

Influência do epigenoma do espermatozóide no metabolismo embrionário

Soraia Henriques Pinto

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Influência do epigenoma do espermatozóide no metabolismo embrionário

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“(...). Os nossos systemas, as nossas theorisações, são provisórias e fallíveis, em face da limitação do nosso saber e das immensas difficuldades da investigação. (...) A verdadeira liberdade de pensamento é a liberdade absoluta da investigação, o direito inviolavel de concluir sobre o que é verdadeiramente acessível á evidencia, e conformar com isso a nossa opinião, independentemente de toda a auctoridade, de toda a ideia preconcebida, de qualquer fanatismo ou superstição. (...) A sciencia verdadeira é, pois, a escola do livre pensamento, e, como tal, eminentemente emancipadora. (...) O homem de sciencia verdadeira tem as suas convicções sobre estas materias, comprehende e respeita as dos outros e faz uma lei de nunca perturbar uma consciencia.”

Comunicações

“A importancia e dignidade da sciencia e as exigencias da cultura scientifica”

Discurso pronunciado na sessão da abertura solemne da Universidade do Porto, no anno lectivo de 1911-1912.

Professor Doutor A. J. Ferreira da Silva

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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 da 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 e auto-plágio constitui um ilícito académico.



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Resumo

A infertilidade é uma realidade cada vez mais evidente na nossa sociedade. O número de casais atingidos por esta doença tem aumentado significativamente. Os factores associados são muitos e variados o que destaca a necessidade de investigação na área. Para os casais que procuram a procriação medicamente assistida (PMA), o processo pode ser física e psicologicamente difícil, particularmente pela impossibilidade de garantia de sucesso.

Nos últimos anos temos assistido ao aumento dos estudos em desenvolvimento embrionário para identificação de biomarcadores preditivos da qualidade do embrião. Tem-se assistido à aplicação de um conjunto de novas tecnologias quantitativas e não invasivas, vulgarmente designadas por “Omics”, que têm vindo a contribuir para o esclarecimento dos mecanismos envolvidos no metabolismo ovocitário e embrionário. Dentro dessas novas tecnologias destaca-se a metabolómica, que se caracteriza pela sua alta capacidade de medições quantitativas de metabolitos presentes em células, tecidos e meios de cultura. Paralelamente, também tem aumentado o interesse pelo estudo do epigenoma do espermatozóide e a sua influência na qualidade espermática. Os microRNAs (miRNAs) são moléculas de RNA não codificantes que desempenham várias funções na proliferação e diferenciação celular, através da regulação pós-transcricional de inúmeros genes. Os miRNAs dos espermatozóides desempenham um papel fundamental nos processos celulares durante a fertilização. Perfis de miRNAs alterados têm sido associados a desenvolvimentos embrionários condicionados.

O principal objectivo deste trabalho foi correlacionar o epigenoma dos espermatozóides com o metabolismo embrionário. Adicionalmente, correlacionou-se os dados obtidos com a qualidade dos embriões, na tentativa de identificar possíveis biomarcadores metabólicos e epigenéticos com relevância para o sucesso das técnicas de PMA. O trabalho repartiu-se por três momentos: inicialmente, analisámos a importância que a preparação e selecção espermática poderia ter nas taxas de sucesso da PMA, de seguida avalámos o exometaboloma embrionário e a sua relação com a qualidade embrionária e, por fim, correlacionámos dois microRNAs espermáticos (miR-34c-5p e miR-191-3p) com a qualidade e o perfil metabólico do embrião.

Na revisão bibliográfica realizada foram discutidas diferentes estratégias de selecção espermática para recuperar os espermatozóides com maior potencial reprodutivo e o seu impacto nos resultados das técnicas de procriação medicamente assistida. Para além disso, são descritas as vantagens e desvantagens de cada método e as técnicas mais adequadas para cada situação clínica. Concluiu-se que, com alguns métodos avançados,

a selecção de espermatozóides é mais eficiente e existe até melhoria de alguns indicadores de PMA (taxa de fertilização e taxa de gravidez). Contudo, a maioria dos métodos ainda está em fase de análise e validação da sua aplicabilidade em contexto laboratorial. Nenhum método, convencional ou avançado, é a solução perfeita podendo até ser aplicados de forma complementar. As técnicas mais utilizadas para a preparação espermática continuam a ser o *swim-up* e os gradientes de densidade.

Para os estudos subsequentes, foram recolhidas amostras obtidas de casais em tratamento no Centro de Genética da Reprodução Prof. Alberto Barros (CGR). Consideramos elegíveis para o estudo os casais referidos para fertilização *in vitro* por injeção intracitoplasmática de espermatozóide (ICSI), para prevenir a interferência de possíveis células de cúmulos ou espermatozóides na quantificação metabolómica. Os procedimentos clínicos, laboratoriais e de estimulação ovária aplicados foram os estabelecidos na prática diária do CGR.

Para a estudo do exometaboloma foram analisados os meios de cultura de 55 embriões em desenvolvimento. As amostras foram recolhidas em dois momentos distintos: 3º (D3) e 5º dia (D5) de cultura embrionária. Obtiveram-se 110 amostras provenientes de 21 casais elegíveis para a investigação (55 amostras de D3 e 55 amostras de D5). Os embriões foram divididos de acordo com a classificação morfocinética atribuída ao D5: embriões de “boa qualidade” (n=26), “atrasados” (n=19) e de “má qualidade” (n=10). O perfil metabólico das amostras recolhidas foi analisado por ¹H-NMR e foram quantificados os seguintes metabolitos: piruvato, lactato, alanina, glutamina, acetato e formato. A análise estatística comparou os diferenciais de consumo e produção dos embriões, entre o 1º e o 3º dia e entre o 3º e o 5º dia de cultura embrionária, de acordo com a sua classificação morfocinética.

Os diferenciais quantitativos dos metabolitos durante os primeiros 3 dias de cultura embrionária mostraram ser preditivos da qualidade do embrião em fase de blastocisto (5º dia de cultura embrionária). Os metabolitos que mais contribuíram para esta discrepância são o piruvato, a alanina, a glutamina e o acetato. Embriões de melhor qualidade morfológica ou com atraso de desenvolvimento exportaram e acumularam piruvato e glutamina no meio, enquanto os embriões de pior qualidade os consumiram. Esta constatação pode sugerir que embriões de “qualidade má” têm um metabolismo menos activo comparativamente aos de “boa e média qualidade”. Do exposto, sugerimos que estes metabolitos possam ser utilizados como potenciais biomarcadores da qualidade embrionária em embriões obtidos por ICSI.

Para o estudo da influência do epigenoma (miRNAs) espermático no desenvolvimento embrionário foram utilizadas as amostras de 13 casais em tratamento. A avaliação espermática foi realizada segundo as orientações da Organização Mundial de Saúde e a

selecção dos espermatozóides foi conseguida com a aplicação de métodos convencionais de preparação espermática (gradientes de densidade e swim-up). A metodologia aplicada para o estudo do exometaboloma foi idêntica à descrita anteriormente. Os microRNAs em estudo - miR-34c-5p e miR-191-3p - foram quantificados por PCR. A análise estatística foi orientada no sentido de explorar a correlação entre a quantidade dos miR-34c-5p e miR-191-3p e todos os restantes dados recolhidos (qualidade seminal, exometaboloma dos embriões e classificação embrionária).

Os níveis de miR-191-3p não apresentam nenhuma relação com nenhum dos parâmetros espermáticos analisados. Contudo, este miRNA aparece associado ao consumo de glucose do embrião (na análise do exometaboloma) durante o seu desenvolvimento *in vitro*. Observámos ainda uma correlação negativa significativa entre os níveis de miR-34c-5p e a motilidade espermática. Também se demonstrou que níveis mais altos de miR-34c-5p nos espermatozóides estavam relacionados com a produção de piruvato, acetato e formato pelo embrião durante o seu desenvolvimento *in vitro*. Estes metabolitos podem estar associados a um melhor desempenho metabólico do embrião. Este trabalho fornece evidência que o conteúdo de miR-34c-5p nos espermatozóides não só está relacionado com a motilidade espermática como também é marcador da competência embrionária, destacando o potencial deste miRNA como biomarcador em PMA.

A avaliação da qualidade embrionária durante o desenvolvimento *in vitro* é um factor crucial para o sucesso da PMA. No entanto, a subjectividade inerente à avaliação morfocinética pode introduzir inconsistências que afectam a escolha do embrião com melhor potencial para transferência ou criopreservação. O estudo do exometaboloma fornece uma avaliação mais completa da viabilidade embrionária e pode contribuir para a melhoria dos resultados das técnicas de PMA. Assim, a possibilidade de integrar a análise do epigenoma do espermatozóide, a tecnologia metabolómica e a avaliação morfocinética embrionária pode permitir a monitorização não invasiva, em tempo real, da viabilidade embrionária. Adicionalmente, a informação recolhida pode ajudar a identificar biomarcadores associados com a qualidade embrionária, o que potencialmente tornariam a selecção embrionária mais consistente e objectiva contribuindo para o aumento da eficácia dos tratamentos de PMA.

