-p-dioxin metabolic process (GO:0018894)
Slc12a3	Solute carrier family 12 member 3	P59158	0.76517	0.079658	Renal sodium ion absorption (GO: 0070294)
Col14a1	Collagen alpha-1(XIV) chain	Q80X19	0.88703	0.080451	Negative regulation of endodermal cell differentation (GO:1903225)
Ap2a2	AP-2 complex subunit alpha-2	P17427	0.29682	0.085329	Postsynaptic neurotransmitter receptor internalization (GO0098884)
Slc5a2	Sodium/glucose cotransporter 2	Q923I7	0.48281	0.087714	Glucose transmembrane transport (GO:1904659)
Actr2	Actin-related protein 2 ATP-binding component of the Arp2/3 complex	P61161	-0.86106	0.090556	Actin nucleation (GO:0033206)
D10Jhu81e	Glutamine amidotransferase-like class 1 domain-containing protein 3A,	Q9D172	-0.28431	0.09265	-
Capza2	F-actin-capping protein subunit alpha-2	P47754	-0.80306	0.094019	Regulation of actin filament depolymerization (GO:0030834)
Hsd11b2	Corticosteroid 11-beta-dehydrogenase isozyme 2	P51661	0.31638	0.095396	Regulation of blood volume by renal aldosterone (GO:0002017)
Gstp1	Glutathione S-transferase P 1	P19157	0.33902	0.09576	Glutathione derivative biosynthetic process (GO: 1901687)
Hsd17b11	Estradiol 17-beta-dehydrogenase 11	Q9EQ06	0.76747	0.096898	Steroide biosynthetic process (GO: 0006694)
Rpl27	60S ribosomal protein L27;	P61358	0.27834	0.097032	Cytoplasmic translation (GO: 0002181)

Hmgcs2	Hydroxymethylglutaryl-CoA synthase, mitochondrial	P54869	-1.0071	0.09861	Fatty acid beta-oxidation using Acyl-CoA oxidase (GO:0033540)

Supplementary table A2.3. List of proteins identified in the MS analysis of kidney fraction enriched with mitochondria of mice from HFD vs. HFDt groups, with corresponding fold change values, p-value and the most relevant biological processes of each protein (according to UniProt and Gene Ontology).

Gene Name	Protein Name	UniProt acession	Fold change	p-value	Biological Process (Gene Ontology)
Acsm3	Acyl-coenzyme A synthetas, mitochondrial	Q3UNX5	-1.3187	0.0007222	Fatty acid beta-oxidation (GO:0006635)
Pdzk1	Na(+)/H(+) exchange regulatory cofactor NHE-RF3	Q9JIL4	-0.66895	0.001271	Positive regulation of inorganic anion transmembrane transport (GO: 1903797)
Pecr	Peroxisomal trans-2-enoyl-CoA reductase	Q99MZ7	-0.5174	0.0021137	Fatty acid beta-oxidation using Acyl-CoA oxidase (GO:0033540)/ Protein import into peroxisome matrix (GO: 0016558)
Slc9a3r1	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	P70441	-0.83121	0.0025854	Positive regulation of inorganic anion transmembrane transport (GO: 1903797)
Keg1	Glycine N-acyltransferase-like protein	Q9DCY0	-0.45938	0.0039938	Glycine metabolic process (GO: 0006544)
Dao	D-amino-acid oxidase	P18894	-0.29661	0.0053651	Glycine biosynthetic process from serine (GO0019264)
Pcx	Pyruvate carboxylase, mitochondrial	Q05920	0.29408	0.0069449	Glycerol biosynthetic process from pyruvate (GO: 0046327)
Acsm1	Acyl-coenzyme A synthetase ACSM1, mitochondrial	Q91VA0	-0.42466	0.0082442	Fatty acid beta-oxidation (GO:0006635)
Hao2	Hydroxyacid oxidase 2	Q9NYQ2	0.48128	0.010462	Glyoxylate metabolic process (GO: 0046487)
Slc22a12	Solute carrier family 22 member 12	Q8CFZ5	-0.87219	0.010941	Renal urate sal excretion (GO: 0097744)
Lap3	Cytosol aminopeptidase	Q9CPY7	-0.15318	0.013178	Glutathione catabolic process (GO:0006751)

Acaa1b	3-ketoacyl-CoA thiolase B, peroxisomal.	Q3TUU3	0.55382	0.013358	Fatty-acid beta-oxidation (GO: 0006635)
Sult1d1	Sulfotransferase 1 family member D1	Q3TV15	0.29129	0.013838	Biphenyl catabolic process (GO: 0070980)
Mep1a	Meprin A subunit alpha	P28825	-0.52812	0.016169	Dipeptidyl-peptidase activity (GO:0008239)
Cndp2	Cytosolic non-specific dipeptidase	Q9D1A2	-0.48022	0.016703	Carnosine metabolic process (GO: 0035498)
Shmt2	Serine hydroxymethyltransferase, mitochondrial	Q9CZN7	-0.29198	0.017183	Histidine biosynthetic process (GO:0000105)
Aadat	kynurenine/alpha-aminoadipate aminotransferase, mitochondrial	Q9WVM8	-0.47266	0.018836	L-Kynurenine catabolic process (GO:0097053)
Rab1	Ras-related protein	P62821	-0.36959	0.019104	Proteins transport from the endoplasmic reticulum to the Golgi apparatus
Aldh1l1	Cytosolic 10-formyltetrahydrofolate dehydrogenase	Q8R0Y6	-0.35276	0.019537	Glycine biosynthetic process from serine (GO0019264)
Ndufa5	ADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5;	Q9CPP6	0.26732	0.019866	Mitochondrial respiratory chain complex I assembly (GO: 0032981)
Gsta3	Glutathione S-transferase A3	P30115	-0.32692	0.023944	Glutathione metabolic process (GO:0006749)
Amacr	Alpha-methylacyl-CoA racemase	O09174	-0.34862	0.02521	Fatty beta-oxidation using acyl-Coa Oxidase (GO:0033540) / Protein import into peroxisome matrix (GO: 0016558)
Aldh2	Aldehyde dehydrogenase, mitochondrial	P47738	-0.28168	0.025432	Monoamine oxidase activity (GO:0097621)
Mut	Methylmalonyl-CoA epimerase, mitochondrial	P16332	-0.40425	0.025695	Succinate metabolic process (GO:0006105)
Prodh	Proline dehydrogenase 1, mitochondrial	Q9WU79	-0.46238	0.02711	Proline catabolic process to glutamate (GO: 0010133)
Gnb2l1	Receptor of activated protein C kinase 1	P68040	-0.28994	0.027464	Cytoplasmic translation (GO: 0002181)
Ivd	Isovaleryl-CoA dehydrogenase, mitochondrial	Q9JHI5	-0.39689	0.027524	Fatty acid beta oxidation using acyl-CoA dehydrogenase (GO0033539)

Gsr	Glutathione reductase, mitochondrial	P47791	-0.31213	0.031209	Glutathione biosynthetic process (GO: 0006750)
Pabpc1	Polyadenylate-binding protein 1	P29341	-0.36988	0.032241	Positive regulation of cytoplasmic translation (GO: 1900152)
Acaa2	3-ketoacyl-CoA thiolase, mitochondrial	P42765	0.31193	0.03245	Fatty acid beta oxidation (GO:006635)
Hibadh	3-hydroxyisobutyrate dehydrogenase, mitochondrial	Q99L13	0.19876	0.032729	Valine catabolic process (GO:0006574)
Eef1a1	Elongation factor 1-alpha 1	P10126	-0.32132	0.033302	Cytoplasmic translation (GO: 0002181)
Dak	ATP-dependent dihydroxyacetone kinase	P10126	0.52793	0.033649	Glycolytic process (GO0061625)
Atp6v1h	V-type proton ATPase subunit H	Q8BVE3	-0.23157	0.035841	Vacuolar proton-transporting V-type ATPase (GO:0030641)
Ggt1	Glutathione hydrolase 1 heavy chain;	Q60928	-0.65407	0.036163	Glutathione biosynthetic process (GO: 0006750)
Glyat	Glycine N-acyltransferase; Mitochondrial acyltransferase	Q91XE0	-0.46497	0.038065	Benzoyl-CoA metabolic process (GO: 1901787)
Lactb2	Endoribonuclease LACTB2	Q99KR3	-0.2581	0.038231	Endonuclease activity (GO: 0004521)
Rps13	40S ribosomal protein S13	P62301	-0.40068	0.04139	Cytoplasmic translation (GO: 0002181)
Hspa9	Stress-70 protein, mitochondrial	P38647	-0.17385	0.042023	Protein import into mitochondrial matrix (GO: 0030150)
Fahd2	Fumarylacetoacetate hydrolase domain-containing protein 2A	Q3TC72	-0.29198	0.043155	Tyrosine catabolic process (GO: 0006572)
Pter	Phosphotriesterase-related protein	Q60866	0.30686	0.046611	Hydrolase activity, acting on ester bonds and endodeoxyribonuclease activity
Ckmt1	Creatine kinase U-type, mitochondrial	P30275	-0.25239	0.047109	Mitochondrial ATP/ADP transmembrane transport (GO1990544; 0140021)
Atp5a1	ATP synthase subunit alpha, mitochondrial	Q03265	0.21628	0.048146	Proton motive force-driven mitochondrial ATO synthesis (GO: 00422776)

Pck1	Phosphoenolpyruvate carboxykinase, cytosolic [GTP]	Q9Z2V4	0.42424	0.049642	Citrate metabolic process (GO: 0006101)
Ezr	Ezrin	Q4KML7	-0.32166	0.052285	Membrane to membrane docking (GO: 0022614)
Ahcy	Adenosylhomocysteinase	P50247	-0.26568	0.055151	Cysteine biosynthetic process via cystathione (GO: 0019343)
Rab7a	Ras-related protein Rab-7a	P51150	-0.33646	0.058084	Endosome to plasma membrane protein transport (GO:0099638)
Ddx3x	ATP-dependent RNA helicase DDX3X	Q62167	-0.25591	0.058498	NLRP3 inflammasome complex assembly (GO:0044546)
Acadm	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	P45952	-0.28239	0.058511	Fatty acid beta-oxidation using Acyl-CoA oxidase (GO:0033540)
Atp5f1	ATP synthase subunit alpha, mitochondrial;	Q9CQQ7	-0.14231	0.059406	Proton - transporting ATP synthase activity ATP synthase activity (GO:0042776)
Acsm2	Acyl-coenzyme A synthetase ACSM2, mitochondrial	D3YXB1	-0.35802	0.059962	Citrate metabolic process (GO: 0006101)
Mep1b	Meprin A subunit beta	Q61847	-0.61471	0.065692	Epidermal growth factor receptor ligand maturation (GO:0038004)
Acly	ATP-citrate synthase	Q91V92	-0.40711	0.066716	Citrate metabolic process (GO: 0006101)
Aldh4a1	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	Q8CHT0	-0.14801	0.07255	Proline catabolic process to glutamate (GO: 0010133)
Ace	Angiotensin-converting enzyme	P09470	-0.71228	0.073148	Renin-angiotensin regulation of aldosterone production (GO:0002018)
Prdx1	Peroxiredoxin-1	P35700	-0.40585	0.073411	Serine phosphorylation of STAT protein (GO:0042501)
Gpd2	Glycerol-3-phosphate dehydrogenase, mitochondrial.	Q64521	0.33782	0.080059	Glycerol-3-phosphate metabolic process (GO:0006072)
Rab5c	Ras-related protein Rab-5C	P35278	-0.44806	0.082129	Small GTPase mediated signal transduction (GO: 0007264)

Ppa2	Inorganic pyrophosphatase 2, mitochondrial	Q91VM9	-0.15781	0.087221	Cellular potassium ion homeostasis (GO: 0030007)
Gm10260	Small ribosomal subunit protein uS13	F6YVP7	-0.20804	0.090538	Ribosomal small subunit assembly (GO:0000028)
Myo1c	Unconventional myosin-Ic	Q9WTI7	-0.25096	0.090826	Negative regulation of calcium ion export across plasma membrane (GO: 1905913)
Actb	Actin, cytoplasmatic 1	P60710	0.10675	0.093504	Regulation of transepithelial transport (GO:0150111)
Etfb	Electron transfer flavoprotein subunit beta, mitochondrial	Q9DCW4	0.31422	0.093908	Fatty acid beta oxidation using acyl-CoA dehydrogenase (GO0033539)
Ldha	L-lactate dehydrogenase A chain;	P06151	0.3612	0.094943	Glutamate catabolic process (GO:0019551)
Atp6v1d	V-type proton ATPase subunit H	Q8BVE3	-0.27053	0.096586	Vacuolar proton-transporting V-type ATPase (GO:0030641)
Aass	Alpha-aminoadipic semialdehyde synthase, mitochondrial	Q99K67	-0.76189	0.096753	Glutamine family amino acid biosynthetic process (GO: 0009084)
Ywhaz	14-3-3 protein zeta/delta	P63101	-0.15245	0.099429	Signal transduction; neuronal development; apoptosis

Supplementary table A2.4. List of proteins identified in the MS analysis of fraction enriched with mitochondria of kidney of mice from CTR vs. HFDt groups, with corresponding fold change values, p-value and the most relevant biological processes of each protein (according to UniProt and Gene Ontology).

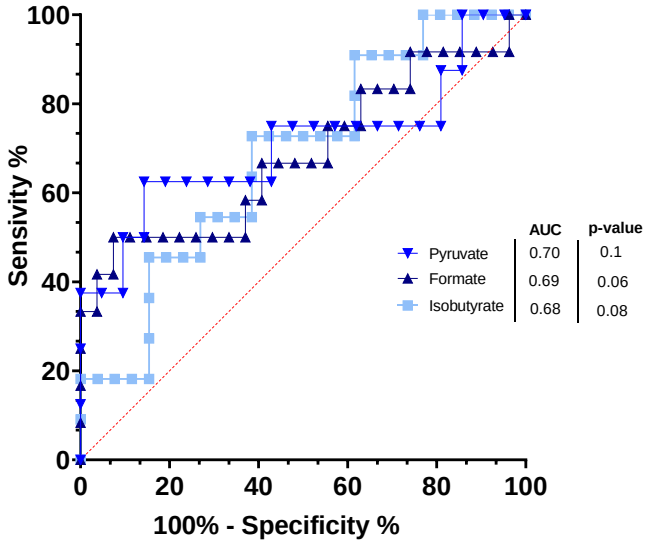
Gene Name	Protein Name	UniProt acession	Fold change	p-value	Biological Process (Gene Ontology)
Pdzk1	Na(+)/H(+) exchange regulatory cofactor NHE-RF3	Q9JIL4	-0.57502	0.0003504	Positive regulation of inorganic anion transmembrane transport (GO: 1903797)
Acaa2	3-ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	-0.33381	0.0012689	fatty acid beta oxidation (GO:006635)
Eef1a1	Elongation factor 1-alpha 1	P10126	-0.46769	0.0021335	Cytoplasmic translation (GO: 0002181)

Slc9a3r1	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	P70441	-0.87864	0.0021399	Positive regulation of inorganic anion transmembrane transport (GO: 1903797)
Aldh7a1	Alpha-aminoadipic semialdehyde dehydrogenase	Q9DBF1	-0.29732	0.0026748	Glycine betaine biosynthetic process from choline (GO:0019285)
Naca	Nascent polypeptide-associated complex subunit alpha	Q60817	-0.26326	0.015318	Cytoplasmic translation (GO: 0002181)
Tst	Thiosulfate sulfurtransferase	P52196	-0.32314	0.02338	Aspartate biosynthetic process (GO: 0006532), Glutamate catabolic process (GO:0019551)
Iars2	Leucine--tRNA ligase, mitochondrial	Q8VDC0	0.28058	0.02349	Prolyl-tRNA aminoacylation (GO:0006433), methionyl-tRNA amino acylation (GO:0006431)
Slc3a2	4F2 cell-surface antigen heavy chain	P10852	0.33689	0.02366	L-leucin import across plasma membrane (GO: 1903801); valine transport (GO:0015829), Phenyalanine transport (GO: 0015823)
Rab5c	Ras-related protein Rab-5C	P35278	-0.4016	0.024009	Small GTPase mediated signal transduction (GO: 0007264)
Pcbp1	Poly(rC)-binding protein 1	P60335	-0.42265	0.02503	Viral RNA genome replication (GO:0039694)
Acsm3	Acyl-coenzyme A synthetase, mitochondrial	Q3UNX5	-0.37931	0.025468	Fatty acid beta-oxidation (GO:0006635)
Dab2	Disabled homolog 2	Q3UHC7	0.43158	0.032351	Postsynaptic neurotransmitter receptor internalization (GO:0098884)
Acox3	Peroxisomal acyl-coenzyme A oxidase 3	Q9EPL9	0.2748	0.032448	Fatty acid beta-oxidation (GO:0006635)
Rab1	Ras-related protein	P62821	-0.2738	0.033365	Proteins transport from the endoplasmic reticulum to the Golgi apparatus
Q922Q1	Mitochondrial amidoxime reducing component 2	Q922Q1	0.3658	0.033669	Nitrate metabolic processo (GO:0042126)
Pabpc1	Polyadenylate-binding protein 1	P29341	-0.3699	0.037117	Positive regulation of cytoplasmic translation (GO:20000767)
Fgg	Fibrinogen gamma chain	Q8VCM7	-0.60268	0.042321	Blood coagulation (GO:0072377)
Ftl1	Ferritin light chain 1	P29391	-0.95237	0.043284	Iron ion transmembrane transport (GO:0034755)

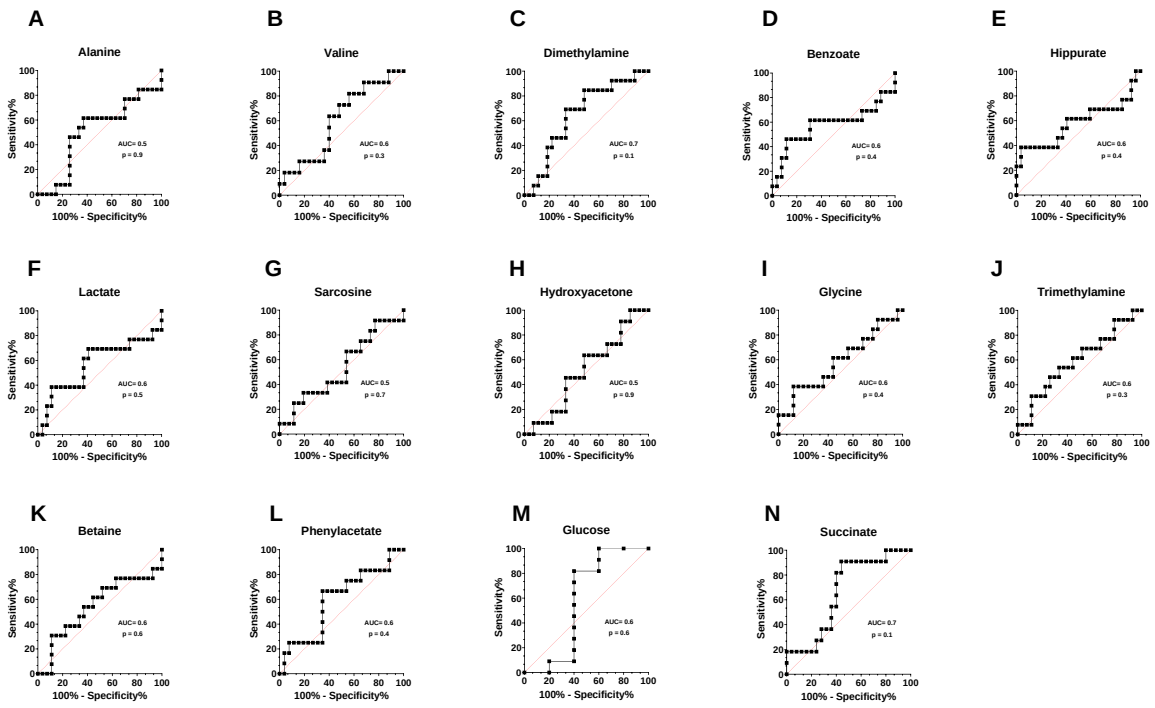
Hsd11b2	11-beta-hydroxysteroid dehydrogenase type 2	P51661	0.30655	0.043791	Aldosterone biosynthetic process (GO: 0032342)
Cltc	Negative regulation of hyaluronan biosynthetic process	Q68FD5	0.34156	0.044011	Negative regulation of hyaluronan biosynthetic process (GO: 1900126)
Slc9a3r2	Na(+)/H(+) exchange regulatory cofactor NHE-RF2	Q9JHL1	-1.143	0.044557	Membrane to membrane docking (GO:0022614)
Acy3	N-acyl-aromatic-L-amino acid amidohydrolase	Q91XE4	-0.32505	0.047853	Aminoacylase activity (GO: 0004046)
Vim	Vimentin;	P20152	-0.48753	0.052171	Epithelial to mesenchymal tansition involved in endocardial cushion formation (GO: 0003198)
Ncl	Nucleolin	P09405	-0.96578	0.060438	CDR-mediated mRNA stabilization (GO: 0070934)
Hk1	Hexokinase-1	P17710	0.27465	0.06352	Glucose 6-phosphate transport (GO:0015760)
D10Jhu81e	Glutamine amidotransferase-like class 1 domain-containing protein 3A	Q9D172	-0.29591	0.067253	-
Vps26a	Vacuolar protein sorting-associated protein 26A	P40336	-0.94742	0.070301	Early endosome to Golgi transport (GO:0034498)
Dlst	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	Q9D2G2	-0.28386	0.07526	Histone succinylation (GO:0106077)
Gm8797	Predicted pseudogene 8797	A0A0A6YW67	-0.30145	0.076615	Cytoplasmic translation (GO: 0002181)
Opa1	Dynamin-like protein	P58281	0.66823	0.080522	Positive/negative regulation of mitochondrial fission (GO:0010636/0090258)
Rab2a	Ras-related protein Rab-2A	P53994	-0.48765	0.081017	Late endosome (GO: 0005770)
Capza2	F-actin-capping protein subunit alpha-2	P47754	-0.84615	0.08244	Regulation of actin filament depolymerization (GO:0030834)

Acss1	Acetyl-coenzyme A synthetase 2-like, mitochondrial	Q99NB1	0.40054	0.08445	Malonyl-CoA biosynthetic process (GO:2001295)
Cycs	Cytochrome c, somatic	P62897	-0.31453	0.085184	Activation of cysteine-type endopeptidase activity involved in apoptotic process by cytochrome C (GO:0008635)
Cyp2a5	Nuclear factor interleukin-3-regulated protein	O08750	0.41626	0.087672	Dibenzo-p-dioxin metabolic process (GO:0018894)
Ca3	Carbonic anhydrase 3	P16015	-1.1543	0.088664	Seryl/leucyl/isoleucyl/cysteinyl-tRNA aminoacylation (GO:0006434)

A3. Supplementary data from Chapter 6



Supplementary Fig A 3.1 - ROC curves of the main metabolites identified in the urine with p value ≤ 0.1, between patients considered non-albuminuric and albuminuric patients. Isobutyrate ( ), Formate ( ) and Pyruvate ( ).



Supplementary Fig A. 3.2 - ROC curves of the remain metabolites identified in the urine with p value > 0.05, between patients considered non-albuminuric and albuminuric patients. Alanine (A), Valine (B), Dimethylamine (C), Benzoate (D), Hippurate (E), Lactate (F), Sarcosine (G), Hydroxyacetone (H), Glycine (I), Trimethylamine (J), Betaine (K), Phenylacetate (L), Glucose (M), and Succinate (N).

Supplementary Table A3.1. Pathway enrichment and topological analysis of the metabolites identified in urine through ^{1}H-NMR using Metaboanalyst online tool in healthy patients when compared to patients with diabetic kidney disease (DKD).

Pathway name	p-value	-log(p)	FDR	Impact
Starch and sucrose metabolism	6.6 E-14	13.2	5.3 E-13	0.4
Galactose metabolism	6.6 E-14	13.2	5.3 E-13	0.03
Neomycin, kanamycin and gentamicin biosynthesis	6.6 E-14	13.2	5.3 E-13	0
Purine metabolism	5.6 E-8	7.3	3.4 E-7	0.02
Glyoxylate and dicarboxylate metabolism	3.7 E-7	6.4	1.8 E-6	0.2
Glutathione metabolism	3.5 E-4	3.5	0.001	0.1
Polyphryrin metabolism	3.5 E-4	3.5	0.001	0

Citrate cycle (TCA cycle)	0.001	2.7	0.005	0.1
Alanine, aspartate and glutamate metabolism	0.001	2.7	0.005	0
Phenylalanine metabolism	0.003	2.5	0.008	0
Valine, leucine and isoleucine degradation	0.01	1.9	0.03	0
Valine, leucine and isoleucine biosynthesis	0.01	1.9	0.03	0
Pantothenate and coA biosynthesis	0.06	1.9	0.03	0
Glycine, serine and threonine metabolism	0.09	1.2	0.2	0.4
Primary bile acid biosynthesis	0.1	1.0	0.1	0.02
Lipoic acid metabolism	0.5	1.0	0.1	0.001
Pyruvate metabolism	0.5	0.3	0.7	0.2
Glycolysis/ Gluconeogenesis	0.5	0.3	0.7	0.1
Cysteine and methionine metabolism	0.6	0.2	0.7	0
Arginine and proline metabolism	0.6	0.2	0.7	0

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- Bernardino RL, **Braga PC**, Oliveira PF, Alves MG (2024). Chapter 19 - Determination of glycolysis flux by extracellular flux measurements. In: Ferreira R, Oliveira PF, Nogueira-Ferreira R, eds. *Glycolysis: Academic Press*; 2024: 443-54. https://doi.org/10.1016/B978-0-323-91704-9.00004-5

- Braga PC, Rodrigues AS, Oliveira PF and Alves MG (2024). Chapter 20- Mitochondrial transplantation in the Kidney. In: Ostojic SM, Kubat GB, Ulger O, Gunaydin S, eds. *Mitochondrial Transplantation and Transfer: Academic press*; 2024 :411-434. https://doi.org/10.1016/B978-0-443-18858-9.00020

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Review

Mitochondrial Pathophysiology on Chronic Kidney Disease

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Abstract: In healthy kidneys, interstitial fibroblasts are responsible for the maintenance of renal architecture. Progressive interstitial fibrosis is thought to be a common pathway for chronic kidney diseases (CKD). Diabetes is one of the boosters of CKD. There is no effective treatment to improve kidney function in CKD patients. The kidney is a highly demanding organ, rich in redox reactions occurring in mitochondria, making it particularly vulnerable to oxidative stress (OS). A dysregulation in OS leads to an impairment of the Electron transport chain (ETC). Gene deficiencies in the ETC are closely related to the development of kidney disease, providing evidence that mitochondria integrity is a key player in the early detection of CKD. The development of novel CKD therapies is needed since current methods of treatment are ineffective. Antioxidant targeted therapies and metabolic approaches revealed promising results to delay the progression of some markers associated with kidney disease. Herein, we discuss the role and possible origin of fibroblasts and the possible potentiators of CKD. We will focus on the important features of mitochondria in renal cell function and discuss their role in kidney disease progression. We also discuss the potential of antioxidants and pharmacologic agents to delay kidney disease progression.

Keywords: chronic kidney disease (CKD); electron transport phosphorylation (ETC) impairment; epithelial-mesenchymal transition (EMT); fibrosis; mitochondria; oxidative stress (OS)

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1. Introduction

Chronic Kidney disease (CKD) is a major global health concern that has high morbidity and mortality [1]. The continuous decline of renal function is closely associated with progressive aberrant accumulation of Extracellular matrix (ECM) proteins such as fibronectin and collagen that culminates in fibrosis. This progressive interstitial fibrosis is thought to be a common pathway for CKD [2]. Even though the extent of fibrosis does not correlate with the glomerular filtration rate (GFR) measure, it gives a hint to the future progression of the disease with greater certainty [3]. Mechanisms underlying fibrogenesis is a matter that has grown in scope and understanding in the present. However, the mechanisms in which this process can halt, or even reverse fibrosis remains poorly understood.

Transforming growth factor-β (TGF-β) has been recognized as the canonical ligand and some strategies to halt fibrosis passes by controlling their precursors that drive pathological fibrotic responses [4]. Nevertheless, metabolic, and innate immune responses, both processes related to mitochondrial functioning, are emerging as plausible strategies to target CKD progression. Therefore, it is remarkably important to understand mitochondrial biology and pathophysiology for effective therapeutic options in renal diseases [5]. Reactive oxygen species (ROS) are not necessarily harmful to the cells. At moderated concentrations, ROS act as second messengers and regulate intracellular signal transduction pathways.

A dysregulation between prooxidant/antioxidant equilibrium results in oxidative damage in cells, tissues, and organs [6,7], as the kidney a highly vulnerable organ, since it has a high energetic demand [8]. This review will discuss the role of Epithelial-Mesenchymal Transition (EMT) in interstitial fibrosis, a process that is associated with multiple kidney diseases that result in irreversible loss of renal function. The paradigm of EMT plasticity will be elucidated, as well as signaling mediators and potential therapeutic targets to avoid progressive kidney disease at earlier stages, even those that await deeper biochemical understanding. Furthermore, we will unveil the possible role of mitochondria dysfunction in the pathogenesis of kidney disease and summarize the recent development of mitochondria-targeted therapies.

2. Chronic Kidney Disease

The kidney has a high energy demand, due to its role in filtering the blood to remove accumulated toxins along with concentrating the urine to avoid dehydration [8]. To balance that activity, the kidney has an active repair capacity since they are able to restore in terms of function and structure after episodes of acute lesions. However, as it is a highly demanding organ, it is susceptible to multiple injuries that with time may result in major problems, such as culminating in a partial or even complete kidney failure. At these stages, the only solution is dialysis and ultimately, kidney transplant [9]. When it happens, kidneys fail to purify the body of metabolic end products, which are normally excreted into the urine and retained in the urea. Renal dysfunction can be determined when the glomerular filtration rate (GFR) is below 60 mL/min/1.73 m^2 for more than three months and when albuminuria, defined as an albumin-to-creatinine ratio above 30 mg/g per day, is present [10]. Uremic retention is usually classified according to its removal pattern by dialysis and can be classified in three different groups, according to the reduced levels in patients at end-stage renal disease (ESRD): (1) small water-soluble compounds with a Molecular weight (MW) < 500 D, (2) middle- to large molecules, mostly peptides; MW > 500 D, and (3) protein-bound compounds with a low MW (<500), where these solutes are difficult to remove by any type of dialysis. In this last group, this type of retention is a trigger for kidney fibrosis in CKD [4].

Kidney disease can be divided into two major categories: acute kidney injury (AKI) and CKD. AKI is characterized by a fast renal function decline while CKD is linked to a gradual loss of renal function over time [11]. CKD is a worldwide health issue with consequences for the cardiovascular immune system which may lead to premature mortality [12,13]. Diabetes is the predominant pathophysiologic driver of CKD, although other conditions, such as hypertension, metabolic disorders, sepsis, ischemia/reperfusion injury, and exposure to nephrotoxins may also lead to CKD [14–16]. Depending on the initial site of injury, it will cause different subsequent pathologies. If there is a renal disorder that affects glomerular structure and function, it is characterized as glomerular fibrosis, whereas infections or proteinuria will lead to tubulointerstitial fibrosis [17]. Curiously, the development of fibrosis in the tubulointerstitium is the foremost predictor for the progression to ESRD in diabetic nephropathy (DN) [18]. DN is considered to be a syndrome [19] characterized by leakage of albumin, metabolites, and ions into the urine, alterations in the GFR, and a higher prevalence of stroke and cardiovascular diseases [20,21]. DN is one of the diseases that is the major cause of CKD and the absolute number of patients affected is still rising, demonstrating that this is a matter that deserves more attention, since diabetes unfortunately is also rising in astronomic proportions [22].

3. Fibrosis in the Kidney

In healthy kidneys, interstitial fibroblasts are responsible for the maintenance of renal architecture, which includes a balance between production and turnover of the ECM, mediating inflammatory processes [23,24]. However, prolonged injury in the kidney sponsors the progression of fibrosis in response to multiple nephropathies, including glomerulonephritis, spontaneous lupus nephritis, chronic allograft nephropathy, and DN [25–27]. The progression

of fibrosis in the kidney affects 1 in 6 people globally annually per 100,000 individuals [28]. By definition, fibrosis is associated with significant alterations in the matrix, whereby functional tissue is replaced by connective tissue. In the fibrotic kidneys, the interstitial spaces are filled with predominantly type I and III collagen, and fibronectin [29,30]. Tubulointerstitial fibrosis is characterized by an excessive connective tissue accumulation during a reparative or reactive process that continues despite the resolution of the primary inflammatory process, leading to the gradual destruction of renal tubules and functional nephrons [31].

Possible Origin of Fibroblasts

The source of interstitial fibroblasts remains a matter of debate. Some genetic mapping studies in mice pointed to a mesenchymal origin, resident in the kidney from nephrogenesis. These precursors are mostly attached to capillaries, specifically at branching points of the peritubular capillary plexus, and some investigators called these cells "pericytes" or "perivascular cells", while others preferred to call these cells resident fibroblasts [31]. However, other studies identified that fibroblasts can be derived from leukocytes [32] or even that endothelial cells can be differentiated into "active" fibroblasts [33]. Theories that support the leukocytes as main precursors of fibroblasts rely on the fact that biomarkers used to detect fibroblasts, such as vimentin, labels macrophages and injured epithelial cells as well as fibroblasts. In support to this, Fibroblast-specific protein 1 (FSP-1), another common biomarker used to identify a supposed fibroblast formation, is also identified in macrophages in rodent models, but this expression is not observed in the fibroblasts [34]. Furthermore, the detection of collagen inside macrophages arrives in a possible ability of this type of leucocytes to produce elements for the pathological matrix.

All of these data reveal that the real source of fibroblasts has to be recapitulated as well as the proper markers used to identify fibrogenesis processes. Additionally, the absence of epithelial to EMT as a source of fibroblast has been reformulated in multiple organs, and some conjectures were observed regarding leucocytes [35]. However, the contribution of circulating leucocytes to matrix-producing fibroblasts continues to generate discordance. Despite the advances that have been made, the studies were performed using an animal model, not in humans, and therefore the exact fibroblast origin population in human kidney disease remains unclear. Additionally, this gap of knowledge is mainly due to a lack of biomarkers available. Myofibroblasts possess the features of both fibroblasts and smooth cells and actively produce collagen in the repairing tissue [36,37]. Although the role of myofibroblasts is well documented, the basis of fibroblast origin, heterogeneity, and abundance in vivo is still a matter of debate with a lot of drawbacks. It is believed that fibroblasts are derived from multiple parental lineages, including endothelial, epithelial cells. A small number can also be derived from bone marrow cells [38]. In this review, we discuss the biomarkers available, including the mitochondria and metabolic fingerprints, and how they act to prevent kidney disease progression.

4. Epithelial–Mesenchymal Transition (EMT)

In response to injury, epithelial cells undergo through EMT process, in which there is a presence of mesenchymal markers instead of epithelial markers [39]. EMT is a process highly coordinated composed of specific key events. The first phase of EMT is characterized by the loss of cell-cell contact and apical-basal polarity leading to a cellular destabilization. This destabilization is caused by the stimulation of tubular epithelial cells with cytokines such as epidermal growth factor (EGF), basic fibroblast growth factor (FGF-2) and TGF-β1, secreted mainly from infiltrating mononuclear cells, resulting in partial loss of epithelial membrane junction and tight junction proteins, such as E-cadherin and zonula occludin. The intermediated phase is related to the de novo expression of mesenchymal proteins, including α-smooth muscle actin (α-SMA) and synthesis of FSP-1 (Figure 1). At this point, there is an actin reorganization, with the reestablishment of the cytoskeletal proteins, and up-regulation of matrix metalloproteinases (MMP-2 and MMP-9) [27]. In the last phase of

EMT transition, the fibroblasts are activated and become myofibroblasts, with an enlarged spindle-shaped morphology, with a higher capacity of cell migration and invasion into the tubulointerstitium [40].

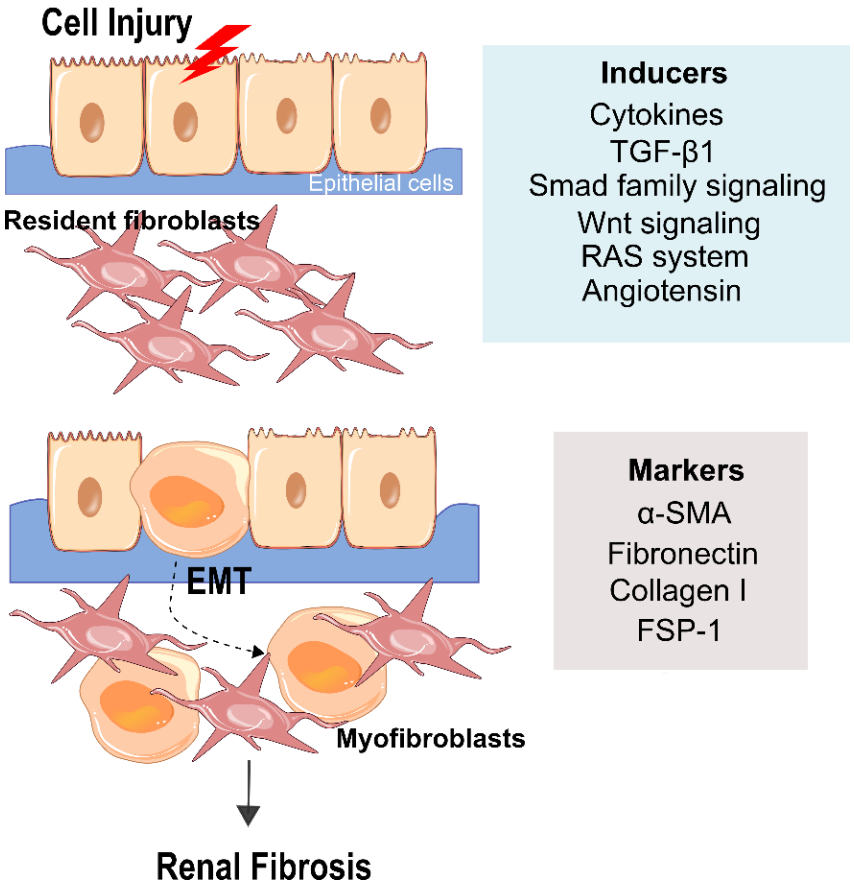

Figure 1. Epithelial and Mesenchymal transition. After injury, epithelial cells suffered a process called epithelial-mesenchymal transition (EMT), in which cells start expressing mesenchymal markers rather than epithelial markers. A wide number of factors have been pointed to as a potentiator of tubular EMT. The most potent inducer is Transforming growth factor-beta 1 (TGF-β1). Furthermore, signaling pathways that cooperate with TGF-β1 are also considered to be important mediators for EMT and consequently to renal fibrosis, such as Smad family, Renin-angiotensin system (RAS), and Wnt/β-catenin signaling. EMT is a highly coordinated process, characterized by the loss of cell-cell contact leading to cellular destabilization. Then, there is a de novo expression of mesenchymal proteins, such as α-SMA, and where it produced extracellular matrix proteins, including fibronectin, collagen I, and Fibroblast-specific protein 1 (FSP-1.) In the last phase of EMT, fibroblasts are activated and called myofibroblasts. These myofibroblasts share the same place that resident fibroblasts, whose proliferation is increased. This accumulation in the interstitium between tubules culminates in tubulointerstitial fibrosis.

Several cytokines and growth factors regulate EMT. Yet, it is believed that stimulation by cytokines is not enough to induce complete EMT. Several studies demonstrated that complete EMT only occurs when the integrity of the tubular basement membranes is disturbed [24,41]. Indeed, EMT is an extreme manifestation of epithelial cell plasticity but also a key event in the regenerative process of the kidney. EMT has emerged as a booster, yet highly debated, to renal fibrosis. Concerning epithelial cells, it is reasonable to hypothesize a switch to a mesenchymal phenotype since these cells during embryogenesis, has a mesenchymal origin. In 1995, Strutz and collaborators elucidated, for the first time, the EMT concept in adult solid organs, such as in the kidney [42]. Myofibroblasts, after injury, participated in the proliferation of resident interstitial fibroblasts. It is believed that cells

such stem cells derived from bone marrow, epithelial cells, vascular smooth muscle cells, endothelial and pericytes, are associated as a source of myofibroblast development [32,43,44].

EMT participate in the pathogenesis of CKD progression in vivo and in certain conditions, it was reversible, i.e., mesenchymal cells acquired again epithelial phenotype. For that a dedifferentiation process is needed. Some shreds of evidence showed that renal tubular epithelial cells cope with damage and have the ability to acquire a stem-like phenotype, expressing CD24, CD133, and vimentin [45]. Like stem cells, these cells were able to proliferate and regenerate [46]. Another report suggests that an endogenous pool of stem cells that are present within the tubule mediates the repair and confer the epithelial phenotype back [47]. However, there is still a lot of controversy surrounding these theories.

Renal tubular epithelial cells can differentiate, proliferate, and re-epithelize after being injured. Recent studies pointed to N-acetyltransferase-8 (NAT8) as a protective enzyme in the kidney. This enzyme is highly expressed at the kidney and is suggested to have a role in differentiation, and re-epithelization processes during kidney injury. Grácio and collaborators demonstrated that in prolonged injury, there was a decrease in NAT8 expression followed by a late rescue. This data indicated that NAT8 can be a possible target, once it maintains the regenerative potential when renal tubular epithelial cells dedifferentiate [48].

Main Potentiators of EMT That Culminate in CKD

There is no doubt that EMT is present in the development and progression of the commonest renal diseases [49]. A wide number of factors have been suggested as crucial potentiators of tubular EMT. It was described that TGF-β1 has a critical role in the basic process of the EMT process, as it is considered to be the most potent inducer [50]. TGF-β1 is a profibrogenic cytokine that mediates the differentiation of fibroblasts into myofibroblasts. Mechanistically it suppresses collagen degradation and stimulates matrix-producing cell proliferation and collagen synthesis in the repairing tissue, leading to the fibrous formation [51]. TGF-β is secreted in an inactive state that requires processing before it can exert its effect, and meanwhile, it is stored on the surface of cells and the ECM, where it is transformed to active TGF-β [52]. At this point, TGF-β induces the differentiation of fibroblasts into myofibroblasts [53]. Along with this, TGF-β has pleiotropic effects, which simultaneously can regulate cellular proliferation, migration and differentiation, modulation of the immune system [54], and stimulation of oxidative stress [55]. In mammals, there are three TGF-β isoforms, nonetheless, the isoform TGF-β1 is the most associated with renal fibrosis [56]. Indeed, the kidney is highly susceptible to its overexpression [57]. It is widely expressed in all kidney cells, particularly in glomeruli [51], and even expressed in macrophages that invade the kidney [52].

In addition to the molecular pathways between the cooperation of TGF-β1 and cytokines that drive EMT, the Smad family of transcriptional activators are considered to be the most important mediators of TGF-β1 signaling. The Smad family is comprised of three different subgroups considering their role in TGF-β family signal transduction: Receptor-regulated Smads, known as R-Smads (which includes Smad 1, 2, 3, 5 and 8), common Smad4 (Co-Smad), and Smads 6 and 7, considered to be Inhibitory Smads (I-Smad). The role of Smads is intimately related to the progression of CKD. The most important are Smads 2 and 3, which control TGF-β in renal fibrosis, being fibroblasts highly activated in DN, hypertensive nephropathy, and obstructive kidney disease [58–60]. Smad 7 controls renal inflammation [61]. TGF-β signaling is initiated with serine/threonine kinase receptor oligomerization and R-Smad phosphorylation. After that an R-Smad/Co-Smad complex is translocated into the nucleus to control transcription of TGF-β responsive genes. I-Smads are responsible for dissipating TGF-β signaling by blocking R-Smads [62–64]. Multiple in vivo studies of different renal-related diseases revealed that after silencing Smad 3, the fibrosis process was alleviated, suggesting that Smad 3 can be a critical pathogenic mediator of TGF-β/Smad signaling in renal fibrosis [65–68]. In concordance with these studies, Li and collaborators demonstrated that MAPK-Smad signaling can directly activate Smads 2

and 3 to mediate diabetic injury [58]. Moreover, Smad 3 is associated with fibrotic genes, such as collagen [69,70]. Moreover, Bone morphogenic protein (BMP-7), a member of the TGF-β superfamily, can also inhibit TGF-β induced EMT in Smad dependent fashion [71] and Smad-independent pathways such as ERK and MAPK through Smad 1 and 5 activation [72–74]. It is highly expressed in the kidney, and in experimental animal models revealed to be capable of halting and reverting renal fibrosis [75]. Additionally, BMP-7, through Smad signaling, plays a protective role in podocyte differentiation [76].

Tubulointerstitial fibrosis, as previously mentioned, is associated with hypertension, inflammation, and activation of some signaling pathways and systems, such as TGF-β1/Smad and Wnt/β-catenin and renin-angiotensin system (RAS) [77,78]. They are closely linked as they form a vicious cycle in which the RAS stimulate tubulointerstitial fibrosis by triggering TGF-β/Smad and Wnt/β-catenin signaling. In a limiting renal blood flow situation, tubulointerstitial fibrosis augments arterial blood pressure and consequently activates RAS, which in turns activates TGF-β/Smad and Wnt/β-catenin signaling [79]. Wnt/β-catenin is activated in multiple kidney diseases, such as DN, polycystic kidney disease and obstructive nephropathy [80,81]. Furthermore, β-catenin has a critical role in the kidney in terms of controlling the expression of the intrarenal RAS. A RAS cascade event starts with the conversion of angiotensinogen into angiotensin (Ang) I through renin. Ang I is subsequently converted to Ang II, the major effect of the RAS, through the angiotensin-converting enzyme (ACE). Renin is the rate-limiting step in RAS activation [82]. All the components of RAS are expressed in kidney tissues, and its activation is intimately related to kidney injury [77,83]. Ang can cause pro-fibrotic event in proximal tubular epithelial cells, podocytes, and mesangial cells of the kidney [79]. Multiple studies already exposed the interaction between Ang II and TGFβ-1 that influences ECM production [79]. It also induces EMT in tubular epithelial cells [84], by stimulating the collagen and fibronectin expression [85]. Therefore, simultaneous blockade of RAS activation and inhibition of TGF-β/Smad and Wnt/β-catenin signaling present are cornerstone therapies against the progression of CKD.

FGF-2 is a mitogen for renal fibrosis. It induces proliferation in primary cortical fibroblasts and stimulates the expression of fibroblastic markers [39]. Additionally, the induction of EMT is magnified when conjugated with TGF-β1 [86]. Connective Tissue Growth Factor (CTGF) is a modulator of TGF-β and MBP-7 antifibrotic signals. CTGF binds to TGF-β1 and potentiates myofibroblast activation, fibronectin accumulation, and α-SMA expression [87]. It was initially reported in renal cells in 1997, and over the years has been correlated with the degree of tubulointerstitial fibrosis [71,88]. In multiple human and animal models of kidney fibrosis, including DN, CTFG has been found overexpressed [89,90]. The anti-CTGF antibody, FG-3019, was described to be effective and well-tolerated in phase I clinical trial for the treatment of microalbuminuria DN [91].

5. The Possible Mechanisms to Delay Fibrosis Progression—Pharmacological and Metabolic Approaches

In healthy kidneys, there are endogenous signals that maintain the normal epithelial phenotype in tubular cells and keep homeostasis. However, with the progression of CKD, these homeostatic mechanisms are disrupted, resulting in fibrosis progression. Given the impact of EMT in CKD progression, there is an urgency to find novel ways to delay, inhibit or even reverse this process. To cope with this problem, some strategies have been developed to slow down or reverse the fibrogenesis process.

5.1. Mechanisms Associated with Signaling Pathways

A pharmacologic approach to delay the progression of fibrosis through TGF-β1 blockage has not revealed the best therapeutic approach. Indeed, LY2382770, an anti-TGF-β1 antibody that blocks all the three forms, in phase II study revealed no efficacy [35]. Fresolimumab is a human monoclonal antibody able to neutralize the isoforms of TGF-β. It was administrated in a single infusion in patients with primary focal segmental glomerulosclerosis.

It has advanced to phase I clinical trial; however, its effectiveness was not demonstrated [92]. In phase II clinical trial, another human monoclonal antibody against TGF-β1, is performed in diabetic patients. Nonetheless, no inhibitory effects were observed [93].

Under the importance of Smad signaling in kidney disease, it is valuable to target these downstream mediators of TGF-β signaling. Selective Smad 3 inhibitors have demonstrated the ability to block excessive ECM production and EMT, halting the development of diabetic renal fibrosis. Indeed, an in vivo study with streptozotocin (STZ)-induced diabetic nephropathy mice, demonstrated to be able to halt the development of diabetic renal fibrosis [94]. In this study, the authors found that a specific inhibitor of Smad 3 was able to inhibit TGF-β1. However, no significant changes were observed on albuminuria levels. This study demonstrates, again, the importance of understanding the molecular mechanisms that are behind slight processes that can advance in kidney disease, more specifically in DN, once albuminuria is no longer a biomarker plausible to detect this pathology, specifically when diabetes disease is present.

Wang and his collaborators used Human Kidney (HK-2) cells and mice with unilateral ureteral obstruction (UUO) and tested novel tetracyclic triterpenoid compounds (PZC, PZD, and PZE), in which they act on the inhibition of the RAS, TGF-β/Smad, and Wnt/β-catenin signaling. These compounds were revealed to selectively inhibit TGF-β1 and Ang II-induced Smad3 phosphorylation by blocking the interaction of TGF-β1 with Smad3 [95]. Moreover, ACE2, was discovered as a homolog of ACE, with a higher catalytic efficiency for degrading Ang II than ACE [96]. This enzyme is highly distributed in the kidney, especially in the brush border of proximal tubules [97]. However, the distribution is altered when kidney disease is present. Notably in DN, the RAS system is a contributor to the progression of the disease. ACE2 expression, has been shown to be altered in diabetes. Its gene deletion expands fibrosis, albuminuria, and glomerular hypertrophy in Akita mice [98]. Additionally, the evaluation of Ace2 in urine could provide a better picture of renal injury and disease progression. It was noticed that ACE2 in urine was higher in patients with CKD compared to healthy patients, and even higher in patients with DN, compared to patients with other renal diseases [99]. Furthermore, patients with urinary ACE2 have been described as variable and can be dependent of the stage of the disease, once it is more present in patients with longstanding diabetes type 1 and CKD that with patients with only diabetes [100]. Collectively, these studies demonstrated that ACE has a renoprotective role in experimental models of diabetes and can act as a promising early clinical marker for DN. The administration of human recombinant ACE has been demonstrated to prevent Ang II-mediated hypertension and renal injury [101,102] and reduce the progression of DN in mice [103]. Clinical trials are already ongoing, demonstrating positive effects, where the administration was well-tolerated in healthy individuals [104] and multiple pharmacologic solutions were tested in order to modulate the RAS system and decrease the progress of renal diseases [105].

Another possible approach found was through the inhibition of signaling pathways that are intimately related to immune signaling that activate the pathological fibroblast. Genetic studies already demonstrated that there is a predisposition to fibrotic kidney disease through dysregulation of the innate immune system. Some polymorphisms in Toll-like receptor 4 (TLR4) [106], Interleukin (IL) IL1β, and IL1 [107,108] are associated with CKD. This correlation unveils the possibility to dampen pro-fibrotic responses and inflammation, without blocking downstream signaling pathways that have homeostatic functions such as NOTCH, WNT, TGFβ, or HEDGEHOG. One example was the inhibition Janus kinase-signal transducer and activator of transcription pathway (JAK-STAT) in DN, through Baricitinib. This study demonstrated a reduction in proteinuria levels [35]. Receptor tyrosine kinases (RTKs) are important players in the pathogenic process of kidney fibrosis. In 2019, Park and his collaborators revealed that the absence of macrophage-stimulated 1 receptor (RON), a RTK receptor that attenuated the expression of EMT and fibrosis-related proteins through control of Src and Smad pathways in HK-2 and fibroblast (NRK49F) cells [109]. Among other studies, RTK inhibition aid in the prevention and

treatment of kidney fibrosis. Imatinib, which is a platelet-derivated growth factor receptor (PDFGR) inhibitor, diminished pathological alterations in different models of CKD [110]. Furthermore, AG1296, the inhibitor of fibroblast growth factor receptor (FGFR) can block the proliferation of kidney fibroblasts [39]. RTK is also linked to TGF-β signaling. That is shown when epidermal growth factor receptor (EGFR), another RTK related to kidney fibrosis, is inhibited, and lower fibrogenesis mediated by TGFβ occurs [111].

Angiotensin II, as previously described, can induce TGF-β1 synthesis in tubular epithelial cells and fibroblasts. Some studies claim that blocking RAS can delay the progression of renal disease. Losartan [112] and Captopril [113] were already described as a possible pharmaceutical solution to delay renal diseases such as chronic allograft nephropathy and diabetes mellitus. BMP-7 is described to have the ability to reverse EMT, through reinduction of the epithelial cell adhesion molecule, E-cadherin. Intraperitoneal injection of human recombinant BMP-7 in mice with nephrotoxic serum nephritis, halt the disease progression [71]. There are currently no antifibrotic drugs approved for the treatment of CKD, representing a major unmet medical need [114].

In recent years, metabolic alterations are recognized as an important pathogenic process that underlies fibrosis. Therefore, metabolically targeting therapies could become important strategies for fibrosis reduction [115].

5.2. Metabolic Targeting Therapies

One possible approach is to follow the disordered metabolism of carbohydrates, lipids, proteins, and hormones. Indeed, ECM is intimately related to glucose metabolism. Targeting glucose metabolism has demonstrated interesting results in halting fibrosis. Ding and collaborators developed a study where they administered 2-Deoxy-D-Glucose (2-DG), a hexokinase inhibitor in rat renal fibroblast cells, which resulted in a diminished expression of TGFβ-associated biomarkers [116]. Furthermore, several clinical outcomes demonstrate that glucagon-like 1 receptor agonist (GLP-1 Ras) have beneficial effects on renal outcomes, being particularly beneficial in terms of oxidative stress and inflammation. Mechanistically, GLP1-Ras improved glucose metabolism by increasing insulin secretion and suppression of glucagon [117]. Within this class of pharmacologic options, some clinical trials showed promising results in diminishing albumin. For instance, the LEADER trial, which involves liraglutide, demonstrated that 52 weeks of treatment led to an increase in glomerular filtration rate and reduction of albuminuria [118]. The SUSTAIN-6 trial was a double-blind trial in which each type two diabetes patient received 0.5 mg once-weekly of semaglutide, and this drove a reduction in new onset macroalbuminuria when compared to the patients that received the placebo [119].

DN, an in vitro study, using HK-2 cells, demonstrated a new mechanism for the amelioration of renal fibrosis. Here, the authors used exendin-4, and it was able to down-regulate miR-192 expression during high glucose treatment. Thus, GLP1R seems to be a key in the regulation against renal fibrosis during diabetic kidneys [120], resulting in a decrease in mesangial and endothelial dysfunction, as well as a decrease in podocyte loss [121].

Furthermore, the role of the kidneys in glucose homeostasis gained attention once glucose transport through sodium glucose transporter-2 (SGLT2), consumed a high proportion of oxygen and ATP in the kidney [122]. In patients with long-standing diabetes, inhibition of SGLT2 by empagliflozin administration halted the advance of renal disease [123]. Along with this, inhibition of SGLT2, in rats with early diabetes, decreased tubular Na+/glucose cotransport, which increased the sodium delivery to the macula densa, resulting in a diminished glomerular hyperfiltration [124].

Targeting fatty acid metabolism and/or receptors also yielded beneficial effects in reducing fibrosis. Free fatty acid receptor 1 (FFA1R) and GPR84 are G-protein-coupled receptors that modulate metabolism through insulin secretion and cellular respiration. In Ffa1r−/− or Gpr84−/− knockout mice used as models of kidney fibrosis, FFA1R revealed to have a protective effect against renal fibrosis, whereas GPR84 had the opposite effect [125]. After this discovery, in this study, the authors used PBI-4050, a synthetic fatty

acid ligand that has agonist affinity to FFA1R and antagonist affinity to GPR84. Therefore, the authors concluded that this compound significantly attenuated fibrosis, being effective for managing inflammatory and fibrosis-related diseases observed in multiple fibrosis models [125]. Additionally, managed carnitine *O*-palmitoyltransferase 1 (CPT1) may be useful for preventing and treating CKD. Human and mouse models with tubulointerstitial fibrosis had lower expression of key enzymes and regulators of fatty acid oxidation (FAO) and hence higher lipid deposition. Kang and collaborators used a synthetic compound, C75, which is a CPT1 activator and fatty acid synthase inhibitor and demonstrated that it was able to preserve renal cell viability and decreased renal fibrosis in human kidney tubule samples [126].

Endothelin-1 (ET-1) is a vasoconstrictor peptide with a role in hypertension and kidney injury [127,128]. It promotes proteinuria and has been implicated in renal inflammatory and fibrotic pathways [129]. In addition, ET-1 is increased in diabetes, being an increased risk of renal disease [130]. In diabetic rats, administration of atrasentan or avosentan, an ETA receptor blockage, reduced albuminuria and renal fibrosis [131,132]. Unfortunately, the administration of avosentan in combination with RAS blockade in patients with DN, after 4 months of treatment has not successfully progressed due to excess cardiovascular adverse events [133].

5.3. Natural Approaches

Natural compounds were already tested in CKD, however, all of them revealed an insufficient quality. Emodin is a natural extract from rhubarb and has beneficial effects on renal function in vivo [134]. To improve the low water solubility and kidney nonspecificity, Huang and collaborators encapsulated emodin in a self-micro emulsifying drug delivery system, which resulted in an improvement of its oral bioavailability, leading to an inhibition of fibronectin, TGF-β1, and intercellular adhesion molecule 1 expression [135]. In sum, drug delivery and therapeutic effects need to be recapitulated, once some pharmacological approaches need the administration of high doses daily, which leads to drug-related toxicity side effects.

Celastrol is a triterpene derived from traditional Chinese medicine, with potent anti-fibrotic proprieties. It is known to suppress cardiac [136] and pulmonary fibrosis [137]. Tang and his collaborators explored its possible effects on renal fibrosis development. They concluded that celastrol was able to attenuate the progression of renal fibrosis by up-regulating cannabinoid receptor 2 expression, which is an anti-fibrotic factor, through Smad3 signaling inhibition on HK-2 cells [138].

Low Vitamin D level is correlated with patients with CKD. It is linked to RAS activation and podocyte injury [139,140]. Additionally, it is believed to have a key role in EMT transition [141]. Vitamin D may also have positive effects on oxidative stress, through restoration of nuclear factor erythroid 2-related factor 2 (Nrf2) levels [142]. Additionally, in the VITAL study, type 2 diabetics randomized to a synthetic D2 agonist (paricalcitol) were able to reduce albuminuria, in a 24-week treatment [143].

5.4. miRNA Approaches and Others

Hormonal therapy has been recognized to have a beneficial role in fibrosis. As happens in lung fibrosis [144] and skin fibrosis [145], administration of thyroid hormone through aerosolized active thyroid hormone (T3), prevented renal fibrosis. In this model, T3 increased the expression of the microRNA miR34a, inhibiting the EMT induced by TGBβ-1 [146]. This study, along with others, demonstrated the possibility of improving the delivery of miRNA or siRNA into the kidney. The common modality for the administration of siRNA is through intravenous injection [147]. In mice, intravenous administration of Smad4 siRNA prevented folic acid-induced in renal fibrosis process by inhibiting α-SMA expression [147].

Gene therapy using Smad microRNA is another solution. miR-21 [148], miR-29 [149], and miR-192 [150] expression are Smad3-dependent and acted to decrease the fibrotic

marker levels. Indeed, inhibition of miR-21 in unilateral ureteral obstruction (UUO) kidneys decreased collagen deposition and reduced fibronectin, α-SMA, and plasminogen activator inhibitor 1 and increased the levels of TGF-β in injured kidneys [151].

One curious way to take advantage of miRNA-based therapeutics is when is transported via exosomes. miR-let7c is down-regulated in fibrogenesis in vivo and in vitro [152]. Wang and collaborators injected mesenchymal stem cells overexpressing miR-let7c to the renal parenchyma via exosomes and resulted in an attenuation of kidney injury, by down-regulating the expression of collagen, MMP9, and TGF-β1 in NRK52E cells and UUO kidneys [153].

Another possible approach is using Lysozyme (LZM) for kidney-specific delivery for targeting kidney cells [154]. This approach takes advantage of the fact of being a low molecular weight protein, which passes freely through the glomerulus during filtration and subsequently reabsorbed by proximal tubular epithelial cells. Allied to this, it is biodegradable and considered non-immunogenic [155]. However, this technique needs improving since it has cardiovascular side effects [156].

Moreover, stem cells revealed potential benefits in DN. Park and collaborators developed a study, in which they treated diabetic Sprague-Dawel rats with human umbilical cord blood-derived stem cells (hUCB-SC) had treatment for four weeks. Intravenously administration of hUCB-SC reduced proteinuria, renal fibronectin, α-SMA and E-cadherin down-regulation [157]. Notwithstanding, some studies point out that stem cells may be a mechanism to reduce kidney disease progression, but there is no evidence on this approach in human studies yet [158].

Human kidney organoids that derive from stem cells are pointed out as promising tools for the personalized evaluation of factors important for the development and prevention of fibrosis. However, these organoids fail in some aspects, such as a lack of blood supply, in glomerular filtration, they do not have immune responses, and hence, they may recapitulate only some of the molecular and cellular responses seen in adult kidneys after AKI [159]. Although several studies were successful at the pre-clinical level, only limited advances have been made so far in the translation of these findings to the level of patient treatment [89].

Taken all the strategies adopted above together, there are still mechanisms to unveil.

Furthermore, there are some tubular biomarkers in urine used to evaluate the development of CKD, such as KIM-I [160,161], NGAL [162], L-FABP [163], and Cystatin C [164]. However, these biomarkers have been inconsistent, with no valuable results on the prediction of CKD in humans. It should be noted that several clinical trials are already underway, to circumvent kidney diseases, such as DN, as well as all the mechanisms associated with it (i.e., fibrosis). However, they all have a flaw. The therapies used, as detailed in Table 1, are adapted from other pathologies, have a slight effect or take a long time before that effect is pronounced [158]. Thus, the search for more reliable renal biomarkers, with more sensitivity and specificity is extremely important for early prediction of the onset and monitoring of the progression of kidney-related diseases.

In the past few years, more attention has been pained to the role that mitochondria can have on CKD, and how its dysregulation can be related to a start point of the disease.

Table 1. Therapeutic options available to delay kidney fibrosis.

Therapeutic Options	Target	Function	Effect	References
LY2382770 (phase II study)	TGFβ-1	Modulator of TGFβ-1	-	[35]
Barcitinib	JAK-STAT pathway	↓ JAK-STAT pathway	↓proteinuria levels	[35]
FG-3019	CTGF	Modulator of TGFβ-1	↓microalbuminuria	[91]
Triterpenoid compounds (PZC, PZD, PZE)	RAS	Smad3 signaling and TGFβ-1 signaling	↓ TGFβ-1	[95]
ACE2	TGFβ-1 with Smad3 signaling	Degrade Angiotensin II	↓renal fibrosis ↓albuminuria ↓glomerular hypertrophy	[98]
Imatinib	RTK	PDFGR inhibitor	↓proliferation of fibroblasts	[110]
AG1296	RTK	FGFR blockage	-	[111]
Losartan	RAS	Angiotensin II	Delay of DN	[112]
Captopril				[113]
Liraglitude semaglutide	GLP-1	insulin secretion and suppression of glucagon	↓albuminuria ↑GFR	[117–119]
Empagliflozin	SGLT2	SGLT2 inhibition	Delay the advance of renal disease	[123]
PBI-4050	FFA1R	Anagonist of FFA1R	↓renal fibrosis	[125]
C75	CPT1	CPT1 activator	↓renal fibrosis	[126]
Atrasentan	ET_A blockers	-	↓renal fibrosis ↓albuminuria	[131]
Avosentan				[132]
Emodin	-	-	↓ fibronectin ↓ TGFβ-1	[135]
Celastrol	Smad3	↑cannabinoid receptor 2 expression	↓renal fibrosis	[138]
microRNAs: siRNA	Smad4	Silecing of Smad4	↓fibrosis ↓ α-SMA expression	[147]
microRNAs: miR-21, miR-29, miR-192	TGF-β1/SMAD signaling	Smad3 inhibitors	↓collagen deposition ↓ fibronectin ↓α-SMA expression	[148–150]
microRNAs: miRlet7c	-	-	↓fibrosis ↓ TGFβ-1 ↓MMP-9	[153,154]
hUCB-SC	-	-	↓ fibronectin ↓α-SMA expression ↓proteinuria levels ↓E-cadherin	[157]

Abbreviations: TGF-β1—transforming growth factor beta; α-SMA—alpha smooth muscle actin; MMP-9—matrix metalloproteinase; DN—diabetic nephropathy; RAS—renin-angiotensin system; ACE-ACE2—angiotensin-converting enzyme; CTGF—Connective Tissue Growth Factor; JAK-STAT—Janus kinase-signal transducer and activator of transcription pathway; RTK—Receptor of tyrosine kinase; PDFGR—platelet-derivated growth factor receptor; FGFR—fibroblast growth factor receptor; SLGT2—Sodium glucose transporter 2; FFA1R—Free fatty acid 1-receptor; CPT1—carnitine *O*-palmitoyltransferase 1; ETA—Endothelin receptor; GLP-1—Glucagon-like receptor-1; GFR—Glomerular Filtration Rate; ↑—increased; ↓—decreased; - data unavailable.

6. Energy Generation in the Kidney

As stated previously, the kidneys have a high density of mitochondria and thus a high consumption of oxygen. This oxygen is metabolized within the mitochondria by oxidative phosphorylation (OXPHOS), through the electron transport chain (ETC), to produce ATP [165]. In a very simple way, a wide range of fuels (glucose, fatty acids, and amino acids) are converted into molecules of ATP. In this process, oxygen acts as an electron acceptor in highly regulated reactions carried out by protein complexes I–IV. The ATP synthase (also called complex V) couples the electrochemical proton gradient

generated by electron transport to ATP synthesis. The protons that are pumped into the intermembrane space as electrons move through complexes I, II, and IV to ATP synthase (complex V) to drive the conversion of ADP to ATP [165] (Figure 2). Additionally, the Krebs cycle (TCA) cycle plays pivotal roles in mitochondrial energy production [166]. TCA is responsible for the production of the following coenzymes in the mitochondrial matrix, which englobes three molecules of nicotine adenine dinucleotide (NADH), and one molecule of flavin adenine dinucleotide ($FADH_2$), which is converted from pyruvate after the glycolysis pathway [167].

The fuel preferences are different and tend to mirror ATP demands at these sites. In the healthy kidney, especially in the thick loop of Henle, glomerular cells, and in the vasculature, about 90% of renal ATP production occurs via OXPHOS with yielding of 36 molecules of ATP per glucose molecule. Furthermore, ATP generated via OXPHOS, is used for the reabsorption of glucose, ions, and other metabolites from filtered urine [168]. OXPHOS machinery can also be used for hormone and protein synthesis and glomerular filtration, including contraction of mesangial cells and podocytes [169]. However, in a more hypoxic renal medulla, what prevails is anaerobic metabolism, where the ATP profit is only two molecules per glucose [170]. Free fatty acid (FFA) oxidation is another fuel that can be used in kidney cells, particularly in the tubulointerstitial compartment (excluding the glomeruli). Oxidation of palmitic acid produces 106 molecules of ATP per molecule of substrate, being more efficient than glycolysis [170]. The accumulation of FFA in AKI and DN exhibit abnormalities in lipid metabolism, increasing the formation of lipid droplets inside the cells [171]. Podocytes can be damaged due to FFA accumulation, which can sense disruption of the glomerular filtration barrier, through the free passage of FFA [172,173]. In rodents, high circulation of FFA revealed susceptibility to renal injury [174]. Lipid-lowering therapies are considered to have protective effects concerning kidney fibrosis [126]. Nevertheless, these mechanisms are controversial once they can be considered to be pathogenic or compensatory responses to renal injury [175–177]. As happens with FFA, ketone bodies require higher amounts of oxygen to produce ATP [178]. Notwithstanding, ketone bodies are an important fuel for OXPHOS within the kidney cortex [179]. However, little is known about their contribution to the kidney. The use of ketone bodies along the nephron is reflected in the mitochondrial density, which is high at this site [180]. Moreover, increased ketogenesis also occurs in glomerular cells in diabetes; however, the rate of intake of ketones in the kidney is not altered in diabetes [181], and so ketone bodies may be an endogenous process, with no relationship with diabetes, but with renal injury only. However, more studies are needed, as well as the contribution of these metabolites and what is the consequence of its increase in mitochondrial dysfunction in the kidney.

Proximal tubular cells need ATP to produce glucose via gluconeogenesis [182] (Figure 2), in which glucose is synthesized from precursors such as lactate, glutamate, and glycerol [183]. Glutamine is the most common amino acid in the human body, and in the kidney, makes a higher contribution to gluconeogenesis than does alanine [184].

Renal gluconeogenesis, in normal individuals, represents nearly 60% of endogenous glucose release during the post-prandial period, while this proportion is half reduced in a patient with type 2 diabetes [183,185].

In diabetic kidneys, these values are exacerbated via stimulation of the malate aspartate mitochondrial shuttle [186], increasing the generation of NADH, FADH2, and ATP, and ultimately increases in renal gluconeogenesis [187]. Moreover, glutaminase, the enzyme responsible to convert glutamine into glutamate [178], is expressed at high concentrations in the tubular compartment of the kidney [188]. Nonetheless, in the diabetic kidney, the impairment of autophagy increases the use of less favorable fuel sources. For instance, glucose is used by proximal tubular cells and fatty acids are used by glomerular epithelial cells, leading to glucotoxicity and lipotoxicity, respectively [189].

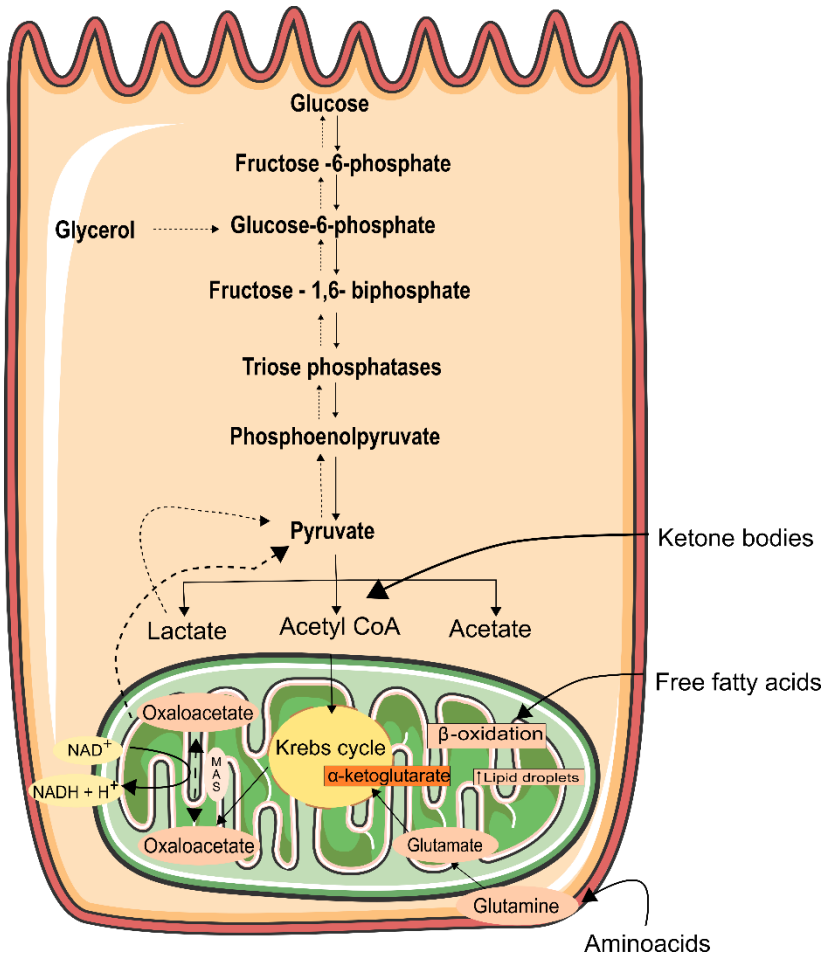

Figure 2. Schematic overview of the major metabolic pathways in renal epithelial cells. Principal substrates used to produce ATP. In renal cells, the uptake of free fatty acids, ketone bodies, glucose, and glutamine lead to increased oxygen consumption and ATP production, through ATP synthase. Glucose, in distal tubules in the healthy kidney, is metabolized to glucose-6-phosphate which is converted into Fructose-1,6-biphosphate. Then, Fructose-1,6-biphosphate is cleaved into two phosphorylated three-carbon compounds glyceraldehyde 3-phosphate and dihydroxyacetone phosphate), known as triose phosphatases. Afterward, glyceraldehyde 3-phosphate is converted into phosphoenolpyruvate and finally into pyruvate. Free fatty acid oxidation is a more efficient substrate to use than glucose in terms of energy production; however, in diabetic nephropathy disease, some complications occurred, i.e., increasing of lipid droplets. Within the cortex, ketones bodies are also a substrate used for ATP production. Glycerol becomes an important gluconeogenic precursor in diabetes. It is broken into glucose-6-phosphate and then be transformed into pyruvate and continues to follow the same path as glucose metabolization. Gluconeogenesis (up dashed arrows), which occurs in proximal tubules cells, needs ATP to produce glucose, which can be synthesized from lactate, and glycerol. Glutamine is degraded and converted to glutamate, which can be transaminated via glutamate-oxaloacetate-transaminase or glutamate-alanine-transaminase, which bot reaction yield α-ketoglutarate, an intermediate of Krebs cycle. Pyruvate resulted from lactate, enters into the mitochondria, and is converted to oxaloacetate. Oxaloacetate, in the cytoplasm of the mitochondria, can be reduced to malate and be exported out and/ or in the cytoplasm. Here, through malate aspartate shuttle (MAS), first, malate is oxidized to oxaloacetate and then converted to phosphoenolpyruvate and subsequently converted to fructose-6-phosphate. After a phosphatase, fructose-6-phosphate is dephosphorylated, which results in glucose-6-phosphate, which then is converted into the release of glucose.

7. Mitochondrial Function in the Kidney

Kidney disease is a worldwide health issue with an unpredictable prognosis that lacks therapeutic options. Mitochondrial biogenesis, fission, fusion, and mitophagy form an essential axis of mitochondrial quality control, to the optimal production of ATP. Mitochondria is responsible for the regulation of cellular oxidative stress, apoptosis-signaling pathway, aging, in all cells and tissues, and calcium homeostasis [168,190]. Renal diseases, such as AKI and DN, can induce an imbalance in mitochondrial homeostasis [168].

Renal tubular cells, which constitute nearly 90% of the outer kidney cortex, have important functions associated, such as reabsorption of nutrients, secretion of the waste from the blood, regulation of the fluid, and electrolyte balance [191]. These functions require a high expenditure of energy, in which mitochondria plays a vital role in energy production. Mitochondria must maintain their normal function to cellular homeostasis in normal organs, and the kidney is not an exception [192,193]. Indeed, the kidney is considered, among other organs, to be one of the most energy-demanding in the human body. In fact, it has the second-highest mitochondrial content and oxygen consumption [194,195]. Within the kidney, the renal proximal tubule accounts for the greatest proportion of oxygen consumption, being a highly ATP-dependent process [196]. The requirement of ATP in glomerular cells is lower since it required lower energy for filtration and other passive processes [197]. Persistent tubulointerstitial injury, with time, is directly associated with mitochondrial dysfunction [198,199]. To further support this, a report in the early stages of DN, in a rat model, described ultrastructural changes in renal proximal tubular mitochondria, which were correlated with disturbance in the main function of renal tubular cells [200].

8. Oxidative Stress and Electron Transport Phosphorylation Impairment in Kidney Function

8.1. Oxidative Stress (OS) Percussions in the Kidney

An imbalance between reactive oxygen (ROS) and nitrogen (RNS) species and cellular antioxidant defenses is termed oxidative stress (OS) [201]. OS plays a critical role in CKD progression, by inducing glomerular and tubular damage or is indirectly associated with inflammation, hypertension, and/or endothelial dysfunction [202]. ROS disturb regulatory mechanisms of the kidney, such as RAS system, tubular glomerular feedback, and myogenic reflex in the arteriole. Therefore, the kidney fails to compensate for water-electrolyte and acid-base imbalances, resulting in an additional increase of OS [203].

Sources of ROS formation include the mitochondrial electron transport chain, catecholamine oxidation, metabolism of arachidonic acid, endothelial cells (xanthine oxidase reaction), and inflammatory cells (myeloperoxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase) [202]. NADPH oxidase 4 (NOX4) is the most important isoform, located in renal fibroblasts, renal tubules, glomerular mesangial cells, and podocytes in the kidney and in the membrane of mainly endothelial cells and fibroblast in the vasculature [201]. The enhanced Nox4 expression leads to an increase in ROS levels. These ROS reduced cytochrome C oxidase activity and caused mitochondrial dysfunction, by inhibiting mitochondrial oxidative phosphorylation [204]. Several kidney cells are under changes when OS imbalance occurs. For instance, high glucose induces intracellular ROS in proximal tubular epithelial cells. In addition, ROS are induced in glomerular mesangial by advanced glycation end products and cytokines [205]. High levels of glucose induce micro-vesicle generations through ROS/Nox4 pathway in mouse podocyte clones cells. The treatment with *N*-acetylcysteine (NAC), a powerful antioxidant, diminished podocyte injury by increasing nephrin expression and inhibiting apoptosis [206].

Indeed, ROS production in response to several stimulus, activates the NADH/NADPH oxidase system in renal cells. Nicotinamide adenine dinucleotide (NAD+) is essential not only for the harvesting of energy in the form of ATP from different fuels, but also to protect the kidney tubule against acute stressor. NAD^+ augmentation may alleviate AKI triggered by ischemia-reperfusion (IRI), systemic inflammation, and toxic injury. Indeed, data from experimental models and clinical studies have pointed to NAD+ homeostasis as a key factor

of kidney health [167]. Metabolic analysis of mouse urine samples identified an increase in quinolinic acid following IRI. Quinolinic acid serves as a precursor for NAD^+ biosynthesis. Accumulation of this acid in the urine suggested a reduction in the levels of the enzyme that uses QPRT substrate. This reduction led Mehr and collaborators to suggest that reduced QPRT expression increased AKI susceptibility in mice [207]. Moreover, in another study, quinolinic acid and tryptophan were measured in urine samples obtained from 215 patients within the first 24 h of intensive care unit admission. Fifty-one patients developed AKI and the increased urinary quinolinic acid: the tryptophan ratio was independently associated with increased risk of AKI, which raises the need for renal replacement therapy and adverse outcomes [167]. The NAD^+ metabolism suggests having a protective effect on exogenous Nam, a member of the water-soluble vitamin B family, in a murine UUO model of renal fibrosis. Nam treatment reduced multiple outcomes that follow UUO, including fibrosis, apoptosis, and tubular atrophy. It inhibited the expression of TGF-β1. This result may target exogenous Nam administration as a potential therapeutical approach to halt CKD progression [208].

Morphological abnormalities and loss of function in mitochondria, through swelling and fragmentation, results in ROS production such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), leading to OS and inflammation, mechanisms that trigger the progression of renal fibrosis [209,210]. Moreover, OS is predominantly induced by excessive cytoplasmic and mitochondrial ROS emission [190].

8.2. Mitochondrial Oxidative Phosphorylation Impairment in the Kidney

Electron transfer at complexes I and III has been suggested as the principal source of ROS overproduction [211] (Figure 3). In addition, several studies have been conducted to evaluate mitochondrial dysfunction as a potentiator and contributor to AKI [212]. Indeed, Emma and collaborators reported a decrease in ATP production related with an increase in ROS production, a release of Cytochrome C, and a disruption of mitochondrial cristae [213]. Histologically, a loss of cristae structure is also observed in AKI, leading to the dissipation of the mitochondrial membrane potential that consequently halted ATP production [213]. In line with these observations, several studies in animal models demonstrated the loss of mitochondrial respiratory proteins, particularly Peroxisome proliferator-activated receptor-gamma coactivator-1 α (PGC1-α) [214]. As expected, renal mitochondrial and ATP production are altered by diabetes. Curiously, early in diabetes, ATP generation is increased, yet it decreases as the disease progresses [215–217]. Oyarzún and collaborators identified an alteration of adenosine signaling in renal cell dysfunction. Here, in early diabetic rats, the authors observed an increased expression of the ecto 5′-nucleotidase (CD73), which hydrolyzes AMP to adenosine, at the renal proximal tubules, leading to tubulointerstitial fibrosis initiation [218]. Thus, higher levels of CD73 lead to the production of high levels of adenosine and emerges as a new tool for the early diagnosis of tubular injury in DN, being less invasive than normal biopsies [218].