Abstract

Infertility is an increasingly evident reality in our society. The number of couples affected by this condition has been significantly increasing. The associated factors are many and varied, highlighting the need for research in the field. For couples seeking assisted reproductive technologies (ART), the process can be physically and psychologically challenging, particularly due to the lack of guarantee of success.

In recent years, we have witnessed an increase in embryo development studies to identify predictive biomarkers of embryo quality. New quantitative and non-invasive technologies have been applied, commonly referred to as “Omics”, which have been contributing to the elucidation of the mechanisms involved in oocyte and embryo metabolism. Among these new technologies, metabolomics stands out for its high capacity for quantitative measurements of metabolites present in cells, tissues, and culture media. Additionally, there has been a growing interest in studying the sperm epigenome and its influence on sperm quality and embryo development. MicroRNAs (miRNAs) are non-coding RNA molecules that play various roles in cell proliferation and differentiation through the post-transcriptional regulation of numerous genes. Sperm miRNAs play a fundamental role in cellular processes during fertilization. Altered miRNA profiles have been associated with conditioned embryo development.

The main objective of this study was to correlate sperm epigenome with embryo metabolism. Additionally, the data obtained were correlated with embryo quality in an attempt to identify potential metabolic and epigenetic biomarkers relevant to the success of assisted reproductive technologies. The study was divided into three phases: initially, we analyzed the importance that sperm preparation and selection could have on the success rates of ART; next, we evaluated the embryo exometabolome and its relationship with embryo quality; and finally, we correlated two sperm microRNAs (miR-34c-5p and miR-191-3p) with embryo quality and metabolic profile.

In the literature review conducted, different sperm selection strategies were discussed to recover spermatozoa with the highest potential and their impact on the results of assisted reproductive technologies. Additionally, the advantages and disadvantages of each method are described, as well as the most suitable techniques for each clinical situation. We concluded that with some advanced methods, sperm selection is more efficient and there may even be improvement in some ART indicators (fertilization rate and pregnancy rate). However, most methods are still in the evaluation and validation stage of their applicability in a laboratory context. No method, conventional or advanced, is the perfect solution and

they may even be applied in a complementary manner. The most commonly used techniques for sperm preparation continue to be the swim-up and density gradient methods.

For subsequent studies, samples were collected from couples undergoing treatment at the Reproductive Genetics Clinic Prof. Alberto Barros (CGR). We considered eligible for the study couples referred for *in vitro* fertilization with intracytoplasmic sperm injection (ICSI) treatment to prevent interference from possible cumulus cells or spermatozoa in the metabolite quantification. The clinical, laboratory, and ovarian stimulation procedures applied were those established in the daily practice of CGR.

For the exometabolome study, culture media from 55 developing embryos were analyzed. The samples were collected at two different time points: on the 3rd (D3) and 5th day (D5) of embryo culture. A total of 110 samples were obtained from 21 couples eligible for the investigation (55 - D3 samples and 55 - D5 samples). The embryos were divided according to the morphokinetic classification assigned on D5: "good quality" embryos (n=26), "delayed" embryos (n=19), and "poor quality" embryos (n=10). The metabolic profile of the collected samples was analyzed by ¹H-NMR, and the following metabolites were quantified: pyruvate, lactate, alanine, glutamine, acetate, and formate. Statistical analysis compared the differences in consumption and production of embryos between the 1st and 3rd days and between the 3rd and 5th days of embryo culture, according to their morphokinetic classification

The quantitative differences in metabolites during the first 3 days of embryo culture were found to be predictive of embryo quality at blastocyst stage (on the 5th day of embryo culture). The metabolites that most contributed to this discrepancy were pyruvate, alanine, glutamine, and acetate. Embryos of better morphological quality or with developmental delay exported and accumulated pyruvate and glutamine in the medium, while embryos of poorer quality consumed them. This finding may suggest that embryos of poor quality have a less active metabolism compared to those of good and fair quality. From the above, we suggest that these metabolites could be used as potential biomarkers of embryo quality in embryos obtained by ICSI.

For the study of the influence of sperm epigenome (miRNAs) on embryo development, we used samples from 13 couples undergoing treatment. Sperm evaluation was performed according to the guidelines of the World Health Organization, and sperm selection was achieved using conventional sperm preparation methods (density gradients and swim-up). The methodology applied for the exometabolome study was identical to that described previously. The microRNAs under investigation - miR-34c-5p and miR-191-3p - were quantified by PCR. Statistical analysis aimed to explore the correlation between the quantities of miR-34c-5p and miR-191-3p and all other collected data (sperm parameters, embryo exometabolome, and embryo classification).

The levels of miR-191-3p do not show any relationship with any of the analyzed sperm parameters. However, this miRNA is associated with the embryo's glucose consumption (in exometabolome analysis) during its *in vitro* development. We also observed a significant negative correlation between miR-34c-5p levels and sperm motility. It was also demonstrated that higher levels of miR-34c-5p in sperm were related to the production of pyruvate, acetate, and formate by the embryo during its *in vitro* development. These metabolites may be associated with better metabolic performance of the embryo. This study provides evidence that the content of miR-34c-5p in sperm is not only related to sperm motility but also serves as a marker of embryo competence, highlighting the potential of this miRNA as a biomarker in ART.

The evaluation of embryo quality during *in vitro* development is a crucial factor for the success of ART. However, the inherent subjectivity in morphokinetic assessment can introduce inconsistencies that affect the embryos selection with the best potential for transfer or cryopreservation. Exometabolome analysis provides a more comprehensive assessment of embryo viability and can contribute to the improvement of ART techniques. Thus, the possibility of integrating sperm epigenome analysis, metabolomic technology, and morphokinetic embryo evaluation may allow for non-invasive, real-time monitoring of embryo viability. Additionally, the information gathered can help identify biomarkers associated with embryo quality, potentially making embryo selection more consistent and objective, thus contributing to increased efficacy of ART treatments.

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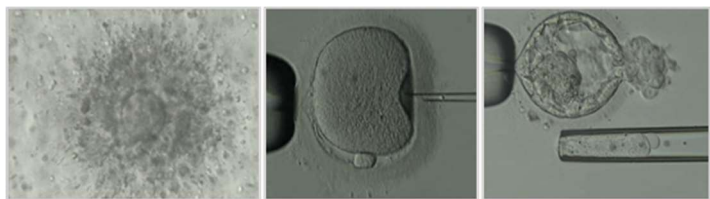
¹H-NMR	Proton Nuclear Magnetic Resonance
ART	Assisted Reproductive Technologies
ATP	Adenosina Trifosfato
BMI	Body Mass Index
cAMP	Cyclic Adenosine Monophosphate~
CASA	Computer-Assisted Semen Analysis Systems
COC's	Complexos ovócito-cúmulos
D3	Terceiro dia de cultura embrionária
D5	Quinto dia de cultura embrionária
DNA	Deoxyribonucleic acid
E₂	Estradiol
EIM-ESHRE	European IVF-Monitoring Consortium da European Society of Human Reproduction and Embryology
EOC	Estimulação Ovária Controlada
ESHRE	European Society of Human Reproduction and Embryology
FIV	Fertilização <i>in vitro</i>
FSH	Hormona Folículo Estimulante
GnRH	Hormona Libertadora de Gonadotrofinas
hCG	Gonadotrofina Coriônica Humana
HIV	Human Immunodeficiency Virus
hMG	Gonadotrofina Menopáusica Humana
HOST	Hypo-Osmotic Swelling Test
ICM	Inner cell mass
ICMART	International Committee for Monitoring Assisted Reproductive Technology

ICSI	Injecção Intracitoplasmática de Espermatozóide/ Intracytoplasmic Sperm injection
IMSI	Intracytoplasmic Morphologically Selected Spermatozoa Injection
IUI	Intrauterine Insemination
IVF	<i>in vitro</i> Fertilization
LAISS	Laser-assisted Immotile Sperm Selection
LH	Hormona Luteinizante
MACS	Magnetic Activated Cell Sorting
MCI	Massa Celular Interna
miRNAs	MicroRNAs
MSOME	Motile Sperm Organelle Morphology Examination
OCCs	Oocyte–Cumulus Complexes
OMS	Organização Mundial de Saúde
P	Progesterona
PCR	Polymerase Chain Reaction
PGT	Teste Genético Pré-Implantação
PGT-A	Teste Genético Pré-Implantação para o estudo aneuploidias
PGT-M	Teste Genético Pré-Implantação para o rastreio de doenças monogénicas
PGT-SR	Teste Genético Pré-Implantação para despiste de rearranjos estruturais cromossómicos
PICSI	Physiological Intracytoplasmic Sperm Injection
PMA	Procriação Medicamente Assistida
qPCR	Quantitative real time polymerase chain reaction
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
TCA	Tricarboxylic Acid Cycle
TE	Trofoectoderme

TEC	Transferência de embrião congelado
TrE	Transferência embrionária
WHO	World Health Organization
β-HCG	Gonadotrofina Coriônica Humana

CAPÍTULO 1

Introdução Geral



1 Introdução Geral

1.1 Enquadramento

Nos últimos anos, temos assistido a um aumento do número de casais inférteis. Este facto pode ser associado a factores sociais, como o adiamento do projecto de parentalidade, e a factores biológicos e bioquímicos, como a exposição crescente a disruptores endócrinos.

A infertilidade caracteriza-se pela impossibilidade de estabelecer uma gravidez clínica após 12 meses de relações sexuais regulares e desprotegidas, ou pela existência de condicionantes na capacidade de reprodução de uma pessoa, individualmente ou com o/a seu/sua parceiro(a) [1]. Há muito que a Organização Mundial de Saúde (OMS) considera a infertilidade como um problema de saúde pública e aponta algumas estratégias para aumentar a acessibilidade dos casais inférteis a tratamentos de procriação medicamente assistida [2]. Segundo dados publicados pela mesma entidade, uma em cada seis pessoas já teve um diagnóstico de infertilidade e, mundialmente, apesar de alguma variabilidade geográfica, a prevalência estimada da infertilidade ao longo da vida é de aproximadamente 17% [3]. Em Portugal, o único estudo realizado, em 2009, revela uma prevalência desta doença entre 9-10% [4].

No último relatório publicado pelo International Committee for Monitoring Assisted Reproductive Technology (ICMART), em 2018, foram realizados, a nível mundial, mais de 3 milhões de tratamentos de procriação medicamente assistida (PMA), com o nascimento de cerca de 760.000 crianças [5]. A nível europeu, segundo o European IVF-Monitoring Consortium da European Society of Human Reproduction and Embryology (EIM-ESHRE), de 1997 até 2019, foram realizados cerca de 12 milhões de tratamentos de PMA, que resultaram em mais de 2 milhões de crianças nascidas [6]. Em Portugal, no ano de 2020, nasceram 2797 crianças com recurso a várias técnicas de PMA. Este valor representa 3.3% do número total de crianças nascidas nesse ano [2].

Do exposto, pode constatar-se a importância que a procriação medicamente assistida já apresenta em termos médicos e sociais. Em países como Portugal, com uma baixa taxa de natalidade e onde o envelhecimento da população é uma preocupação, a PMA pode contribuir para o equilíbrio da pirâmide etária e para o aumento da população. É uma área em crescimento, que nos últimos anos apresentou uma evolução médica e tecnológica acentuada, que se traduziu numa melhoria das taxas de sucesso. Contudo, apesar dos avanços nos protocolos de estimulação ovárica e nas técnicas laboratoriais, a percentagem de casais que vê o seu projecto de parentalidade alcançado por PMA está muito aquém do desejável. Analisando os dados de tratamentos já realizados, sem recurso a doação de

gâmetas ou teste genético pré-implantação, a taxa de nado-vivo por transferência embrionária ronda os 25%, a nível europeu; em Portugal, é de aproximadamente 23% [2, 6]. Estes valores são reveladores da necessidade de alargar e aprofundar o conhecimento em biologia e bioquímica da reprodução, contribuindo assim para a optimização dos tratamentos de PMA.

1.2 A Procriação Medicamente Assistida

O termo PMA engloba um conjunto de procedimentos médicos e de técnicas laboratoriais que têm como objectivo auxiliar na concepção de uma criança quando a gravidez não ocorre de forma natural. Actualmente, com a mudança da lei em Portugal, as mulheres sem parceiro e os casais de mulheres podem recorrer à PMA. Contudo, a maioria das pessoas que recorrem aos tratamentos continuam a ser casais heterossexuais com problemas de infertilidade.

Da definição de infertilidade está implícito o conceito de que um casal só deve procurar ajuda médica após um ano de insucesso na obtenção de gravidez de forma natural, mas os estudos sugerem que, dependendo da idade do elemento feminino, o início do estudo da infertilidade no casal poderá ter de ser antecipado [1]. A idade materna é crucial na obtenção de uma gravidez, quer de forma natural quer com recurso a tratamentos de PMA [7]. No caso de mulheres acima dos 35 anos, a procura de ajuda médica deve iniciar-se ao fim de 6 meses de ausência de gravidez espontânea. Em mulheres acima dos 40 anos, e nos casos em que já existe uma condição médica que pode condicionar a fertilidade, a avaliação clínica deve ser iniciada de forma mais imediata [5]. A avaliação do casal infértil é um processo de investigação clínica que, como qualquer outra avaliação médica, assenta numa anamnese detalhada e em exames complementares de diagnóstico. O objectivo principal é determinar qual a causa da infertilidade (diagnóstico) e, em consequência, estabelecer o plano de tratamento mais adequado.

Os factores de infertilidade podem ser de causa masculina ou feminina, e incluem as disfunções ovulatórias (25%), os factores tubários (20%), os factores masculinos (30%) e as disfunções uterinas e tubo-peritoneais (10%). Apesar dos avanços médicos, em 25% dos casais, a causa de infertilidade não é identificada, sendo classificada como inexplicada ou idiopática [8]. É importante salientar que a infertilidade devida a factores masculinos representa 30% dos casos, mas quando associada a factores femininos esse valor sobe para 50%. Isto significa que o casal é a unidade fundamental de estudo para um diagnóstico de infertilidade, devendo os dois elementos serem avaliados em simultâneo para otimizar o processo [9, 10].

Existem várias técnicas utilizadas em PMA e que são aplicadas de acordo com o diagnóstico de infertilidade que o casal apresenta. A menos invasiva é a inseminação intrauterina, considerada uma técnica de primeira linha, cuja utilização é limitada a casos de infertilidade menos graves. Como técnicas de segunda linha, e tecnicamente mais exigentes, existem a fertilização *in vitro* (FIV) e a injeção intracitoplasmática de espermatozóide (ICSI). De realçar que, se um dos elementos do casal é estéril ou se um, até ambos, tem gametogénese condicionada, é possível a realização de todas as técnicas mencionadas, mas com recurso a gâmetas doados (Figura 1.1).

Uma das principais causas de perdas embrionárias muito precoces e de abortamentos no primeiro trimestre de gravidez são os desequilíbrios cromossómicos [11]. A alta incidência de aneuploidias (número anormal de cromossomas) em gâmetas e embriões humanos é uma das principais causas de insucesso nos tratamentos de PMA [12]. O facto de actualmente a maternidade ser um projecto de vida muitas vezes adiado em muito contribui para esta alta incidência, uma vez que a idade materna avançada confere um risco acrescido para as aneuploidias ovocitárias [13].

O teste genético pré-implantação (PGT) é uma técnica utilizada em PMA, que permite analisar o material genético de embriões antes de serem transferidos para o útero. O objectivo principal é identificar os embriões sem alterações genéticas específicas e com isto aumentar a probabilidade de obtenção de uma criança saudável. Para os casais em que existe um risco elevado de transmissão à descendência de doença genética (cromossómica ou génica), a realização do teste genético pré-implantação, com transferência de embriões não portadores da patologia, é uma alternativa ao diagnóstico pré-natal, que em algumas situações culmina com a interrupção médica da gravidez [10].

As unidades de PMA são compostas por equipas multidisciplinares de profissionais (médicos de várias especialidades, embriologistas, enfermeiros e psicólogos) que acompanham o casal desde o diagnóstico até à realização do tratamento. Os laboratórios de PMA são estruturas altamente especializadas e monitorizadas. São equipados com tecnologia avançada para a realização de todos os procedimentos inerentes à área de embriologia, andrologia e criobiologia. O objectivo principal de um tratamento de PMA é a obtenção de uma gravidez evolutiva de acordo com as melhores práticas clínicas, salvaguardando, simultaneamente, a segurança e o bem-estar dos pacientes.