A dysfunctional Electron Transport Chain (ETC) undergoes an increase in glycolysis, where there is an increase of lactate and pyruvate levels, as well as hexokinase activity [219,220]. This suggests that the kidney, after injury, can maintain its function by altering its metabolic preferences [221]. Glucose oxidation is recognized as the golden pathway adopted for ATP production in the kidney. Within the kidney, glomeruli, podocytes [222], endothelial cells [223], and mesangial cells [216] are highly dependent on glucose oxidation for energy production yet can shuttle substrates such as lactate into oxidative energy production following the conversion to pyruvate. Nevertheless, the delivery of metabolic substrates for ATP production, such as fatty acids and ketones, are changed by diabetes [224]. Alterations in metabolic fuel sources in diabetes provide a great contribution to renal hypoxia. In diabetes, there are some animal studies that describe that renal sites are damaged by hypoxia. One example is a study done in 2017 in pre-diabetic pigs, in which was observed hypoxia and mitochondrial dysfunction in the medullary ascending loop of

Henle [225]. Moreover, chronic hypoxia of the proximal tubules has been characterized as a final common pathway in the progression of CKD to ESRD [226].

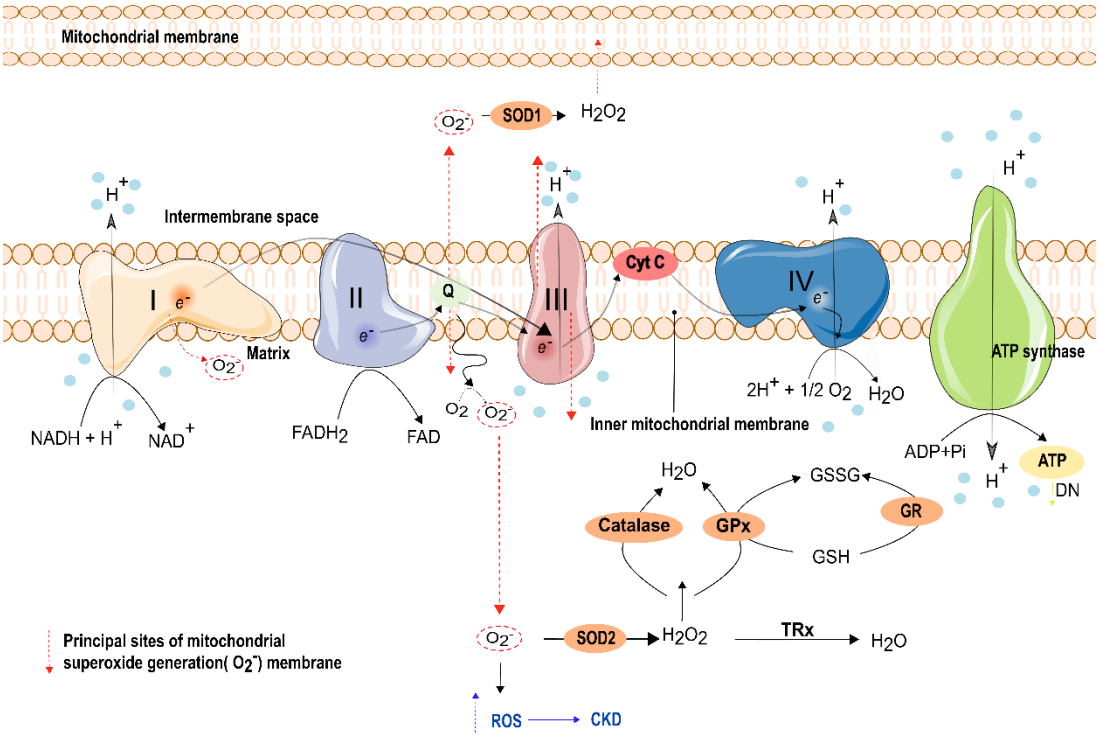

Figure 3. Mitochondrial oxidative phosphorylation and the mechanism of the main antioxidant enzymes present in the kidney. Reducing equivalents (NADH, $FADH_2$) produced through the Krebs cycle donate electrons to the electron transport system at complex I and complex II. These electrons are subsequently transferred to other electron carriers, including coenzyme Q, complex III, cytochrome C (Cyt C), and complex IV. In complex IV, the oxygen is reduced into water. The donation of electrons provides the energy to pump protons (H^+), which is responsible for the establishment of the electrochemical proton gradient at the inner mitochondrial membrane. This phenomenon generates the proton motive force that forces the protons back inside the matrix at ATP synthase to regenerate ATP from ADP and Pi. In diabetic nephropathy (DN), the production is reduced. With physiological conditions, some part of the O_2 is converted into reactive oxygen species (ROS), where complexes I and III are considered the principal sites of ROS production. In the kidney, when the electron transport chain and mitochondria functioning is impaired, there is an imbalance between ROS and antioxidant activity, which may be a potentiator and contributor of kidney-related diseases such as chronic kidney disease (CKD), and DN. The main antioxidant enzymes are superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase (GR). SOD1 in the intermembrane space and SOD2 is released to the matrix. Levels of H_2O_2 are reduced to water through the cooperation between the antioxidant enzymes schematically represented in the scheme.

Several studies already demonstrated that renal disease can have an origin in deficiencies in OXPHOS as a result of mutations in Coenzyme Q10 biosynthesis monooxygenase 6 [227], cytochrome C oxidase 10 [228], Pdss2 [229], and p-53-controlled ribonucleotide reductase [230], in human and animal models. To have a deeper knowledge of the degree that ETC is affected, the evaluation of defects in the activity of the mitochondrial complexes, individually, may contribute to understanding how mitochondrial dysfunction and renal impairment are possibly correlated.

Gene mutations that result in complex I deficiency are the most common oxidative phosphorylation disorders in humans [231]. Forbes and collaborators studied mice with a knockdown of the complex I gene, Ndufs6. Heterozygous mice, with an approximately 40% impairment of complex I activity in the kidney, had a kidney disease that precedes

cardiac abnormalities, whereas homozygous mice died of cardiomyopathy and advanced chronic kidney disease [232]. In line with these studies, complex I dysfunction was already identified in humans with diabetes. Here, it was observed a reduction by approximately 50% in complex I renal cortical activity [233]. Complex III deficiencies, associated with increased ROS production, were also detected in the diabetic kidney [234]. It is not a new theme of debate that mitochondria network changes, including bioenergetic alterations, are implicated in DN. Coughlan and collaborators, in a study of diabetic rat mitochondria, at four weeks of age, reported that these rats revealed a decrease in ATP content and mitochondrial fragmentation in the kidney, within proximal tubule cells. By eight weeks of age, they detected a generation of glomerular damage, due to an increased capacity for mitochondrial capacity. Later, worsened effects were observed. At 16 weeks of age, there was tubular damage and mitochondrial uncoupling. This study unveils that changes in mitochondrial bioenergetics and dynamics may precede the development of renal lesion in diabetes and not the other way around [235]. To complement this idea, a study in nondiabetic mice demonstrated that increasing kidney metabolism is not sufficient to induce kidney damage as it is in the presence of hyperglycaemia and hypertension [236].

Furthermore, complex I deficiency is also associated with an increased mitochondrial superoxide production in skin fibroblast mitochondria. Skin fibroblasts, obtained from young individuals with DN, also have mitochondrial dysfunction [237]. In mitochondrial ATP generation, the electron can leak from the respiratory chain and form superoxide, through the binding with oxygen [198]. Indeed, Complex I and Complex III are the main sites of superoxide production in renal mitochondria. Complex I generated superoxide is released to the mitochondrial matrix, while complex III generated superoxide is released to the matrix and intermembrane space [238,239] (Figure 2). Coenzyme Q10 (CoQ10) plays a crucial role in the mitochondria respiratory chain and protects against damage from ROS [240–242]. CoQ10 deficiencies have been correlated with severe renal pathologies, including glomerular and tubular interstitial disease [227,243,244]. Mutation in genes related to the biosynthesis of CoQ10, such as PDSS2, COQ2, COQ6, COQ8, and COQ9, have been related to the genetic nephrotic syndrome in humans [227,242,243]. Regarding COQ2 nephropathy, a study done in the Drosophila model revealed that the silencing of genes related to Q10 resulted in multiple abnormalities. Coq2-deficient nephrocytes showed elevated levels of autophagy and mitophagy that increased the levels of ROS. It also decreased respiratory chain activity at complexes II and III [242]. Additionally, disturbed regulatory miRNAs, such as miR21, were reported in a patient that carried an impaired IV activity and with CKD [245,246]. These studies highlight that mitochondria are intimately related to renal diseases.

Urinary markers of mitochondrial dysfunction were evaluated in an individual with DN. This study quantified 94 urine metabolites in patients with diabetes mellitus (DM), and patients with CKD and DM, and in healthy controls. Thirteen metabolites were significantly reduced in DM+CKD patients, in which 12 of these 13 are linked to mitochondrial metabolism demonstrating a global suppression of mitochondrial activity in DN. Additionally, urine exosomes from patients with diabetes and CKD had a lower expression of PGC1-α (a master regulator of mitochondrial biogenesis) and presented less mitochondrial DNA [226]. PGC1-α is considered a booster for mitochondrial biogenesis [247] and is a key player in fuel switching [248]. An experimental model of kidney-related disease [214] demonstrated that the increase of PGC1-α activity is associated with renoprotection and mitochondrial biogenesis. Proximal tubules exposed to a diabetic environment, overexpression of the GTPase repressor/activator protein 1 homolog (RAP1) improved mitochondrial function through PGC1-α activation [249]. Additionally, overexpression of PGC1-α protected renal tubular cells against oxidant stress, with an increase in mitochondrial number and respiratory capacity [250].

As expected, Mitophagy is also altered in ischemic AKI. Mitochondria are dynamic organelles that constantly undergo fission and fusion, in response to cellular energy requirements and to maintain mitochondrial integrity [251]. Furthermore, the fusion of

mitochondria is also a mechanism to overcome damage, by acquiring healthy proteins to improve their function [252]. During fission, mitochondria are split into two, where fusion is the combination of two mitochondria, to produce one larger organelle [251]. Succinctly, the fission process is mediated by dynamin-related protein (Drp1) and its receptors which allow for it to be properly recruited to the outer mitochondrial membrane to carry out fission, such as mitochondrial fission 1 (FISP1). Moreover, fusion is mostly regulated by the interaction between mitofusin 1 and 2 (MNF1/2). MFN1 and MFN2 are located on the outer mitochondrial membrane and are a key player for outer membrane fusion. Protein dynamin (also known as OPA1) resides in the inner membrane and is important for inner membrane fusion [253]. Dysregulation on these processes results in an impairment of ATP production [254]. Moreover, the NAD^+-dependent SIRT family deacetylases, particularly the mitochondrial matrix-resided SIRT3 protein, play an important role in regulating mitochondrial dynamics and function under AKI [255]. Yet, the underlying mechanism of mitochondrial dysfunction in renal fibrosis warrants further research.

9. Antioxidant Capacity in the Kidney

Oxidative damage in the mitochondria alters DNA transcription, which affects the normal functioning of mitochondrial proteins, antioxidants, and oxidative phosphorylation enzymes [256]. This OS-induced injury may be derived from ischemia/reperfusion, energy shortage from impaired biogenesis, ATP energetics, or even defects in the mitophagy process [190].

The imbalance between the generation of ROS levels and local antioxidant capacity is a biomarker of mitochondrial dysfunction in DN [217,235]. To overcome the excessive production of ROS, mitochondria have an antioxidant system. Indeed, homeostasis is maintained through a complex set of antioxidantal mechanisms that prevent oxidative stress-induced injury (Figure 3). The physiologic formation of ROS is detoxified by endogenous antioxidants, which are classified into two groups: enzymatic and non-enzymatic. Enzymatic antioxidants include superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione S-transferase (GST), and glutathione reductase (GR), while non-enzymatic pathways include ETC, glucose autoxidation, glycation products (AGEs), GSH, and other pathways [257]. Additionally, exogenous antioxidants obtained from daily food intake can be hydrophilic (Ascorbate/vitamin C and flavonoids) or lipophilic (α-tocopherol/vitamin E and carotenoids), being classified as nonenzymatic antioxidants [258]. Under physiological conditions, mitochondria have an efficient antioxidant system to cope with ROS [259], which includes, superoxide dismutase that reduces O_2^- in H_2O_2 faster than the rate of O_2^- production [168]. Levels of H_2O_2 lead to glutathione peroxidase activation which reduces H_2O_2 into water (Figure 3) [260]. Increased levels of ROS have multiple consequences. It can cause breaks in mitochondrial DNA, which leads to mutations in the next generation, and it can also trigger apoptosis (such as Cytochrome C) [261]. In an OS situation, NRF2 can boost an answer to ROS overproduction [261]. The association of Nrf2 with kidney disease has been reported in some studies. In mice with DN, Nrf2−/− showed increased ROS levels and higher oxidative DNA damage. Along to this, increased TGF-β1 and ECM accumulation were found. These aspects were all decreased when Nrf2 was activated, which significantly prevented the progression of AKI to CKD transition [262].

Nrf2 can activate transcription of heme oxygenase 1 (HO-1), and ferritin that can mitigate AKI and renal injury, on glomerular endothelium, cortical peritubular capillaries, and interstitial leukocytes [263]. Natural bioactive compounds demonstrated kidney protective effects by activating Nrf2 in experimental CKD models [264]. HO-1 inducers were applied in multiple diseases, including CKD. Sulforaphene, an Nfr2 activator, was able to reduce ROS induced by PI3K/Akt/GSK3β activity and EMT, in HK-2 cells, after high glucose exposure [265]. Again, in a high glucose situation, Astaxanthin attenuated OS and fibronectin accumulation in glomerular mesangial cells and improved the metabolic performance and kidney morphology in STZ-induced diabetic rats [266].

Furthermore, curcumin was able, through activation of Nrf2 signaling, to ameliorate albuminuria levels, in a rat model of CKD, by attenuating inflammation [267]. In another study, curcumin was also suggested to prevent EMT of podocytes, proteinuria and kidney injury in DN [268]. In line with this study, 14 randomized controlled studies demonstrated that curcumin markedly lowers mesangial area, proteinuria, serum creatine, and protects the kidney of rats or mice with diabetes [269].

Silibin was also able to induce biochemical changes in the kidney of rats, by reducing lipid peroxidation and improved antioxidant defense that contributed to the preservation of the normal histological architecture of the renal tissue [270].

Factors that cause OS are activation of NADPH oxidases, uncoupled endothelial nitric oxide synthase, and mitochondrial dysfunction together with decreases antioxidant defenses, such as a decreased expression of antioxidant enzymes and intracellular GSH content.

The glutathione redox cycle is the critical antioxidant mechanism found in several intracellular organelles, such as mitochondria [260]. Mitochondrial glutathione (mGSH) can be oxidized to glutathione disulfide (GSSG) by superoxide anions [260] (Figure 3). In addition, GSSG can be reversed again in mGSH, through glutathione reductase, which requires NADPH from the pentose phosphate pathway. To maintain the oxidative balance in the cells, the cell must contain low levels of GSSG and high levels of GSH [201]. Furthermore, severe OS can overcome the ability of the cell to reduce GSSG to GSH, resulting in the accumulation of GSSG [271]. Hence, the ratio of GSH and GSSG is a marker of OS [272]. Furthermore, mutual maintenance of ascorbate and GSH can occur in vivo, since ascorbate can maintain intracellular GSH [273]. Thus, these mechanisms are considered pivotal process for preserving mitochondrial functions.

An increasing number of studies demonstrated that enhancement of antioxidant enzyme activity resulted in a protective effect in animal models of AKI [274]. SOD is a key enzyme in the detoxification of free radicals and all three isoforms are highly expressed in the kidney [275]. It converts O_2^- to H_2O_2 and oxygen, and in turn, the catalase or glutathione peroxidase system reduces H_2O_2 to water [201]. Administration of SOD or catalase can diminish ROS in proximal tubule injury after hypoxia in vitro [276]. It can also attenuate oxygen radicals in vivo after renal ischemia in rabbits [277]. In rats, SOD was able to improve renal function and reduced tissue injury and cortical mitochondrial lipids peroxidation [278]. Long-term treatment with a SOD mimetic (MnTMPyP) alleviated oxidation parameters in kidney fibrosis ischemic [279]. MnTMPyP blocked O_2^- and peroxynitrite formation, and reversed functional kidney deficits, suggesting that antioxidant intervention is beneficial, and halting ROS formation can alleviate microvascular failure and renal injury [280]. Another similar study is in early experimental DM in hypertensive rats, where the administration of tempol, another SOD mimetic, corrected the oxidative imbalance and improved oxidative stress-induced renal injury, decreasing albuminuria and fibrosis [281].

Decreases in renal superoxidase are accompanied by mitochondrial biogenesis and less mitochondrial glucose oxidation in diabetic mice [282]. Mitochondrial biogenesis can occur as a cellular response to oxidative stress [283]. Regarding the progression of DN, the contribution of mitochondrial superoxide production is not clear. However, some data led to the theory that superoxide production can be an indicator of mitochondrial healthy activity [284].

There is no doubt that hyperglycemia causes high production of ROS. Hence, future studies are needed to provide a better insight into the diabetic kidney. Indeed, particularly in this class of patients, where albumin is no longer considered a biomarker of the disease, there is an urgent need to find, if possible, a new biomarker, especially at earlier stages, to inhibit the onset and the progression of DN. In recent years, mitochondria-target therapeutics have attracted much interest as they demonstrated that attenuating mitochondrial dysfunction can prevent the progression of kidney diseases [190,285].

10. Targeting Kidney Mitochondria—Therapeutic Approaches

Mitochondria-targeted antioxidants are based on the mechanism of delivery of known redox agents to the mitochondrial matrix through conjugation with TPP+ (triphenylalkyphosphonium cation) moiety [286]. As aforementioned, CoQ10 has renoprotective and prevents detrimental changes in mitochondrial function and morphology. A derivative of CoQ10, known as mitoquinone (MitoQ), is studied in multiple clinical trials, including Parkinson's disease [287] and CKD [288]. This molecule could be delivered and concentrated at mitochondria matrix to function as ROS scavenger [286]. Administration of MitoQ mitigated mitochondrial OS that resulted in the prevention of DN in a murine model of inherited diabetes [288] and experimental models T2DM [289]. These studies revealed an improvement of renal function, a decreased glomerular hyperfiltration, and albuminuria. MTP-131, a cationic peptide with antioxidant properties that increases the efficiency of OXPHOS, demonstrated an improvement in kidney function and fibrosis in models of AKI [289], and diabetes [290]. Recently, its efficiency on mitochondrial myopathies is being tested in Phase I and II clinical trials [188,291].

Furthermore, MitoTEMPO is another selective mitochondria-targeted antioxidant that could be uptaken and accumulated in the energized mitochondria matrix and hence, modulate CoQ10. Sun and collaborators developed a study where they co-administrated MitoTEMPO (3 mg/kg/day) with CoQ and ML385 (an antagonist, used to inhibit Nrf2) (30 mg/kg/day) in db/db mice. Here, they observed a restoration of the mitophagy mechanism (through the activation of the Nfr2/ARE pathway) and alleviation of kidney dysfunction in glomeruli [292]. In line with these studies, in another study performed in nephrectomy mice, MitoTEMPO administration (1 mg/kg/day), was able to improve renal function and alleviate renal fibrosis by reducing cytokines, mitochondrial dysfunction, ER stress, and profibrotic factors through the Sirt3-SOD2 pathway [293]. Surprisingly, renal MnSOD inactivation associated with increased mitochondrial O_2^-, can be attenuated with the mitochondrial-targeted antioxidant Mito-TEMPO [294].

Mito-CP is a SOD mimetic that targets mitochondria and protects against cell dysfunction, apoptosis, injury, and inflammation in mice administered cisplatin. Additionally, it reduced NOX2/4 mRNA and protein, lipid oxidation, protein nitration, and pro-inflammation markers (MPO, ICAM-1) [295]. Moreover, mitochondrial-targeted peptides protected cardiolipin from cytochrome C peroxidation, demonstrating efficacy against oxidative stress, tubular cell damage, and dysfunction in IRI [296].

Edaravone (MCI-186) is a small molecular weight compound that in rats was able to attenuate ROS radical generation in kidney tubular cells in vitro, lipid peroxidation, and mitigated renal dysfunction in IRI [297]. In dogs, similar results were observed, in which there were improvements in renal function and reduced renal tubular cell damage, lipid, and oxidation [298].

NAC is a synthetic derivative of cysteine and precursor of GSH that revealed to be protective in the Rhabdomyolysis (RM)-induced AKI animal model [299] improving kidney function, renal GSH, and systemic oxidative stress. However, in some clinical trials in humans, no positive effects were achieved in AKI, due to its heterogeneity and to the confounding serum creatinine levels as possible contributors to the neutral effects [300].

Another possible approach is to benefit from dietary antioxidants. For example, vitamin E and selenium attenuate nephrotoxicity [301]. Surprisingly, selenium, which can enhance the activity of GSH-dependent antioxidant enzymes, and hence can inhibit renal oxidative damage, had no renoprotective effects in an animal model of RM-mediated AKI [302]. Vitamin C, also known as ascorbate, is also able to attenuate oxidative damage and renal injury in some animal models [300]. It has multiple actions in human renal injury, such as renoprotection in AKI, a reduction in multi-organ failure, an improvement in renal function in sepsis and severe burns and enhancement of endothelial-dependent vasodilation in renal allograft [274].

Vitamin C is an essential nutrient obtained from the diet and is a highly effective non-protein reducing agent capable of donating electrons in widely enzymatic and non-

enzymatic reactions [303]. Interestingly, vitamin C can be recycled via GSH and /or GSH or NADH-dependent enzymes, enhancing the potential protective action of this vitamin [273]. Thus, vitamin C is proposed to have an important physiological role as an effective in vivo antioxidant once it has a low reduction potential, which allows direct interaction with a wide range of physiological ROS/RNS [304]. Vitamin C scavenges radicals (O_2^-) and non-radical oxidants and reduces levels of α-tocopheroxyl radical in lipids and membranes, allowing recycling of vitamin E and inhibition of lipid peroxidation [305]. Another positive effect is that it can be administrated in large quantities, with minimal adverse effects [306,307]. Low plasma vitamin C is a risk for mortality in patients with AKI co-morbidities, such as diabetes [308]. Groebler and collaborators compared treatment with vitamin C alone or in combination with a synthetic polyphenol. Both compounds decreased plasma and kidney oxidative markers when administrated alone or in combination. Nevertheless, only vitamin C revealed a potential clinical effect on reducing proteinuria, plasma urates, and renal tubule casts [309].

These results demonstrate that vitamin C intake may prevent ROS-mediated renal damage in AKI.

11. Conclusions

Interstitial fibrosis is the most common outcome of all forms of CKD. Given the high morbidity and mortality associated with AKI and CKD, there is an important unmet medical need to recover renal function. There is no doubt that it is necessary to pay better attention to the condition of diabetes, even more when this pathology is associated with kidney problems, as these patients end up having a clinical worsening much sooner. Here, diabetic nephropathic patients deserve better attention, as their diagnosis fails in the sense of being early, due to the lack of biomarkers. Henceforth, it is important to find new disease markers, preferably at earlier stages. Mitochondria seems to be a plausible way forward, as deficiencies at various levels have been shown to be indicators of the development of kidney disease. The correction and or the prevention of mitochondrial dysfunction may be pivotal in the prevention and treatment of kidney disease. Therefore, it may be prudent to consider ROS levels as an indicator of mitochondrial function rather than an endpoint marker of damage. Cells have their own antioxidant defense system against multiple disease, where redox imbalance has a critical role in the pathogenesis of CKD progression. This disturbance already starts in an early phase of CKD, and so, intervention at this point can help to attenuate deleterious effects. Many promising approaches are currently being investigated. Thus, it is necessary to develop a deeper knowledge, to detect whether it is a structural abnormality that trigs the gradual loss of renal function, or if it is a bioenergetic issue. Hence, the detection of mitochondrial dysfunction, both in structure or in terms of complexes activity, may promote the development of a more personalized therapy, which can act at the level of a single complex, or in a specific protein to improve renal function. Likewise, it is needed to investigate the detailed molecular mechanism of the renoprotection of the kidney, such as new advances in drug delivery systems, in order to avoid the poor bioavailability of some pharmacologic approaches. It should be considered to give the mitochondria a more important role in kidney disease prognosis since it seems to be an attractive approach given to their possibility of targeted therapy, than the currently used.

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Review

Mitochondria Quality Control and Male Fertility

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Simple Summary: Mitochondria play a crucial role in numerous cellular processes, including energy production, apoptosis, and calcium homeostasis. In the male reproductive system, mitochondria are particularly important for the development and maintenance of germ cells, which ultimately lead to the production of healthy sperm. Dysfunction in mitochondrial physiology can lead to an imbalance in reactive oxygen species, which can have detrimental effects on sperm quality. Thus, mitochondrial quality control can ultimately define male reproductive capacity. Studies have shown that non-communicable diseases such as obesity, diabetes, and cardiovascular disease can have a negative impact on mitochondrial function in sperm, leading to decreased sperm motility, concentration, and viability. Therefore, understanding and managing mitochondrial quality control could be a valuable approach to developing new strategies to combat male infertility. Herein we discuss the relevance of mitochondria quality control to male fertility, particularly the role of oxidative stress and the parameters needed to be evaluated.

Abstract: Mitochondria are pivotal to cellular homeostasis, performing vital functions such as bioenergetics, biosynthesis, and cell signalling. Proper maintenance of these processes is crucial to prevent disease development and ensure optimal cell function. Mitochondrial dynamics, including fission, fusion, biogenesis, mitophagy, and apoptosis, maintain mitochondrial quality control, which is essential for overall cell health. In male reproduction, mitochondria play a pivotal role in germ cell development and any defects in mitochondrial quality can have serious consequences on male fertility. Reactive oxygen species (ROS) also play a crucial role in sperm capacitation, but excessive ROS levels can trigger oxidative damage. Any imbalance between ROS and sperm quality control, caused by non-communicable diseases or environmental factors, can lead to an increase in oxidative stress, cell damage, and apoptosis, which in turn affect sperm concentration, quality, and motility. Therefore, assessing mitochondrial functionality and quality control is essential to gain valuable insights into male infertility. In sum, proper mitochondrial functionality is essential for overall health, and particularly important for male fertility. The assessment of mitochondrial functionality and quality control can provide crucial information for the study and management of male infertility and may lead to the development of new strategies for its management.

Keywords: male fertility; mitochondrial quality control; non-communicable diseases; spermatozoa

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1. Introduction

Mitochondria, commonly known as the "powerhouses of the cell", are intricate organelles present in all eukaryotic cells. They consist of two membranes, the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM), which separate the mitochondrial matrix from the cytoplasm of the cell and define the intermembrane space and mitochondrial matrix [1]. While OMM is highly permeable, IMM is selectively impermeable to most substances. This property is essential for the development of specific transport systems that regulate the movement of molecules to and from mitochondria [1]. The IMM can be further divided into two subdomains, an inner boundary membrane which is in contact with the OMM and the cristae membrane [2]. The cristae membrane is responsible for enclosing the oxidative phosphorylation (OXPHOS) respiratory chain complexes, including complexes I, II, III, and IV [3].

Mitochondria play a critical role in maintaining cellular homeostasis, by facilitating distinct and essential physiological processes such as bioenergetics, biosynthesis, and cell signalling. One of its primary functions is the synthesis of ATP, which occurs through cellular respiration and involves the conversion of ADP and phosphate ions into ATP by the mitochondrial ATP synthase. ATP is the primary energy currency of the cell and is required for various cellular processes, including muscle contraction, protein synthesis, and signal transmission [2]. ATP is also used in a variety of cellular functions, including biosynthesis and degradation of proteins, maintenance of membrane potentials [4] and also to many other physiological functions. Furthermore, mitochondria are known to be a major source of reactive oxygen species (ROS). Specifically, ROS are primarily generated in the respiratory complexes I and III of the electron transport chain (ETC) [5]. Mitochondria regulate the production of ROS through several mechanisms, including the modulation of mitochondrial membrane potential (MMP), the redox state of the ETC complexes, and the availability of oxygen [2,6]. These organelles are considered cell signalling centres and may participate in stress responses [7]. In addition to their primary functions, mitochondria are also involved in several other biological processes. They play a role in aging, thermogenesis, and calcium storage, thereby contributing to the maintenance of cellular calcium homeostasis [2]. Mitochondria use fission and fusion as the primary processes to regulate multiple aspects, including their distribution in the cytoplasm, as well as their size, shape, and number [2]. Mitochondrial quality control is maintained through various processes, including mitochondrial fission and fusion, biogenesis, mitophagy and apoptosis [8]. All these processes are essential for several physiological functions, including male reproduction.

Mitochondria play a crucial role in the male reproductive tract, contributing to the occurrence of spermatogenesis (sperm production) and oocyte fertilization. Spermatogenesis occurs in the seminiferous tubules, where germ cell proliferation and differentiation processes lead to the production of a high quantity of spermatozoa, which heavily rely on mitochondrial function [9]. It can be divided into three phases—spermatogonia mitosis (amplification of spermatogonial stem cells (SSCs)), spermatocytes meiosis (reduction in the chromosome number), and spermiogenesis where the round shaped spermatids are transformed into a more elongated shape [10]. Mitochondria found in spermatogonia and early spermatocytes are small and conventional, exhibiting limited OXPHOS activity. As spermatogenesis progresses to later stages, such as spermatocytes, spermatids, and spermatozoa, the mitochondria undergo a transformation to become more condensed, elongated, and efficient in their OXPHOS activity [11].

Changes in mitochondria morphology depicted in Figure 1 appear to occur prior to their structural association with the sperm flagellum. While most mitochondria are lost during spermiogenesis, some are arranged within the developing spermatid tail [12] and are metabolically more efficient [13] (Figure 1).

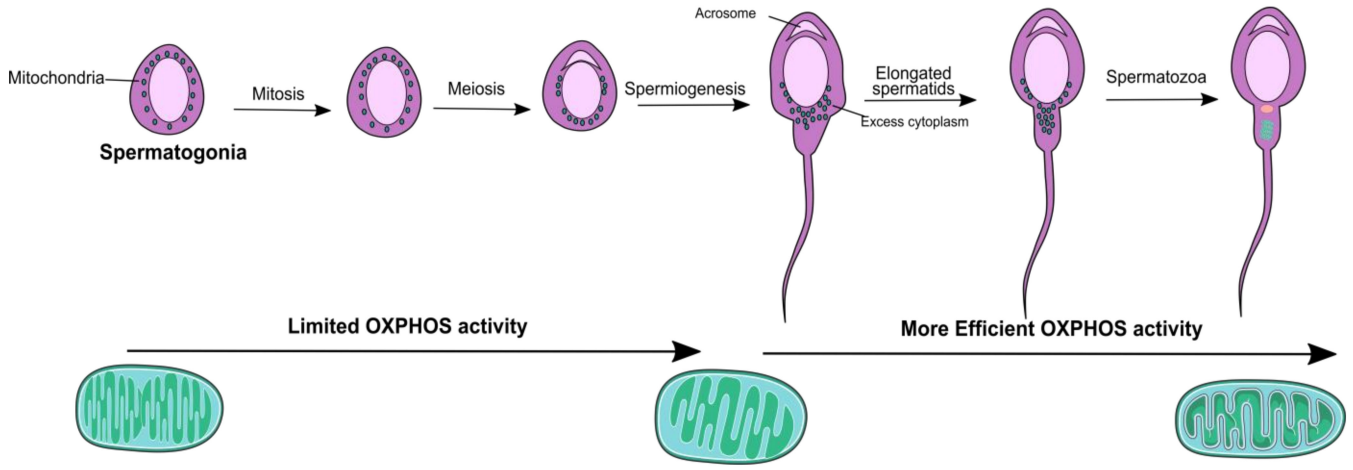

Figure 1. Schematic representation of the stages of spermatogenesis and the corresponding alterations in mitochondrial morphology. Mitochondria has a more orthodox shape in the early stages and become increasingly elongated and condensed as spermatogenesis progresses exhibiting a more efficient Oxidative Phosphorylation (OXPHOS) activity.

In testicular tissue, mitochondria have multiple functions that include energy production [14], steroid hormones production in the testis [15], the maintenance of cell proliferation [16] and cell death [17]. Moreover, in spermatozoa, mitochondria-generated ROS play a vital role in the physiological processes that enable these cells to fertilize an oocyte. These processes include biochemical changes linked to tyrosine phosphorylation, cholesterol release, and the interaction between sperm and egg. However, excessive ROS production can lead to oxidative stress (OS) and contribute to several deleterious events [18]. Human spermatozoa contain only one copy of mtDNA per mitochondrion [19] and their mtDNA sequence is identical to that found in somatic cells. However, the absence or weakness of DNA repair mechanisms in sperm mtDNA leads to a higher mutation rate [2]. Mitochondria in spermatozoa play a crucial role in ATP production, which is essential for promoting sperm motility [20], thus human spermatozoa motility depends on the correct performance of the OXPHOS [21]. Overall, the appropriate functionality of the ETC is inexorable to mitochondrial performance which, in turn, is associated with sperm function [2]. Any alterations that affect mitochondrial quality and performance in sperm have the potential to cause male infertility. Therefore, this review aims to explore the role of mitochondria throughout the male reproductive tract and how their quality control, including dysfunction in response to various metabolic cues such as metabolic disorders and environmental pollutants, may affect male fertility.

2. Mitochondria Physiology throughout the Male Reproductive Tract

As stated, spermatogenesis is an event that can be divided in three phases to transform spermatogonial stem cells (SSCs) into spermatozoa and occurs in the seminiferous tubules of the testis [13]. In the spermatozoa, there are between 70–80 mitochondria located in the midpiece [12,13]. Notably, there are metabolic and morphological changes in mitochondria physiology along the different cellular microenvironment of the testis which encloses SSCs, spermatocytes and spermatids [13]. Sertoli cells play a vital role in maintaining the homeostasis of the testis by forming the blood–testis barrier (BTB), which creates a specialized microenvironment in the adluminal space of the seminiferous epithelium [22]. The BTB is a highly specialized tight junction located in the adluminal compartment of the seminiferous epithelium. Its main function is to prevent autoantigens present in spermatogenic cells from being recognized by immune cells of the host [22,23]. The SSCs are located at the base of the BTB, which enables them to utilize nutrients and glucose from the blood supply to produce ATP [24]. Mitochondria in SSCs are immature and highly

vacuolated, with a spherical shape and a low electron-lucid matrix and few cristae. In contrast, spermatocytes and spermatids possess a large number of mature mitochondria [25]. Throughout spermatogenesis, germ cells go through several microenvironments with varying concentrations of glucose and metabolites as SSCs are turned into mature sperm [26]. During this process, the mitochondria not only increase in number but also change shape, particularly in meiotic prophase I [26]. These variations in mitochondrial size and shape depend on the proper functioning of fusion/fission and mitophagy, which are essential for maintaining intracellular homeostasis [27], as it will be explained further in detail. Moreover, as spermatogonia and SSCs are located in the basal compartment, they have direct contact with blood and interstitial fluid, providing them with access to glucose and other metabolites. However, spermatocytes and spermatids have limited access to these nutrients [26]. The differential access to metabolites between the basal compartment and adluminal compartment reflects the changing metabolic needs of male germ cells during the various stages of spermatogenesis. It appears that spermatogonia primarily rely on glucose to produce energy through glycolysis [28], while spermatocytes and spermatids rely on lactate provided by Sertoli cells, as well as pyruvate, as their main energy sources [29]. The lactate produced by Sertoli cells is converted to pyruvate by lactate dehydrogenase. Pyruvate is then utilized in mitochondria to produce ATP, indicating that spermatocytes and spermatids in the adluminal compartment are dependent on mitochondrial OXPHOS activity for energy generation [26]. The high energy demand of meiotic spermatocytes, particularly during the meiotic prophase I (MPI), requires a substantial supply of lactate and pyruvate. This demand is expected given the significant energy needed to complete meiosis, including DNA replication, recombination, and chromosomal segregation [26]. This phase represents approximately 90% of meiosis, a quarter of the spermatogenic process, and can be further divided into four different stages (leptotene, zygotene, pachytene, and diplotene) [30]. Electron microscopy studies conducted in rodent testes have shown that there is an increase in OXPHOS activity during the MPI stage of meiosis. Specifically, during pachytene, mitochondria present in the spermatocytes assume a more elongated shape while the cristae are more compact [31]. This type of mitochondrial organization has been shown to be associated with increased OXPHOS activity [32]. Conversely, in spermatids that have completed the meiosis process, the mitochondria are fragmented, and the cristae do not have either an orthodox or a condensed shape, indicating a return to reliance on glycolysis [31].

Sertoli cells play a crucial role in regulating the number of germ cells in the testis. They do this by modulating apoptosis during mitosis, which prevents the overproduction of germ cells and maintains the homeostasis of the testis [33]. After phagocytosis of apoptotic spermatogenic cells, the lipids from these cells are degraded into fatty acids which undergo β-oxidation to generate ATP in Sertoli cells. The degradation of the apoptotic cells into lipids results in a significant increase in long-chain acyl-CoA dehydrogenase, an enzyme involved in β-oxidation, in the mitochondria of Sertoli cells. This process of utilizing lipids from apoptotic germ cells for energy production is an important mechanism for maintaining the metabolic activity of Sertoli cells and for supporting the development of germ cells [34]. In addition to promoting β-oxidation of lipids, Sertoli cells also play a role in regulating mitochondrial biogenesis and OXPHOS activity in germ cells. Activin A, a protein secreted by Sertoli cells, has been shown to promote mitochondrial biogenesis and the formation of elongated cristae-rich mitochondria in spermatocytes and spermatids. This is achieved through transcriptional regulation of key genes involved in mitochondrial biogenesis, including peroxisome proliferator-activated receptor-γ coactivator 1α, nuclear respiratory factor (NRF) 1, and NRF2 [24,31]. These transcription factors activate the expression of genes involved in mitochondrial DNA replication, transcription, and translation, as well as those encoding components of the electron transport chain, leading to increased OXPHOS activity in germ cell [35].

Leydig cells are another crucial somatic cell type found in the testis. Their role in male germ cell differentiation is less well studied than that of Sertoli cells [13]. Leydig cells are

in the interstitial tissue of the testis and are responsible for the production and secretion of steroid hormones, particularly androgens such as testosterone. These hormones are synthesized and released into the bloodstream in response to the stimulation of luteinizing hormone (LH) from the pituitary gland [36]. While the exact role of Leydig cells in male germ cell differentiation is not fully understood, it is known that androgens play an important role in the regulation of spermatogenesis and male fertility. Testosterone plays an essential role in spermatogenesis as it is required for the development of spermatogonia and the initiation of meiosis. It also regulates the release of spermatids from Sertoli cells into the lumen of the seminiferous tubules [37]. When circulating LH binds to its receptor on Leydig cells, it triggers a signalling cascade that increases the production of cyclic AMP (cAMP). This increase in cAMP promotes the transport of cholesterol to the inner mitochondrial membrane (IMM) and initiates the first step of testosterone production. This step involves the conversion of cholesterol into pregnenolone by the P450 cholesterol side-chain cleavage enzyme (P450scc). Pregnenolone then undergoes further enzymatic reactions to eventually produce testosterone [38]. When referring to cholesterol in mitochondria, its typical functions are biogenesis, membrane maintenance and the production of steroids [39]. Although the primary function of mitochondria is to produce ATP, this is not the case for Leydig cells. The morphology of the cristae in Leydig cells does not allow the connection between the F1 complexes of ATP synthase in the mitochondrial matrix when they are close to the mitochondrial membrane [40]. Therefore, Leydig cells produce less ATP, and their mitochondria are mainly responsible for the production of steroid hormones, such as testosterone, through the steroidogenesis pathway.

After leaving the testicular seminiferous tubules, spermatozoa have to mature in the epididymis where they gain the capacity to reach and fertilize the oocyte [41]. Epididymal cells create a unique microenvironment in the epididymal lumen to support sperm maturation. This microenvironment is maintained by epithelial cells, which keep the pH acidic and the bicarbonate concentration low. This ensures that the spermatozoa do not become prematurely activated while they are maturing in the epididymis [41]. The acidic pH is supported by proton secretion by V-ATPases, which are abundant in the apical membrane and intracellular vesicles of the narrow and clear cells in the epididymis [41]. The narrow and clear cells present in the epididymis are considered mitochondria-rich cells and these abundant mitochondria are linked to the acidification of the lumen due to their carbonic anhydrase activity, endocytic activity and most importantly, proton secretion through V-ATPase [42].

It is important to mention the importance of ROS in spermatozoa capacitation, since protein tyrosine phosphorylation is regulated by ROS production [43]. The presence of ROS triggers a cascade of biochemical reactions that enhance sperm motility. First, ROS induces the conversion of ATP to cAMP by the enzyme adenylyl cyclase. Next, cAMP activates protein kinase A (PKA), which further stimulates ROS production and the enzyme NADPH oxidase. PKA also phosphorylates serine and tyrosine residues, leading to activation of protein tyrosine kinase (PTK). Finally, PTK triggers phosphorylation of tyrosine residues in the sperm flagellum axoneme, resulting in increased motility [43]. Another key event during capacitation is the mobilization of calcium ions, which is also triggered by ROS. This increase in calcium ions leads to the cleavage of PIP2 (phosphatidylinositol-4,5-biphosphate), producing DAG (diacylglycerol). The presence of DAG and PKC (protein kinase C) induces the phosphorylation of phospholipase A2, a membrane enzyme that plays a critical role in sperm function. This phosphorylation event increases the fluidity of the spermatozoa membrane, enabling it to fuse with the oocyte. This membrane fusion is a crucial step in fertilization and requires precise coordination between sperm and oocyte. [44]. Overall, the interplay between ROS, calcium ions, PKC, and phospholipase A2 during capacitation is a finely tuned process that ensures successful fertilization.

Spermatozoa are highly vulnerable to the harmful effects of ROS, which include superoxide anion, hydrogen peroxide, hydroxyl radical, nitric oxide, and peroxynitrite. Elevated levels of ROS can lead to oxidative damage, impairing sperm function [45]. This

impairment translates into a loss of sperm motility and mitochondrial activity and also the possibility of losing the ability to fertilize oocytes [46]. Spermatozoa have limited capacity to repair oxidative damage, as their chromatin is highly compacted and lacks the necessary machinery to synthesize new proteins. Therefore, they rely on antioxidant enzymes that are produced during spermatogenesis and epididymal maturation to provide some degree of protection [46]. During epididymal maturation, the plasma membrane of spermatozoa becomes enriched with polyunsaturated fatty acids (PUFAs) [13]. While PUFAs contribute to the integrity of the sperm membrane and improve its ability to fuse with the oocyte during fertilization, they also increase the sperm susceptibility to OS. Therefore, the balance between ROS and antioxidants is critical for maintaining proper sperm function and fertility [47].

As discussed above, to fertilize an oocyte, spermatozoa must be prepared through morphological alterations, such as the remodelling of the plasma membrane. Those changes will assist the penetration of the oocyte and the survival of sperm in the female reproductive tract. This process, known as capacitation, is mainly characterized by the activation of tyrosine kinase via cAMP [13]. Capacitation is related to the improvement of sperm characteristics such as membrane fluidity, motility, and calcium inflow, facilitating spermatozoa to penetrate the oocyte [48]. Glycolysis and mitochondrial OXPHOS are the main metabolic pathways that support capacitation, as ATP is required for this process to occur [49]. In a study by Carrageta and colleagues, human spermatozoa were incubated under in vitro capacitation conditions with varying glucose concentrations. Spermatozoa incubated without glucose exhibited a lower viability, while those incubated with glucose maintained viability over time [49]. The study also revealed that the sperm cells incubated with glucose exhibited higher levels of tyrosine residue phosphorylation, which is a commonly used biomarker for sperm capacitation. This finding demonstrates the importance of glucose in promoting human sperm capacitation [49]. ATP production via OXPHOS occurs in the midpiece of spermatozoa, primarily supporting sperm motility. However, it is also crucial for maintaining chromatin structure and regulating the acrosome reaction in spermatozoa [50]. Zhang and colleagues investigated sperm MMP in young college students, using the N-α-benzoyl-DL-arginine-para-nitroanilide HCl (BAPNA) substrate method to measure acrosin activity and DNA fragmentation index (DFI) to assess chromatin integrity. Their findings indicated that individuals with low MMP had reduced acrosin activity and lower DFI when compared to those with high or moderate MMP [50]. This study also found that sperm MMP dissipation led to ROS overproduction and reduced ATP content [50]. Overall, mitochondria play a vital role throughout the male reproductive tract, and their dysfunction can cause errors during spermatogenesis, sperm capacitation and oocyte fertilization, leading to male infertility.

3. Relevance of Mitochondrial (Dys)function to Male Fertility

Mitochondrial activity plays a crucial role in all stages of male reproductive potential, from spermatogenesis to oocyte fecundation. In spermatozoa, mitochondria are located in the midpiece and form the mitochondrial sheath that surrounds the axoneme [51]. This structure is connected to the axoneme by a reticulum of filaments and synthesizes ATP, which is necessary for the proper sperm function [52,53]. Recent comparative studies have shown that the amount of mitochondria in the sheath is directly related to sperm velocity and ATP production. For instance, Gu and colleagues studied sperm morphology and mitochondrial functions in 10 mammalian species, including humans, and found that a higher number of mitochondria in the sheath was positively correlated with increased sperm motility and ATP production [54]. In humans, studies have shown that low sperm motility is associated with smaller midpieces, abnormally assembled mitochondria, and mitochondrial membranes with structural defects [51]. These findings suggest a direct correlation between the correct mitochondrial structure and fertilization rates [55].

Sperm quality is closely linked to proper mitochondrial function, and disruptions in the ETC pathway can adversely affect sperm quality [51]. In humans, the MMP, which serves

as an indicator of energy and mitochondrial functions, has been found to be associated with sperm viability [56] and the ability to perform acrosome reaction [57] as well as the capacity to fertilize oocytes naturally or in vitro [58]. When measuring the enzymatic activity of the respiratory chain complexes, Ruiz-Pesini and colleagues used specific modulators of the ETC complexes and found that their correct functioning influenced sperm parameters such as motility and vitality [21]. They were also able to demonstrate a direct correlation between mitochondrial enrichment of complex II and more efficient spermatogenesis, leading to a higher number of ejaculated spermatozoa [21]. Lastly, mitochondrial respiration and oxygen consumption have been linked to higher sperm motility and capacitation [59], further highlighting the direct relationship between mitochondrial function and healthy sperm physiology, as we summarized in Table 1.

Mitochondria are also involved in other processes that can affect sperm quality, such as generation of ROS, calcium control and cell signalling [51] (Figure 2). The overproduction of ROS by leukocytes, immature germ cells, and defective spermatozoa can exceed the physiological threshold leading to oxidative stress, which is very deleterious for the sperm [51]. Therefore, it is crucial to maintain a delicate balance of ROS levels in the male reproductive system. Oxidative stress can have a negative impact on sperm quality and function, including reducing sperm motility, affecting mitochondrial activity, and ultimately, reducing the ability of sperm to successfully fertilize the oocyte [60]. The high levels of PUFAs in the sperm membrane make them highly susceptible to lipid peroxidation caused by ROS. This peroxidation of lipids can also trigger the generation of ROS by sperm mitochondria. PUFAs penetrate the IMM and inhibit the proper flow of electrons in the ETC, leading to the production of superoxide anions, oxidative DNA damage [61] and DNA base oxidation [62]. When OS is present, there is increased amount of ROS that stimulates more generation of ROS [2]. This is due to the formation of aldehyde–protein adducts, which are by-products of lipid peroxidation and bind to proteins in the mitochondrial ETC, causing the generation of more ROS in the mitochondria [63] (Figure 2). This perpetuates a cycle of ROS generation and the resulting OS, which ultimately leads to apoptosis of spermatozoa. This occurs because sperm mitochondria are extremely sensitive to oxidative stress, despite being the main intracellular ROS generator [51]. Therefore, the maintenance of ROS levels within the physiological range is crucial for preserving sperm quality and function.

Koppers and colleagues demonstrated that mitochondrial ROS generated from complex III can cause the release of hydrogen peroxide into the extracellular space without detectable peroxidative damage. In contrast, the induction of mitochondrial ROS from complex I results in leakage into the mitochondrial matrix, leading to peroxidative damage to the IMM [61]. This damage to the IMM caused by mitochondrial ROS from complex I may result in the opening of the mitochondrial permeability transition pores and fragmentation of the IMM, leading to activation of the mitochondrial intrinsic apoptotic pathway [64]. Since the chemical triggers for apoptosis that exist in somatic cells are not present in the spermatozoa, these will automatically undergo truncated apoptosis unless pro-survival factors are able to prevent this process [65]. However, the specific signalling pathways that mediate these processes in spermatozoa are not well understood. An important signalling molecule is calcium, which plays a crucial role in sperm function. Calcium is a messenger that participates in a variety of cellular processes. In humans, it has been demonstrated to be crucial in sperm function, being involved in the movement of the flagellum, capacitation, acrosome reaction and chemotaxis [66]. Calcium-dependent pores in the sperm IMM open in response to high intracellular calcium levels, allowing calcium to enter mitochondria and reduce MMP [67], activating the apoptotic pathway [51].

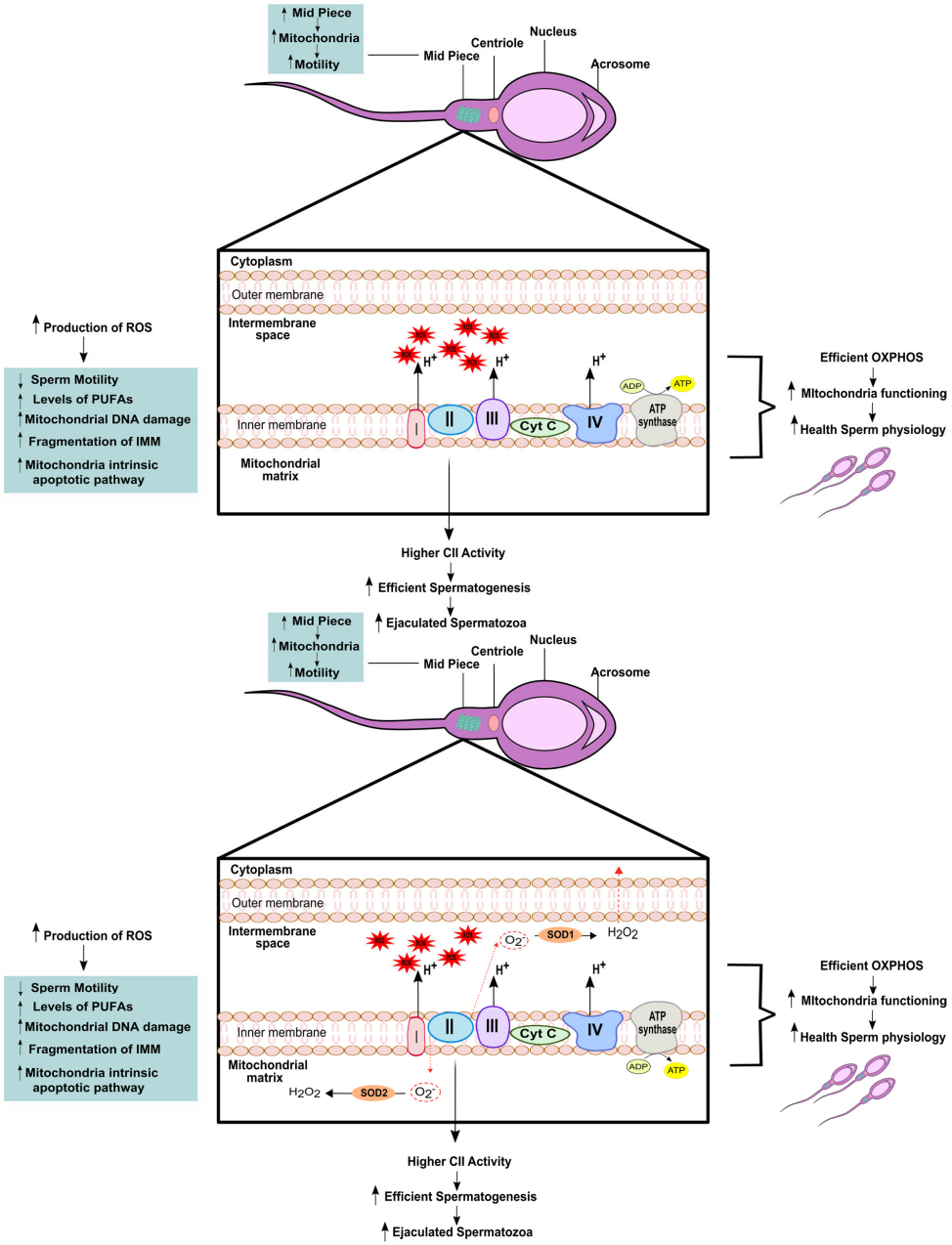

Figure 2. Schematic summary of the complex relationship between mitochondrial quality and spermatozoa health. An efficient oxidative phosphorylation (OXPHOS), particularly at the complex II (CII) level in the electron transport chain, will lead to more ejaculated spermatozoa presenting a better health sperm physiology. Furthermore, a higher mid piece of the spermatozoon is directly linked with a higher motility. However, an imbalance between reactive oxygen species (ROS), will lead to lower sperm motility as well as an increase in mitochondrial DNA damage, an increase in polyunsaturated fatty acids (PUFAs) and increase in the mitochondrial apoptotic pathway. The enzymes superoxide dismutase 1 and 2 (SOD1 and SOD2, respectively) composed the metabolic machinery that handles reactive oxygen species (ROS) in the mitochondrial matrix. Specifically, these enzymes are able to convert superoxide radical anion ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2).

Research on sperm apoptosis has demonstrated that apoptotic markers in sperm are similar to those found in somatic cells. In both cases, phosphatidylserine is externalized in the plasma membrane, mitochondrial integrity is compromised, caspase is activated, and DNA damage occurs [68–71]. Despite the similarities, understanding and explaining the apoptotic process in spermatozoa remains a challenge due to the unique physiological

conditions of these cells [51]. Apoptotic markers of immature sperm, such as blebbing of the plasma membrane, formation of apoptotic bodies, impaired mitochondrial integrity, defects of the nuclear envelope, and fragmentation of the nucleus, have been associated with infertility in men with reproductive issues [72]. Conversely, these markers are not present in fertile men and mature sperm [73]. The mitochondria, responsible for the intrinsic apoptotic pathway, are thought to play a crucial role in triggering apoptosis in spermatozoa. For example, a study conducted on infertile men with spermatic alterations showed a direct positive correlation among ROS, cytochrome c outside the mitochondria, and the induction of caspases 9 and 3. In another study by Paasch and collaborators it was shown that inducing apoptosis in human sperm leads to higher activity of caspase 9 and 3, along with lower MMP and sperm motility [74]. Activation of caspases 9 and 3 is associated with low sperm quality, higher DNA fragmentation, and reduced fertilization capabilities. Furthermore, when caspase 3 is activated together with disrupted MMP, it leads to the release of phosphatidylserine in spermatozoa [75]. These findings are consistent with the notion that the intrinsic apoptotic pathway involving mitochondria plays a role in regulating sperm apoptosis.

The phosphatidylinositol 3-kinase (PI3K)/AKT signalling pathway plays a crucial role in regulating cell cycle progression, growth, proliferation, survival, and migration [2]. Under stress, the activation of AKT promotes cell survival [76]. Activation of the PI3K enzyme leads to the phosphorylation of AKT1, which, in turn, silences apoptotic pathway promoters, helping to maintain the functionality of spermatozoa. [65]. Inhibition of the PI3K enzyme in this pathway results in the dephosphorylation of AKT1, leading to the initiation of the intrinsic apoptotic pathway in spermatozoa [2]. Consequently, this leads to caspase activation, increased production of mitochondrial ROS, oxidative DNA damage, and reduced sperm motility [77]. Due to the unique architecture of the sperm head, which separates the nucleus from the mitochondria and cytoplasm in the sperm midpiece, endonucleases that are activated during apoptosis are unable to cleave nuclear DNA. As a result, DNA fragmentation in nuclear DNA is not a consequence of apoptosis [77].

Multiple changes can occur in mtDNA at the molecular level, including deletions, substitutions, and other point mutations. These changes can result in poor sperm quality and subsequently male infertility [14]. Mouse models have been used to study the role of OXPHOS in spermatogenesis. Specifically, mice with error-prone mtDNA replication and a mutation in the subunit of the mtDNA polymerase γ have been found to have infertility issues [78]. The "mtDNA mutator" mice presented early degradation in multiple organ systems, particularly the testes, where severely degenerated seminiferous tubes and germ cell depletion were observed at 10 months of age [78]. Jiang and colleagues demonstrated that altering the expression levels of the mtDNA regulator, mitochondrial transcription factor A (Tfam), could affect infertility in mtDNA mutant mice. Increasing Tfam expression levels mitigated the infertility phenotype, while decreasing levels worsened the phenotype [79]. Additionally, mitochondrial dysfunction has been shown to affect MPI in mice. Mice with a high level (4696 bp) pathogenic deletion in mtDNA had low OXPHOS activity, which resulted in meiotic arrests at the transition between zygotene and pachytene [15]. In mice lacking the testis-specific adenine nucleotide translocator 4 (Ant4), a genetic ablation caused spermatogenic arrest in the leptotene phase of MPI [80].

In humans, it has been reported that males with poor sperm quality have a higher prevalence of sperm mitochondrial DNA deletions compared to normozoospermic males [81]. Large-scale mtDNA deletions have been reported to occur more frequently in males with obstructive azoospermia compared to fertile and infertile men with non-obstructive azoospermia [2]. This can be attributed to blockage in the reproductive tract, which creates a higher level of OS and mitochondrial dysfunction. These deletions can affect the OXPHOS system, causing a decrease in ATP production, which can lead to poor sperm motility and decreased fertilization capacity. In addition, these deletions can also cause oxidative damage to sperm DNA, leading to increased levels of sperm DNA fragmentation and subsequent infertility. Additionally, large deletions or a high number of deletions can lead to disruption of ETC,

reducing the efficiency of oxidative phosphorylation and impairing ATP synthesis [82]. This mitochondrial dysfunction can affect sperm quality and motility. Indeed, one of the most sensitive biomarkers of male fertility is sperm mtDNA copy number [83]. When the copy number of human mtDNA is altered, both sperm motility and fertilization capacity are be affected [84]. It has been demonstrated that infertile males or those with abnormal semen parameters have a decline in mtDNA integrity when copy number increases [85]. Conversely, a decrease in mtDNA copy number in spermatozoa has been associated with lower sperm motility [86]. The regulation of proteins in sperm has been shown to be differentially affected in patients with decreased sperm motility [87], particularly proteins involved in the fibrous sheath and energy production [88], as well as those involved in spermatogenesis, sperm maturation, sperm tail structure and motility, and mitochondrial quality control [89]. In samples from patients with reduced sperm motility, it has been shown differential protein expression of several proteins. Proteins involved in energy and metabolism, such as triose-phosphate isomerase, glycerol kinase 2, and succinyl-CoA:3-ketoacid co-enzyme A transferase 1, are expressed at higher levels compared to those involved in sperm motility and structure, as well as stress response [90]. This suggests a possible shift in energy production towards glycolysis rather than OXPHOS, which may be a compensatory mechanism to maintain ATP levels and reduce OS in spermatozoa with impaired motility. Additionally, proteins involved in mitochondrial quality control, such as Lon peptidase 1 and prohibitin, were downregulated, suggesting a possible role of mitochondrial dysfunction in reduced sperm motility. In many cases, mitochondrial dysfunction leading to errors in spermatogenesis, sperm capacitation, and oocyte fertilization, which affect sperm quality, motility, and fertilization capacity, are caused by external factors such as metabolic diseases. Thus, a deeper understanding of the impact of metabolic diseases on mitochondrial quality control is imperative to manage and ensure adequate spermatogenesis and prevent male infertility. Appropriate management of metabolic diseases may include lifestyle modifications, pharmacological interventions, or other treatments aimed at reducing mitochondrial dysfunction and improving overall metabolic health. By doing so, we can improve male fertility outcomes and reduce the incidence of infertility caused by metabolic diseases.

Table 1. Spermatozoa fitness alterations due to mitochondrial dysfunction.

Mitochondrial Associated Mechanism	Spermatozoa Outcomes	References
↓ MMP	↓ sperm viability ↓ Acrosome reaction	[57]
↓ ETC	↓ sperm motility ↓ sperm vitality	[21]
↑ amount of mitochondria in the sheath	↑ sperm morphology ↑ ATP production	[53]
Enrichment of complex II	More efficient spermatogenesis ↑ ejaculated spermatozoa	[21]
↑ Oxidative stress	↓ sperm motility ↓ fertilization capacity	[59]
Damage in IMM	↑ apoptotic pathway ↓ MMP	[63]
↓ mtDNA integrity	↓ sperm motility Poor sperm quality	[14,83,85]

4. Non-Communicable Diseases and Environmental Impact on Mitochondrial Quality of Testicular Cells and Sperm

When analysing mitochondrial quality in male fertility, it is crucial to take into account both internal mitochondrial parameters and cues that may alter the mitochondria's proper operation. Indeed, non-communicable diseases (NCDs) are an example of a condition that can impact male fertility by altering mitochondrial function. NCDs refer to chronic,

non-infectious and non-transmissible diseases that can have a significant impact on overall health and well-being [91]. By studying the relationship between NCDs and mitochondrial function, we can gain a deeper understanding of how these diseases can affect male fertility and develop targeted strategies to mitigate their negative effects. Among the most common NCDs are cardiovascular and neurological diseases, as well as cancer and diabetes. Obesity, which is associated with a group of other NCDs including diabetes mellitus (DM), hypertension, metabolic syndrome, non-alcoholic fatty liver disease, and cardiovascular diseases, is also a major risk factor [91]. In addition, recent studies have linked obesity to some types of cancer such as colorectal, liver, and prostate [92].

Obesity is associated with mitochondrial dysfunction, which results in lower energy metabolism. When nutrient supply is abundant, cells produce more mitochondria. However, if nutrient supply remains excessive, the mitochondrial system becomes overloaded, leading to dysfunction and accumulation of non-oxidized lipid products. This causes accumulation of fat and OS, which leads to mitochondrial damage and a further decline in energy metabolism [91]. Excess adipose tissue is known to increase aromatase activity, which converts testosterone to oestradiol, leading to decreased testosterone levels in men [93]. Since testosterone is crucial for regulating spermatogenesis, this reduction in testosterone levels can significantly reduce sperm production [94]. Moreover, decreased testosterone levels are also accompanied by mitochondrial dysfunction in Leydig cells, resulting in oxidative damage to lipids, proteins, and mtDNA, promoting the production of ROS and reduced ATP levels [95]. Obesity is often correlated with a diet rich in saturated fats (SFA) and low in PUFAs [96]. In humans, a direct correlation between increased amounts of SFA and decreased sperm count and concentration has been described [97]. Conversely, a higher intake of omega-3 PUFAs has been linked to better sperm quality. These findings highlight the importance of a balanced and healthy diet in maintaining mitochondrial function and male fertility, as described in Figure 3.

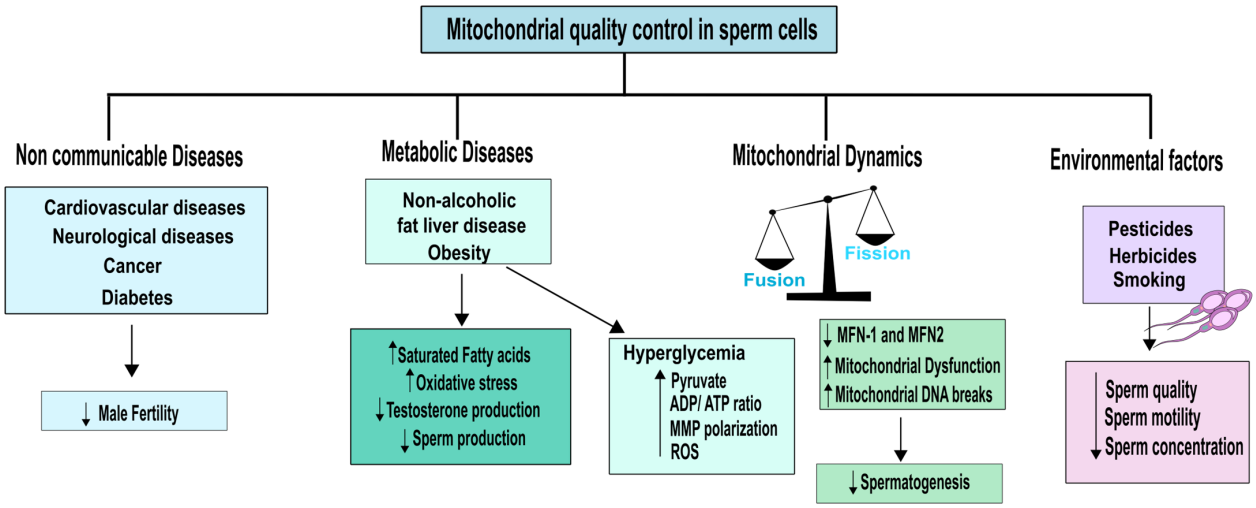

Figure 3. Overview of the interconnected homeodynamics of sperm cells and mitochondrial quality control. Various factors can influence this delicate balance, including non-communicable diseases, metabolic diseases, mitochondrial dynamics, and environmental factors. An imbalance in any of these conditions can result in unhealthy spermatozoa, compromising male fertility. Abbreviations: ROS- Reactive oxygen species; MMP—mitochondrial membrane potential; MFN-1—mitofusin 1 and MFN-2—mitofusin 2.

Regarding PUFAs, it has been suggested to play a role in modulating sperm bioenergetic pathways. Indeed, PUFAs are important components of cell membranes and play a role in regulating mitochondrial function, oxidative stress, and inflammation [98]. There is a isoenzyme form of sperm lactate dehydrogenase (LDH-C4) that is crucial for the nutritional regulation of omega-3 PUFA [98]. This enzyme is present in both the mitochondrial

matrix and the cytosol of spermatozoa, and plays a crucial role in the energy metabolism of spermatozoa by catalysing the conversion of pyruvate to lactate and the adjuvant oxidation of NADH [20,99]. LDH-C4 also enables the concurrent advancement of OXPHOS and glycolysis transporting reducing equivalents from the cytosol into the mitochondria and by regenerating NAD+, respectively [94]. It has also shown that a that a diet rich in omega-3 PUFAs can reduce OS in sperm cells by increasing the ratio of aconitase to fumarase activity [98]. The activity ratio of these two Krebs cycle enzymes is considered a marker for mitochondrial production of ROS [100]. On the other hand, a diet high in SFA and low in PUFA has been linked to decreased activity of LDH-C4, pyruvate dehydrogenase, and respiratory enzymes [100].

Despite growing interest in the relationship between diet and reproductive health, the potential effects of dietary carbohydrates on sperm quality remain largely unexplored by scientific research [94]. Glucose is the main fuel for glycolysis in sperm cells, where it is metabolized to pyruvate and/or lactate to produce ATP, the essential energy source for sperm motility. Thus, any reduction in glucose uptake and metabolism by sperm can lead to a decrease in ATP levels, impairing their ability to swim and fertilize an egg [94]. Elevated blood glucose levels have been associated with decreased testosterone production and increased OS in the body [101]. These factors can negatively impact sperm health by impairing mitochondrial function, which is crucial for generating energy and maintaining motility [102]. As a result, high blood glucose levels may lead to reduced sperm motility, highlighting the importance of controlling blood glucose levels for optimal reproductive health. Diabetes mellitus (DM) refers to a group of metabolic disorders characterized by chronic hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. It is a complex and heterogeneous condition, with multiple subtypes and underlying mechanisms, but chronic hyperglycaemia is the defining feature of all forms of DM [103]. The pathogenesis of DM involves a complex interplay of various factors, including an imbalance between free radical formation and the antioxidant defence mechanisms in the body. This OS can result in damage to cellular components, leading to impaired insulin signalling and glucose metabolism [104]. Hence, the dysregulation of the redox balance, characterized by increased free radical formation and/or decreased antioxidant defences, plays a crucial role in the development and progression of DM. DM is associated with a range of vascular and organ-related complications that arise due to overproduction of ROS induced by hyperglycaemia. ROS can cause oxidative damage to various tissues and organs, leading to inflammation, cell injury, and impaired organ function [105]. Mitochondrial dysfunction is a critical factor in the pathogenesis of diabetes, as evidenced by the lower rates of ATP synthesis observed in individuals with a family history of the disease, even before the onset of poor glucose tolerance [106]. Hyperglycaemia promotes the synthesis of pyruvate and increases the flow of reducing equivalents into the ETC [105], resulting in a higher increased ATP/ADP ratio and MMP polarization. However, the significant electrochemical potential difference created by the proton gradient can partially inhibit complex III of the ETC, leading to coenzyme Q accumulation in its reduced form and subsequent generation of superoxide [106]. This increased reduction in coenzyme Q and the resulting production of ROS is suggested to be responsible for mitochondrial dysfunction, which is a key factor in the metabolic abnormalities and tissue histopathology associated with DM [106]. Normally, mitochondria maintain a slightly reduced MMP that generates less ROS, but in hyperglycaemia, hyperpolarization of the MMP leads to increased production of ROS [105].

It was found that exposure to high glucose concentrations causes rapid fragmentation of mitochondria, leading to increased production of ROS [107]. Incubating sperm with high glucose concentrations prevented periodic fluctuations in ROS production by inhibiting mitochondrial fission [107]. This suggests that hyperglycaemic conditions induce dynamic changes in mitochondrial morphology that contribute to the overproduction of ROS. Thus, the mitochondrial fission/fusion machinery represents a potential target for monitoring and regulating acute and chronic ROS production in hyperglycaemia-related disorders [105].

Taken together, these findings indicate that one of the main complications of obesity and DM is the overproduction of ROS and the resulting OS. Understanding the mechanisms underlying ROS production and regulation is critical for developing effective interventions to mitigate the negative consequences of hyperglycaemia and OS. As discussed above, both spermatozoa and germ cells are vulnerable to oxidative stress (OS), which can negatively impact sperm quality by reducing sperm count, motility, and increasing the incidence of abnormalities [108]. Furthermore, oxidative damage to mitochondrial DNA can increase the frequency of large-scale deletions and DNA strand breaks, accelerating germ cell apoptosis and reducing sperm quantity, which is associated with male infertility and lower semen quality [86]. The importance of mitochondrial respiratory activity in mammalian spermatogenesis has also been highlighted by studies linking defects in mitochondrial respiration to meiotic arrest and abnormal sperm morphology [15]. These findings emphasize the crucial role of mitochondrial function and OS regulation in maintaining sperm quality and male reproductive health.

In recent years, there has been an increase in life expectancy, leading to an increase in the age of parents and a corresponding rise in infertility issues. However, there is still limited research on the effects of aging on male fertility and how advanced paternal age affects offspring [109]. Aging in males is associated with changes in the hypothalamic–pituitary–gonadal axis and alterations in the testis, penis, and prostate [12]. Testosterone levels decrease, sperm motility slows, and erectile dysfunction becomes more common in older men [12]. In addition, there is an increase in chromosomal defects and DNA damage, which may have consequences for the offspring [110]. In cells, mitochondrial dysfunction also has a direct correlation with age, where respiratory chain function is affected with advancing age [12]. Studies in mice expressing defective polymerase gamma (POLG), which is essential in mitochondrial DNA replication, have shown premature aging [111]. Using nuclear magnetic resonance (NMR) spectroscopy, Jarak and colleagues were able to identify metabolic changes associated with different stages of reproductive maturity in mice. A notable finding was a significant decrease in testicular creatine content in older mice, indicating changes in the conditions required for male germ cell development [112]. Creatine is a crucial component in the energy metabolism of tissues with high energy demands, including the testis, and assists in ATP replenishment. Therefore, alterations in creatine levels may suggest metabolic changes associated with aging in the testis. In addition, the study showed that advanced age in mice was associated with increased levels of complex I protein, which is linked to ROS overproduction, as well as increased expression levels in the other OXPHOS complexes [112]. However, when comparing 24-month-old mice to 12-month-old mice, there was a significant decrease in the expression levels of the OXPHOS complexes with age, suggesting a decline in mitochondrial function [112].

While NCDs and aging can contribute to mitochondrial dysfunction in the male reproductive tract, there are several environmental factors that can also impact male fertility. In particular, there is growing interest in the potential impact of toxicants, such as herbicides on sperm quality [113]. Despite increased research in this area, our understanding of how these chemicals affect the molecular processes that influence sperm quality remains limited [114]. Anifandis and colleagues demonstrated the negative impact of herbicides on human sperm motility and mitochondrial function by treating spermatozoa with 1 mg/L of Roundup, whose primary active component is glyphosate (GLY) [113]. Using mitochondrial staining, the researchers observed a decrease in sperm motility and mitochondrial staining after one hour of exposure to Roundup, compared to control cells. This suggests that GLY may cause reduced sperm motility by inducing oxidative stress in mitochondria and increasing the production of mitochondrial apoptotic signals [113]. In a more recent study, Ferramosca and colleagues investigated the effects of GLY and glufosinate ammonium (GA) on the efficacy of mitochondrial respiration in human sperm mitochondria. Their findings showed that GLY significantly reduces mitochondrial functionality by lowering oxygen in both the active and passive stages of mitochondrial respiration. In addition, GA may induce mitochondrial permeability by altering the PI3K/AKT complex phosphorylation

status, resulting in the loss of motility in human sperm mitochondria [114]. These examples underscore the impact of environmental factors such as pesticides and herbicides on mitochondrial quality control in sperm, which can compromise male fertility.

Cigarette smoking is one of the major health concerns in people of fertile age, and nicotine is its main component. [115]. Detection of nicotine and its major metabolite, cotinine, in the seminal plasma of smokers has demonstrated that tobacco chemicals can penetrate the blood–testis barrier and cause harm to spermatozoa [116]. Components of cigarette smoke are known to be toxic and their intake can lead to testicular microcirculation, DNA, and chromosomal damage in germ cells [117]. Numerous studies have shown that cigarette smoking reduces semen volume, sperm concentration, motility, and normal physiology [118–120], while also decreasing the sperm's ability to fertilize [121,122]. Additionally, sperm from smokers have higher levels of oxidative DNA damage and aneuploidy compared to non-smokers [123]. Chohan and Badawy used phosphorescent analysis to measure oxygen concentrations in sperm suspensions and compared sperm respiration rates in smokers and non-smokers. Their findings revealed that cigarette smoking significantly influences sperm respiration by reducing mitochondrial oxygen consumption [115]. Overall, cigarette smoking has a detrimental effect on male fertility, and its negative impact on sperm motility, morphology, and DNA integrity is well-established. It is important for individuals of reproductive age to avoid cigarette smoking to protect their reproductive health.

While the health advantages of physical activity have been extensively studied for a variety of medical conditions, the impact of exercise on male fertility remains unclear [95]. Several studies indicate that the type, duration, and intensity of exercise have varying impacts on male fertility [95]. Vigorous exercise has been found to reduce male reproductive capacity [124], whereas aerobic, resistance, or combined exercises have been shown to improve male fertility [125]. Although the effects of physical exercise on sperm quality are well established, the underlying mechanisms remain unclear [95]. However, some compelling evidence suggests that high-intensity exercise can induce OS [126,127], which may play a role on impacting male fertility. Aerobic exercise leads to increased oxygen consumption, which is associated with a higher rate of electrons passing through the mitochondrial respiratory chain complexes, possibly causing OS [128,129]. Additionally, catecholamines released during exercise, prostanoid metabolism, xanthine oxidase, and NAD(P)H oxidase are sources of ROS [95]. Regular exercise or chronic anaerobic training can enhance endogenous antioxidant defence mechanisms, reducing oxidative damage [129,130]. However, it has been suggested that rigorous training or prolonged competition periods may increase OS [131]. This is because ROS release can dysregulate the inflammatory and neuroendocrine systems, which may exceed the capacity of the antioxidant system to protect against damage [132]. In healthy young adults who do not exercise regularly, any type of exercise can increase free testosterone concentrations. However, for athletes, only high-intensity exercise appears to lead to an increase in testosterone levels [95]. Recent studies among elite athletes have revealed that their testosterone levels are significantly lower than the physiologically normal range, which could be a consequence of chronic exposure to high levels of aerobic exercise volume and intensity [133,134]. This puts athletes at risk of overtraining and persistent fatigue [95]. Unfortunately, low testosterone levels have been associated with OS, which can result in a decline in sperm quality, thus affecting male fertility [18].

5. Mitochondria Quality Control Parameters Essential to Evaluate

Mitochondria play a crucial role in male fertility, contributing to spermiogenesis, capacitation of the spermatozoa, and oocyte fecundation [13]. Therefore, when assessing the quality of mitochondrial functions as a biomarker or sentinel for sperm quality, several factors should be considered along the male reproductive tract. As already explored, mitochondria morphology differs significantly along spermatogenesis. Specifically, mitochondria in SSCs are heavily vacuolated, spherical, and lack cristae. Furthermore, fusion of OMM and IMM is regulated by proteins anchored to the membrane, including mitofusin

(MFN)-1,2 and optic atrophy (OPA)-1. These proteins facilitate the fusion of the OMM and IMM, and when they are non-functional, they can lead to the disintegration of the mitochondria [135]. On the other hand, fission is controlled by proteins such as cytosolic dynamin, Fission1 protein (Fis-1), mitochondrial fission factor (MFF), and dynamin-related protein 1 (Drp1). Drp1 is transported from the cytosol to the mitochondria, and a Drp1 deficiency can result in mitochondria hyperfusion [136]. A recent study conducted by Varuzhanyan and colleagues aimed to investigate the significance of mitochondrial fusion in mouse spermatogenesis. They found that double mutants for MFN-1 and MFN-2 were unable to produce any sperm, indicating that mitochondrial fusion is necessary for proper spermatogenesis functioning [137]. In Drosophila melanogaster, a mutation in the mitofusin homolog (Marf) has been associated to male sterility, being essential for the maintenance of male germline stem cells [138]. Therefore, it is evident that mitochondrial fusion/fission is essential for the correct functioning of spermatogenesis and these mechanisms could pointed as a possible biomarker on mitochondrial fitness that can be correlated with spermatozoa health (Figure 3). Additionally, recent findings have highlighted the critical role of autophagy in post-meiotic spermatids for cellular remodelling [139]. Although excess mitochondria are removed during spermatogenesis, the involvement of mitophagy in maintaining mitochondrial quality in the male reproductive tract is still under intense debate and requires further understanding [26].

Throughout the male reproductive tract, a significant amount of energy is required to complete the complex processes involved in spermatogenesis and oocyte fertilization. The primary source of energy is derived from OXPHOS [140]. Sperm contains substrates such as glutamic acid (GLU) [141], pyruvate, and lactate that serve as potential energy sources [142]. GLU can be converted into α-ketoglutarate through reactions involving alanine aminotransferase (ALT) and aspartate aminotransferase (AST). In this process, pyruvate and oxaloacetate are transformed into alanine and aspartate, respectively, which contribute to OXPHOS [141]. Alternatively, pyruvate can be reduced to L-lactate by lactate dehydrogenase, generating NAD+ for glycolysis. Pyruvate can also enter the Krebs cycle to support OXPHOS [99]. LDH activity in the mitochondrial matrix of sperm was first discovered in rabbit epididymal spermatozoa and has since been reported in several species, including humans [143]. In humans, LDH-C4 is present in the sperm mitochondrial matrix and facilitates the conversion of lactate to pyruvate, thereby aiding in mitochondrial energy production [144]. Studies have shown that supplementation of sperm media with lactate and pyruvate improves mitochondrial function compared to media containing only glucose [145]. However, this finding has sparked debate over the preferred metabolic pathway for maintaining optimal sperm function [146]. Both oxidative and glycolytic energy metabolism pathways work in tandem to generate energy [145]. Despite the ongoing debate, the crucial question remains whether sperm mitochondria should operate at their maximum capacity, as increased mitochondrial activity leads to increased ROS production [145]. As previously mentioned, ROS are considered harmful by-products of mitochondrial metabolism. When the level of ROS exceeds the antioxidant defences of the cell, it leads to cell damage and oxidative stress [147]. However, mild oxidative stress is necessary for sperm functions such as fertilization, motility, and capacitation, despite causing damage to sperm structure and function [148]. For example, ROS can promote capacitation by regulating tyrosine phosphorylation through redox, which enhances the sperm's ability to bind to the zona pellucida [149]. Research on stallion spermatozoa has associated increased ROS production with rapid mitochondrial activity in sperm [142]. In another study, higher ROS levels were observed in stallion spermatozoa with good freezability after cryopreservation, compared to those with poor freezability, indicating increased mitochondrial activity [150]. Therefore, while excessive ROS production can cause OS and impair sperm function, a moderate amount of ROS is required for proper sperm function. The optimal balance between ROS generation and antioxidant defence should be maintained to support healthy sperm function [145]. In conclusion, mitochondria play a vital role in male fertility, and their quality and proper functioning in sperm are maintained

not only through biogenesis mechanisms, but also by balancing oxidative and glycolytic metabolic pathways and by balancing the overproduction and underproduction of ROS. The goal is to prevent cell damage, oxidative stress, and apoptosis, and to enable all phases of the male reproductive tract to function properly. Maintaining optimal mitochondrial function is essential for healthy sperm and successful fertilization. Therefore, future research should focus on understanding the precise mechanisms that regulate mitochondrial metabolism and identifying potential therapeutic targets to enhance mitochondrial function and improve male fertility.