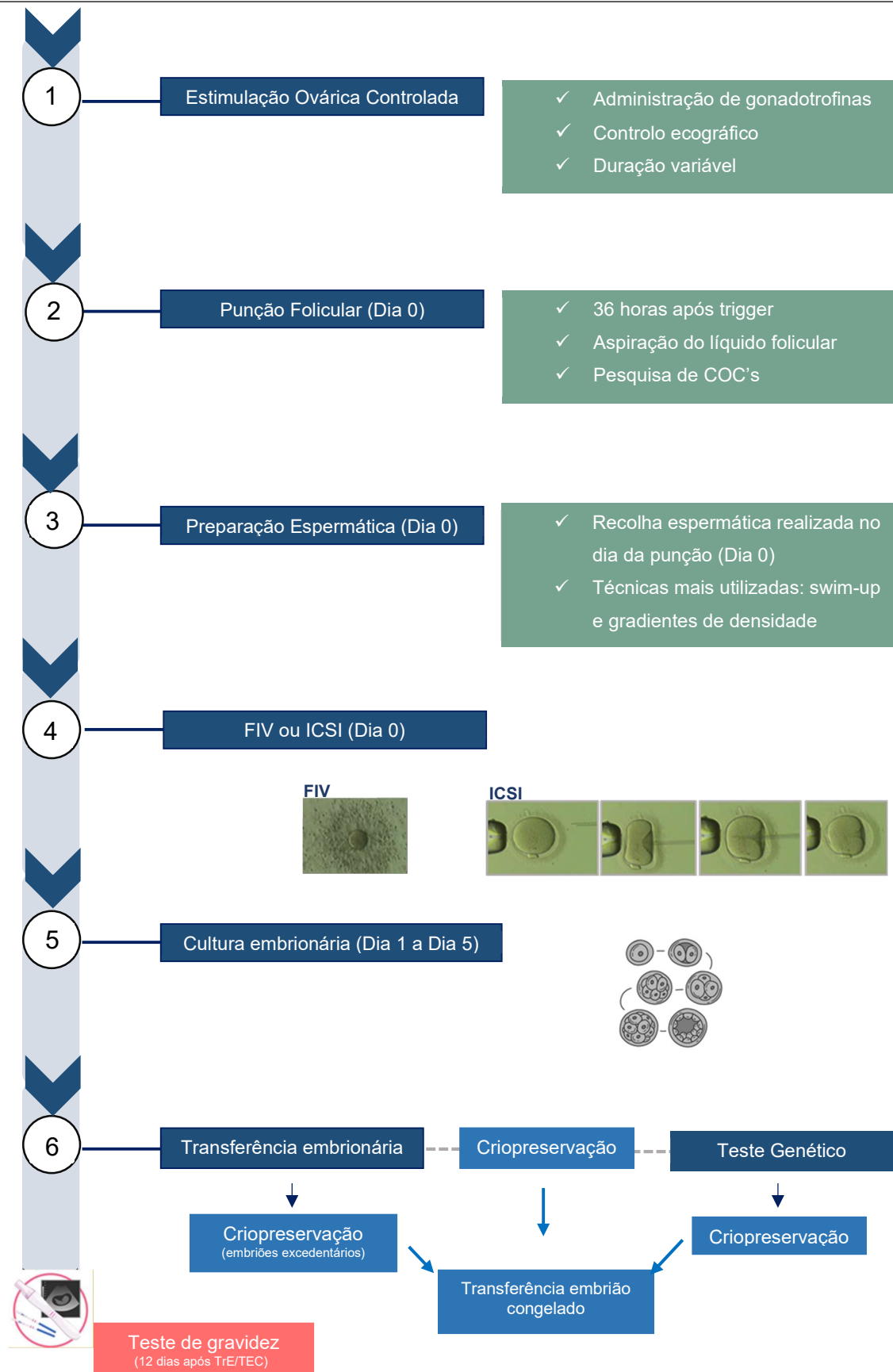


Fig. 1.1 Esquema das etapas de um ciclo de tratamento de PMA. Desde o dia da punção folicular até à TrE, criopreservação ou PGT os embriões são mantidos em cultura controlada (5 dias). Nos casos de PGT e/ou criopreservação de embriões a transferência dos embriões é realizada em TEC (ciclo posterior).

1.3 Desenvolvimento folicular

Um dos aspectos fundamentais de todo o processo de PMA é a estimulação ovárica com recurso a vários protocolos. A gestão da estimulação ovárica controlada medicamente, e monitorizada ecograficamente, assenta no conhecimento da endocrinologia básica de um ciclo normal de ovulação. É durante o desenvolvimento fetal que fica estabelecida a reserva de ovócitos a ser utilizada durante toda a vida reprodutiva da mulher. As ovogónias são as células precursoras dos ovócitos e a sua actividade mitótica é crucial para o estabelecimento da reserva inicial de ovócitos. É a partir destas células germinativas que os ovócitos se vão desenvolver e diferenciar [14]. Grande parte das células germinativas sofre apoptose antes da formação dos folículos ováricos. Aos cinco meses de desenvolvimento fetal, os ovários contêm aproximadamente 6 milhões de ovogónias e esse valor decresce acentuadamente até ao nascimento [15]. As restantes ovogónias iniciam o processo de divisão meiótica. Após o início da meiose, a proliferação mitótica já não é possível e fica estabelecido o número finito de ovócitos (ovócitos primários), que nesta fase estão rodeados por uma camada de células epiteliais - folículo primordial. Os ovócitos primários passam por várias fases transitórias da primeira meiose e ficam retidos na prófase I (fase de diplóteno). Estes ovócitos podem entrar em atresia ou retomar a meiose, após a activação hormonal existente na ovulação. Assim, os folículos primordiais podem ficar quiescentes durante anos, até ao recrutamento folicular, constituindo a reserva folicular que vai diminuindo ao longo da vida da mulher (0,5 milhões de folículos primordiais na menarca) [14, 16-18].

A regulação de todos os processos, envolvendo a oogénese e o desenvolvimento folicular, depende de um sistema hormonal que é coordenado por uma interacção complexa entre o hipotálamo, a hipófise e os ovários. Na puberdade, o aumento da produção da Hormona Libertadora de Gonadotrofinas (GnRH) induz a maturação do eixo hipotálamo-hipófise e o ovário torna-se receptivo à acção das hormonas hipofisárias, iniciando-se o recrutamento folicular. A GnRH é segregada de forma pulsátil pelo hipotálamo e induz a produção de gonadotrofinas pela hipófise anterior. As gonadotrofinas são essenciais para o desenvolvimento dos folículos ováricos e para a ovulação. A hormona folículo-estimulante (FSH) é uma hormona que actua a nível do ovário, estimulando o desenvolvimento folicular, e a hormona luteinizante (LH) é uma hormona que regula o ciclo menstrual e a ovulação [19].

Do ponto de vista fisiológico, durante a vida reprodutiva da mulher, a maioria dos folículos vai sofrer atresia por apoptose e apenas alguns vão ser recrutados para desenvolvimento, diferenciação e ovulação. Apesar do processo acontecer de forma contínua, o ciclo de vida de um folículo de Graaf ou dominante, pode ser dividido em três fases: inicial, que ocorre desde o nascimento e é independente de suporte por gonadotrofinas; a fase de

crescimento e recrutamento, que é dependente da presença de FSH; a fase de maturação folicular, que é dependente da presença de LH e que culmina com a ovulação [20].

Quando um folículo primordial inicia o seu crescimento, passa por diversas transformações: o aumento de tamanho do ovócito e a proliferação e diferenciação das células foliculares em células da granulosa. Há a formação de uma membrana glicoproteica protectora a envolver o ovócito (zona pelúcida) e o folículo passa a designar-se por folículo primário. Segue-se uma intensa actividade mitótica das células da granulosa, com formação de uma estrutura vascularizada adjacente ao folículo (teca interna), dando origem ao folículo secundário ou pré-antral. Todas estas fases da foliculogénese, desde folículo primordial até à formação do pré-antral, são independentes do suporte das gonadotrofinas. O desenvolvimento folicular, a partir do folículo pré-antral, é hormono-dependente e, portanto, só acontece na presença de gonadotrofinas [21].

As alterações hormonais características do início do ciclo menstrual induzem o crescimento de alguns folículos (recrutamento folicular). O aumento das concentrações de FSH e LH, libertadas pela hipófise anterior, marca o início da fase folicular. Nesta fase os folículos apresentam um diâmetro de 0.2-0.4 mm com múltiplas camadas de células da granulosa. Sob a influência da FSH, o número de receptores de FSH nas células da granulosa aumenta e, concomitantemente, as células da granulosa iniciam a produção de estradiol (E_2). A produção de estradiol também está dependente da LH, uma vez que é através do estímulo desta hormona que as células da teca convertem colesterol em androgénios. Estes, por sua vez, são convertidos em estrogénios nas células da granulosa, por acção da enzima aromatase. A contínua proliferação das células da granulosa leva à produção de líquido folicular entre as células, o que origina a formação de uma cavidade denominada antro - folículo antral. Os folículos antrais produzem quantidades crescentes de estradiol e inibina B, que, por retroalimentação negativa no hipotálamo e na hipófise, provoca o declínio na produção de FSH. Enquanto os folículos antrais, que ainda dependem da FSH para o crescimento, entram em atresia, o folículo dominante ou de Graaf, que já apresenta receptores para a LH nas células da granulosa, continua a crescer apesar dos níveis em declínio da FSH. A selecção de um único folículo dominante, de entre o total de folículos recrutados, está associada com o seu desenvolvimento mais acentuado e à sua maior capacidade de conversão de androgénios em estrogénios. O folículo dominante continua o seu crescimento e a sua produção de estradiol até à concentração mínima requerida para desencadear o pico de LH que antecede a ovulação. Aquando da ovulação, antes da libertação do ovócito, termina a primeira e inicia-se a segunda divisão meiótica com a formação de um ovócito secundário retido em metáfase II. Após a ovulação, na fase lútea, as células da granulosa que permanecem no folículo remanescente passam por uma alteração estrutural e funcional dando origem ao corpo lúteo responsável pela

produção de inibina B, de estrogénios e de progesterona (esta última em maior concentração). Os níveis elevados de progesterona associados à concentração de inibina B e estrogénios impedem, por retroalimentação negativa do eixo hipotálamo-hipófise-ovário, a produção de gonadotrofinas para que não exista crescimento folicular, no mesmo ciclo, após a ovulação. Na ausência de gravidez, o corpo lúteo regride. Com a diminuição da concentração sérica de estrogénios e progesterona, daí resultante, a inibição da hipófise deixa de existir o que permite nova produção de FSH e LH, dando-se início a novo ciclo de recrutamento folicular [21-25].