6. Conclusions

Mitochondria are essential organelles for male fertility, and their correct functioning is critical for the success of the reproductive process. Mitochondria play an essential role in generating ATP, the energy required for the physiological mechanisms involved in spermatogenesis and fertilization. However, mitochondria also produce ROS, which can lead to cellular damage, oxidative stress, and apoptosis if not properly maintained. One of the crucial functions of mitochondria in sperm is to produce ATP, which is necessary for various sperm processes, including motility, hyperactivation, capacitation, and acrosome reaction. The correct functioning of mitochondria in sperm is directly related to sperm quality, and defects in mitochondrial function can lead to male infertility. Mitochondria are susceptible to damage from both internal and external factors. Internal factors include overproduction of ROS by leucocytes, immature germ cells, and defective spermatozoa, which can cause oxidative stress and damage mitochondria. External factors that can damage mitochondria include NCDs, such as obesity and diabetes, aging, and poor health habits such as lack of exercise and smoking. Mitochondria have quality control mechanisms to prevent malfunctions. One such mechanism is biogenesis, which ensures that only functional mitochondria are retained, and dysfunctional ones are eliminated. Metabolic pathways in sperm also play a crucial role in maintaining mitochondrial function, by balancing oxidative and glycolysis metabolic pathways and maintaining a physiological equilibrium in the production of ROS. Assessing mitochondrial functionality and quality control is crucial to understanding and managing male infertility. Measuring mitochondrial DNA quality and assessing mitochondrial function in sperm can provide valuable information for diagnosing and treating male infertility. Additionally, developing interventions that target the mitochondrial pathways involved in sperm function may lead to improved treatments for male infertility. In conclusion, mitochondria play a critical role in male fertility, and their correct functioning is essential for the success of the reproductive process. Maintaining a balance between ATP generation and ROS production is necessary to prevent damage to the sperm and maintain sperm quality. By understanding the mechanisms involved in mitochondrial function and quality control, we can develop effective interventions for the treatment of male infertility.

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Research article

The progression from mild to severe hyperglycemia coupled with insulin resistance causes mitochondrial dysfunction and alters the metabolic secretome of epithelial kidney cells

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ABSTRACT

Diabetic nephropathy (DN) and insulin resistance (IR) in kidney cells are considered main causes for end-stage renal failure. However, it is unclear how IR affects early stages of the disease. Here, we investigate the impact of mild (11 mM) and severe (22 mM) hyperglycemia, with and without induced IR, on cellular metabolism and mitochondrial bioenergetics in a human kidney cell line (HK-2).

IR in HK-2 cells was induced with palmitic acid and cellular cytotoxicity was studied. We evaluated the impact of mild and severe hyperglycemia with and without IR on the metabolic secretome of the cells, their live-cell mitochondria function, mitochondrial membrane potential, and mitochondrial complex activities. Furthermore, we measured fatty acid oxidation and lipid accumulation.

Cells cultured under mild hyperglycemic conditions exhibited increased mitochondrial bioenergetic parameters, such as basal respiration, ATP-linked production, maximal respiration capacity, and spare respiration capacity. However, these parameters decreased when cells were cultured under higher glucose concentrations when IR was induced. Our data suggests that progression from mild to severe hyperglycemia induces a metabolic shift, where gluconeogenic amino acids play a crucial role in supplying the energy requirements of HK-2.

To our knowledge, this is the first study to evaluate the progression from mild to severe hyperglycemia allied to IR in human kidney cells. This work highlights that this progression leads to mitochondrial dysfunction and alters the metabolic profile of kidney cells. These results identify possible targets for early intervention in DN.

1. Introduction

Diabetic nephropathy (DN) is considered a major cause of morbidity and mortality in patients with diabetes and is associated with the leading cause of chronic and end-stage renal disease [1]. Elevated plasma glucose levels can trigger cellular dysfunctions, leading to changes in energy utilization and mitochondrial dysfunction, both of which play a critical role in disease initiation [2]. The kidney is an energetically demanding organ that requires large amounts of energy in the form of ATP due to the re-absorption process [3]. In the renal cortex, proximal tubules depend on the efficiency of oxidative phosphorylation (OXPHOS) to produce ATP, while glomerular cells have a lower oxidative capacity. Since proximal renal tubular cells have high mitochondrial content they are particularly susceptible to metabolic and mitochondrial injury from hyperglycemia [4]. The role of mitochondrial (dys)function in kidney physiology remains largely unknown, although it has been

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suggested that the detection of mitochondrial dysfunction may promote the development of personalized therapies or the identification of specific proteins to improve renal function (as reviewed by Braga and collaborators [5]). Furthermore, after approximately 15 years following a diabetes diagnosis, nearly 40% of patients do not develop albuminuria, a commonly used biomarker for renal disease, which suggests that the disease can progress silently and that albuminuria may no longer be a reliable biomarker [6]. In addition, chronic hyperglycemia is associated with excessive inflammation, which accelerates insulin resistance (IR), a hallmark that contributes to renal injury [7]. Indeed, IR is present in non-diabetic patients already in the early stages of chronic kidney disease (CKD), being more prevalent as the disease progresses [8,9]. IR is a pathophysiologic state where there is a decline in the response to the normal actions of the hormone insulin, leading to high levels of blood glucose [8]. Several clinical trials have shown that IR can be considered as an independent risk factor in the development of CKD [10–12]. However, the exact mechanism by which IR affects kidney disease, even in non-diabetic patients, remains poorly understood [13]. Elevated circulating free fatty acids (FFA) and triglycerides are typical symptoms of dyslipidemia in diabetic patients. Indeed, one of the main causes of IR, including in the kidney, is lipotoxicity caused by high FFA levels [14,15]. FFA and their metabolites interfere with insulin signaling by inhibiting glucose uptake and glycogen synthesis [16]. Palmitate is the most abundant saturated fatty acid present in human serum and elevated levels in the bloodstream have been described to promote lipid accumulation, IR, oxidative stress as well as cell death [17]. Nonetheless, its impact in DN is still not well comprehended, especially in combination with IR. Studies investigating mitochondrial bioenergetics and its association with the progression of DN are scarce. To our knowledge, the ones that exist only explore the differences between physiological and diabetic states [18,19]. Herein, we explore the impact of mild to severe hyperglycemia as well as IR under normo and different hyperglycemic *in vitro* conditions in renal epithelial cells' metabolic and mitochondrial bioenergetics profiles.

2. Materials and methods

2.1. Cell line culture

Human kidney cell line 2 (HK-2), an immortalized proximal tubule epithelial cell line from the adult human kidney, was purchased by ATCC (Manassas, VA, USA), and was cultured in DMEM:F12 medium (1:1, pH = 7.4 with a final concentration of glucose of 7.7 mM) (Sigma -Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS, Sigma), 50 mg/mL gentamicin, 1% Penicillin-Streptomycin (Pen-Strep), 1% amphotericin B, 15 mM HEPES and 14 mM $NaHCO_3$. The cells were incubated at 37 °C with a 5% (v/v) CO_2 humified atmosphere.

2.2. Experimental groups

HK-2 cells were grown until reaching a confluence of 80–90%. Then, the culture medium was replaced by DMEM:F12 medium with Insulin-Transferrin-Selenium supplement (ITS, in mg/mL: insulin 10, transferrin 5.5 and selenium 0.0067), pH 7.4. To assess the effects of glucose and IR, cells were treated for 24 h with varying concentrations of glucose (5 mM, 11 mM, and 22 mM) to mimic physiological, mild, and severe hyperglycemic conditions, respectively. In addition to these 3 experimental groups, we also evaluated the effect of different glucose concentrations (in mM: 5, 11 and 22) combined with IR. To induce IR, 250 μM of palmitic acid (PA) was used as described by Ge and collaborators [20]. The preparation of palmitate-BSA conjugated was adapted from Qin and collaborators [21]. After 24 h, extracellular media was collected for further analysis by proton nuclear magnetic resonance (^{1}H NMR) and the cells were detached with trypsin-ethylenediamine tetraacetic acid (EDTA) solution. After detachment, the cells were counted with a Neubauer chamber and stored at −20 °C for protein, RNA and mitochondria isolation. Moreover, to assess insulin sensitivity and stimulation, we conducted two additional groups in our study. The first group was treated with 5 mM of glucose, while the second group was treated with 5 mM of glucose along with PA. Both groups were subjected to a 100 nM insulin stimulation for a duration of 3 h. Subsequently, the cells were collected for analysis using the slot blot technique.

2.3. Western blot and slot blot

Cell lysates were homogenized using RIPA buffer (PBS 1×, 1% NP–40, 0.5% Sodium deoxycholate and 0.1% SDS 10%), supplemented with 1% of protease inhibitor mixture (AEBSF, Aprotinin, Bestatin, E−64, Leupeptin and Pepstatin A), 100 mM of sodium orthovanadate and 100 mM of phenylmethylsulphonyl fluoride (PMSF). Then the final supernatants were obtained by centrifugation at 14 000 g for 20 min at 4 °C. Protein concentration was calculated using BCA protein assay kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. Total protein (20 μg) from HK-2 cells was prepared with electrophoresis sample buffer (0.125 M Tris–HCl pH = 6.8; 4% SDS; 20% glycerol, 10% 2-mercaptoethanol), fractioned on a SDS-PAGE system with TGX™ Stain- Free Acrylamide gel (Bio-Rad), 90 V gel for 90 min, at room temperature and transferred to polyvinylidene difluoride membranes in a Trans-Blot® Turbo™ transfer System (Bio-Rad; 1.3 A, up to 25 V, 10 min). Total protein was measured using fluorescence, which eliminates the need for a housekeeping protein control that is often found to be erroneous used in the literature, as explained by Maloy and colleagues [22].The membranes were blocked in a Tris-buffered saline solution with 0.05% Tween 20 (TBS-T) containing 5% nonfat dry milk. Thereafter, membranes were incubated with the primary antibodies, anti-*p*-IRS1(Ser1001) (#2385, Cell Signalling, dilution 1:2500) and anti-PI3 kinase, p85 (#06–195, Millipore, dilution 1:2500), diluted in TBS-T containing 1% nonfat dry milk, overnight, at 4 °C. Afterward, membranes were incubated with the respective peroxidase-conjugated secondary antibody (anti-rabbit, A3687, Sigma, 1:5000), for 90 min, at room temperature. The immunoreactive bands were observed using a WesternBright™ ECL Chemiluminescent HRP Substrate (Bio-RAD) and then exposed to ChemiDoc™ MP Imaging system (Bio-Rad, California, USA). Each band was quantified using BIO-PROFIL Bio-1D Software from Quantity One (VilberLourmat, Marne-la-Vallée, France). The value of band density was normalized with the value of total protein and expressed as fold variation to the control group (5 mM). To evaluate insulin sensitivity/stimulation, slot blot technique was performed. The serine residues of p-IRS1 were evaluated as described by Oliveira PF and collaborators [23].

2.4. RNA extraction, cDNA synthesis and quantitative real-time PCR (qPCR)

The total RNA extraction of HK-2 cells was performed using Total RNA isolation kit (NZYTech), following the manufacturer's instructions. Total RNA was reversed transcribed with M-MuLV Reverse Transcriptase (NZYTech), resulting in cDNA synthesis (1 μg/μL) according to the manufacturer's instructions. To analyze Insulin receptor (IRS), mRNA expression was performed by qPCR assay. In brief, to each sample containing 0.07 μg of cDNA, was added 0.64 μL of forward and reverse primer (10 μM), and 8.0 μL of Speedy NZYtaq 2× Green Master Mix (NZYthech). The volume was adjusted with RNase-free water until 16 μL. The protocol for qPCR was the following: 2 min at 95 °C for polymerase activation, 5 s at 95 °C for DNA denaturation and, 30 s at 72 °C for the extension. The melting curve was determined by an additional step: 1 min at 95 °C, 30 s at 55 °C, and 30 s at 95 °C. qPCR reactions were carried in duplicate, and the optical density was assessed by a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The primer sequences, the annealing temperatures, and the number of cycles required for the exponential phase of amplification in qPCR are listed in Table 1.

All conditions were optimized for each set of primers. The quantitative analyses were calculated according to the $2^{-\Delta\Delta Ct}$ expression formula [24].

2.5. Cell viability using LDH and MTT assays

HK-2 cells were cultured in a 48-well plate at a density of 4×10^4 cells per well and incubated overnight. After that, cells were treated with different glucose concentrations (in mM: 5, 11 and 22) with or without PA for 24 h. Then, LDH release was determined by measuring the extracellular activity using a commercial assay kit following the manufacturer's instructions (LDH Citox Assay Kit, Biolegend, San Diego, CA, USA). In brief, 100 μL of extracellular media were collected into a 96 well plate, to which were added 100 μL of working solution. LDH assay substrate was added to all samples in a dark environment and this mixture was incubated at 37 °C for 15 min. After that time, a 50 μL stop solution was added to stop the enzymatic activity and absorbance was read at 490 nm using the BioTek Synergy HT (BioTek, Winooski, VT, USA) microplate reader. Subsequently, the metabolic toxicity of PA was assessed through the MTT colorimetric assay [25]. Cells were washed with PBS, and incubated with 3-(4,5-dimethylthiazole-2-yl)-2, 5-biphenyl tetrazolium bromide (MTT, Merk Millipore, Burlington, MA, EUA), in a final concentration of 5 mg/mL, for 2 h, in a dark environment. Then, the medium was removed, and it was added DMSO to each well, to dissolve the formazan crystals formed inside the cells and 100 μL of each well was transferred to a 96-well plate culture and a blank was made with DMSO. The absorbance was read at the wavelengths of 570 nm and 650 nm using the BioTek Synergy HT (BioTek, Winooski, VT, USA) microplate reader. Metabolic viability was calculated by the difference between 570 nm and 650 nm.

2.6. Nuclear magnetic resonance (NMR) spectroscopy

^{1}H NMR spectra of the extracellular media collected were acquired using a Brucker Avance 600 MHz spectrometer equipped with a 5 mm QXI probe with a z-gradient (Bruker Biospin, Karlshruhe, Germany), at 14.1 T and 25 °C, using standard methods [26]. Sodium fumarate was used as internal reference (singlet, 6.50 ppm.), in a final concentration of 2 mM, to quantify the following metabolites present in solution (chemical shift: ppm): lactate (quartet, 4.10), alanine (doublet, 1.45), glutamate (multiplet, 2.34), isoleucine (dublet, 0.93), pyruvate (singlet, 2.35), glutamine (multiplet, 2.44) and H1-α glucose (doublet, 5.22) were determined when present in medium. The spectra were manually phased, and baseline corrected. NUTS-ProTM NMR software (Acorn NMR, Inc, Fremont, CA, USA) was used to integrate the chosen metabolite peaks. Metabolite consumption or release (production) was determined for each condition by the difference between pre and post treatment. The concentration of the metabolites is expressed as μmol/10^6 cells.

2.7. Mitochondrial respiration

Mitochondrial oxygen consumption (OCR) of cultured HK-2 cells, was measured using Seahorse XFe24 Analyzer (Agilent Technologies), following the manufacturer's instructions. Two days before the analysis, 6×10^4 cells were seeded in each well of the 24-well culture plate. On the next day, the cells were treated with conditions of the different experimental groups. On the day of the experiment, the injections ports were loaded. For standard mitochondrial oxygen consumption assessment, it was used Mito Stress Test Kit, and the injections ports were filled with the following compounds: oligomycin (an inhibitor of oxidative phosphorylation), fluorocarbonylcyanide phenylhydrazone (FCCP, a mitochondrial uncoupler), and rotenone/antimycin A (inhibitors of complex I and III, respectively). We used sequential injections of oligomycin (1.5 μM), FCCP (1 μM) and Rotenone/antimycin A (0.5 μM). Concerning cell plate, on the day of the experiment, cells were washed with PBS and the medium culture was replaced with assay medium, supplemented with Pyruvate (10 mM) and Glutamine (20 mM) (DMEM medium, pH = 7.4, Agilent). To have access to long chain fatty acid oxidation, we used Etomoxir to inhibit this pathway. We further used Mito Stress Test Kit, and sequential injections of Etomoxir (4 μM), injections of oligomycin (1.5 μM), FCCP (1 μM) and rotenone/antimycin A (0.5 μM). This assay was performed under conditions of saturating palmitate and L-carnitine by adding them to each well at the time of reading, avoiding the end of substrate. Between each injection, three measurements of each OCR were recorded. All recordings were 8 min apart and the running time was nearly 100–120 min. Test analysis provided measurements of basal respiration, ATP production, maximal respiration, spare or reserve capacity, proton leak and non-mitochondrial respiration. OCR values were normalized to protein concentrations, using a standard BCA assay.

Table 1
Primer sequences and qPCR conditions used to assess mRNA abundance in human kidney cells. Human Insulin Receptor (IRS) and Beta- 2 microglobulin (hβ2M). hβ2M was used as a housekeeping gene. Ann. T(°C): Annealing temperature. C: Number of cycles.

Gene (Accession number)	Sequence (5′-3′)	Ann. T (°C)	C
hIRS (NM_001185098.1)	FWD: CTGCATCAGAAGAGGCCATCA RVS: CCCCACATGCTTCACGAGC	63	30
hβ2m (NM_004048.4)	FWD: AGATGAGTATGCCTGCCGTG RVS: TCATCCAATCCAAATGCGGC	58	35

2.8. Mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was evaluated using the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; T3168, Invitrogen™, Carlsbad, CA, USA). To sum up, 1.5×10^4 cells were seeded in a 96-well plate culture, with a black plate and clear bottom (Corning Incorporated costar®). When a 60–70% cell confluence was reached, cells were treated with different concentrations of glucose and/or with PA for 24 h. After treatment, cells were washed with PBS and incubated in culture medium with JC-1 (2 μM), at 37 °C for 30 min, in a dark environment. The media with JC-1 was then replaced by fresh media without JC-1. As a positive control, cells were incubated with 20% DMSO to decrease the MMP. The fluorescence of the JC-1 monomers (ex 485/530 nm; excitation/emission) and JC-1 aggregates (535/590 nm; excitation/emission) were assessed by a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). The ratio between JC-1 aggregates/monomers was calculated and used as an MMP marker and the results were expressed as fold variation to the control group (5 mM). JC-1 aggregates/monomers fluorescence was observed in a Nikon Eclipse Ci-plus microscope equipped with DS-Fi3 microscope camera, a CI-FL-2 Epi-Fluorescence Attachment, and pE-300 Lite Multi Band Power Supply (Nikon, Shinagawa, Tokyo, Japan). Nuclei were stained with Hoechst 33 342 (H3570, Thermo Scientific, Waltham, MA, USA). Pictures were captured with a 200 × magnification and a DAPI filter (395/425 nm; excitation/emission) was used for observing the nuclei, FITC filter (480/510 nm; excitation/emission) for JC-1 monomer, and G-2A filter (510/575 nm; excitation/emission) for JC-1 aggregates. All pictures were captured with the same exposition time and gain. The pictures obtained were processed using the NIS-Elements Br 4.60.00 software (Nikon, Shinagawa, Tokyo, Japan).

2.9. Mitochondrial isolation and mitochondrial complexes I and II activity

HK-2 cells were homogenized in ice-cold extraction buffer (in mM: 130 sucrose, 50 kC l, 5 KH_2PO_4, 5 HEPES, pH = 7.4) using a glass-Teflon Potter Elvejhem (Kimble, Millville, NJ), and then centrifuged at 1000 g

for 10 min, at 4 °C. The resulting supernatant was then centrifuged at 12 000 g for 15 min, at 4 °C to obtain a mitochondria-free cytosolic fraction and a mitochondria-enriched fraction, as described by Monteiro-Cardoso and collaborators [27]. The pellet obtained corresponded to the mitochondria-enriched fraction, which was resuspended in a washing buffer (250 mM sucrose, 5 mM HEPES, pH = 7.2), and centrifuged at 12 000 g, for 15 min at 4 °C. The final protein content of this fraction was determined by the BCA method. Activities of the mitochondrial complexes were assessed at 37 °C, in a 96-well plate, according to well-established protocols [28]. Complex I (CI) activity was determined following NADH oxidation. The reaction occurred in a potassium phosphate buffer (25 mM KH_2PO_4, 5 mM $MgCl_2$, pH = 7.5), supplemented with 1 mM KCN, 193.8 μM decylbiquinone,4 μM antimycin A, 10 μM rotenone (or an equal volume of buffer) and the volume corresponding to 5 μg of mitochondrial protein. The reaction was triggered by the addition of NADH (100 μM), and fluorescence at 450 mM (excitation: 366 nm) was measured every 30 s for 15 min. CI activity was determined by the difference between NADH oxidation rate in rotenone-inhibited wells and noninhibited wells (nmol NADH.min^{-1}.mg protein^{-1}). Complex II (CII) activity was evaluated by monitoring the reduction of 2,6-dichlorophenolindo-phenol (DCPIP) at 600 nM (ϵ = 20.7 mM^{-1} cm^{-1}) in a potassium buffer (25 mM KH_2PO_4, pH = 7.5), supplemented with 250 μM DCPIP, 1 mM KCN, 193.8 μM decylbiquinone, 10 μM rotenone, 4 μM antimycin A, 162.5 μM and the volume corresponding to 5 μg of the mitochondrial enriched fraction. The negative control wells were additionally supplemented with 5 mM oxaloacetate (CII inhibitor). The reaction was initiated by adding 2 mM succinate and absorbance was measured every 30 s for 20 min. CII activity was expressed as nanomoles of DCPIP per minute per milligram protein (nmol DCPIP.min^{-1}.mg protein^{-1}). Citrate synthase (CS) activity was assessed spectrophotometrically, by monitoring the reduction of 5,5-dithio-bis-2-nitrobenzoic acid (DTNB). The reaction buffer consisted of 200 mM Tris-HCl buffer (pH = 8.0), 0.02% (m/v) Triton X-100, which was supplemented with 0.4 mM DTNB, 2 mM oxaloacetate, and the corresponding volume of 5 μg of protein. The reaction was triggered by the addition of acetyl-CoA (122 μM), and the absorbance at 412 nm was recorded at 30 s for 10 min. CS activity was calculated by DTNB (ϵ = 13.6 mM^{-1} cm^{-1}) reduction rate, determined in the linear range of the plot and expressed as nanomoles per minute per milligram of protein (nmol.min^{-1}.mg protein^{-1}). The activity of mitochondrial redox chain complexes were normalized with CS activity.

2.10. Oil Red O staining

To assess intracellular lipid accumulation, cells were stained with Oil Red O staining (ORO) [29]. Briefly, 5×10^4 cells were seeded in 24-well plate. After the treatment, cells were fixed with 4% formalin for 1 h at room temperature. Then, after rinsing twice with PBS supplemented with Ca^{2+} and Mg^{2+}, cells were incubated for 5 min with 60% isopropanol. Then, cells were stained with freshly diluted (in a final concentration of 0.02%) ORO working solution at room temperature for 20 min. After that, cells were washed firstly with 40% of isopropanol and then with PBS. For the quantification of lipid accumulation, it was added to the stained samples, 200 μL of 100% isopropanol to each well, and the plates were slowly agitated at room temperature for 10 min to dissolve the ORO staining. Thereafter, 100 μL of the samples of each well were transferred to a 96-well plate, and the absorbance was measured at 510 nm using a BioTek Synergy HT (BioTek, Winooski, VT, USA) microplate reader. The results are expressed as fold variation to the control group (5 mM).

2.11. Statistical analysis

Analysis was performed using GraphPad 8 (GraphPad Software, Inc). The distribution of the data was tested, and groups were compared using two-way ANOVA with Bonferroni's multiple comparisons tests. Statistical significance was considered at $p < 0.05$ and data are presented as mean ± SEM (N = 3 to N = 8; N refers to distinct passages of the HK-2 cell line, where each condition that was performed in cell culture plates were done in triplicates).

3. Results

3.1. Palmitic acid (PA) induces IR and downregulates insulin signaling pathway in HK-2 cells

To investigate the molecular mechanisms underlying palmitic acid (PA)-induced IR in HK-2 cells, we assessed insulin signaling in both non-exposed and PA-exposed cells. Specifically, we first examined the expression of the insulin receptor. As demonstrated in Fig. 1 (Panel A), HK-2 cells of all experimental groups expressed the insulin receptor (INSR). Activation of INSR by insulin typically leads to the recruitment and phosphorylation of insulin receptor substrate proteins (p-IRS), which then activates the PI3K cascade. Our results demonstrated that, upon INSR activation, the levels of p-IRS in HK-2 were lowered in the cells of the groups treated with PA (Fig. 1, Panel B). Between the groups of HK-2 exposed only with glucose, there was a decrease in the p-IRS1 protein expression after these cells were cultured in severe hyperglycemia condition (22 mM; 0.46 ± 0.12, fold variation to control) in comparison to the mild hyperglycemia condition (11 mM; 1.59 ± 0.34, fold variation to control). Moreover, we observed a similar decrease in p-IRS1 levels in cells treated with 11 mM glucose along with PA treatment (0.51 ± 0.15, fold variation to control) and in those exposed to glucose in physiological conditions (5 mM) with PA (0.34 ± 0.01, fold variation to control). PI3K levels were also affected in the cells of the groups treated with PA (Fig. 1, Panel C). However, this decrease was more pronounced when the cells were treated with severe hyperglycemic conditions and PA (0.58 ± 0.07, fold variation to control) compared with those from the groups treated with increasing concentrations of glucose, although with no statistical differences.

3.2. PA-induced IR in HK-2 cells induces an increase in LDH release and a decrease in the cells metabolic viability

To evaluate the effect of PA-induced IR on cell membrane integrity in HK-2 cells, we performed the LDH assay. When the cells were cultured with PA, all glucose concentrations tested induced an increase in LDH release in comparison to the control group (1.00 ± 0.11, fold variation to the control) (Fig. 2, Panel A). Furthermore regarding the experimental groups where the cells were cultured with PA, the highest concentration tested of glucose led to a decrease in LDH release (2.53 ± 0.19, fold variation to the control) in comparison to the lowest concentration of glucose tested (3.00 ± 0.04, fold variation to the control). In addition, in PA-induced IR condition, when the cells were exposed to 11 mM of glucose there was an increase in LDH release (3.79 ± 0.33, fold variation to control) in comparison to the cells only treated with 11 mM of glucose (1.07 ± 0.03, fold variation to the control group). The same results were obtained when we compared the cells treated with 22 mM of glucose and PA (2.54 ± 0.20, fold variation to control) and with only 22 mM of glucose (1.37 ± 0.18, fold variation to the control). When we assessed the metabolic viability, by using the MTT assay (Fig. 2, Panel B), all the concentrations of glucose tested in combination with PA had a decrease in metabolic viability in comparison to the control group (1.00 ± 0.09, fold variation to the control). Furthermore, when the cells were exposed to a mild hyperglycemia condition and PA (11 mM) there was a significant decrease of the metabolic viability (0.33 ± 0.01, fold variation to the control) in comparison to that of cells treated only with 5 mM of glucose, 11 mM of glucose (0.73 ± 0.05, fold variation to the control) and 22 mM of glucose (0.82 ± 0.04, fold variation to the control). The same results were obtained when the cells were treated with 5 mM of glucose with PA, which also presented a decrease in metabolic viability (0.48 ± 0.05, fold variation to the control). Furthermore, the group

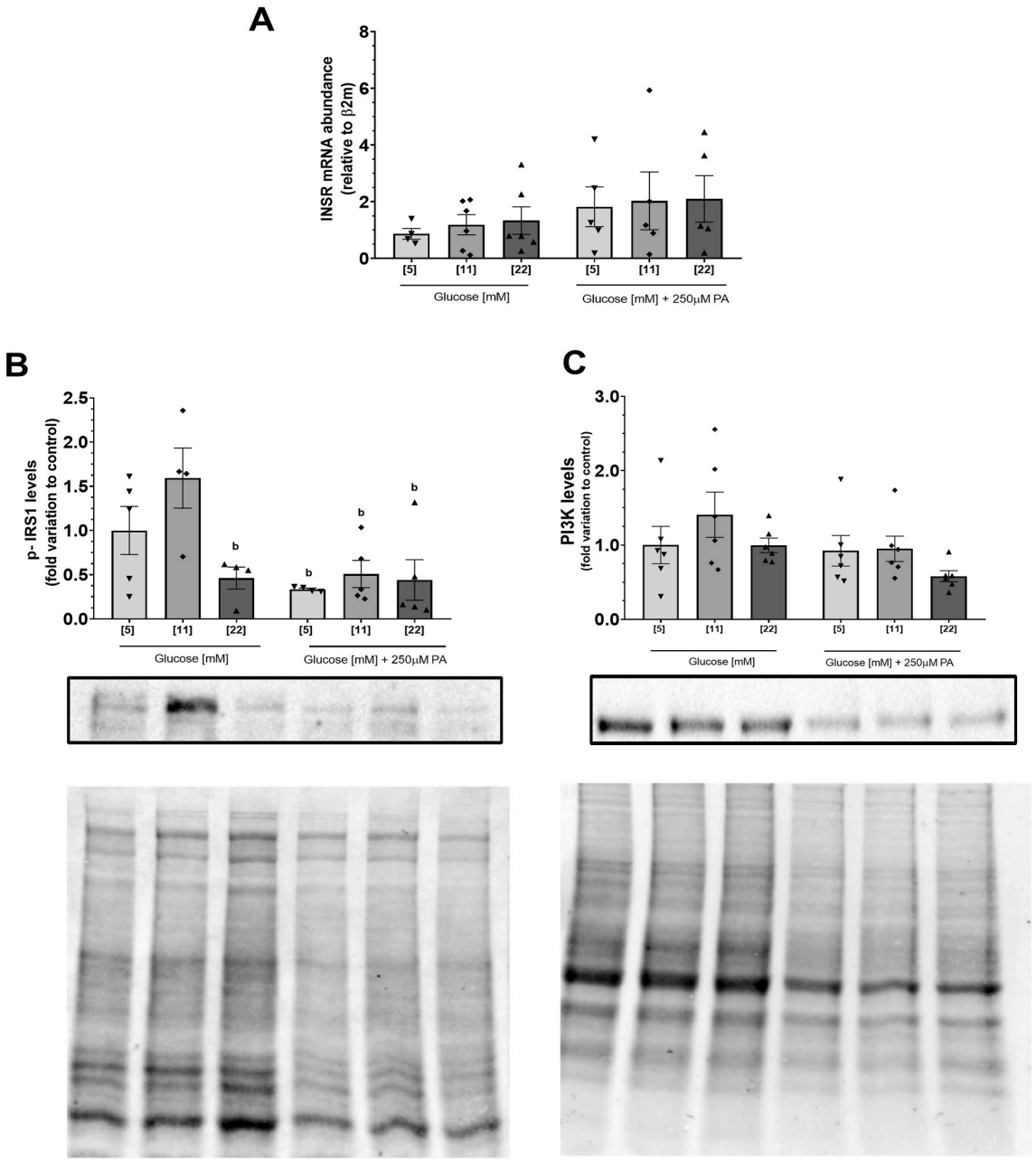

Fig. 1. Insulin pathway inhibition in human kidney cells by exposure to palmitic acid (PA). Quantification of mRNA abundance of Insulin Receptor (INSR) (Panel A), protein levels of p-IRS1 (Panel B) and PI3K (Panel C) and total protein in human kidney cells (HK-2), with and without treatment with PA. Results were tested by two-way ANOVA with Bonferroni's multicomparison test. The results are expressed as mean ± standard error of the mean (SEM) (N = 6, in triplicates each experimental group). Significantly different results ($P < 0.05$) are indicated as: b-relative to 11 mM. Fold variation to the control represented as 5 mM of Glucose.

treated with 22 mM of glucose and PA (0.56 ± 0.03, fold variation to the control) presented a decrease in metabolic viability compared with the control group (5 mM of glucose) and with the cells treated with only 22 mM of glucose.

3.3. The combination of PA-induced IR with mild to severe hyperglycemia alters the metabolic profile of HK-2 cells

In HK-2 cells, glucose consumption was increased when the cells were treated with increasing concentrations of this hexose and when IR was induced by PA (Fig. 3, Panel A). Concerning only the treatment with glucose, cells in mild hyperglycemia demonstrated an increase (32.51 ± 3.18 μmol/10^6 cells) in glucose consumption in comparison to those cultured under normoglycemia (18.07 ± 2.05 μmol/10^6 cells). In addition, this increase was also observed in PA-induced IR conditions, in cells treated with mild (43.89 ± 5.04 μmol/10^6 cells) to severe hyperglycemic conditions (44.12 ± 3.79 μmol/10^6 cells). Furthermore, the higher increase was observed between matched groups of glucose (22 mM), where the highest glucose consumption was observed when the cells were cultured with PA (44.12 ± 3.79 μmol/10^6 cells vs. 29.31 ± 5.47 μmol/10^6 cells).

Kidney cells utilize glutamine as an important source to meet their metabolic demands, and this amino acid also plays a crucial role in maintaining acid-base homeostasis. Among all our experimental conditions, glutamine consumption in these cells (Fig. 3, Panel B) was higher when the cells were treated with 11 mM of glucose, with PA-induced IR, followed by the cells treated with the same concentration of glucose but without PA (8.62 ± 1.56 μmol/10^6 cells and 5.44 ± 0.89

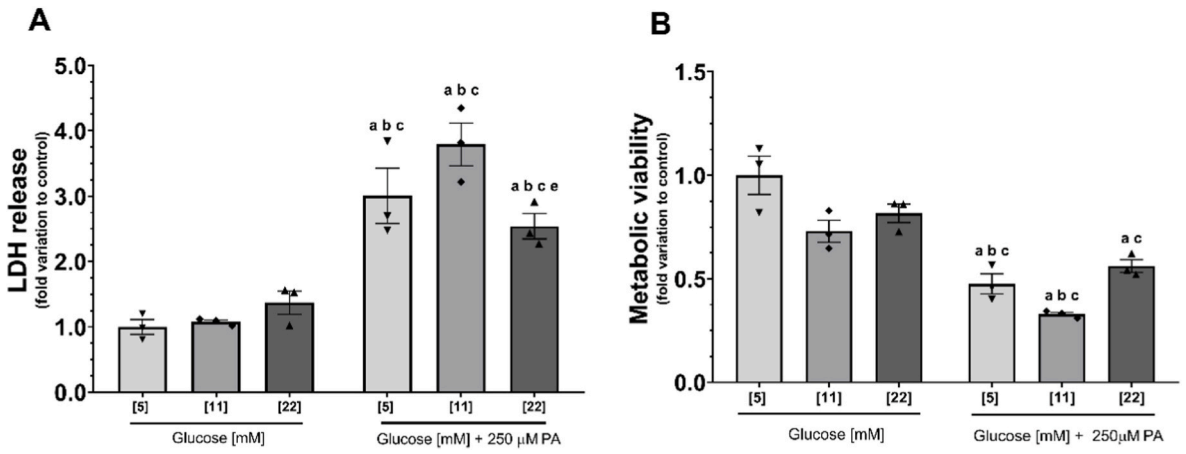

Fig. 2. Evaluation of Lactate Dehydrogenase (LDH) release (Panel A), and metabolic viability (Panel B) of HK-2 treated with different glucose concentrations (5 mM, 11 mM and 22 mM) without and in the presence of palmitic acid (PA). Results were tested by two-way ANOVA with Bonferroni's multicomparison test. The results are expressed as mean ± standard error of the mean (SEM) (N = 3, in triplicates each experimental group). Significantly different results ($P < 0.05$) are indicated as: a-relative to 5 mM Glucose, b-relative to 11 mM Glucose, c-relative to 22 mM Glucose and e−relative to 11 mM + PA. Fold variation to the control represented as 5 mM of glucose.

μmol/10^6 cells, respectively), particularly in comparison to that of cells under normoglycemia, with or without PA-induced IR (3.41 ± 0.77 μmol/10^6 cells and 1.96 ± 0.33 μmol/10^6 cells, respectively). The cells also presented an increased consumption of glutamine when cultured also under severe hyperglycemic condition after PA-induced IR (5.89 ± 0.61 μmol/10^6 cells).

Similar results were obtained when we evaluated pyruvate consumption by HK-2 cells (Fig. 3, Panel C). The highest consumption of pyruvate was observed in cells cultured in mild hyperglycemia with or without PA-induced IR (2.09 ± 0.40 μmol/10^6 cells and 1.80 ± 0.32 μmol/10^6 cells, respectively), particularly in comparison to the normoglycemia with or without PA-induced IR (0.35 ± 0.04 μmol/10^6 cells and 0.31 ± 0.03 μmol/10^6 cells, respectively). Regarding the groups treated only with different concentrations of glucose, a decrease in pyruvate consumption was observed when the cells were cultured under severe hyperglycemia (0.90 ± 0.18 μmol/10^6 cells) in comparison to the mild hyperglycemic condition (1.80 ± 0.33 μmol/10^6 cells). Furthermore, comparing only the PA-induced IR groups, the severe hyperglycemic condition caused an increase in pyruvate consumption (1.15 ± 0.23 μmol/10^6 cells) in comparison to the cells under mild hyperglycemic condition.

Moreover, isoleucine consumption was also altered in our experimental design (Fig. 3, Panel D). The highest consumption of isoleucine was observed when the cells were treated with 11 mM of glucose, without and with PA-induced IR (1.47 ± 0.40 μmol/10^6 cells and 1.57 ± 0.30 μmol/10^6 cells, respectively).

Lactate is considered a major precursor of gluconeogenesis including in the kidney. We observed increased production of lactate (Fig. 3, Panel E) when the cells were treated with increasing concentrations of glucose (11 mM and 22 mM) and even higher after PA-induced IR, however with no statistical significance among the groups.

Alanine is also an amino acid that contributes to gluconeogenesis. Its production was increased in a hyperglycemic condition plus PA (2.51 ± 0.26 μmol/10^6 cells) in comparison to the normoglycemic and mild hyperglycemic condition without PA (1.59 ± 0.09 μmol/10^6 cells, 1.54 ± 0.18 μmol/10^6 cells, respectively) (Fig. 3, Panel F). Glutamate production was also affected after PA-induced IR (Fig. 3, Panel G). In fact, this effect was noticed in the cells cultured under physiological condition with PA-induced IR (3.62 ± 0.53 μmol/10^6 cells), when compared to cells without PA-induced IR (0.71 ± 0.11 μmol/10^6 cells), and this increase was more noticed in the higher concentrations of glucose (11 mM: 0.96 ± 0.12 μmol/10^6 cells; 11 mM with PA-induced IR: 3.41 ± 0.62 μmol/10^6 cells; 22 mM: 1.89 ± 0.26 μmol/10^6 cells; 22 mM with PA-induced IR: 3.88 ± 0.52 μmol/10^6 cells).

3.4. Mitochondrial performance of HK-2 cells is affected by PA-induced IR in a glucose-dependent manner

The data above demonstrated that PA was able to affect HK-2 cells metabolic pathways to obtain energy. Therefore, we examined whether this condition could affect the cellular energy production by mitochondria. To examine oxygen consumption rates (OCR) and bioenergetic parameters in all experimental groups, we used the Seahorse XFe24 analyzer in a live cellular environment (Fig. 4). OCR including basal respiration, ATP production, maximal respiration, spare reserve capacity, proton leak and non-mitochondrial respiration were evaluated using specific modulators. HK-2 cells showed decreased basal respiration after PA-induced IR in a glucose-dependent manner (Fig. 4, Panel A). Concerning the groups only treated with glucose, the cells cultured in 11 mM of glucose (1.38 ± 0.15 μmol/min/μg protein) had higher basal respiration in comparison to those cultured in 5 mM (0.82 ± 0.06 μmol/min/μg protein). Comparing the groups treated with 22 mM of glucose, the minimal rate of basal respiration was observed when the cells were treated with PA, (0.63 ± 0.08 μmol/min/μg protein vs. 1.08 ± 0.14 μmol/min/μg protein). Following the injection of oligomycin, the electron flow through electron transport chain (ETC) decreases due to inhibition of ATP synthase (Fig. 4, Panel B). The ATP production by HK-2 cells associated to respiration was significantly higher in the groups treated with 11 mM (0.80 ± 0.11 μmol/min/μg protein) and 22 mM (0.75 ± 0.07 μmol/min/μg protein) of glucose in comparison to the normoglycemia group (5 mM; 0.41 ± 0.06 μmol/min/μg protein). Moreover, there was also an increase between glucose-matched groups in ATP production when the cells were treated with 5 mM of glucose with PA-induced IR (0.75 ± 0.12 μmol/min/μg protein). It was also observed a decrease in ATP production parameter when cells were treated with 22 mM of glucose and PA in comparison to the matched group, however without statistical significance ($p = 0.074$). Injection of FCCP, a mitochondrial uncoupler, caused an increase in OCR and allowed the measurement of the maximal mitochondrial respiration (Fig. 4, Panel C) and spare respiratory capacity (Fig. 4, Panel D). The maximal respiration was higher in the cells treated with only 11 mM of glucose (3.00 ± 0.67 μmol/min/μg protein) than in the cells cultured with only 5 mM of glucose (1.78 ± 0.17 μmol/min/μg protein). Additionally, in PA-induced IR condition, there was a decrease in the mild hyperglycemia condition (1.79 ± 0.12 μmol/min/μg protein). The lowest value on maximal respiration OCR was observed when the cells

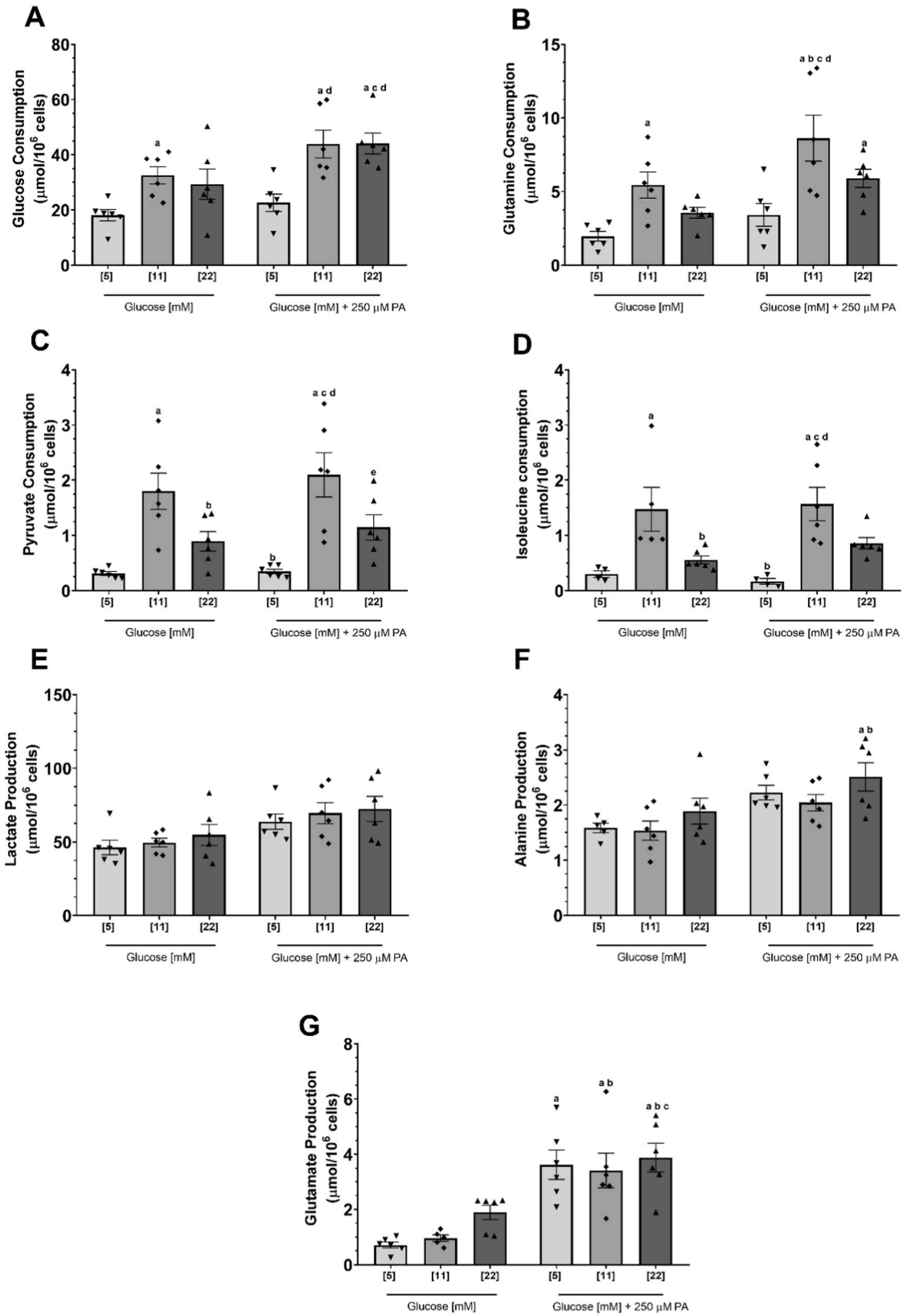

Fig. 3. Evaluation of the consumption and production of metabolites by HK-2 cells treated with different glucose concentrations (5 mM, 11 mM and 22 mM) without or with palmitic acid (PA)-induced IR. Evaluation of glucose (Panel A), glutamine (Panel B), pyruvate (Panel C), and isoleucine (panel D) consumption after 24 h of treatment. Quantification of lactate (Panel E), alanine (Panel F) and glutamate (Panel G) production. Results were tested by two-way ANOVA with Bonferroni's multicomparison test. The results are expressed as mean ± standard error of the mean (SEM) (N = 6, each N corresponds to the medium collected of one Petri dish per each experimental condition). Significantly different results ($P < 0.05$) are indicated as: a-relative to 5 mM Glucose, b-relative to 11 mM, c-relative to 22 mM Glucose, d-relative to 5 mM + PA and e−relative to 11 mM + PA.

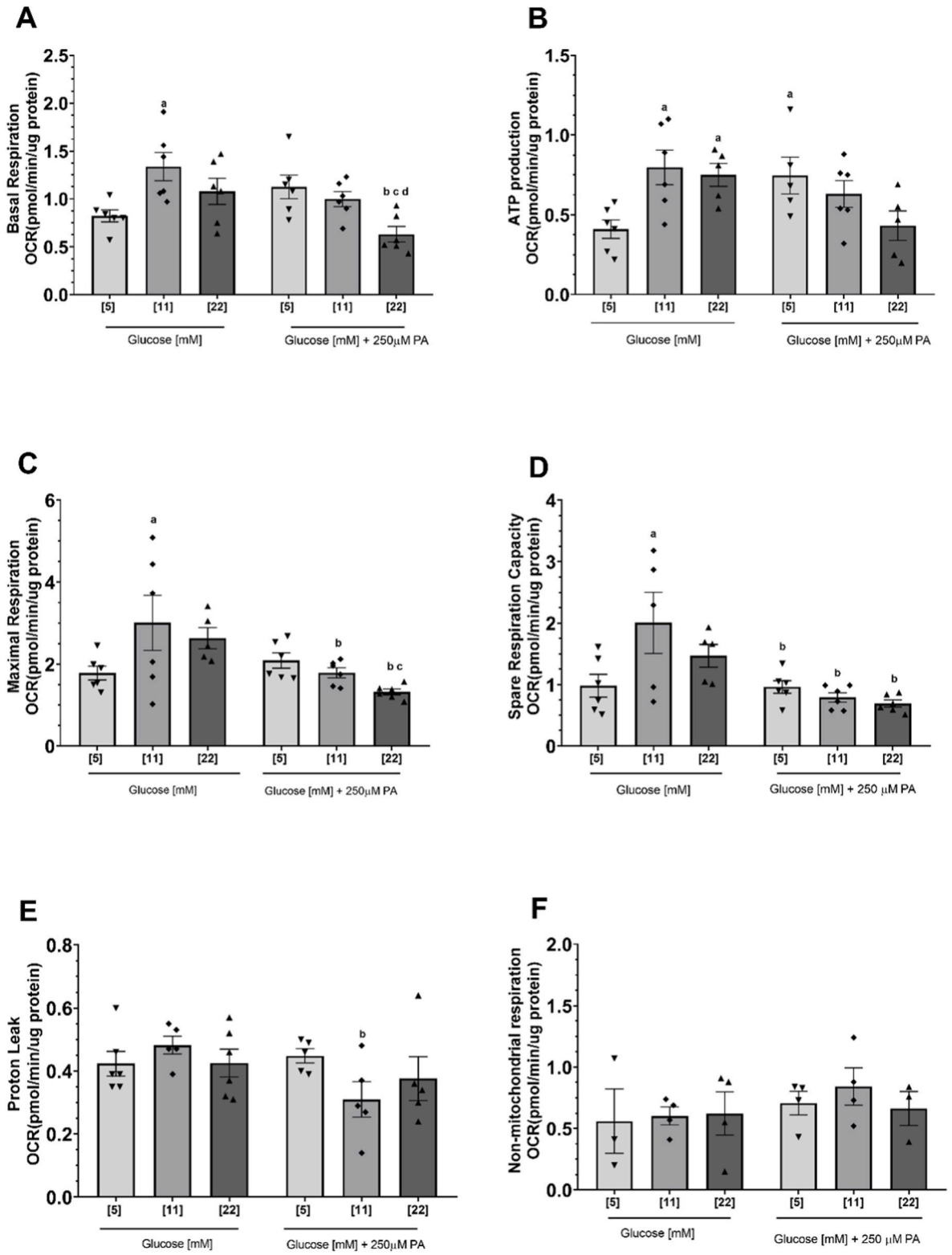

Fig. 4. Mitochondrial profile of HK-2 cells treated with different concentrations of glucose and/or IR induced with palmitic acid (PA). Oxygen consumption rates are reported in Basal Respiration (Panel A), ATP production (Panel B), Maximal Respiration (Panel C), Spare Respiration Capacity (Panel D), Proton Leak (Panel E) and non-mitochondrial respiration (Panel F). Results were tested by two-way ANOVA with Bonferroni's multicomparison test. The results are expressed as mean ± standard error of the mean (SEM) (N = 6, in triplicates each experimental group). Significantly different results ($P < 0.05$) are indicated as: a-relative to 5 mM Glucose, b-relative to 11 mM, c-relative to 22 mM Glucose and d-relative to 5 mM + PA.

were treated with 22 mM of glucose plus PA (1.32 ± 0.07 μmol/min/μg protein), when compared with the matched group and with the group where the cells were treated with 11 mM of glucose and PA. The same tendency was observed in the spare respiration capacity (Fig. 4, Panel D), since the cells treated with 11 mM of glucose (2.00 ± 0.50 μmol/min/μg protein) demonstrated an increase in comparison to the cells cultured with only 5 mM of glucose (0.98 ± 0.18 μmol/min/μg protein), but this parameter decreased when compared to the matched group of glucose treatment (0.79 ± 0.08 μmol/min/μg protein). The lowest value was observed when PA-induced IR was present in the concentration 22 mM of glucose (0.68 ± 0.06 μmol/min/μg protein) when compared to the cells treated only with 22 mM of glucose (1.47 ± 0.19 μmol/min/μg protein; p = 0.052). We also measured the energy dissipation as proton leak (Fig. 4, Panel E) and we found that only cells from the groups where IR was induced presented a decrease in proton leak. More specifically, we observed a significant decrease in the cells cultured with a concentration of 11 mM of glucose (0.31 ± 0.06 μmol/min/μg protein) when compared to the those from the group treated with the same concentration of glucose but without PA-induced IR (0.48 ± 0.03 μmol/min/μg protein). No alterations were observed in non-mitochondrial respiration (Fig. 4, Panel F), except for the cells grown under the concentration of 11 mM of glucose plus PA (1.34 ± 0.51 μmol/min/μg protein), that presented a slight increase in comparison to the cells grown only with 5 mM of glucose (1.17 ± 0.63 μmol/min/μg protein), although with no statistical significance.

3.5. PA-induced IR in HK-2 cells reduces mitochondrial membrane potencial and disrupts complex I activity

As previously described, PA-induced IR was able to reduce the mitochondria functional parameters. Thus, we evaluated the possible mechanism that could mediate those alterations. Mitochondria

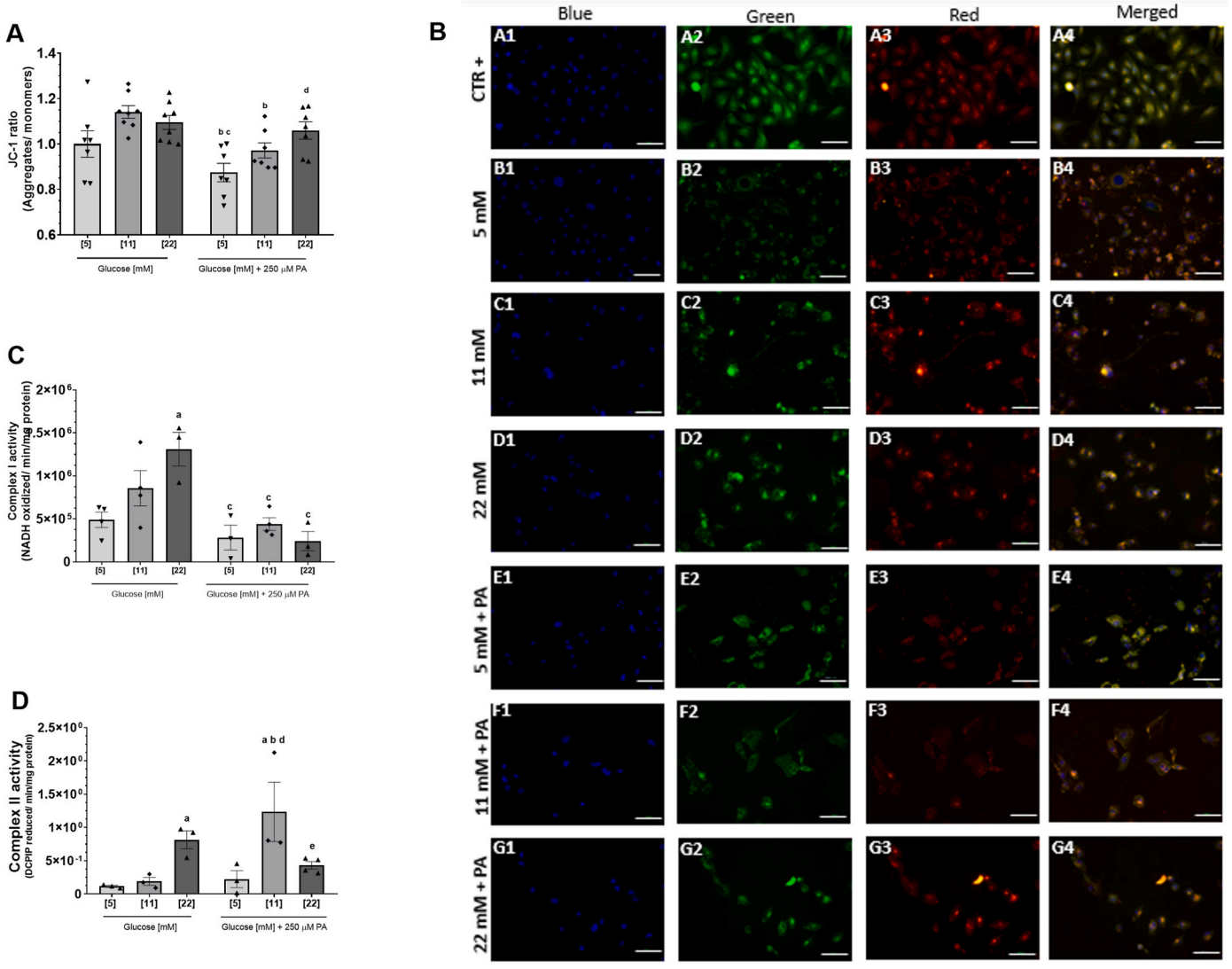

Fig. 5. Effect of increasing concentration of glucose and/or IR in HK-2 cells in mitochondrial membrane potential, through JC-1 assay (Panel A). Analysis of JC-1 J-aggregates (red) or J-monomers (green) in HK-2 cells, by fluorescence microscopy (Panel B). In A1, B1, C1, D1, E1, F1 and G1 are represented nuclei (DAPI filter), in A2, B2, C2, D2, E2, F2 and G2 are represented monomers (green, FITC filter), in A3, B3, C3, D3, E3, F3, G3 and H3 are represented JC-1 J-aggregates (red, G-2A filter) and in A4, B4, C4, D4, E4, G4 and H4 are represented the overlap between JC-1 aggregates and monomers, with a scale bar of 100 μm. Panel C represents the activity of the complex I and panel D represents the complex II of the electron transport chain in mitochondria. Results were tested by two-way ANOVA with Bonferroni's multicomparison test and expressed as mean ± standard error of the mean (SEM) (N = 8 for JC-1 assay, and N = 3 for mitochondrial complexes, in triplicates for each experimental group). Significantly different results (P < 0.05) are indicated as: a-relative to 5 mM Glucose, b-relative to 11 mM and c-relative to 22 mM Glucose, d-relative to 5 mM + PA and e−relative to 11 mM + PA. Abbreviations: CTR + - positive control of the experiment of JC-1 (20% of DMSO).

Membrane potential (MMP) is an indicator of mitochondrial functionality and can be used as a measure of ETC activity. Our results demonstrated that, when IR was induced by PA, there was a decrease in the MMP of HK-2 cells (Fig. 5, Panel A and Panel B), particularly in the physiological glycemic concentration and mild hyperglycemic condition (0.87 ± 0.04, fold variation to the control and 0.97 ± 0.03, fold variation to the control, respectively). Additionally, the cells treated with 11 mM of glucose plus PA have a decreased MMP in comparison to the cells treated with only 11 mM of glucose (1.14 ± 0.03, fold variation to the control). Furthermore, still in PA-treated groups, the hyperglycemic condition demonstrated an increase of MMP (1.06 ± 0.04, fold variation to the control) when compared to the cells treated with 5 mM of glucose (0.87 ± 0.04, fold variation to the control). The complexes of ETC maintain the MMP, which is essential for ATP production. Our results demonstrated that PA-induced IR allied to severe hyperglycemia had an impact on the activity of specific complexes of ETC (Fig. 5, Panel C and D). The activity of CI in HK-2 cells after PA-induced IR decreased, particularly in severe hyperglycemia condition ($2.41 \times 10^5 \pm 1.13 \times 10^5$ NADH oxidized/min/mg protein) in comparison to the matched group ($1.31 \times 10^6 \pm 1.96 \times 10^5$ NADH oxidized/min/mg protein). Furthermore it was also observed a decrease in cells treated with normoglycemia and mild hyperglycemia condition ($2.83 \times 10^5 \pm 1.44 \times 10^5$ NADH oxidized/min/mg protein, and $4.40 \times 10^5 \pm 7.35 \times 10^4$ NADH oxidized/min/mg protein, 5 mM + PA and 11 mM + PA respectively). Moreover, regarding the groups treated with different concentrations of glucose, cells treated with 22 mM presented a higher CI activity in comparison to those in physiological condition ($1.31 \times 10^6 \pm 1.44 \times 10^5$ NADH oxidized/min/mg protein (Fig. 5, Panel C). The activity of mitochondrial complex II (CII) (Fig. 5, Panel D), was also evaluated, and similar results were obtained in the groups treated with glucose, where the cells treated in severe hyperglycemia conditions demonstrated an increase in CII activity in comparison to the normoglycemia condition (0.81 ± 0.14 DCPIP reduced/min/mg protein vs. 0.12 ± 0.01 DCPIP reduced/min/mg protein, respectively). Moreover the highest increase of CII activity was observed when cells were cultured in the mild hyperglycemic condition (1.23 ± 0.45 DCPIP reduced/min/mg protein) when compared to those in normoglycemic condition with PA (0.22 ± 0.14 DCPIP reduced/min/mg protein) or without PA, and with the matched group (0.19 ± 0.06 DCPIP reduced/min/mg protein).

3.6. PA-induced IR in HK-2 cells does not promote lipid oxidation but it leads to the accumulation of lipids in the cells under mild to hyperglycemic conditions

Kidney cells can utilize not only conventional amino acids, but also lipids, specifically fatty acids, as a source of energy, particularly in the tubulointerstitial compartment. However, despite their ability to use lipids as a fuel source, these cells can also accumulate them. Our results demonstrated that the cells in PA-induced IR conditions, independently of the concentration of glucose tested, increased lipid accumulation (Fig. 6, Panel A) in comparison to the same concentration without IR induced (5 mM: 2.04 ± 0.25 vs. 1.00 ± 0.32,11 mM: 2.13 ± 0.28 vs. 0.89 ± 0.07 and 22 mM: 1.97 ± 0.25 vs. 0.92 ± 0.11, respectively). Thus, we next intended to investigate in our experimental groups subjected to PA-induced IR, if FFA were being used to obtain energy, through the seahorse Mito Stress kit and Etomoxir (FFA oxidation inhibitor) (Fig. 6, Panel B). Our results demonstrate that FFA are not the preferential substrate to produce ATP when cells are treated with 5 mM of glucose (0.84 ± 0.11 μmol/min/μg protein), in comparison to the same concentration when the pathway is inhibited (2.64 ± 0.63 μmol/min/μg protein) since the production of ATP was higher. No differences were observed in the matched group, when lipid oxidation pathway was inhibited. It was only observed a decrease in mild (1.18 ± 0.53 μmol/min/μg protein) and severe hyperglycemic (0.98 ± 0.16 μmol/min/μg protein) condition, when FFA oxidation was inhibited.

4. Discussion

DN is a significant but under-recognized contributor to the global burden of diabetes [30]. Furthermore, impaired insulin sensitivity is strongly associated with alterations in renal cellular metabolism, which can lead to kidney damage [31]. It is generally accepted that IR is related with CKD or complications of CKD cause IR; however, the development of IR is not restricted to DN or specific kidney disease [32]. Nonetheless, IR is reported as a predictor of DN, since patients without diabetes can present IR and still develop CKD [33]. Thus, the exact impact of IR on kidney disease pathophysiology remains a topic of debate requiring further investigation. Our experiment aimed to uncover the metabolic and mitochondrial mechanisms underlying kidney cell performance under different glucose stimuli, with and without PA-induced IR. In

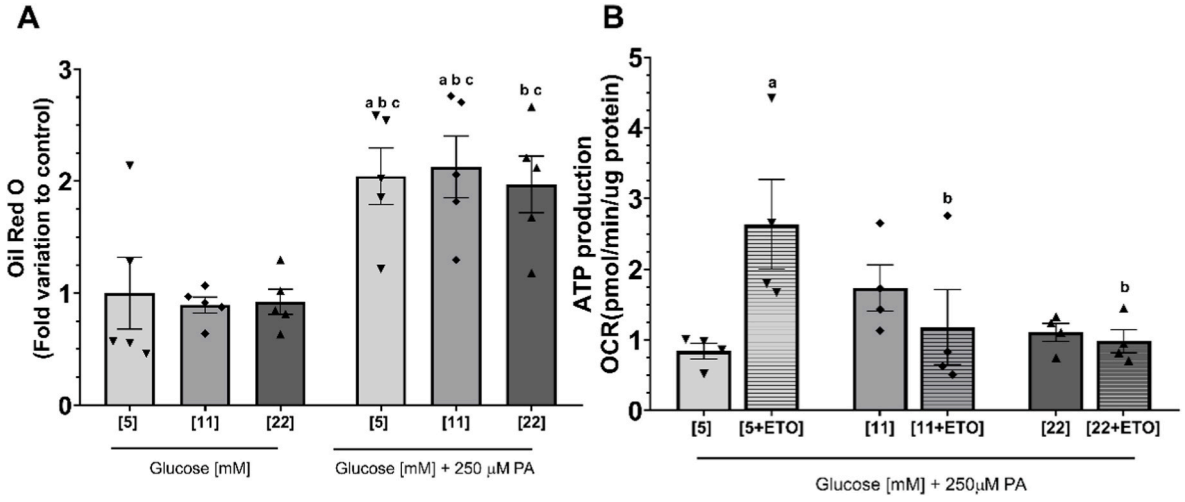

Fig. 6. Lipid accumulation through Oil Red O staining (Panel A), in HK-2 cells with or without PA-induced IR in increasing glucose concentrations (5 mM, 11 mM and 22 mM) , and ATP production in increasing glucose concentrations (5 mM, 11 mM and 22 mM) with IR induced with palmitic acid (PA), with or without free fatty acid (FFA) oxidation inhibition, through Etomoxir (ETO) (Panel B). Results were tested by two-way ANOVA with Bonferroni's multicomparison test and are expressed as mean ± standard error of the mean (SEM) (N = 5 and N = 4, panel A and B respectively, in triplicates each experimental group). Significantly different results ($P < 0.05$) are indicated in Panel A: a-relative to 5 mM glucose, b-relative to 11 mM glucose and c-relative to 22 mM Glucose. Fold variation presented as 5 mM of Glucose. Panel B as: a-relative to 5 mM + PA, b-relative to 5 mM + PA + ETO.

addition, IR may inhibit β-oxidation of fatty acids [34], resulting in the accumulation of free fatty acids, such as palmitic acid, being reported in Diabetes Mellitus or DN. As described by Ge and collaborators [20], PA induced IR through a downregulation on insulin signaling pathway in HK-2 cells. Our findings provide compelling evidence that IR was induced through the downregulation of the active form of insulin substrate (p-IRS1) and subsequent inhibition of downstream signaling, specifically affecting the expression level of PI3K. Furthermore, the impact of palmitate on insulin sensitivity was evident in our supplementary data (see Figure S1 in Supplementary Material 1), where insulin stimulation at basal glucose concentration revealed a reduction in p-IRS1 expression, clearly highlighting palmitate's role in inducing IR. In addition to the aforementioned signaling pathway, it is worth noting that IR has been associated with an increase in the content of branched-chain amino acids [35]. This enhancement is associated with the activation of the mTOR/S6K1 kinase signaling pathway and subsequent phosphorylation of serine on IRS-1 residues, which further cause IR [36]. Our results indicate changes in cell membrane integrity, as observed through LDH release, and metabolic viability, suggesting the induction of lipid toxicity. These findings are highly associated with DN progression and IR. Therefore, the PA-induced IR model used in this study appears to be an excellent approach to achieve our research goals since our results demonstrated an increase of specific amino acids, that are correlated with the downstream of p-IRS1 signaling pathway. Our next goal was to investigate the metabolic and mitochondrial changes that may occur. Previous studies have shown that in diabetes, high glucose levels due to nutrient overload can affect nutrient transport, sensing, and metabolism in proximal tubular cells (PTCs) [37,38]. Our results indicate that under mild to severe hyperglycemic conditions, glucose uptake increases when IR is induced. Previous studies have demonstrated that downregulation of insulin signaling leads to decreased glucose uptake [13]. However, in the kidney, particularly in the proximal tubules (from which HK-2 cells are derived), glucose reabsorption is mediated through glucose transporters that are not sensitive to insulin [39,40]. Furthermore, Mueckler and collaborators demonstrated that in diabetic patients, the expression of these glucose transporters were increased [41]. Interestingly, our results pointed that the uptake of glucose increased in mild glycemic condition and even higher when PA was used, being this effect dissipated in a severe hyperglycemic condition. The kidney is a metabolically complex organ, with PTCs tuned to gluconeogenesis rather than glycolysis [42], where the main renal gluconeogenic substrates are glutamine (20%), alanine (5%), glycerol (10%) or lactate (60%) [43,44]. Renal gluconeogenesis makes a greater contribution during starvation conditions, where it is estimated to be responsible for 40–45% of total glucose production [45]. Our findings show that glutamine consumption is significantly increased under mild hyperglycemia conditions, with an even higher increase observed when IR is induced. Glutamine is one of the most abundant amino acids in the human body, and in the kidney, it is estimated to contribute more to gluconeogenesis than alanine [46]. Our results showed that, in our experimental design, HK-2 cells secrete alanine instead of consuming it, particularly when IR is induced and under the stimuli of the highest glucose concentration. In 1996, Fouque and colleagues concluded that the human kidney-cortex tubule could adapt its metabolism to the available carbon source. Following incubation with increased amounts of alanine, a higher contribution of this amino acid to the production of not only glucose but also lactate and glutamate was observed. These findings suggest that the metabolism of alanine may be adjusted in response to the metabolic needs of the kidney for anabolic and catabolic purposes [47]. In our study, the experimental conditions maintained constant and elevated levels of glucose, which prevented HK-2 cells from metabolizing alanine and instead resulted in its production and export to the extracellular environment. The increased alanine production observed in cells under IR condition may be related to altered consumption of glucose and glutamine. Indeed, it was reported elevated plasma concentrations of alanine in insulin-resistant states [48] suggesting that PTCs might contribute to that effect, leading to the overall exacerbation of the IR status. Glutamate can act as a respiratory substrate in the oxidative deamination direction of glutamate dehydrogenase (GDH), which also generates α-ketoglutarate for the Krebs cycle. In a cross-sectional study, Ogawa and colleagues found that individuals with diabetes had increased urinary excretion of glutamate, while the blood glutamate concentration decreased, and the reabsorption of glutamate declined [49]. They also proposed that the depletion of glutamate in PTCs is compensated by an increase in glutamine levels from the blood, which is consistent with our results in IR conditions, showing that lower levels of glutamate are accompanied by increased glutamine consumption. Furthermore, we observed increased consumption of isoleucine in the presence of mild hyperglycemia, with or without induced IR. A recent follow-up study of diabetic patients with DN found that higher baseline levels of isoleucine were associated with an increased risk of rapid decline in glomerular filtration rate, and was identified as an independent risk factor for development of DN [50]. Our data also suggest that isoleucine may play a role in mild hyperglycemia, independently of IR. Indeed, as DN progresses, there is a noticeable disruption in the metabolism of certain amino acids. Among these, branched-chain amino acids (BCAAs) such as isoleucine play a crucial role in glucose metabolism. An insightful study by Wang and collaborators explored the metabolic changes in plasma samples from healthy volunteers, diabetic patients, and DN patients, aiming to identify potential biomarkers for DN. Their study revealed a significant increase in the levels of valine, leucine, and isoleucine in the plasma of diabetic patients compared to the control group. However, interestingly, these BCAAs did not exhibit the same increase in DN patients [35]. This observation underscores the potential utility of amino acids as biomarkers for diabetes in the body. Moreover, the distinctive trend of these amino acids being upregulated in diabetes and downregulated in DN could serve as an early warning sign for the onset and progression of DN. These findings hold promise for better understanding the metabolic changes associated with diabetes and DN and could pave the way for the development of targeted therapies and early detection strategies to manage and treat DN effectively. Additionally, lactate can be converted to pyruvate and utilized in gluconeogenesis after entering the mitochondria. *In vivo* and *in vitro* tracer studies of early renal changes associated with diabetes mellitus have reported an increase in glycolysis-driven lactate production or pyruvate-to-lactate conversion [51,52]. Our experimental findings indicate that lactate production remains relatively stable under the given experimental conditions. However, we observed a noteworthy increase in pyruvate consumption, particularly in mild hyperglycemic conditions, irrespective of PA treatment. This intriguing observation suggests that the excess pyruvate generated could potentially be converted into lactate. Notably, lactate serves as one of the renal gluconeogenic carbon sources for renal metabolism. These results shed light on the intricate metabolic pathways and adaptations that occur in response to hyperglycemic conditions, providing valuable insights into the renal metabolism's dynamic nature. The conversion of pyruvate into lactate may have implications for glucose homeostasis in the kidneys, and further investigation into these mechanisms could enhance our understanding of renal physiology and potential therapeutic approaches for managing hyperglycemic-related disorders. In addition, these results may also reflect an impaired mitochondrial function in DN [53]. That is expected since the kidney is an organ that demands high amount of energy in the form of ATP to fuel membrane pumps that are needed for active transepithelial transport [54]. Since the ATP is mainly provided through oxidative phosphorylation, kidney function depends on mitochondria, being highly susceptible to damage, especially in DN [55]. Taking in consideration the metabolic dysfunction detected after the different glucose stimuli and PA-induced IR, we speculated that cellular respiratory chain (dys) function in the kidney could be a good indicator for disease progression in response of glucose. Thus, we assessed the bioenergetic profile of HK-2 cells to explore the mechanisms by which different glucose stimuli

and/or IR can affect cellular respiration. Our results showed that exposure to hyperglycemia negatively impacted the bioenergetic profile of the cells, with greater susceptibility observed when IR was induced. In the early stages of DN, renal ATP demand is raised [56], but with the progression of the disease ATP synthesis [57], glucose oxidation [58], and fatty acid oxidation [59] start to decline. Our findings suggest that mild hyperglycemia, which mimics the onset of the disease, enhances all mitochondrial bioenergetic parameters, including basal respiration, ATP production, maximal respiration, and spare respiratory capacity. However, these parameters decrease when cells are cultured in higher concentrations of glucose and when IR is induced. This is consistent with studies that reported changes in mitochondrial activity after exposure to high concentrations of glucose. It has been shown that exposure to high glucose concentrations can result in a decreased mitochondrial respiration, an increased ROS production, and ultimately mitochondrial dysfunction [60–62]. Czajka and collaborators demonstrated concurrent results, where, after 4 and 8 days of exposure to 25 mM of glucose, HK-2 cells presented a decrease in mitochondrial oxygen uptake [62]. Curiously, they also demonstrated that decreased mitochondrial respiratory capacity coincides with increased glycolysis in HK-2 cells. Our results also support that the increased consumption of glucose and pyruvate and the subsequent increase in lactate production can be a compensatory mechanism when mitochondrial activity is diminished. To further understand the decline in mitochondrial respiratory capacity (MRC) observed, we evaluated individual activity of CI and CII of the ETC. Our results showed that both complexes increased their activity in severe hyperglycemic condition, and the activity was decreased when IR was induced. This damage was more pronounced in CI, especially in cells exposed to the severe hyperglycemic condition, which led to a depolarization of MMP, when compared to the matched group. This finding is in agreement with a study by Forbes and collaborators, which showed that knockdown of CI in mice was associated with the progression of CKD [63]. Contrary to the previously mentioned results, an interesting finding was the observed increase in the activity of CII when cells were exposed to a mild concentration of glucose in combination with PA. This observation aligns with the simultaneous increase in gluconeogenic amino acids under the same condition, implying an overall boost in metabolic activity. However, despite this metabolic upregulation, it appears that the increase was insufficient to elevate the overall energy status. Our data strongly indicate that the decline in MRC could serve as a crucial indicator of DN progression. These findings suggest that mitochondrial performance plays a pivotal role in the context of DN. Targeting the mitochondrial machinery, especially focusing on CI functionality, emerges as a promising therapeutic strategy. By addressing these specific mitochondrial components, we might uncover potential avenues to manage and mitigate the progression of DN [64]. Understanding the link between mitochondrial dysfunction and DN could offer crucial insights into the disease's underlying mechanisms. Consequently, targeting mitochondrial pathways might pave the way for innovative treatments that can effectively intervene and halt the progression of DN, ultimately improving patient outcomes and quality of life. Further studies in this area are warranted to explore the full therapeutic potential of modulating mitochondrial function in DN.

Finally, our experimental findings provide compelling evidence that FAO is not the primary energy pathway utilized by HK-2 cells in response to glucose stimuli under our specific experimental conditions. However, inhibiting FAO during induced IR in a hyperglycemic environment resulted in several notable effects. Firstly, there was a significant decrease in ATP-linked production, suggesting the importance of FAO as a potential compensatory energy source during IR. Additionally, the inhibition of FAO led to the accumulation of fatty acids as lipid droplets within the cells. These observations align with previous research, which has shown that enzymes and regulators of FAO are reduced in PTCs of patients with DN and in animal models of DN. This reduced FAO capacity contributes to intracellular lipid accumulation, mitochondrial dysfunction, and the development of tubulointerstitial fibrosis—important hallmarks of kidney disease progression [55,65,66]. These findings shed light on the intricate metabolic alterations that occur in response to IR and hyperglycemia in the context of DN. Understanding the role of FAO and its impact on cellular lipid metabolism and mitochondrial function is crucial for identifying potential therapeutic targets to intervene in the progression of DN. Further research in this area could pave the way for the development of novel treatment strategies aimed at preserving renal function and ameliorating kidney disease in diabetic patients.

5. Conclusion

The current study provides important insights into the pathogenesis of kidney disease progression, particularly DN, and its association with hyperglycemia and IR. Our findings demonstrate that these conditions have a negative impact on the bioenergetic profile of kidney cells, leading to a metabolic shift that is characterized by the altered metabolism of gluconeogenic amino acids. Our findings reveal a notable increase in multiple metabolic and bioenergetic parameters under mild hyperglycemic conditions, whereas these effects were not observed in the severe hyperglycemic condition. This intriguing observation implies that hyperglycemia might offer a critical window of opportunity to identify potential biomarkers in the early stages of the disease. The distinct response of metabolic and bioenergetic parameters in mild hyperglycemia indicates the dynamic nature of cellular adaptations during the early phases of the disease process. This insight could hold significant implications for early detection and intervention strategies for DN. Identifying reliable biomarkers during the early stages of DN is of paramount importance as it allows for timely diagnosis and targeted therapeutic interventions. Our research contributes valuable evidence to the potential significance of hyperglycemia as a pivotal factor in the search for biomarkers that can aid in the detection and management of DN at its onset. Further exploration of these metabolic changes and their correlation with disease progression could provide essential insights into the underlying mechanisms of DN. Ultimately, this knowledge may lead to the development of more effective treatment approaches aimed at halting or slowing the progression of DN, thereby improving patient outcomes and quality of life.

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Credit authors statement

Patricia .C.Braga: Writing-original draft, methodology, formal analysis, data curation, investigation and writing-review; **Raquel .L. Bernardino:** conceptualization, methodology and writing-review and editing; **Bárbara Guerra-Carvalho:** formal analysis and writing review; **David.F.Carrageta:** formal analysis and writing review. **Pedro.F.Oliveira:** conceptualization, methodology, writing-review and editing, project administration and funding acquisition; **Anabela.S.Rodrigues:** conceptualization, methodology, writing-review and editing funding acquision and project administration. **Marco.G.Alves:** conceptualization, methodology, writing-review and editing, project administration and funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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CHAPTER 19

Determination of glycolysis flux by extracellular flux measurements

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19.1 Summary

Glycolysis is a metabolic pathway that breaks down glucose into different products and generates energy. The final product of glycolysis is lactate in anaerobic conditions and pyruvate in aerobic conditions. These reactions can change the composition of the medium surrounding the cells and, consequently, lead to variations in pH. An alteration of this pathway could indicate a change in cellular differentiation, proliferation, or survival. Glycolysis measurement by some biochemical assays provides low resolution, does not offer a comprehensive picture of metabolic pathway utilization, and elucidates only one parameter at a time. With the Seahorse extracellular flux (XF) analyzer, these limitations are overcome. Both glycolysis and oxidative phosphorylation pathways can be measured simultaneously. Moreover, the users do not need to use multiple instruments, hazardous chemicals and compounds, and a high number of cells or tissues or spend much time analyzing the data. Furthermore, the technique is very versatile since it is possible to analyze these parameters in adherent or suspended cells and/or in tissues. The glycolysis stress test kit allows us to examine different parameters, such as glycolysis, glycolytic capacity, glycolytic reserve, and nonglycolytic acidification, which is translated in alterations of extracellular acidification rate (ECAR) of the medium, giving a more complete overview of the behavior of the cells or tissues in a specific situation. So, the evaluation of the metabolic profile of the cells and tissues through the Seahorse XF analyzer offers numerous advantages relative to other methods. The flexibility and ability to perform real-time measurements by this technique can often allow one to deduce pathway engagement.

Glycolysis
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19.2 Introduction

Energy-producing metabolic pathways mainly include glycolysis and mitochondrial oxidative phosphorylation that are used to support cellular functions, such as cell proliferation (Vander Heiden, Cantley, & Thompson, 2009). Glycolysis is a series of 10 reactions that convert one molecule of glucose into two molecules of pyruvate, with the concomitant production of a small amount of adenosine triphosphate (ATP) (TeSlaa & Teitell, 2014). The first step of glycolysis involves the transfer of a phosphate from the ATP to glucose, resulting in glucose-6-phosphate, by the action of hexokinase (Harris & Harper, 2015). Regulation of a pathway is achieved by modulation of the rates of reactions that control the overall flux through the pathway. For example, hexokinase activity is regulated by product inhibition. If flux through the glycolytic pathway is diminished, the concentration of glucose 6-phosphate increases, blocking the hexokinase active site by the product saturation (Harris & Harper, 2015). Indeed, managing with hexokinase activity is considered to be a possible way to evaluate the glycolytic function of a cell, once this enzyme is considered as a control point of the pathway, as we will describe later.