A diferenciação folicular, desde folículo primordial até ao folículo pré-ovulatório, pode durar vários meses e só as duas últimas semanas são dependentes da presença de gonadotrofinas. Por essa razão, só nesta fase é que é possível existir alguma manipulação exógena do crescimento folicular [18].

1.4 Estimulação ovárica controlada (EOC)

A primeira criança nascida por PMA foi o resultado de um tratamento de fertilização *in vitro*, com um ovócito recuperado por laparoscopia em ciclo natural, ou seja, sem qualquer tipo de estimulação ovárica [26]. A constatação que a optimização do tratamento teria de passar obrigatoriamente pela obtenção de mais do que um ovócito maduro induziu o desenvolvimento da utilização de fármacos para a estimulação do crescimento folicular e maturação ovocitária [27]. A estimulação ovárica consiste num tratamento farmacológico, que tem como objectivo o desenvolvimento de folículos ováricos, e que permite a obtenção de múltiplos ovócitos aquando da aspiração folicular [1]. Dos vários factores que podem condicionar a qualidade ovocitária, no decorrer de um tratamento de PMA, o tipo de protocolo usado na estimulação ovárica é um dos mais relevantes [28].

Num ciclo de estimulação ovárica controlada (EOC), a administração exógena de gonadotrofinas ajuda a manter as concentrações de FSH acima do limiar mínimo até ao final da fase folicular, ampliando a janela de tempo para o recrutamento folicular. Desta forma, é possível estimular o crescimento de vários folículos simultaneamente. Na estimulação controlada para PMA, uma das preocupações é que o estradiol produzido por estes folículos pode resultar num pico precoce de LH endógeno [29]. Por esta razão, na EOC, para além da administração de gonadotrofinas exógenas para estimular o desenvolvimento folicular, é necessária a administração concomitante de substâncias agonistas ou antagonistas da GnRH para supressão da hipófise [30].

O protocolo de estimulação ideal deve ser o mais personalizado possível, por forma a reduzir os potenciais riscos e os efeitos secundários sem comprometer o sucesso do tratamento. Ao longo das últimas décadas, os protocolos de estimulação têm evoluído ao

ritmo do conhecimento da biologia da reprodução e do desenvolvimento tecnológico. Actualmente, existem dois tipos de gonadotrofinas: as urinárias e as recombinantes. As moléculas recombinantes são as mais utilizadas. Contudo, a gonadotrofina menopáusica humana (hMG), extraída da urina de mulheres pós-menopáusicas, é uma gonadotrofina urinária que ainda se utiliza com alguma frequência. As gonadotrofinas recombinantes, isto é, sintetizadas através de técnicas de engenharia genética, são várias e incluem a folitropina, a corifolitropina e a lutropina [31]. A EOC, num mesmo ciclo, pode ser obtida com o recurso a apenas uma molécula ou utilizando várias moléculas combinadas.

A dose inicial de gonadotrofinas é definida clinicamente de acordo com a idade da mulher, índice de massa corporal, reserva ovária (determinada pela contagem dos folículos antrais e/ou pela quantificação sérica da hormona anti-mülleriana) e a resposta ovária a estimulações anteriores [32]. Existem vários protocolos para a EOC (Figura 1.2). Os mais utilizados são o protocolo agonista da GnRH longo luteínico, o protocolo agonista da GnRH curto e o protocolo antagonista da GnRH. O protocolo agonista longo inicia-se com a administração do agonista de GnRH, no 21º dia do ciclo anterior, e a estimulação é iniciada após verificada a inibição ovária (aproximadamente no 2/3º dia do ciclo menstrual subjacente). O protocolo agonista curto é iniciado ao 2º dia do ciclo com a administração simultânea do agonista da GnRH e da gonadotrofina. Em ambos, a administração do agonista da GnRH e da gonadotrofina é mantida até à decisão do desencadeamento da maturação final ovocitária. No protocolo antagonista da GnRH, a estimulação com gonadotrofinas é iniciada no 2º/3º dia do ciclo menstrual. Após o 6º dia de estimulação, quando existe pelo menos um folículo de 12/14mm, é iniciada a administração do antagonista da GnRH. A administração do antagonista da GnRH e da gonadotrofina é mantida até à indução da maturação final ovocitária. Em todos os protocolos, a resposta ovária à estimulação é avaliada através da monitorização ecográfica transvaginal e da quantificação dos níveis séricos de E_2 [29, 33].

A última fase da EOC é a maturação final do ovócito (trigger) que se realiza 36 a 38 horas antes da punção folicular. A maturação final ovocitária necessita da actuação da LH, o que é alcançado com a administração da gonadotrofina coriónica humana (hCG) ou com a administração do agonista da GnRH. A hCG tem sido usada como indutor da maturação final ovocitária porque exerce funções semelhantes às da LH endógena (estrutura molecular análoga) e o agonista de GnRH actua induzindo o pico endógeno de LH a nível da hipófise [29, 34].

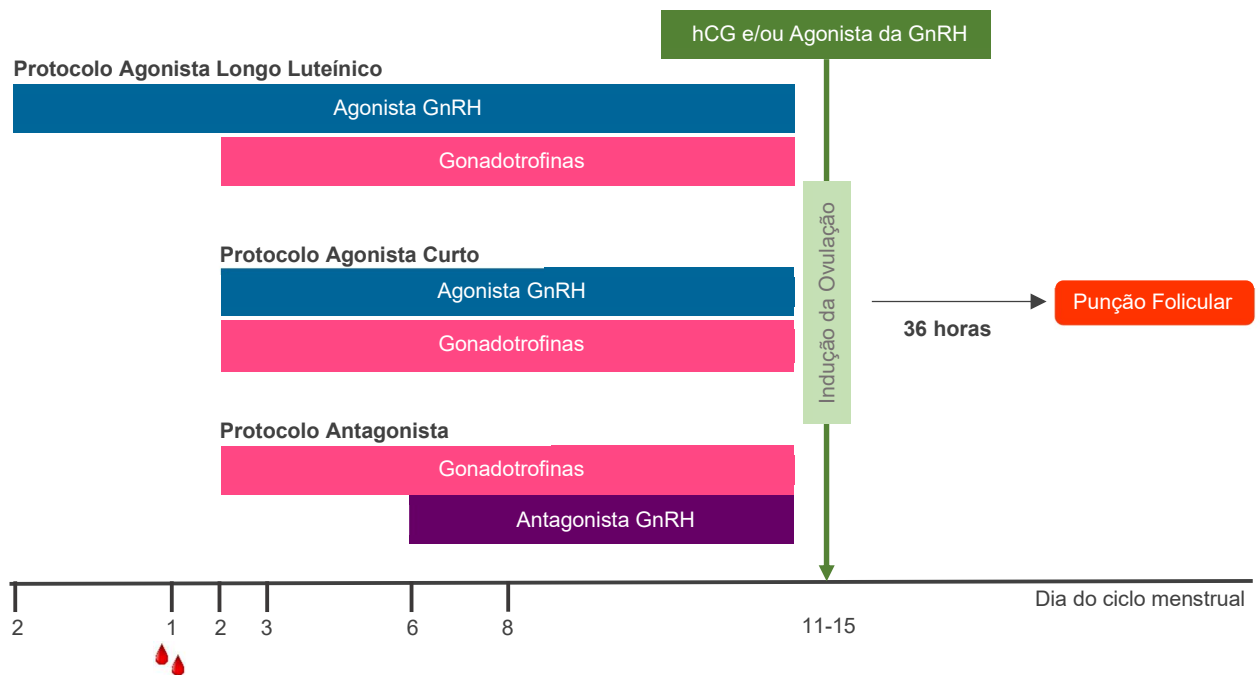


Fig. 1.2 Protocolos de EOC. Protocolo agonista longo luteínico, Protocolo agonista curto e Protocolo antagonista. O dia da maturação final ovocitária (trigger) depende da resposta ovárica que é avaliada ecograficamente. A recolha dos ovócitos é realizada 36 horas após a maturação final ovocitária independentemente do tipo de EOC.

1.5 Punção Folicular

A punção folicular é um procedimento médico que tem como objectivo a aspiração dos folículos e é habitualmente realizada sob sedação [35]. Após as 36 horas da maturação final ovocitária (etapa final da EOC), a punção é executada sob controlo ecográfico e através de uma agulha acoplada à sonda ecográfica transvaginal; o líquido folicular é aspirado para posterior pesquisa no laboratório dos complexos ovócito-cúmulos (COC's) recolhidos.