Furthermore, glycolysis can supply biosynthetic intermediates for cell development. Glucose 6-phosphate can also be oxidized via the pentose phosphate pathway to carbon dioxide and ribulose 5-phosphate, in which carbon flux generated will contribute to nucleotide biosynthesis that is essential for DNA replication and RNA transcription.

In cells that use aerobic respiration as the primary energy source, oxidative phosphorylation is the energetic preferential pathway. Pyruvate can be used by mitochondria to produce substrates for oxidative phosphorylation, producing 34 molecules of ATP per one glucose (Nolfi-Donegan, Braganza, & Shiva, 2020), through the ATP synthetase complex (also known as Complex V). Glycolysis can be used as an emergency backup for energy production or as the preparation step before oxidative phosphorylation, being markedly less efficient in ATP production when compared to oxidative phosphorylation (Koppenol, Bounds, & Dang, 2011).

Furthermore, pyruvate has different fates, such as the production of lactate (Jin & Zhou, 2019; Matschinsky & Wilson, 2019). Pyruvate can be converted into lactate in the cytosol by lactate dehydrogenase with oxidation of NADH to NAD^+. The Seahorse Analyzer can be used to measure cellular responses to modulators of glycolysis and oxidative phosphorylation. Seahorse technology uses fluorescent probes sensitive to oxygen and protons to determine the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), respectively (Yang, Chadwick, Dart, Kamishima, & Quayle, 2017). The conversion of glucose to pyruvate, and subsequently lactic acid, results in the production and extrusion of protons into the extracellular medium, resulting in the acidification of the medium, this acidification can be evaluated through the ECAR (Perez, Hill, Benavides, Dranka, & Darley-Usmar, 2010; Wu et al., 2007).

ECAR is a measure of lactic acid production, generated by anaerobic glycolysis, in a general way representing the extracellular glucose that is taken up by the cell and

converted into acidic end products, such as lactate, translating medium pH as an indirect measurement of the glycolytic pathway (Newell, Franchi, Pouyssegur, & Tannock, 1993).

With Seahorse XF Glycolysis Stress Test, it is possible to evaluate the different parameters, such as glycolysis, glycolytic capacity, glycolytic reserve, and nonglycolytic acidification (Fig. 19.1). For that, different modulators that are injected sequentially in cells or tissues are used, and it is possible control the time of injection in real time. First, glucose is injected to supply the substrate for glycolysis and then oligomycin is injected, an inhibitor of ATP synthase in the mitochondria, shifting the burden of ATP supply entirely in glycolysis. Finally, 2-deoxyglucose (2-DG) is injected, which blocks the hexokinase and consequently the glucose-6-phosphate production (Chen & Gueron, 1992).

19.3 Materials, equipment, and reagents

- Seahorse XFe24 Analyzer
- Agilent Seahorse XF Glycolysis Stress Test Kit
- XF24 Cell Culture Microplates
- XFe24 Sensor Cartridge
- Seahorse XF Calibrant Solution
- Seahorse XF 200 mM glutamine solution

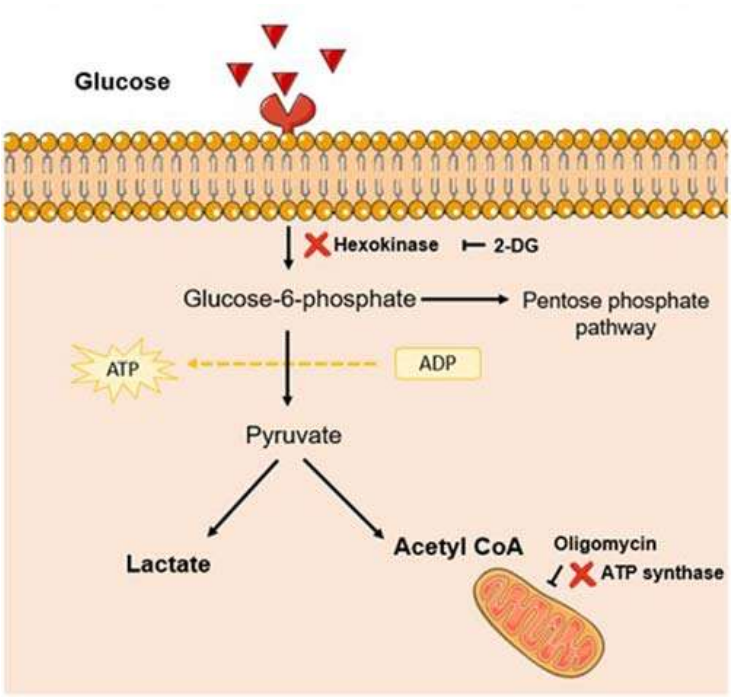

Figure 19.1 Glycolysis stress test modulators of glycolysis. Glucose feeds glycolysis. Oligomycin inhibits oxidative phosphorylation of the electron transport chain through ATP synthase inhibition, therefore preventing aerobic ATP generation and forcing cells to increase glycolytic flux. 2-deoxyglucose (2-DG) is used as a competitive inhibitor of glucose, resulting in a decrease in the glycolysis pathway.

- Seahorse XF DMEM medium, pH 7.4
- Cell culture medium
- Phosphate-buffered saline (PBS)

Alternatives: Seahorse XFe96 Analyzer (for this equipment, it is necessary to adjust the volumes of all components)

19.4 Protocols

19.4.1 Day before the experiment

19.4.1.1 Seed cells and prepare XFe24 sensor cartridge

1. For adherent cells, trypsinize the cell culture until it detaches from the culture flask.
2. Add culture medium with serum to inhibit trypsin action, break up cell clumps with a pipette to create a cellular suspension, and count cells (e.g., automatic counter orhemacytometer).
3. Resuspend the desired number of cells in the culture medium to the volume of 100 μL per well.

   Note: The number of the cells seeded in culture microplates needs to be adjusted to the cell type.
4. Pipette 100 μL of the cell suspension into each well of the XF24 Cell Culture Microplates.
5. Incubate the XF24 Cell Culture Microplates with cells seeded at 37°C, 5% CO_2 to allow cells to adhere for 1–6 hours. Add an additional volume of 400 μL of culture media and allow cells to grow overnight in an incubator.

   Note: The cell plate will likely need to be prepared one or many days in advance, depending on cell proliferation and a number of seeded cells.

   Note: Optimization of the cell number is required. Before the experiment, different numbers of cells per well should be seeded to assess growth and the desired confluence. Experimental cell density should be optimized to obtain the ECAR value of at least 20 mpH/min, which corresponds to the lowest detection range of the machine.
6. The XFe24 sensor cartridge needs to be hydrated overnight to obtain stable results. Add Seahorse XF Calibrant Solution to each well of the XFe24 well microplate. The cartridge contains fluorophores associated with O_2 and H^+ for each well.
7. Incubate the XFe24 sensor cartridge with 1 mL of Seahorse XF Calibrant Solution to hydration at 37°C without CO_2 for at least 6 hours.

   Note: Make sure that the calibrant solution level is enough to maintain the sensor submerged. Lower the sensory cartridge back onto the utility plate gently and avoid creating air bubbles.
8. Turn on the Seahorse XFe24 Analyzer equipment before the assay, so that it reaches the correct temperature before the assay.

19.4.2 Day of the experiment

1. Preparation of glycolysis stress assay medium and cell plate:
   a. The Seahorse XF DMEM medium supplemented with 2 mM L-glutamine (glycolysis stress assay medium) need be warm until 37°C and adjusted pH to 7.4.

      Note: 2 mM L-glutamine is the recommended concentration, but the composition of the medium may be different depending on the cell type or culture conditions.
   b. Remove the cell culture medium from each well of the plate, where the cells are seeded, and wash all wells with saline solution (PBS) heated to 37°C.

      Note: Washing should be done carefully so as not to disrupt the cell monolayer.
   c. Add 500 μL of glycolysis stress assay medium to each well and incubate the plate for 1 hour at 37°C in an incubator without CO_2.

      Note: CO_2 can alter the pH of the medium and consequently alter ECAR measurements.
   d. After 1 hour, the glycolysis stress assay medium needs to be replaced by a new fresh medium (37°C).
2. Preparation of inhibitors
   a. The inhibitors will be added through the injection ports in the cartridge (Fig. 19.2), and they should be at a concentration of 10x higher than the desired final concentration. First, it is necessary to prepare the stock solution with a glycolysis stress assay medium and follow the dilutions shown in Table 19.1.

      Note: Vortex for approximately 1 minute to ensure full resuspension of compounds.

      Note: If you are performing a standard assay, that is, with no additional injections, load the compounds into the appropriate ports (glucose at port A; oligomycin at port B; and 2-DG at port C). However, if you inject an additional compound before glucose, use port A for the desired compound and then load glucose at port B, oligomycin at port C, and 2-DG at port D.

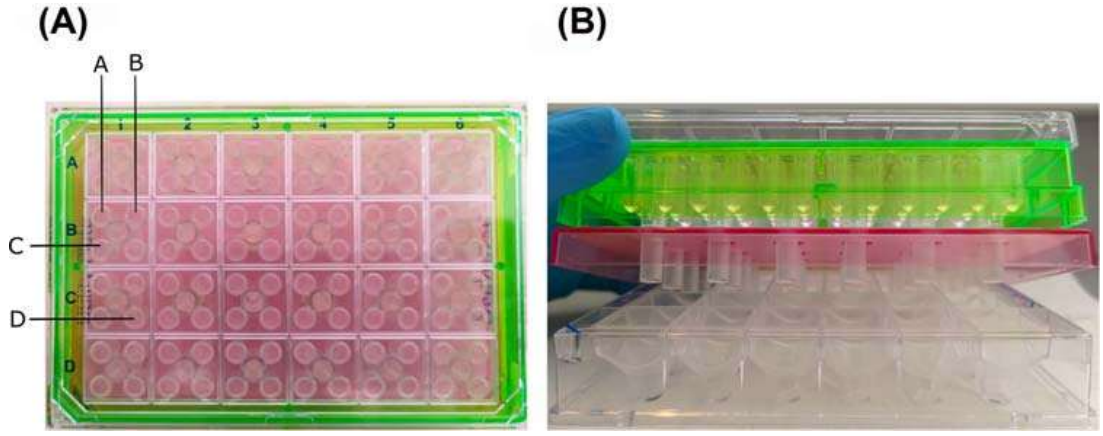

Figure 19.2 Seahorse cartridge layout. (A) Ports of the cartridge: A, B, C, and D. (B) Cartridge constitution: Utility plate, pink plate, cartridge, and lid.

Table 19.1 Preparations of stock solutions and work solutions for loading sensor cartridge ports.

Compound	Volume of assay medium (AM)	Stock solution concentration (SS)	Work solution (10x)	Volume to add to the port (uL)	Final well concentration
Glucose port A	3000 μL	100 mM	3000 μL (SS) + 0 μL (AM)	56	10 mM
Oligomycin port B	720 μL	100 μM	300 μL (SS) + 2700 μL (AM)	69	1.0 μM
2-DG port C	3000 μL	500 mM	3000 μL (SS) + 0 μL (AM)	69	50 mM

Note: The protocol outlined is for the 24-well format of the instrument. Volumes will need to be adjusted if another format is used. Work solution concentration needs to be adjusted to the cell and tissue type.

b. After cartridge loading, place the utility plate with the loaded sensor cartridge on the Seahorse XFe24 Analyzer to perform calibration. It will take approximately 20 minutes.

Note: Before placing the cartridge, remove the cover and the pink plate that is between the utility plate and the cartridge (Fig. 19.2).

3. Preparation of the protocol

a. Open the Program Wave and select Glycolysis stress test on the Templates window and set up the program.

b. At the plate map tab, click "add group" and select corresponding wells, in which each group has a different color associated (Fig. 19.3).

Note: Each sample should be measured in triplicate.

c. At the Protocol tab, the parameters are as follows: 3 cycles—of injection of port A, 3 cycles of—injection of port B, and 3 cycles of—injection of port C.

Note: All protocol parameters can be modulated, like the number of cycles between each injection. These parameters need to be adjusted related to cells or tissue in analysis.

d. After cartridge calibration, the utility plate is replaced by a cell or tissue culture plate.

4. Results analysis

a. Through the results obtained by graphs provided by Seahorse XF Glycolysis Test Report Generator (Fig. 19.4), we can export all values to an Excel or prism file to calculate the principal parameters:

- **Glycolysis** = ECAR after addition of glucose - ECAR after 2-DG injection
- **Glycolytic capacity** = ECAR after addition of oligomycin - ECAR after 2-DG injection
- **Glycolytic reserve** = ECAR after addition of oligomycin - ECAR after addition of glucose
- **Nonglycolytic acidification** = ECAR after 2-DG injection.

Note: The results should be normalized to cell number, protein concentration, or other methods.

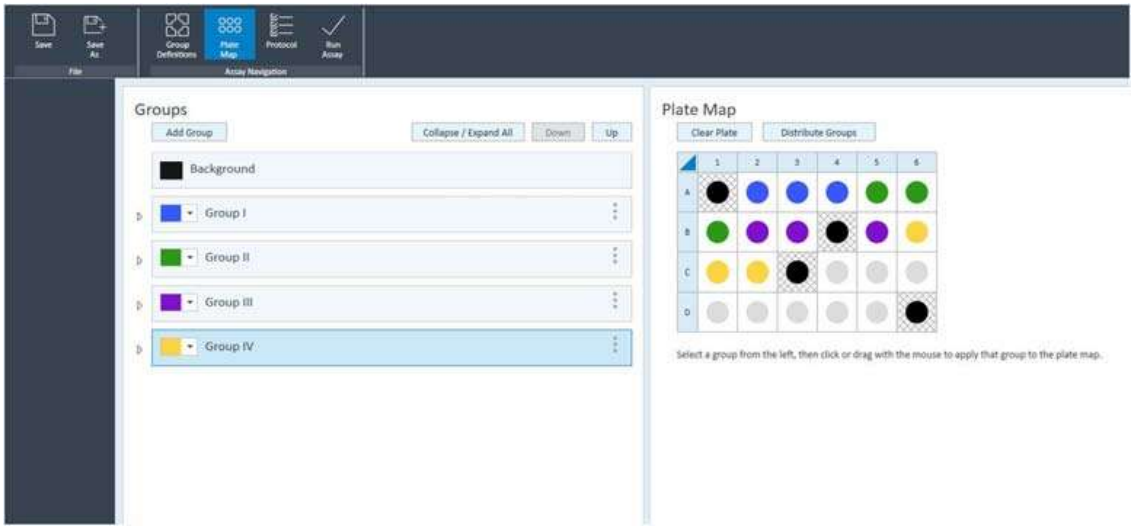

Figure 19.3 Seahorse plate layout. Triplicated of different experimental groups.

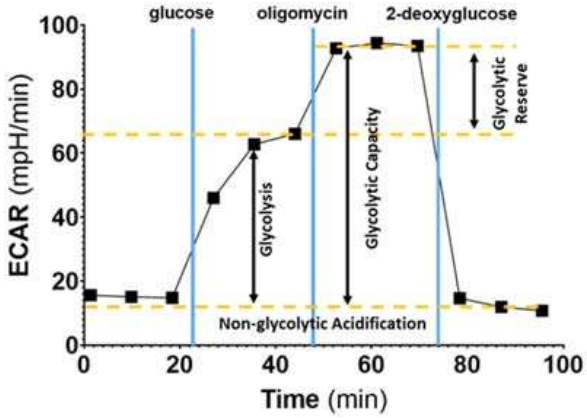

Figure 19.4 Schematic representation of the extracellular acidification rate (ECAR) during glycolysis extracellular flux assay, after injection of different modulators through time. The different parameters can be measured by the ECAR values as a function of time, where glycolysis is the difference between ECAR after addition of glucose and ECAR after 2-deoxyglucose (2-DG) treatment; glycolytic capacity is the difference between ECAR after addition of oligomycin and ECAR after 2-DG treatment; glycolytic reserve is the difference between ECAR after oligomycin injection and ECAR after addition of glucose; and nonglycolytic acidification corresponds to the ECAR value after 2-DG treatment.

19.5 Analysis and statistics

The glycolysis stress test begins with a baseline measurement of the ECAR in the cells with a glucose free-medium (Fig. 19.4). At this point, cells are at their basal minimum, a stage that is considered as **nonglycolytic acidification**. This acidification corresponds to CO_2 generated in the tricarboxylic acid cycle, being converted to HCO_3^- and H^+. The injection of glucose activates **glycolysis**. This process is characterized as the conversion of glucose to pyruvate that can be converted into lactate, acetyl-CoA, or acetate.

The catabolism of one glucose to two lactate generates two H^+, which presents an increase in extracellular acidification. The cells are then challenged with the injection of oligomycin, which shuts down the generation of ATP through oxidative phosphorylation. Cells respond to this decrease in ATP production by driving the cells to use glycolysis to its maximum capacity, resulting in an increase in ECAR levels, being characterized as glycolytic capacity. Depending on cellular models, glycolytic capacity can be a predictor of drug sensitivity, like in tumors (Issaq, Teicher, & Monks, 2014) and dendritic cells (Malinarich et al., 2015). It can also be associated with cell damage, where for instance, a decline in glycolytic capacity is observed in a heart failure model (Phillips et al., 2010). Furthermore, a decrease in glycolytic capacity can be linked with cellular reprogramming and differentiation (Chung, Arrell, Faustino, Terzic, & Dzeja, 2010; Folmes et al., 2011).

Notwithstanding, in a cell with high glycolytic capacity, the glycolytic rate in the absence of oxidative phosphorylation may fully meet the whole of the cell's current ATP demand without being maximal. To evaluate if the cells are operating at their maximum glycolytic level or not, the cellular ATP demand should be increased experimentally, where a lack of glycolytic response will demonstrate that it was running at its full capacity. However, if an increase in glycolytic acidification is observed, it will demonstrate that it was not (Mookerjee, Nicholls, & Brand, 2016).

Finally, the experiment is ended with the injection of 2-DG, which returns ECAR to its nonglycolytic level. With this injection, it is possible to determine the **glycolytic reserve**, which indicates the capacity of a cell to respond to an energetic demand, as well as how close the glycolytic function is to the cell's theoretical maximum (Fig. 19.4).

19.6 Related techniques

For an accurate data interpretation, it is important to do normalization of results and to minimize inconsistency and variations from well to well. First of all, it is important that after the experiment on Seahorse analytics, the plate with the cells should be removed as soon as possible to avoid the detachment of cells and the medium should be gently removed.

To normalize the data, several approaches can be useful. One possible way to normalize is through **cell number density**. However, it is not the most reliable method of normalizing the data, if the cell proliferation is affected throughout the duration of the experiment.

Still, regarding the cell number, another approach is to proceed to DNA quantification. This technique is predicated on the hypothesis that the content of DNA is linearly proportional to the number of cells. The content of nuclear DNA can be easily measured in a standard plate reader by using commercial reagent kits (Angelini, Pi, & Xie, 2022).

It is also possible to normalize by evaluation of cellular density through colorimetric sulforhodamine B (SRB) assay (Martins, Oliveira, & Alves, 2019). Briefly, cells are fixed in 1% acetic acid in methanol for 1 hour at $-20°C$. Next, cells were stained with 0.05% (w/v)

SRB dissolved in 1% of acetic acid for 1 hour at 37°C. Afterward, unbound SRB was removed through washing with 1% acetic acid solution. SRB bound to cell proteins is extracted afterward, with 10 mM Tris solution (pH 10) in a shaker for 10 minutes at room temperature. The optical density of the resulting media is determined at 490 nm.

Furthermore, to correct the mitochondrial content of the samples, OCR can be normalized through **citrate synthase activity** (Gunnewiek et al., 2020). In brief, the background citrate synthase activity is recorded in the absence of its substrate Acetyl-CoA, followed by its addition. In practice, after completion of OCR measurements, the seahorse medium must be replaced by a buffer solution composed with Triton X-100 and Tris—HCl and the plate must be stored at −80°C. After thawing the plate, it is added in each well a solution with Acetyl-CoA, DTNB, oxaloacetate, and Triton X-100. The background reduction of DNTB is measured spectrometrically at 412 nm and 37°C for 10 min, collecting points at every minute. The citrate activity will be determined by the rate that DTNB is reduced in the presence of oxaloacetate, subtracted by the background DTNB conversion rate, using an extinction coefficient of 13600 M^{-1} cm^{-1}, and expressed as nmol/min/mg protein (F Monteiro-Cardoso, Oliveira, Moreira, P., Peixoto, & Videira, R., 2015). Apart from normalization through mitochondrial enzymatic activities, mitochondrial protein content is also an option. To do that, expression of some mitochondrial proteins such as CPT1B, TIMs, and TOMs can be quantified. Nevertheless, with so few cells per well, it might be challenging to determine the mitochondrial content (Yépez et al., 2018).

The most accurate method measure for the normalization of the results is the cellular or tissue lysate **protein quantification**. Total protein can be obtained using RIPA buffer. After that, the total protein concentration can be determined by routine methods in the laboratory.

19.7 Advantages and disadvantages

The advantages and disadvantages of the use of Seahorse Analytic to evaluate the glycolytic capacity are summarized in Table 19.2. In sum, it is important to highlight that effectively this approach is faster and has a high resolution and high sensitivity; however, the costs are too high. So it is important to evaluate if this approach is the one that can provide the necessary information to obtain a more accurate result.

19.8 Alternative methods and procedures

The Seahorse XF analyzer is a powerful tool for measuring glycolysis and oxidative phosphorylation simultaneously in the same cells. However, enzymatic approaches are possible to mimic what happens in seahorse equipment. One example is the kit Glycolysis Stress Test Companion Assay, which has the same rationale that the glycolysis stress test kit of Seahorse does. The principal reagents, like in glycolysis stress kit,

Table 19.2 Advantages and disadvantages to evaluate the glycolytic capacity through seahorse analytics.

Advantages	Disadvantages
Evaluation of OCR and ECAR simultaneously in the same cells.	The cost is high.
Evaluation of different samples in different conditions at the same cell culture plate.	Optimization of the compounds is needed.
Very small quantities of cells are necessary (as few as 1% of cells required by other methods).	Only four injectable compounds are possible to be tested in a single assay.
Most essays can be completed in 1.5–2 h.	Injectable compounds may potentially interfere with sensor fluorescence or the plastic plate and produce misleading data.
High-resolution and sensitive equipment.	Complementary techniques are necessary to normalize the data.
Background correction wells.	

are oligomycin, 2-DG, and glucose oxidase, which have positive control of the assay, and glucose-free respiration buffer. The results are read in a microplate reader, with controlled temperature and fluorescence intensity at 360 ± 40 nm.

Furthermore, there are other approaches, which can indirectly be used to measure the glycolytic rate of a cell. One possible method is measuring the activity of rate-specific glycolytic enzymes. Indeed, specific enzymes are considered key points in the glycolysis pathway, in which their activities control the maximum possible flux. The activity of hexokinase, phosphofructokinase, and pyruvate kinase, the main enzymes studied, can be evaluated. It is usually done through the conversion of NAD^+ to NADH or $NADP^+$ and NADPH by spectrophotometric methods (Vander Heiden et al., 2009).

Metabolite measurements are also a possibility to study glycolytic flux. Unlabeled or ^{13}C labeled metabolites can be quantified by nuclear magnetic resonance spectrometry. Indeed, ^{13}C- labeled glucose provides glycolytic flux data by quantifying ^{13}C-labeled glucose in downstream metabolites. Even though labeled substrates are expensive and sometimes challenging to detect, the identification of ^{13}C-glucose-derived carbons in glycolytic intermediates is robust and, therefore, considered a good method for evaluating glycolysis (Gkiouli, Biechl, Eisenreich, & Otto, 2019).

19.9 Troubleshooting and optimization

During the experiment, several problems could appear, which are more or less easy to solve. Before the experiment, it is highly recommended that the cells and or tissue be at confluency over 80% to obtain a good signal. Nonetheless, some issues can occur

Table 19.3 Problems that can occur during the seahorse analytics experiment and the possible solution.

Problem	Solution
Variability between wells	Test the number of cells to be seeded per well and the time it takes to double, to avoid excess confluence and cell detachment that can lead to variation in the readings.
OCR levels are <40 pmol O_2/min	Increase the seeded cell number.
Minimal response to oligomycin	Cells growing in nutrient-rich media such as Optimem barely use mitochondria to produce ATP. Therefore, incubate the cells in DMEM with a reduced glucose concentration (or galactose-based medium) to promote the use of mitochondria for ATP production.
Post-assay normalization by cell count does not yield a reliable result	Use an alternative cell counting method before seeding, such as trypan blue. This may yield improved accuracy when analyzing sensitive cell lines that are more prone to cell death.
Detachment of permeabilized cells from the microplate	Several changes can be made to improve cell adhesion: 1. Increase cell seeding density; 2. Grow cells for longer (2 days rather than 1); 3. Consider a cell coating (polylysine, gelatin solution, collagen, Cell-Tak, Matrigel, or others)
Intact cells cannot be accessible to the full dose of drugs.	It will be needed to permeabilize the cell membrane. Digitonin can be a solution to permeabilize the cells.

during the injection of the different modulators, which are described in Table 19.3, as well as some solutions that need to be taken into count to solve the troubleshooting.

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CHAPTER

20

Mitochondrial transplantation in kidney injury

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Introduction

Kidney disease is a public health problem that has grown. In 2020, Kidney Disease: Improving Global Outcomes, an independent organization with the mission to improve care and outcomes of patients with kidney disease worldwide, organized a consensus conference to harmonize existing acute kidney disease (AKD) and chronic kidney disease (CKD) definitions.

CKD is defined as a progressive and irreversible loss of kidney function. The principal hallmarks are an estimated glomerular filtration rate (eGFR) < 60 mL/min per 1.73 m^2, the presence of markers of kidney damage (proteinuria, active urine sediments, structural abnormalities, histological damages), for more than 3 months, and/or a history of kidney transplantation (Lameire et al., 2021). AKD and CKD can be distinguished by the duration of the renal outcomes. The term acute defines a condition of a sudden episode (6 h−7 days) of injury that is short and can be reversible, while chronic has a long term and with persistent episodes associated. On the other hand, acute kidney injury (AKI) is a subset of AKD associated with high morbidity and mortality, including an increase in adverse renal outcomes. It is also suggested that AKI is not self-limited, as it serves as an incoming for subsequent AKI episodes and potentially to CKD (Heung et al., 2016). Furthermore, an episode of AKI in patients with CKD can cause further renal deterioration to end-stage kidney disease

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(ESKD). Multiple factors can contribute to AKI-related renal disease progression, including age, diabetes, hypertension and cardiac problems, severe episodes of AKI, and even delayed renal function recovery from AKI (Yan et al., 2021). Several studies already demonstrated that mitochondrial morphological alterations are present during AKI to CKD, including swelling, fragmentation, and loss of cristae. Moreover, impaired mitochondrial functioning results in decreased electron transport chain (ETC) activity, a decrease in membrane potential, and an increase in reactive oxygen species (ROS) generation, particularly in podocytes (Aparicio-Trejo et al., 2019; Funk & Schnellmann, 2012; Li, Li, et al., 2020; Liang et al., 2022). Notably, some of these studies suggest that independently if the renal outcomes are from sudden or persistent episodes, mitochondria functioning is highly affected by kidney diseases. In fact, as the kidney is an extremely metabolically active organ, it needs a high amount of energy to accomplish its functions, such as filtration and reabsorption (Bhargava & Schnellmann, 2017). As there is no effective treatment for kidney disease, and as mitochondria are signalized as having a key role in disease progression, the interest in therapeutic mitochondria targeted therapy has increased, with a high interest in mitochondrial transplantation. In this chapter, we will highlight the importance of the mitochondria in the kidney, unveil, and summarize the principal techniques found in mitochondrial transplantation, particularly those applied or with possible relevance for application in kidney disease.

An overview of acute kidney injury

William MacNider used the term AKI for the first time in 1918 to describe a case of acute mercury poisoning. Recently, the term acute renal failure has been replaced by AKI (Bellomo et al., 2004). AKI is characterized by a sudden loss of renal function, uremia, and dysregulation of extracellular volume and electrolytes, resulting from multiple insults, including sepsis, cardiovascular surgery, and ischemia–reperfusion injury (IRI). AKI has additional causes, such as direct kidney injury and urinary tract obstruction, in addition to decreased blood flow (Lameire et al., 2006). Patients with AKI typically have a heterogeneous etiology, involving extrarenal pathology (such as prerenal azotemia and acute postrenal obstructive nephropathy) and particular kidney diseases (like acute interstitial nephritis, acute glomerular, and vasculitic renal disorders) (Chawla & Kimmel, 2012). As previously mentioned, the diagnosis of AKI is predicated on a rapid decrease in glomerular filtration rate (GFR), which is also accompanied by a decrease in urine production or an increase in serum creatinine levels (Khwaja, 2012). After this first step of diagnosis, AKI can be classified into three main categories: prerenal, intrinsic, and postrenal AKI. Prerenal and postrenal AKI result from a decline in GFR caused by extrarenal disease, whereas "intrinsic" AKI represents true kidney disease (Agarwal et al., 2016). AKI is considered an independent risk factor for CKD and ESKD, where severe AKI may be followed by an irreversible loss in kidney function (Bucaloiu et al., 2012; Coca et al., 2012). But currently, it is known to be strongly linked to interrelated syndromes, where CKD predisposes or sensitizes people to AKI (James et al., 2015), and AKI is one of the most notable variables to speed up the progression of CKD (Palomba et al., 2017). Among these causes of AKI, IRI is the most striking, where renal tissue is compromised.

In addition to mitochondrial damage, energy depletion, and tubular apoptosis, IRI induced AKI (IRI-AKI) also causes a disturbance in the cellular redox balance and intensifies ROS formation in the kidneys during reperfusion, which can lead to renal failure or dysfunction (Bhargava & Schnellmann, 2017; Li, Liao, et al., 2020; Yu et al., 2020). AKI has been described as a state of tubular ATP depletion (Thadhani et al., 1996). In addition, the opening of mitochondrial permeability transition pores due to mitochondrial swelling and dysfunction is a key event that contributes to AKI progression since the release of proapoptotic mediators, such as cytochrome C, can occur and induce renal cell apoptosis (Wakabayashi & Karbowski, 2001). Mitochondria are the main source of intracellular ROS, which are mainly produced at complexes I and III of the ETC when electrons derived from either NADH or $FADH_2$ react with O_2 (Chouchani et al., 2016); thus, the mitochondrial dysfunction caused by AKI potentiates the production of ROS. Thereby, mitochondrial (dys)function has been strongly associated with the pathogenesis of AKI, causing renal tubular dysfunction and cell death (Venkatachalam & Weinberg, 2012); however, targeting mitochondria therapies to improve mitochondria fitness is still a field barely explored and debatable.

Mitochondria function and its importance in the kidney

In mammalian cells, mitochondria are the intracellular organelles in the control of biological oxidation. The primary purpose of mitochondria is to produce ATP through electron transport via oxidative phosphorylation (OXPHOS). This is done in conjunction with metabolite oxidation through the tricarboxylic acid cycle and fatty acid catabolism by β-oxidation to provide energy to kidney basal cell function (Duann & Lin, 2017; Nourbakhsh & Singh, 2014). Indeed, one of the human organs that uses up the most energy is the kidney, which also consumes the most oxygen and has the second-highest mitochondrial content after the heart (Emma et al., 2016).

Mitochondria are crucial for metabolic control, production of ROS, preservation of intracellular calcium homeostasis, thermogenesis, and control of intrinsic apoptotic and proliferative pathways (Brooks & Dong, 2007; Che et al., 2014).

Proximal tubular cells generate ATP mainly through mitochondrial OXPHOS, while podocytes, endothelial, and mesangial cells exhibit a higher glycolytic capacity to generate energy (Bhargava & Schnellmann, 2017; Wirthensohn & Guder, 1986). A growing body of research indicates that a variety of acute and chronic injuries may result in oxidative stress derived from the ETC, ultrastructural defects, abnormal activation of the mitochondrial pathway of apoptosis, unstable mitochondrial DNA (mtDNA), and inadequate clearance of damaged mitochondria (Duann & Lin, 2017). Mitochondrial homeostasis is the balance between the control of mitochondrial biogenesis, mitochondrial dynamics, including mitochondrial fragmentation and mitophagy. An imbalance may induce ROS production and apoptosis. These alterations will, in turn, lead to the development and progression of diseases related to mitochondrial dysfunction. In the kidney, it leads to an increased risk of tubular interstitial disease, cystic kidney disease, podocytopathy, and nephropathy syndromes (Duann & Lin, 2017).

Thus, it is important to understand mitochondrial pathophysiology that is addressed in AKI and CKD. A deeper understanding of the cellular molecular processes that control

mitochondrial activities in kidney disorders ought to contribute to the improvement of treatment approaches.

Mitochondrial quality control and biogenesis in AKI

Mitochondrial turnover is a mechanism adopted to assure mitochondrial quality. In this process, dysfunctional mitochondria are selectively eliminated and replaced through an increase in the number of preexisting mitochondria.

In order to increase the production of metabolic enzymes and increase the mitochondrial metabolic capacity, the nuclear and mitochondrial genomes must coordinate. The mtDNA is a double stranded circular molecule that is roughly 16 kb in size and encodes 37 genes, comprising 13 structural subunits of the mitochondrial respiratory chain (ETC complexes I, III, IV, and V) (Brown et al., 2010). Furthermore, mitochondrial biogenesis can be influenced by multiple external factors, such as exercise, caloric restriction, and oxidative stress (Brown et al., 2010). The peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) protein family is responsible for activating mtDNA transcription. PGC-1α, the key regulator of mitochondrial biogenesis in this family, is foremost expressed in proximal tubules. During tissue damage and healing, the induction of mitochondrial biogenesis is linked to the activation of transcription and several signaling pathways, which includes AMP-activated protein kinase/PGC-1α (AMPK/PGC-1α) pathways used by C1q/tumor necrosis factor related protein-3 (CTRP3) to promote biogenesis, and the Gby-Akt-eNOS-sGC (soluble guanylatecyclase) pathways stimulated by the β2 adrenergic receptor agonists (Puigserver et al., 1998).

Unsurprisingly, the dysregulation of mitochondrial biogenesis has been associated to AKI (Fig. 20.1). Nuclear respiratory factor 1 (NRF1) and nuclear respiratory factor 2 (NRF2) are transcriptional coactivators of PGC-1α that promote mitochondrial biogenesis, and are responsible to control of OXPHOS, fatty acid oxidation (FAO), and the synthesis of nicotinamide adenine dinucleotide (NAD^+), which is a key metabolic coenzyme/cosubstrate involved in cellular energy, that confers renal protection (Gureev et al., 2019; Lynch et al., 2018; Tran et al., 2016). PGC-1α coordinately upregulates the enzymes that synthesize NAD de novo from amino acids, however in AKI there is an attenuation of this de novo pathway (Fig. 20.1). These results are supported by data obtained in PGC-1α knockout mice following IRI. Tran and collaborators demonstrated that PGC-1α knockout mice experienced substantial fat storage, a local deficit of the NAD precursor niacinamide, and an inability to restore normal renal function (Tran et al., 2016). Furthermore, in sepsis associated AKI, there is a suppression of PGC-1α which negatively affects the recruitment of NRF1 and NRF2, leading to an impairment of OXPHOS (Parikh et al., 2015; Tran et al., 2011) (Fig. 20.1).

According to a different study, PGC-1α protein levels rise and PGC-1α mRNA expression decreases on the third and tenth days following unilateral ureteral obstruction, and these changes are strongly linked to fibrosis (Hickey et al., 2011). However, extra advantages are provided by PGC-1α expression. For example, conditional ablation of the renal proximal tubular cells of mammalian target of rapamycin complex 1 causes an increase in the progression of fibrosis following acute ischemic kidney damage, resulting in unfavorable outcomes related to the kidney (Grahammer et al., 2014).

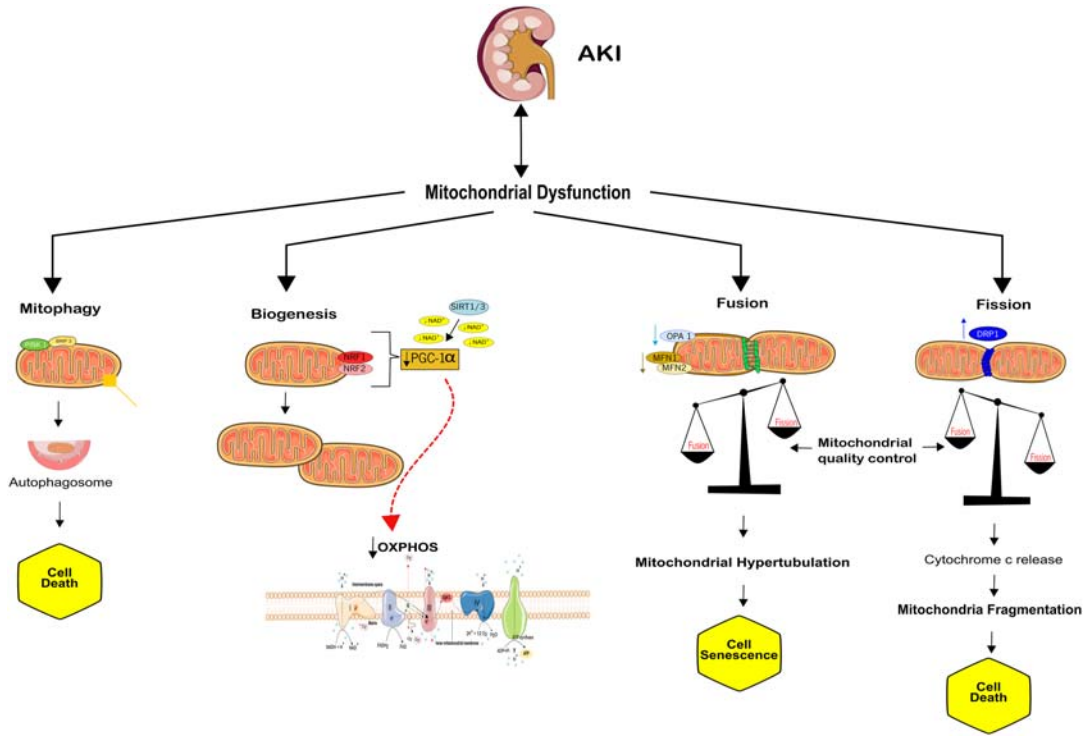

FIGURE 20.1 **Schematic illustration of the pathological processes of mitochondrial dysfunction including biogenesis, fusion, fission, and mitophagy in AKI.** Balance of fission and fusion processes is necessary to maintain the normal mitochondria quality control. In fusion, there is a dysregulation in the GTPases, where OPA1, Mfn1, and Mfn2 have a decreased activity. Excessive fusion is associated with mitochondrial hypertubulation, which leads to cell senescence. Biogenesis is also affected in AKI. There is a downregulation of PGC-1α which is related with the downregulation of the SIRT1 and 3, that will result in a downregulation of NRF1 and NRF2, culminating in a reduced activity of OXPHOS. Fission event is also altered in AKI, where it can be observed an upregulation of Drp1, that results in an increase of cytochrome C release, and further in mitochondrial fragmentation. Mitophagy protects kidney from IRI by removing damaged mitochondria. The two main mechanisms of mitophagy are the PTEN-induced putative kinase 1 (PINK1) and E3 ubiquitin ligase Parkin-dependent pathway and Bcl2/adenovirus E1B 19-kDa protein interacting protein 3 (BNIP3). An imbalance in mitochondrial control can also occur by excessive of fission over fusion, leading the mitochondria to death. Abbreviations: *AKI*, acute kidney injury; *IRI*, ischemia–reperfusion injury; *OPA1*, optic atrophy 1; *Mfn1*, mitofusin 1; *Mfn2*, mitofusin 2; *NRF1*, nuclear respiratory factor 1; *NRF2*, nuclear respiratory 2; *SIRT1/3*, sirtuin 1 and 3; *NAD+*, nicotinamide adenine dinucleotide; *PGC-1α*, PPArgama-coactivator-1 alpha; and *Drp1*, dynamin-related protein 1.

The family of NAD^+- dependent deacetylases known as sirtuins (SIRT1-7) can regulate mitochondrial biogenesis and play a key role in kidney disease (Fig. 20.1).