O líquido folicular aspirado é observado ao estereomicroscópio, por um embriologista, para identificação e recolha dos COC's. Todas as manipulações são realizadas em câmara de fluxo laminar e com superfície aquecida a 37°C. Os COC's encontrados são colocados em placas com meio de cultura pré-equilibrado coberto com uma camada de óleo mineral. São mantidos em cultura, em incubadoras com condições de temperatura, humidade e gasificação controladas (37 °C, 6% CO₂, 5% O₂, e 90% de humidade), até à realização da fertilização *in vitro* [36].

1.6 Técnicas de preparação e selecção de espermatozoides

As técnicas de selecção espermática em PMA têm como objectivo o isolamento de espermatozoides com alta capacidade de fertilização que possam levar à obtenção de uma gravidez evolutiva. Ao longo dos anos, o conhecimento da fisiologia da reprodução levou ao desenvolvimento de técnicas mais sofisticadas de preparação espermática, com vista a separar de forma mais efectiva os espermatozoides viáveis dos imaturos, imóveis ou de menor potencial de fertilização.

A preparação espermática, no contexto do tratamento de infertilidade, pretende eliminar o plasma seminal, eliminar *debris* e contaminantes, concentrar os espermatozoides com mobilidade progressiva rápida e seleccionar os que têm a melhor morfologia. O método de preparação deve ser escolhido em função das características espermáticas da amostra. [37]. As técnicas possíveis são várias, embora as mais utilizadas sejam a lavagem simples com meio de cultura, o *swim-up* e o uso de gradientes de densidade [38]. No caso de indivíduos azoospermicos ou com alterações graves da espermatogénese, convém realçar que existe a possibilidade de recolha de espermatozoides directamente dos testículos, por biópsia, e a preparação da amostra pode ir desde a lavagem simples com meio de cultura até à digestão e lise enzimática [39]. A discussão da importância e das técnicas actualmente disponíveis para a selecção espermática em PMA será detalhada no capítulo 3.

1.7 Técnicas de Fertilização *in vitro*

Em termos laboratoriais, as técnicas de fertilização que podem ser aplicadas são duas: fertilização *in vitro* convencional (FIV) e a fertilização *in vitro* com microinjecção intracitoplasmática de espermatozóide (ICSI). A maioria dos procedimentos clínicos e laboratoriais são comuns nas duas técnicas, diferem apenas na forma como os ovócitos são fertilizados. Na FIV os ovócitos são colocados na presença de uma concentração definida de espermatozoides e, eventualmente, um espermatozóide fertiliza o ovócito, ou seja, a entrada do espermatozóide no ovócito é dependente apenas da competência dos gametas envolvidos. Na ICSI, um embriologista, com recurso a um microscópio invertido acoplado a um sistema de microinjecção, selecciona um único espermatozóide de uma amostra de espermatozoides previamente preparada e injecta-o directamente no citoplasma do ovócito [40]. A FIV é um processo menos invasivo porque há menos manipulação dos gametas o que a torna num processo mais próximo da fertilização *in vivo*. A injecção intracitoplasmática de espermatozóide foi apresentada em 1992 como uma nova ferramenta para melhorar a fertilização em casais que recorriam à PMA, na sequência de um diagnóstico de infertilidade de causa masculina grave [41]. Foi uma técnica

revolucionária, no sentido em que passou a ser possível obter fertilização e gravidez em casais em que a qualidade espermática estava muito reduzida. Ela contorna várias barreiras biológicas, que naturalmente seleccionam os espermatozóides, ao injectar um espermatozóide no ovócito para alcançar o melhor desenvolvimento embrionário e fetal [42]. Acabou por ser utilizada, também, em casos de falha idiopática de fertilização em FIV, e a sua aplicação generalizou-se mesmo na ausência de factor masculino no diagnóstico da infertilidade [43]. Actualmente, cerca de 70% dos tratamentos na Europa e 60% dos tratamentos a nível mundial são com recurso à ICSI, o que a torna a técnica de fertilização *in vitro* mais utilizada nos laboratórios de PMA [5, 6]

1.8 Cultura, avaliação e selecção embrionária

A cultura embrionária inicia-se desde o dia da recolha dos ovócitos (dia 0) e prolonga-se até ao dia da transferência ou criopreservação embrionária, que pode ir até ao sexto dia de cultura. A manutenção dos embriões em cultura é um sistema altamente monitorizado e integra incubadoras, meios de cultura, consumíveis, controlo da qualidade do ar e das temperaturas. O conceito de sistema de cultura embrionária enaltece a importância da qualidade e estabilidade que deve imperar num laboratório de embriologia. A qualidade embrionária depende da qualidade das gâmetas de onde derivam e, apesar dos embriões apresentarem alguma plasticidade que lhes permite a adaptação a várias condições de cultura, o seu desenvolvimento também é condicionado pelas interações de todos os parâmetros laboratoriais [44].

Desde sempre a avaliação morfológica tem sido a ferramenta base para a observação e selecção embrionária. Na última década, na sequência de inúmeros trabalhos que foram sendo publicados sobre o desenvolvimento embrionário, os sistemas de avaliação foram sofrendo alterações e, actualmente, a avaliação é morfocinética, ou seja, para além das características morfológicas do embrião (por exemplo, o número de células, a percentagem de fragmentação, etc.) considera também as etapas temporais dos eventos biológicos (por exemplo, os tempos das divisões celulares, o início da blastulação, etc.) [45].

Segundo os últimos consensos em embriologia, independentemente da técnica aplicada (FIV ou ICSI), todos os tempos de observação estão definidos para avaliação ovocitária ou embrionária, e são executados, na sua maioria, tendo como ponto de referência a hora da realização da inseminação. Os tempos definidos para a observação embrionária reflectem o que acontece na maioria dos casos. Todavia, como existe alguma variabilidade inerente a todos os processos biológicos, pode haver necessidade de observações adicionais [46].

A avaliação de embriões deve ser realizada utilizando microscópio invertido com óptica Hoffman ou equivalente e com recurso a altas amplificações (pelo menos 200x, de

preferência 400x). A avaliação inicia-se desde a verificação da fertilização até à decisão de transferência ou congelação. Nos embriões clivados (entre o dia 2 e o dia 4 de desenvolvimento embrionário) a avaliação deve incluir o número de células, tamanho, simetria e percentagem de fragmentação. Na avaliação de blastocistos (entre o dia 5 e o dia 6 de desenvolvimento embrionário) deve ser avaliado o grau de expansão do blastocisto, a morfologia da massa celular interna (MCI) e a morfologia da trofoectoderme (TE). A avaliação deve ainda incluir alterações embrionárias consideradas relevantes para o sucesso do tratamento, como a existência de vacúolos lisos e multinucleação. O desenvolvimento embrionário pode também ser avaliado usando imagens de time-lapse, mantendo os critérios da avaliação por microscopia [37, 47].

Nos últimos anos assistiu-se à implementação da tecnologia time-lapse a incubadoras de cultura embrionária. Este sistema permite a captura de imagens do desenvolvimento embrionário, em intervalos de tempo que podem ir dos 5 até aos 20 minutos. A recolha de dados que daí resultou permitiu o desenvolvimento de algoritmos para classificação e selecção embrionária. Inicialmente foi apontado como um sistema de selecção embrionária promissor, mas não existe evidência conclusiva que com este tipo de avaliação se obtenham melhores resultados em PMA. Contudo, este tipo de sistema complementa a avaliação embrionária realizada por embriologistas, permite a observação contínua dos embriões sem interferir com a cultura embrionária e continua a gerar dados para o aperfeiçoamento dos algoritmos [48].

Apesar da tentativa de uniformizar critérios, existem vários sistemas de classificação embrionária em uso. A qualidade embrionária é traduzida em escalões de classificação de acordo com a avaliação embrionária observada diariamente. Estes escalões determinam os embriões de qualidade máxima, média e má. É com base nestas classificações que se seleccionam os embriões a transferir, congelar ou descartar [46].

1.9 Transferência embrionária

A transferência embrionária (TrE) é a colocação de um ou mais embriões na cavidade uterina que podem estar em qualquer fase de desenvolvimento [1]. De uma forma geral, três factores podem condicionar o sucesso da implantação embrionária: a qualidade embrionária, a receptividade endometrial e a técnica de transferência [49]. Esta fase é a última etapa do ciclo que se iniciou na estimulação ovárica e é um procedimento crucial para o sucesso do tratamento. Recomenda-se que seja realizada com orientação ecográfica e com recurso a cateteres de transferência macios [50]. O cateter carregado com o embrião (ou embriões) seleccionado(s) para transferência tem de passar o canal cervical e o embrião deve ser colocado na porção distal da cavidade endometrial [51]. A

confirmação da gravidez é realizada 12 dias após a transferência por doseamento serológico da gonadotrofina coriônica humana (beta-hCG).

Quando a transferência embrionária é realizada no seguimento de um tratamento de FIV ou ICSI, estamos perante uma transferência a fresco. Contudo, por razões médicas (por exemplo, o risco de hiperestimulação ovárica e a assincronia embrião-endométrio), por razões técnicas (por exemplo, a realização de análise genética dos embriões), ou pela existência concomitante de embriões excedentários de qualidade, os embriões são criopreservados e a transferência é realizada em ciclo de transferência de embrião congelado (TEC). Nestas situações, a transferência embrionária é realizada num ciclo posterior, em que não é necessária a estimulação ovárica, mas é essencial a preparação endometrial para a transferência de embriões congelados [52].

Para que a implantação ocorra na superfície uterina (endométrio) e o desenvolvimento embrionário aconteça de forma favorável é fundamental que o endométrio esteja numa fase receptiva. Num ciclo ovulatório normal, o desenvolvimento endometrial é controlado pela produção hormonal dos ovários, que estão sob a influência das alterações associadas ao desenvolvimento folicular, à ovulação e ao aparecimento do corpo lúteo. Cada uma destas fases está associada a perfis hormonais que estimulam o endométrio a apresentar uma estrutura proliferativa (fase proliferativa normalmente associada ao desenvolvimento folicular) para depois se transformar num endométrio receptivo a uma eventual implantação embrionária (fase receptiva após a ovulação - fase lútea) [53]. Nos ciclos de tratamento com estimulação ovárica existe uma produção endógena resultante dos corpos lúteos presentes nos ovários após punção, mas que é insuficiente para uma correcta diferenciação endometrial necessária para a implantação [54].

Nos ciclos em que a TrE é realizada no seguimento da estimulação ovárica, independentemente do ciclo de EOC realizado, no suporte da fase lútea é normalmente utilizada progesterona (P) exógena, associada ou não à administração de estrogénios, dependendo de qual o indutor da maturação final ovocitária (hCG ou Angonista da GnRH). No caso de TEC, existem vários tipos de protocolos possíveis para a preparação endometrial: ciclo espontâneo, ciclo natural modificado e ciclo artificial. No ciclo espontâneo, o desenvolvimento do endométrio ocorre por estimulação hormonal endógena e a TEC é definida após o pico espontâneo de LH. No ciclo natural modificado, a TEC é planeada após ovulação induzida e existe suplementação de progesterona. No ciclo artificial, o endométrio é preparado para implantação com administração de estrogénios e progesterona com ou sem supressão da ovulação [49].

O número de embriões a transferir depende de muitos factores, nomeadamente da qualidade embrionária, da idade da mulher, do diagnóstico de infertilidade, da resposta à estimulação ovárica e do resultado de tratamentos de infertilidade já realizados. A

percentagem de gravidezes gemelares foi sempre uma preocupação na PMA. Todavia, na expectativa de aumentar as taxas de gravidez por tratamento, houve uma tendência para a transferência de mais do que um embrião por ciclo. Nos últimos anos, com a melhoria dos sistemas de cultura embrionária, a optimização da congelação de embriões e a melhoria de selecção embrionária, tem havido uma orientação para a transferência electiva de um embrião, contribuindo desta forma para a redução do número de gravidezes gemelares sem condicionar as taxas de sucesso [6, 55, 56].

Em Portugal, por Deliberação do Conselho Nacional de Procriação Medicamente Assistida (CNPMA), expressa nos “Requisitos e parâmetros de funcionamento dos centros de PMA”, o número de embriões a transferir deve ser de um ou dois, e no caso de mulheres com idade inferior a 35 anos e nos tratamentos com recurso a doação de ovócitos deve privilegiar-se a transferência de apenas um embrião.

1.10 Técnicas complementares

1.10.1 Criopreservação

A criopreservação de gâmetas e embriões pode ser aplicada em várias situações, nomeadamente na preservação da fertilidade, nos programas de doação de ovócitos, no teste genético pré-implantação, na criopreservação de embriões excedentários e na transferência embrionária diferida. É uma técnica muito utilizada e uma ferramenta importante para a optimização de certos aspectos técnicos da PMA, melhorando a segurança e a eficácia dos tratamentos [57].

Durante o processo de congelação, a formação de cristais de gelo extracelulares, mas principalmente intracelulares, é a maior causa de danos celulares que levam à lise celular. Para minimizar a formação de cristais de gelo é usada uma mistura de substâncias químicas crioprotectoras, algumas com capacidade para atravessar a membrana das células (crioprotectores permeáveis) e outras que actuam a nível extracelular (crioprotectores impermeáveis). Apesar de necessários, os crioprotectores também podem induzir danos celulares por toxicidade ou por alterações osmóticas [58].

Inicialmente, a criopreservação de ovócitos e embriões era realizada com recurso a uma técnica denominada de congelação lenta. Esta técnica associa uma descida gradual da temperatura a concentrações baixas de crioprotectores. Contudo, devido à formação de gelo, difícil de controlar durante o processo, a técnica foi sendo associada a maiores danos celulares em ovócitos e embriões [59]. Na tentativa de resolução das desvantagens da congelação lenta, surgiu uma nova técnica de criopreservação denominada vitrificação. Neste processo, para prevenir a formação de cristais de gelo, são usadas concentrações

mais elevadas de crioprotectores e uma descida de temperatura ultra-rápida, dos 37°C para -196°C em menos de um segundo, resultando numa curva de congelação extremamente rápida ($>10000^{\circ}\text{C}/\text{min}$) [60]. A utilização de altas concentrações de crioprotectores associada a uma curva de arrefecimento rápida é essencial para a congelação de embriões num estado vítreo [61]. Ao contrário da congelação lenta, em que parte do processo é realizado de forma automatizada, a vitrificação é um método totalmente manual e dependente do operador. Apesar de nos últimos anos existir um esforço para tornar a técnica parcialmente automatizada, isso ainda não é uma realidade nos laboratórios de embriologia.

A superioridade da técnica de vitrificação de ovócitos e embriões tem sido demonstrada em recentes meta-análises publicadas, apresentando melhores resultados em termos de taxa de sobrevivência após descongelação, taxa de gravidez clínica e taxa de gravidez cumulativa [62]. Estes resultados, associados à optimização da técnica em termos laboratoriais, conduziram à substituição progressiva da congelação lenta pela vitrificação. Segundo o último relatório da ESHRE, que reporta a 2019, a transferência de embriões criopreservados é a segunda técnica mais aplicada em PMA (a seguir à ICSI), superando a FIV. Nesse ano, aproximadamente 35% de todas as transferências embrionárias realizadas na Europa foram de TEC; a nível mundial este número subiu para 56.8% [5, 6].

1.10.2 Teste Genético Pré-Implantação

O Teste Genético Pré-Implantação (PGT) permite a avaliação genética de ovócitos (glóbulos polares) e embriões (clivados ou blastocistos) para a determinação de anomalias genéticas. Inclui o PGT-A para o estudo das anomalias numéricas cromossómicas (aneuploidias), o PGT-M para o rastreio de doenças monogénicas e o PGT-SR para despiste de rearranjos estruturais cromossómicos [5, 63]. O objectivo de um tratamento com PGT é a possibilidade de transferência de um embrião sem patologia molecular ou cromossómica que eventualmente poderia ser transmitida pelos progenitores.

O procedimento de biópsia implica a abertura da zona pelúcida e a retirada de glóbulos polares ou de células embrionárias para estudo genético. A biópsia em fase de blastocisto é o método mais frequentemente utilizado em PGT. É necessária a recolha de várias células da trofoectoderme, implica a cultura embrionária prolongada até à fase de blastocisto e a criopreservação dos embriões imediatamente após a biópsia [63, 64]

Havendo indicação para PGT, a biópsia insere-se na fase final de um ciclo de tratamento, o que significa que passa por todos os procedimentos de um ciclo de PMA desde a estimulação ovária. Uma vez que os embriões têm obrigatoriamente de ser congelados, a transferência embrionária é sempre realizada de forma diferida. As indicações para PGT-

M e PGT-SR são avaliadas de acordo com as indicações clínicas para cada caso e implica a avaliação por um geneticista. No caso do PGT-A, existem já algumas indicações definidas que incluem a idade materna avançada, as falhas de implantação recorrentes, a existência de factor de infertilidade masculino grave e a ocorrência de abortamentos de repetição [63].

Em Portugal, o CNPMA deliberou que o PGT-A não carece de autorização prévia desde que se verifique pelo menos uma das seguintes situações: idade superior a 38 anos, falhas de implantação (pelo menos três transferências embrionárias sem sucesso), abortamentos clínicos de repetição (pelo menos dois) de causa desconhecida e gestação anterior com anomalia cromossómica de número ou anomalia estrutural desequilibrada.