It has been shown that SIRT1, a NAD-dependent deacetylase, can control PGC-1α expression and activity. When a SIRT1 activator (SRT1720) was administered to patients with IRI-AKI, the kidneys' expression of PGC-1α was restored. This improved mitochondrial biogenesis, as observed by an increase in the mass and ATP levels of the mitochondria, and ultimately improved renal function (Funk & Schnellmann, 2013). Besides, another

activator of mitochondrial biogenesis can be addressed to recover renal function after an AKI episode (Jesinkey et al., 2014). Another example is formoterol, which is a potent agonist of β2-adrenoreceptor, that can induce mitochondria biogenesis by increasing mtDNA copy numbers, oxygen consumption rate, and PGC-1α expression in renal proximal tubular cells (Wills et al., 2012). SIRT3 is present in the mitochondrial matrix, and is responsible to maintain OXPHOS efficiency (Haigis et al., 2012), mtDNA integrity (Osman et al., 2011), as well as mitochondrial biogenesis and fission (Morigi et al., 2015). In sepsis and cisplatin-induced AKI, SIRT3 overexpression has been shown to have a protective effect (Zhao et al., 2018; Zhao et al., 2016). Moreover, mitochondrial FAO in proximal tubules represents a major source of ATP production. In cisplatin induced AKI, a downregulation of PGC-1α was able to decrease the free FAO genes (like carnitine O-palmitoyltransferase and medium chain-specific acyl-CoA dehydrogenase), resulting in a decreased mitochondrial FAO. Thus, this impairment was reported as linked to ATP depletion in AKI (Jang et al., 2020). There are not many studies, but the ones found in the literature highlight a very important role for mitochondrial quality control and biogenesis in AKI.

Mitochondria dynamics in AKI

Mitochondria, as dynamic organelles, possess the ability to regulate their morphology, orchestrating the formation of a tubular network through coordinated fusion and fission events. An excess of mitochondria fission leads into mitochondrial fragmentation, whereas when there is an enhanced mitochondrial fusion results in mitochondrial hypertubulation (Fig. 20.1). A precise balance between these two events is crucial to maintain mitochondrial fitness (Galvan et al., 2017). The coordination between these two opposite processes is primarily mediated by GTPases belonging to the dynamin family. Mitochondrial fission preserves the genetic, biochemical, and functional integrity of mitochondria. Additionally, it makes it easier for them to communicate to each other and contributes to cell homeostasis. It is a process that allows the division of one mitochondrion in two daughter mitochondria, through dynamin-related protein 1 (Drp1). During the mitochondrial fission, Drp1 is recruited and triggers the recruitment of dynamin 2 to perform the membrane scission. On the other hand, the number and distribution of the mitochondria are regulated by mitochondrial fission (Fig. 20.1) (Roushandeh et al., 2019). This process is also responsible to remove the damaged mitochondria through mitophagy. Mechanistically, mitochondrial fusion is caused by mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2) that contribute to the outer mitochondrial membrane, while optic atrophy 1 (Opa1) contributes to the inner membrane fusion.

It is significant to highlight that changes in the expression of these proteins and the disruption of the balance between fission and fusion can impact the integrity and functionality of mitochondria, which have been highly associated with AKI. Several studies have documented a correlation between AKI and an early-stage rise in mitochondrial fission following renal damage (Nowak et al., 2011; Zhan et al., 2013). In fact, an impaired regulation of Drp1 results in increased mitochondrial fission and subsequent release of cytochrome *c*, contributing to end organ damage (Galvan et al., 2017). Mitochondrial fragmentation results from AKI induced reduction in Mfn and Opa1, fusion regulators, whereas the protein Drp1, which controls mitochondrial fission, is quickly activated. To revert this event, Yan and collaborators demonstrated in a murine model that modulation of Mfn2 through microRNAs

successfully prevented the disturbance of mitochondrial dynamics and the death of tubular cells in ischemic AKI (Yan et al., 2020). In agreement, Perry and collaborators, in an IR induced mouse model, demonstrated that Drp1 causes mitochondrial dysfunction and increases maladaptive repair pathways, resulting in fibrosis (Perry et al., 2018). Additionally in a rodent model with IRI in which was modulated the expression of Drp1 through an inhibitor, mdivi-1, there was an attenuation in mitochondrial fragmentation, cytochrome *c* release, caspase activation, and apoptosis (Brooks et al., 2009).

Subsequently, mdivi-1 treatment showed a renoprotective effect on mitochondrial function in sepsis-induced AKI. This was evident through an elevation in ATP production, a decrease in mitochondrial fragmentation, and a decrease of the inflammasome mediated pyroptosis of renal tubular epithelial cells caused by NOD-like receptor pyrin domain-3 (Liu et al., 2020). The NOD-like receptor is closely related to renal inflammation and fibrosis. These studies were further consolidated with research demonstrating that mitochondrial dynamics are critical to mediate AKI and the progression of fibrosis (Ludwig-Portugall et al., 2016).

Mitophagy is a sort of selective autophagy that targets and destroys damaged or redundant mitochondria. This process prevents cell death from mitochondrial oxidative stress and the release of proapoptotic proteins. There are two main mechanisms of mitophagy identified (Fig. 20.1). One is the PTEN-induced putative kinase 1 and E3 ubiquitin ligase Parkin-dependent pathway and the other is Bcl2/adenovirus E1B 19-kDa protein interacting protein 3 and the Nip3-like protein X (NIX) pathway (Parikh et al., 2015). Through the removal of damaged mitochondria, mitophagy protects the kidney from IRI. Moreover, some studies have shown that protecting mice against IRI-AKI involves the activation of mitophagy through AMPK (Livingston et al., 2019; Tang et al., 2018; Tran et al., 2011).

Furthermore, renal function, tissue damage, and apoptosis were more severe in proximal tubules with a deletion of autophagy gene-related 7 (Atg7), a mitophagy-related protein, in comparison to the wild type mice (Jiang et al., 2012). Furthermore, renal tubular cells have an increase in autophagosomes in septicemia patients' kidney tissues, which is in contrast with hydropic mitochondria with cristae destruction. This pattern shows that damaged mitochondria are removed via mitophagy (Takasu et al., 2013). Further research revealed that p53 binds to Parkin protein, leading to an impairment of its translocation in mouse heart, thereby inhibiting mitophagy (Hoshino et al., 2013). Notably, the activation of p53 has been documented in AKI, with suggestions that the p53 mediated inhibition of mitophagy might play a role in the pathophysiology of AKI (Ishimoto & Inagi, 2016). Furthermore, Gibbs and collaborators, discovered that when serotonin [5-hydroxytryptamine (5-HT)]1F receptor, which is a physiological regulator of mitochondrial biogenesis, is deleted, the renal damage is exacerbated leading to worse recovery during AKI (Gibbs et al., 2018). Regarding Mfn2, Gall and collaborators, demonstrated that a knockdown of this gene in cultured primary proximal tubular epithelial cells resulted in increased apoptosis under metabolic stress (Gall et al., 2012). However, in contrast to this, the same authors later demonstrated that a conditional knockdown of Mfn2 in mice proximal tubule could accelerate renal recovery in IR induced AKI, by increasing epithelial cell proliferation, rather than apoptosis (Gall et al., 2015). All of these reports demonstrated, as reviewed by Braga and collaborators (Braga et al., 2022), that mitochondrial homeostasis is critical to the maintenance of normal kidney function. Thus, mitochondria dynamics represent possible mediators in AKI and can be modulated to counteract or develop effective therapies to this disease.

Mitochondria as a therapeutic target in kidney transplantation

AKI has been associated to the development of CKD and ESKD. For ESKD, kidney transplantation is the recommended treatment option. Kidney transplantation increases patient survival compared to maintenance dialysis and confers a better quality of life (Lo et al., 2020). However, due to a shortage of transplantable kidneys, a higher number of patients continue to be treated by dialysis while they are waiting to a compatible kidney.

The rate of acute transplant rejection and short term graft failure has significantly decreased thanks to advancements in tissue type matching and immunosuppressive regimes; however, maximizing long term graft performance is still difficult, particularly with kidneys from deceased donors (Lo et al., 2020). The outcomes from living donors are better than those from deceased donors. Nowadays, the universal method for preserving kidneys from donors to recipients is cold storage (CS) (Debout et al., 2015; Hosgood et al., 2011; Salahudeen & May 2008). If the donor is deceased, the kidney undergoes long hours of CS, while kidney from living donors is exposed to only a few hours. Regardless of the technique chosen to preserve the kidney (hypothermic machine perfusion or static CS), the kidney decreases its metabolic rate (Kay et al., 2009; O'callaghan et al., 2013).

Ischemia—a condition in which tissues lack blood—is associated with a decrease of oxygen and nutrients supplies that mark the beginning of the early events that cause harm in the deceased donor kidney (Bonventre, 1988). Warm ischemia and reperfusion, also referred to as ischemia−reperfusion damage, are introduced during transplantation via vascular anastomosis. Short and long term graft outcomes are impacted by IRI events during kidney transplantation (Stewart et al., 2017). Through the activation of enzymes such as xanthine oxidase, NADPH oxidase, uncoupled nitric oxide synthase (NOS), and mitochondrial respiratory complexes, IRI itself encourages and enhances ROS generation. Furthermore, the cessation of supplied oxygen during ischemia triggers renal tissue hypoxia (Haase, 2006). Renal function recovery requires enough reperfusion in the transplant to prevent hypoxic tissue harm. Even when molecular oxygen is restored by reperfusion, the vascular injury from IRI causes a drop in renal blood flow of up to 50% following reperfusion (Hellberg et al., 1991; Mason et al., 1984; Schrier et al., 2004; Thiel et al., 1982). This will, in turn, limit the ability to exchange solutes (Rosen et al., 1992) and produce a high rate of aerobic glycolysis (Cohen, 1979). In fact, it has been suggested that one typical route to renal failure following transplantation is chronic hypoxia in the tubulointerstitium (Nangaku, 2006). Few studies highlight the importance of mitochondrial dynamics during renal CS combined with transplantation (CS/Tx). Salahudeen and collaborators, using primary human proximal tubular cells, tried to elucidate the impact in the mitochondria pathophysiology in transplanted kidneys, when exposed to CS and after a rewarming process that is associated with the transplantation event (Salahudeen et al., 2001). The authors observed that, after 24 h of CS exposure, the cells presented mitochondrial swelling and a loss of inner mitochondrial membrane. Given the continued necrosis, the rewarming was linked to significant levels of apoptosis. Additionally, this study found that the antioxidants in the storage solution shielded the cells from the effects of CS and rewarning, which can cause necrosis and apoptosis (Salahudeen et al., 2001). Later on, Yin and collaborators developed a study in Wistar rats, where the main goal was to determinate which renal cells lose their viability (observed using Trypan blue

dye to identify the nuclei of nonviable cells) after warm and cold ischemia. Warm ischemia led to injury of proximal tubular cells, while cold ischemia caused renal failure at podocytes and peritubular endothelial cells level. Podocytes were blebbed, swelled, and their pedicels separated from the basement membrane in cold ischemia-induced damage, while proximal tubule disruption was less severe in comparison to the warm ischemia induced. Furthermore, in warm ischemic, electron microscopy demonstrated shedding of microvilli and marked swelling of mitochondria (Yin et al., 2002). Later on, Lewis rat kidneys were exposed to CS alone or CS combined with transplantation and proteins related to mitochondrial function and mitochondrial dynamics were evaluated (Parajuli et al., 2017). The results demonstrated that the treatment only with CS reduced respiratory complex I and II activities along with reduced expression of Drp1 protein. It also induced the loss of the OPA1 and an alteration of mitochondrial protease regulating OPA1 processing. Furthermore, in CS/Tx group, it was observed a reduction of complex I, II, and III activities as well as a loss of the short forms of OPA1, Mfn1 and Mfn2 proteins. It was also observed that after CS/Tx the expression of Drp1 protein was greatly reduced, along with the mitochondrial fission factor. Remarkably, Drp1 protein levels were unchanged in rats receiving autotransplants (transplants without CS exposure) (Parajuli et al., 2017).

Despite a lack of consistent studies, it is already noticed that mitophagy after CS/Tx is also affected. Indeed, a study performed in rat tubular epithelial cells demonstrated that autophagy is highly affected during CS rewarming. The inhibition of autophagy, through Atg5 siRNA, resulted in increased cleaved caspase-3 and increased apoptosis, demonstrating that this induction of apoptosis may be deleterious as a therapy to decrease delayed graft function (Jain et al., 2015).

Notably, lower ATP levels and ROS accumulation during renal CS/Tx are caused by mitochondrial respiratory dysfunction, which suggests the possibility that mitochondrial function is where "damage" signals from CS arise (Mitchell et al., 2011; Parajuli et al., 2017; Salahudeen, 1999; Salahudeen et al., 2003). Moreover, this mitochondrial respiratory dysfunction impairs the ion exchange/transport channel resulting in calcium accumulation (Devarajan, 2005; Kosieradzki & Rowiński, 2008), which in turn will activate several enzymes that are responsible for the occurrence of apoptosis (Orrenius et al., 2003). To overcome the issues of CS and rewarming, some solutions have been pointed out, also with repercussions on the mitochondrial level.

Schleef and colleagues conducted a comprehensive study involving mice subjected to various protocols. They employed a bilateral renal IRI model to evaluate acute injuries within the initial 24 h, and a less severe unilateral renal IRI model to assess chronic lesions at 1 month. Their objective was to investigate whether the application of mild therapeutic hypothermia (mTH) at 34°C during the ischemic period could offer protective effects on the structure and function of the kidneys against injuries (Schleef et al., 2022). An in vivo bilateral renal IR model led to an increase in plasma urea and acute tubular necrosis at 24 h. Moreover, 1 month following unilateral IR, there was a notable reduction in kidney atrophy and fibrosis. In terms of mitochondria fitness evaluation, mTH protected from mitochondrial dysfunction, through preservation of calcium retention capacity and OXPHOS. mTH effect was maintained in the long term (1 month), by decreasing fibrosis and renal atrophy, and abrogating chronic renal inflammation (IL-6, IL1-β and IL-10) (Schleef et al., 2022).

Since mitochondrial health is highly regulated to maintain kidneys physiology and function, exploring interventions to safeguard renal mitochondria during CS emerges as a valuable avenue for investigation, holding promise as potential therapeutic options. One example is the use of the pharmacological activation (NS11021) of the mitochondrial large conductance Ca^{2+} activated K^{+} (mitoBK) during CS in rats (Shrum et al., 2020). This activator immobilized the impaired renal mitochondrial respiration at the complex's levels, by providing protection against respiratory dysfunction at Complex II and partially protects Complexes III and IV. It also offers protection against protein tyrosine nitration as well as apoptosis (Shrum et al., 2020). All of these findings show that one of the main initiators of CS/Tx-induced kidney impairment is mitochondrial damage, leading to a significant decrease of mitochondrial fitness, that will in turn, result in the aggressiveness of the renal outcomes in kidney diseases.

Mitochondrial "medicine" is a new therapeutic approach focused on clinical use of mitochondrion (Picard et al., 2016). In fact, previous reports, already demonstrated that mitochondrial transplantation can improve the outcomes of multiple diseases, such as Parkinson's disease (Chang et al., 2016), liver IR injury (Hayakawa et al., 2016), brain stroke (Hayakawa et al., 2016), and heart diseases (Emani et al., 2017). Unfortunately, in kidney diseases, the best therapy is limited to preventive strategies, and lately dialysis and fluid management, which is not an effective solution. Therefore, the establishment of new therapeutic approaches is required to enhance the quality of patients' life with renal failure to solve the issues related with hemodialysis and organ transplantation (Chapman et al., 2016; Ibrahim et al., 2016).

Mitochondrial transplantation: A novel technique in evolution

Mitochondrial dysfunction is known to be related in multiple pathophysiology diseases, such as cancer, neurodegenerative diseases, cardiovascular, and renal failure. Thus, mitochondrial medicine reveals to be a novel and innovating therapeutic modality, which the treatment resembles only at the mitochondrial level.

Given that mitochondria are actually transferrable from one cell to another, the idea of mitochondrial transplantation is based on this fact. When mitochondria are harmed, in order to sustain the wounded cells, this can happen (Roushandeh et al., 2019). There are already reports documenting successful cases of mitochondrial transplantation. For instance, in response to cerebral ischemia, astrocytes have been found to secrete mitochondria via membrane vesicles (Falchi et al., 2013), which were subsequently successfully transported into neurons, improving cell survival signaling (Hayakawa et al., 2016).

Different contact modes, such as junction, cell fusion, and tunneling nanotube (TNT) development, allow for the migration of mitochondria between cells (Lu et al., 2017; Paliwal et al., 2018). In a work led by Islam and collaborators, mitochondrial transfer from mesenchymal stem cells (MSCs) to alveolar type II epithelial cells (ATII) restored the bioenergetic profile of ATII and improved survival in a mouse model of lipopolysaccharide-induced pneumonia (Islam et al., 2012). Concerning TNT formation, the transfer of functional mitochondria from healthy pheochromocytoma cells (PCs) to stressed PC cells via F-actin based TNTs reversed stressed cells in early stages of apoptosis in culture (Wang & Gerdes, 2015). In

the context of acute respiratory distress syndrome, the transfer of mitochondria from MSCs to alveolar macrophages through TNTs is recognized as a crucial pathway contributing to the in vivo antimicrobial effect of MSCs. Jackson and collaborators, in 2016, taken advantage of MSCs being self renewing multipotent adult stem cells, which can be isolated from different tissues and organs (Alhadlaq & Mao, 2004; Hoogduijn et al., 2007) where, in vivo conditions, they can be differentiated into multiple cell types (Pittenger et al., 1999).

Furthermore, acute respiratory distress syndrome preclinical models have demonstrated the protective benefits of MSCs. Jackson and collaborators demonstrated that coculture of MSCs with monocyte-derived macrophages enhanced the phagocytic capacity in MSC treatment in mouse with pneumonia *Escherichia coli* associated (Jackson et al., 2016). Additionally, they showed that MSCs underwent a substantial mitochondrial translocation to macrophages, at least in part, through structures like TNT. Hence, with this study, they demonstrated that mitochondrial donation via direct cell contact from MSCs to innate immune cells can be considered as a novel mechanism adopted on MSCs in acute respiratory distress syndrome (Jackson et al., 2016). In vivo models of asthma and COPD have also demonstrated the protective effects of mitochondrial translocation from MCS to bronchial epithelial cells via TNT (Ahmad et al., 2014; Li et al., 2014) as well as in vitro model of reperfusion injury in human umbilical vein endothelial cell culture (Liu, Ji, et al., 2014).

Theoretically, the process of mitochondrial transference seems simple; however it is questionable if the delivering of mitochondria into the cells is active or passive. To overcome some issues related to the mitochondrial internalization, some strategies are being implemented to improve the mitochondrial delivery. Mitochondria transfer can be based on the transfer RNA (tRNA) targeting therapy (Kolesnikova et al., 2004; Mahata et al., 2006; Wang et al., 2012), cell−cell fusion (Ma et al., 2005), microinjection (Pinkert et al., 1997; Yi et al., 2007), or even through peptide mediated mitochondrial delivery (PMD) (Liu, Chang, et al., 2014) (Fig. 20.2). Enhancing mitochondrial uptake and improving transplanting efficiency are the main goals of PMD. Moreover, the PMD and microinjection share some similarities. Because this technique does not involve artificial gene recombination, which is required in the tRNA mitochondrial targeting method, it is simple to apply in clinical practice (Liu, Chang, et al., 2014). Given that human MSCs lack the capacity to transfer mitochondria or fuse with cells that have mitochondrial disease and pathogenic mtDNA mutations, the PMD model seems to be more effective and productive than the cell−cell fusion intervention. Indeed, Cho and collaborators demonstrated that human MSCs can transfer mitochondria to the cells with compromised mitochondrial function. In a coculture of MSCs and 143B derived cells, they induced irreversible damage to the mtDNA and it was noticed that the function was restored through a direct transfer of intact mitochondrial particles rather than mtDNA alone (Cho et al., 2012).

PMD was firstly implemented in cell lines exhibiting myoclonic epilepsy with ragged red fibers (MERF) disease, carrying mtDNA 8344np A to G mutation, in the attempt to rescue mitochondrial disease. In this study, isolated healthy mitochondria from donor were delivered into MERF fibroblast though Pep-1 conjugation (Pep-1-Mito). After 3 days of treatment with Pep-1-Mito, it was observed a restoration of OXPHOS subunits (complex I, II, and IV), mitochondrial membrane potential, ATP synthesis, and a reduction of ROS production (Chang et al., 2013). Therefore, a promising treatment to prevent the pathogenesis of mitochondrial disorders is the transplantation of mitochondria using PMD (Fig. 20.2).

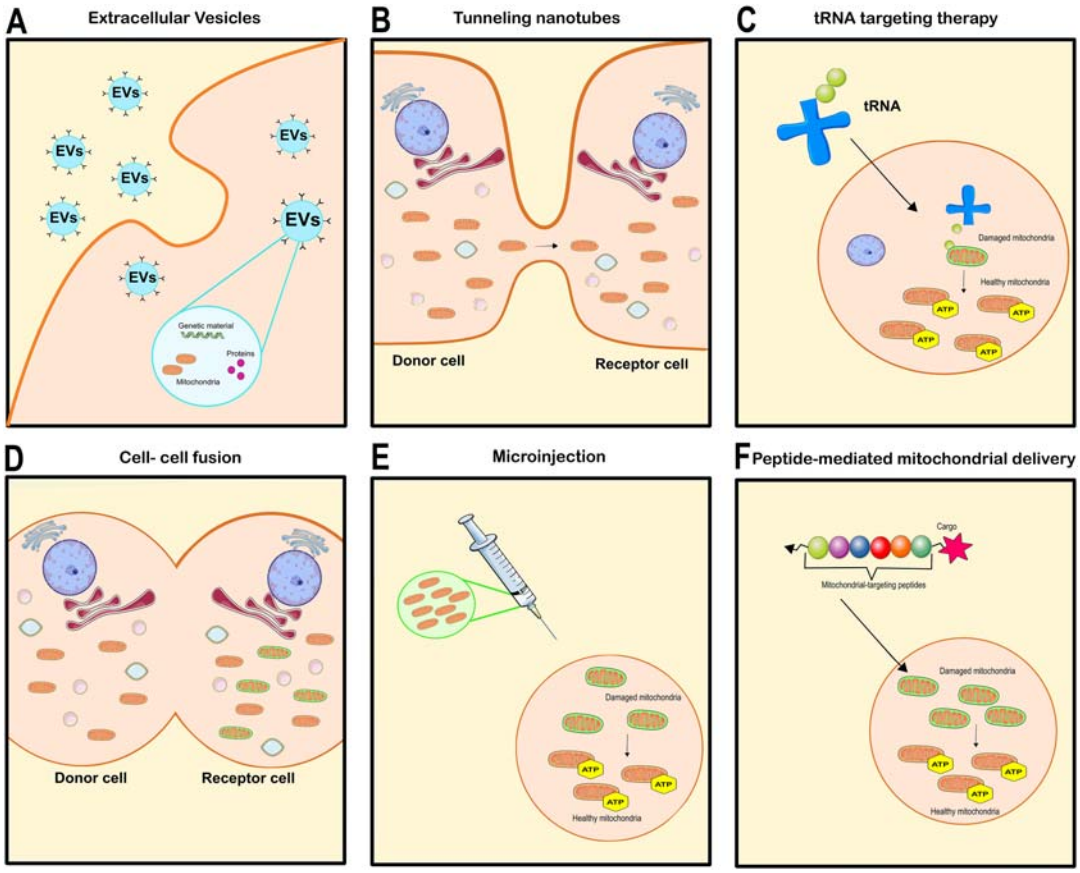

FIGURE 20.2 **Schematic illustration of the potential mechanisms by which mitochondria can be transplanted, including extracellular vesicles (Panel A), tunneling nanotubes (Panel B), tRNA targeting therapy (Panel C), cell–cell fusion (Panel D), microinjection (Panel E), and peptide-mediated mitochondrial delivery (Panel F).** The main objective of all the mechanisms is the turnout of damaged mitochondria into healthy mitochondria to reestablish mitochondrial quality control.

Furthermore, Wu and associates created a biocompatible polymer to improve in vivo applications for cellular transplantation, particularly mitochondrial transplantation into cardiovascular and cancer cells. Herein, the authors demonstrated that the polymer conjugate composed of dextran with triphenylphosphonium onto isolated mitochondria protects the organelles and facilitates cellular internalization compared with uncoated mitochondria, with an increase delivery rate of isolated mitochondria. Additionally, they noticed that in heart and breast cancer cell lines, the coating to preserve the mitotic respiratory function caused a notable change in the bioenergetic phenotype from glycolysis to OXPHOS (Wu et al., 2018).

The kidney, as with cancer and neurologic cells, requires a high amount of energy, and thus a correct mitochondrial functioning. In addition, considering renal diseases, it is important to address to the mitochondria a higher importance, since it has a role in the maintenance of cellular function and homeostasis. The reabsorption and control of water, glucose, and electrolytes like sodium ions are carried out by proximal tubules by active transport, which consumes a significant amount of ATP (Wang & Kestenbaum, 2018). Additionally, cytoskeletal changes brought on by mitochondria degeneration result in brush border destruction, a loss of cell to cell connections, and increased epithelial shedding (Ralto & Parikh, 2016; Tang & Dong, 2016; Zhan et al., 2013). Renal tubule healing requires morphologically improved renal tubular epithelial cells that are complemented by functional endogenous mitochondria (Konari et al., 2019), and so mitochondrial transplantation can be valuable solution to improve mitochondria fitness, which in turn will be reflected in kidney normal function recovery.

Mitochondrial transplantation in the kidney

Renal dysfunction or failure can result from a disruption or cessation in oxygen delivery because mitochondrial function and viability depend on a steady supply of oxygen delivered by the blood. The resulting tissue ischemia and disruption of oxygen delivery impacts mitochondrial energy production (Bhargava & Schnellmann, 2017; Bonventre & Weinberg, 2003; Ishimoto & Inagi, 2016). Mitochondrial dysfunction in the kidney has a key role in the onset of diseases including diabetic nephropathy (DN) and acute and chronic renal failure (Galvan et al., 2017; Tábara et al., 2014).

There are already some studies demonstrating that mitochondrial transplantation has protective outcomes in the heart and brain ischemia, resulting in an enhanced of cardiac function (Huang et al., 2013) and restoration of motor function in an ischemic rat brain (Huang et al., 2016). Furthermore, a growing number of studies indicate that mitochondrial dysfunction plays a significant role in AKI (Forbes, 2016; Ishimoto & Inagi, 2016; Ralto & Parikh, 2016). Several preclinical studies have used mitochondrial transplantation, including in a case of IRI in pig hearts (Guariento et al., 2020), spinal cord injury in rat (Gollihue et al., 2017), and IRI of brain rat (Zhang et al., 2019). Therefore, the rescue of cells and the return of normal function will be facilitated by supplementing or replacing the damaged mitochondria with transplanted viable mitochondria that have been separated from a healthy tissue and then delivered to the injured organ, tissue, or cells.

In stem cell based interventions, Togel and associates looked into the safety and long-term management of autologous and allogenic MSCs following AKI during a 3-month period, which is comparable to 3 to 4 years of follow-up. They showed that both autologous and allogeneic MSCs are effective in the treatment of AKI, particularly in retarding interstitial renal fibrosis and loss of renal function. Furthermore, the protection from chronic renal damage provided by MSCs was shown only in rats with severe AKI, suggesting that the degree of the initial injury may have an impact on this protection (Tögel et al., 2009).

Renal tubular epithelial cells can be damaged by renal ischemia, which can result in the loss of renal function (Emma et al., 2016). Nevertheless, following kidney injury, these tubular epithelial cells have some capacity to repair; however, the source of the cells responsible for this recovery remains unclear. Some studies suggested that there is a group of renal resident scattered tubular cells that undergo proliferation after injury (Ebrahimi et al., 2013; Emma et al., 2016). Extracellular vesicles (EVs) that are released from donor cells to target cells carry proteins and genetic material, making them a key intercellular communication mechanism. It is known that some EV can have and deliver mitochondrial materials that can increase the bioenergetic function in recipient cells (Fig. 20.3) (Boudreau et al., 2014; Guescini et al., 2010; Phinney et al., 2015). Zou and collaborators explored if the EV release could have a positive impact on scattered tubular cells in the repairing of injured tubular epithelial cells. They tested this hypothesis, using a proximal tubular epithelial cell line (PK1 cells), that was cocultured with stem-like cells (STC) isolated from fresh pig kidneys in three different conditions: STC-like cells, STC-like cell derived EV or EV free conditioned medium during 3 days. STC-like cells were intraarterially injected into mice 2 weeks after the induction of unilateral renal artery stenosis. They reported that STC-like cells EV carried and transferred mitochondrial material to injured tubular epithelial cells improvement in renal function and alleviated fibrosis in ischemic kidney injury. Furthermore, oxidative stress and mitochondrial function in tubular epithelial cells have been repaired in unilateral renal artery stenosis model in vivo and antimycin-induced injured proximal tubular epithelial cells in vitro (Zou et al., 2018).

Moreover, the administration of microvesicles derived from MSCs, reported by Bruno and collaborators, enhanced the recovery from glycerol-induced AKI in mice through stimulation of tubular cell proliferation (Bruno et al., 2009). In support to this, Souidi et al. provided evidence in favor of this by showing that the microvesicular structures of MSCs enabled mitochondrial transplantation to contribute to the regeneration of ischemic kidney tissue (Souidi et al., 2013).

Concerning mitochondria transplantation, Yuan and collaborators have also taken advantage from the MSCs and for the first time reported the mechanisms that are involved in the kidney protection in DN through MSCs mitochondrial transference. In streptozotocin (STZ) induced DN mice, it was demonstrated that PGC-1α mediated mitochondria synthesis and PGC-1α/TFEB mediated autophagy, which was able to reduce inflammation and ameliorate kidney damage by simply mitochondria transference from MSCs to macrophages (Yuan et al., 2021). Regarding autologous mitochondria transfer through intraarterial injection, there are few studies on experimental models of kidney focal ischemia. This method involves the injection of viable and respiration-competent autologous mitochondria extracted from the patient's healthy skeletal muscle to restore the functionality of native mitochondria that have been damaged by IRI (Fig. 20.3) (Roushandeh et al., 2019). Doulamis and collaborators investigated the effectiveness and safety of intraarterial autologous mitochondrial transplantation for renal protection in bilateral renal IRI (Doulamis et al., 2020). Following 60 min of ischemia, female Yorkshire pigs received 1×10^9 mitochondria in a single injection into each renal artery. Within the tubular epithelium of the cortex and medulla, the injected mitochondria were rapidly absorbed by the kidney. After 24 h of reperfusion, urine output and eGFR were significantly increased along with a decrease in serum creatine and blood urea nitrogen in pigs that received mitochondria. Furthermore, histopathological analysis revealed that

mitochondrial transplanted kidney presented only patchy mild acute tubular injury, while the kidney treated only with vehicle had extensive coagulative necrosis of the proximal tubules (Doulamis et al., 2020). Additionally, still in the kidney treated only with the vehicle, the renal cortex IL-6 expression was elevated in comparison to those who received mitochondrial transplantation. The authors thus suggested that their results obtained from a clinically relevant model allow a translation to humans, enhancing the knowledge of clinicians for the treatment of AKI (Doulamis et al., 2020). After this study, Jabbari and collaborators followed the same approach in mice, by examined the effect of auto transplantation of healthy mitochondria from muscle cells to injured kidney cells through injection into the renal artery.

They proved that renal tubular cell death could be avoided by mitochondrial transplantation (7.5×10^6 mitochondria/mL), which also increased renal tubules' capacity to regenerate, reduced IRI-induced apoptosis, and restored renal function (Jabbari et al., 2020). Kubat and collaborators explored the beneficial effects of isolated mitochondria transplantation of MSCs in vivo, to mitigate renal damage. The authors used rats as animal model and the kidney damage/renal injury was induced with the administration of a single dose of doxorubicin (6 mg/kg). The mitochondrial transplantation was performed in two separated injections (8×10^6 mitochondrial in 500 μL) on the lateral side of the renal cortex. After 10 days, the animals were sacrificed. The findings showed that proteinuria levels started to decrease 6 days after isolated mitochondria from MSCs had been inserted into the renal cortex. Furthermore, after transplanting mitochondria, tubular cells showed histological evidence of tubular regeneration and it was observed a rising antioxidant stress enzyme levels (SOD and GPx) against oxidative stress (Kubat, Ozler, et al., 2021). Later, using a similar methodology, the same authors investigated the outcomes of the direct injection of mitochondria isolated from MSCs into the renal cortex of rats experiencing renal ischemia (Kubat, Kartal, et al., 2021). In this study, it was administered a single dose of 2×10^7 mitochondria/mL and the rats were monitored for 48 h. This study demonstrated that mitochondrial transplantation, reduced apoptotic markers, including TUNEL and Caspase-3 in injured renal. Moreover, it promoted proliferation and regeneration of tubular cells. In terms of mitochondrial dynamics, the expression of Drp1 protein was decreased in tubular cells after injury (Kubat, Kartal, et al., 2021) (Fig. 20.3).

Konari et al. conducted a study where for the first time it was unveiled the potential therapeutic effects of mitochondrial transplantation from bone marrow derived MSCs in DN. They observed that mitochondria (1×10^4 mitochondria per gram of body weight), within 24 h, were incorporated into proximal tubular cells and affected SOD2, Bcl-2, Bax, and PGC-1α expression in STZ-induced diabetic mice and rats, which suppressed ROS production, and inhibition of apoptosis. Furthermore, it was noted that within 3 days, STZ rats' proximal tubules showed an induced histological improvement that led to the recovery of megalin and SGLT2 expression (Fig. 20.3). Thus, this study demonstrates that mitochondria transplantation plays a key role in the therapeutics of bone marrow−derived MSCs toward DN treatment (Konari et al., 2019).

Overall, all the studies so far undoubtedly highlight that mitochondrial transplantation can have a role in kidney disease. Still, further studies are needed to determine the conditions and the mechanisms by which it can be useful for each particular case of the plethora of kidney diseases.

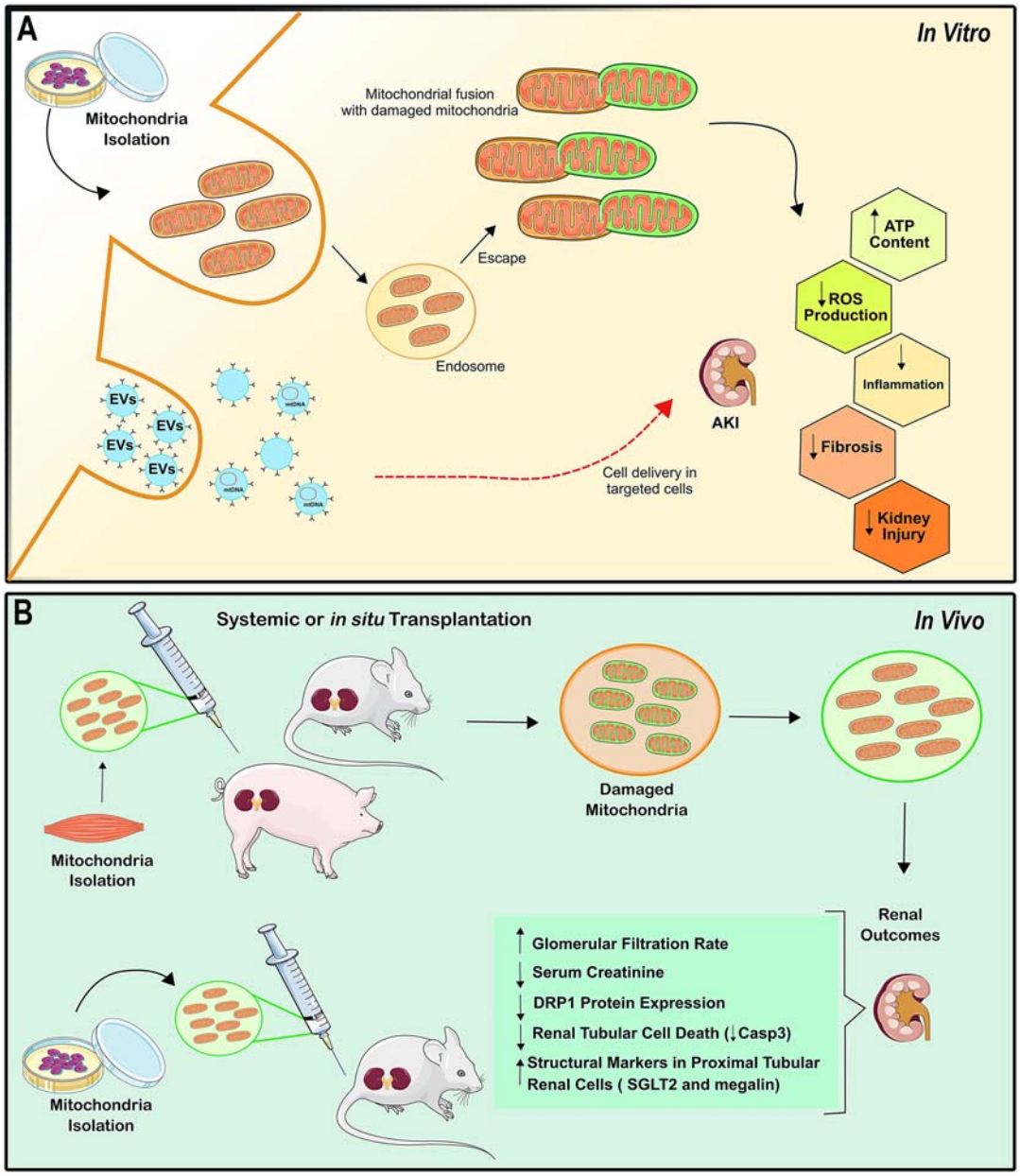

FIGURE 20.3 **Mitochondrial transplantation in different kidney cell models in vitro (Panel A) and in vivo (Panel B).** In vitro, the main mitochondria derived from MSCs, which enter in the target cell through endocytosis, and then there is a fusion between the healthy and the damaged mitochondria. Furthermore, EVs are released from donor cells to target cells that carry proteins and genetic and mitochondrial material that will contribute to an increase of renal function. With this approach, a decrease was observed in kidney injury, associated with an increase of ATP content, ROS production, and with a decrease in fibrosis and inflammation. Mitochondrial transplantation in vivo, in kidney disease, is only reported in mice, rat, and swine model. The isolation of the mitochondria can be from MSCs or even from skeletal muscle. The mitochondria are transplanted through microinjection technique, where their delivery can be systemic or in situ. After the injection of healthy mitochondria, the damaged ones are replaced, leading to a higher mitochondrial quality. The principal renal outcomes observed with this novel technique are an increase in glomerular filtration along with a decrease in serum creatine. A decrease was also observed in the expression of the Drp1 protein, and the renal tubular apoptotic markers, such as Casp3. Furthermore, an increase is observed in structural markers in proximal tubular renal cells including SGLT-2 and megalin. Abbreviations: *AKI*, acute kidney injury; *casp 3*, caspase 3; *EVs*, extracellular vesicles; *MSCs*, mesenchymal stem cells; *SGLT2*, sodium glucose transporter 2.

Conclusion

Mitochondrial dysfunction can be traced in multiple diseases including diabetes, ischemia, liver injury, aging, cancer, skeletal muscles disorders, neurodegenerative diseases, infertility, and even renal failure (Ma et al., 2017; McCully et al., 2016). Since tubular epithelial cells are among the most metabolically active epithelia in the human body, it is not surprising that the first pathological anomalies in kidney illnesses may manifest in tubular epithelial mitochondria following acute renal damage (Bhargava & Schnellmann, 2017). Thus, mitochondrial dysfunction is related to typical pathology in AKI (Thadhani et al., 1996). The proteins related to mitochondrial homeostasis, including biogenesis, fragmentation and mitophagy, which are processes that play important roles in AKI progression, highlight the importance to understanding the pathophysiology of AKI, and the mechanisms that link AKI to the progression of CKD (Wakabayashi & Karbowski, 2001) and mitochondria (dys)function. More specifically, as there is no effective treatment in kidney disease, despite dialysis and kidney transplantation, there is an urgent need to find new approaches that can rely on the mechanisms that link all the described processes in this chapter. Taking in consideration the role of mitochondria physiology and function in kidney, mitochondrial transplantation appears to be a safe approach, without major risks associated, with substantial improvements in mitochondrial functioning, and thus, it can be suggested as an alternative for the treatment of kidney diseases. Nevertheless, more studies are needed to understand how different mitochondrial transplantation procedures can be useful or implemented to treat distinct kidney diseases since this technique has limitations that need a special attention to be overcome. We are only now starting to unveil and understand how these mechanisms occur and can be useful and thus, exciting new works are expected in the years to come.

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