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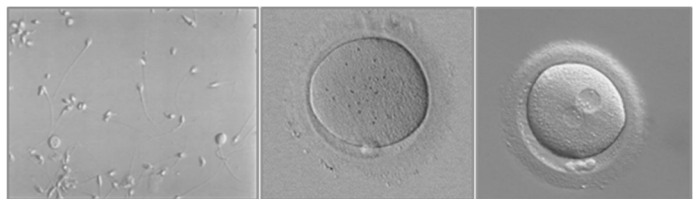
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CAPÍTULO 2

Objetivos



2 Objectivos

O objectivo geral deste trabalho é contribuir para a melhoria das técnicas de selecção de espermatozóides, aprofundando o conhecimento sobre fisiologia molecular destas células. Para isso, propusemo-nos analisar a influência do epigenoma do espermatozóide no metabolismo embrionário, revisitando o impacto das técnicas de selecção espermática, e correlacionando o exometaboloma com a classificação morfológica e o conteúdo de miRNAs no espermatozóide. O objectivo final é o de contribuir para a optimização da selecção de embriões e com isso contribuir para o aumento das taxas de sucesso da PMA, e simultaneamente aprofundar o conhecimento na área da biologia da reprodução.

Tendo em conta este objectivo geral, foram estabelecidos os seguintes objectivos específicos:

1. Rever os métodos convencionais e avançados de preparação espermática para PMA, avaliando se as técnicas mais recentes trazem vantagens para os casos específicos de infertilidade, contribuindo assim para uma maior taxa de fertilização e gravidez clínica.
2. Avaliar o impacto das técnicas de selecção espermática nos resultados de tratamentos de PMA, discutindo se a escolha do método de selecção de espermatozóides influencia a probabilidade de sucesso da fertilização *in vitro* e da gravidez.
3. Identificar biomarcadores da qualidade embrionária através da análise do exometaboloma por $^1\text{H-NMR}$, uma técnica para analisar metabolitos extracelulares, e com isso contribuir para a melhoria da selecção de embriões para transferência ou criopreservação, contribuindo para a redução do tempo até à obtenção de uma gravidez por PMA.
4. Avaliar se a integração da metabolómica com a classificação morfocinética tradicional de embriões, é um modo mais robusto para prever quais os embriões com maior potencial de desenvolvimento e implantação.
5. Investigar a relação entre o conteúdo de miRNAs no espermatozóide e a qualidade do embrião durante o desenvolvimento *in vitro*, e com isso determinar se a presença de miRNAs específicos no espermatozóide afecta a qualidade do embrião durante o desenvolvimento.
6. Avaliar a correlação entre o teor de miRNAs no espermatozóide e o desempenho metabólico do embrião durante o desenvolvimento *in vitro*, e assim estudar se a

quantidade de miRNAs específicos no espermatozóide influencia a actividade metabólica do embrião, e se pode ser um indicativo do seu potencial de desenvolvimento.

7. Com a melhoria da capacidade de avaliar mais objectivamente a qualidade dos gâmetas e de fazer uma selecção de embriões mais eficiente, pretendemos contribuir para o aumento das taxas de sucesso da PMA.

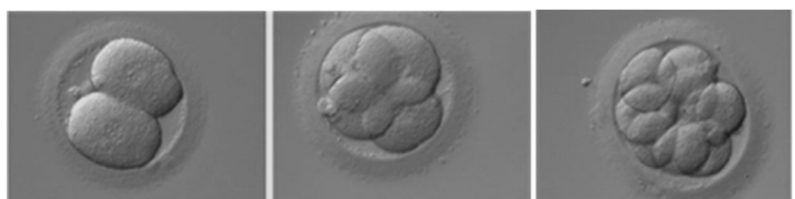
CAPÍTULO 3

Sperm selection strategies and their impact on assisted reproductive technology outcomes

Este capítulo foi adaptado integralmente do seguinte artigo publicado:

Pinto S, Carrageta DF, Alves MG, Rocha A, Agarwal A, Barros A, Oliveira PF. Sperm selection strategies and their impact on assisted reproductive technology outcomes. *Andrologia*. 2021 Mar;53(2):e13725. doi: <https://doi.org/10.1111/and.13725>

O texto integral pode ser consultado no Anexo A desta Dissertação.



3 Sperm selection strategies and their impact on assisted reproductive technology outcomes

3.1 Abstract

The application of assisted reproductive technologies (ART) has revolutionized the treatment of human infertility, giving hope to the patients previously considered incapable of establishing pregnancy. While semen analysis is performed to access whether a sample has an adequate number of viable, motile and morphologically normal sperm cells able to achieve fertilization, sperm selection techniques for ART aims to isolate the most competent sperm which is characterized by the highest fertilizing potential. Based on the semen analysis results, the correct sperm selection technique must be chosen and applied. In this review, different sperm selection strategies for retrieving sperm with the highest fertilizing potential and their impact on ART outcomes are discussed. In addition, advantages and disadvantages of each method and the best suited techniques for each clinical scenario are described.

3.2 Introduction

Assisted reproductive technologies (ART) revolutionized human fertility by providing treatment to patients previously considered incapable for reproductive purposes [1]. In 2017, it was estimated that approximately 6 million infants were born through ART since 1978, when the first baby produced by *in vitro* fertilization (IVF) was born. It has been reported that about half of these births were in the past decade, highlighting the increasing use of these technologies [2].

Initially ART research was mainly focused on comprehending the factors that increase the likelihood of a successful pregnancy and improving outcomes for mothers and their ART-conceived children. Currently, the fathers health and sperm selection have deserved a higher attention from clinicians and researchers [3]. Sperm are complex cells, whose fertilizing potential can be jeopardized by small morphologic, physiological, or even biochemical alterations [4, 5]. It is estimated that 40-50% of the infertility cases are due to male factors, mainly caused by defective spermatogenesis, retrograde ejaculation, hypogonadism, or erectile dysfunction [6]. However, the majority of these cases are classified as idiopathic [7, 8]. Some of these cases are associated with lifestyle (such as sedentarism and smoking) or metabolic disorders (as obesity or diabetes mellitus), although the molecular links remain to be disclosed [9-11].

The selected methods for sperm preparation depends of the results on sperm analysis and fresh sample in the day of the ART treatment. Sperm selection techniques for ART aim to isolate sperm cells that are characterized by a high fertilizing potential and, subsequently, may lead to a successful pregnancy. Over the past years, the understanding on sperm physiology has been improved and more sophisticated techniques were developed in order to effectively isolate physiologically normal sperm from those that are immature, immotile, or have poor fertilizing potential. Conventional methods remain an effective tool for sperm selection, however, more advanced methods are employed to address specific and complex infertility cases to attain higher take home baby rate. In this review, sperm selection techniques for the isolation of the most competent sperm and their impact on ART outcomes will be discussed. Furthermore, each method and technique will be briefly presented to discuss which are the best for each clinical scenario.

3.3 Standard semen analysis

The guidelines for human semen analysis are described in the 5th edition of the World Health Organization (WHO) Manual for the Examination and Processing of Human Semen [12], which provides reference values for semen volume, pH, liquefaction, sperm count, total and progressive motility, vitality, morphology, and leucocytes concentration (references values are summarized in Table 3.1). Upon analysis, the patient is diagnosed accordingly and the most appropriate sperm selection technique is chosen. The main goal of sperm selection techniques is to isolate the spermatozoa with the most fertilizing potential for use in ART. Generally, the chosen technique depends on the characteristics of the sample and human semen analysis.

Although widely applied as a general indicator, the semen analysis has several limitations. Simon et al. reported that 80% of men seeking fertility treatment had idiopathic infertility beyond diagnosis confirmed by established standard testing protocols [13]. Sperm are complex cells influenced by a myriad of internal and external stimuli (such as pH, ionic concentration, or temperature), with several factors that may compromise its fertilizing potential still remaining elusive to identify. In fact, new devices for diagnosis are developed based on the novel data produced by the scientific community, such as computer-assisted semen analysis (CASA) systems, analysis of the DNA damage, accessing sperm telomere length, or the adoption of stricter criteria for sperm morphology evaluation [14, 15]. In particular, assays for analysing sperm DNA integrity have been extensively investigated over the past decade [16, 17]. Sperm DNA integrity is essential for fertilization and early stages of the embryo development [18]. Sperm cells do not express natural DNA repair mechanisms and are not able to repair DNA post-spermiogenesis [19, 20]. DNA damage

is inevitable due to sperm physiology and the environment that they are exposed but upon fertilization it can be repaired to some extent. When the damage exceeds the oocyte's repair capacity, it may result in infertility [21]. Clinical data has shown that the chance of both natural and artificial conception is reduced if DNA fragmentation index is more than 30% [20, 22, 23]. For this purpose, several techniques have been developed with the finality of analysing sperm chromatin and DNA integrity although standardization or guidelines to uniformize results remain absent.

Table 3.1 Reference values for normal sperm parameters according to the 5th WHO Manual for the Examination and Processing of Human Semen [12].

Semen parameter	Reference Value
Semen volume	≥ 1.5
Sperm concentration	≥ 15 ($10^6/\text{mL}$)
Total sperm number	≥ 39 ($10^6/\text{ejaculate}$)
Total motility	≥ 40 (%)
Progressive motility	≥ 32 (%)
Normal morphology	≥ 4 (%)
Viability	≥ 58 (%)
Leucocytes concentration	< 1 ($10^6/\text{mL}$)

3.4 Preparation of semen samples

Seminal plasma and its constituents are helpful for sperm to achieve fertilization in natural conditions, however they are obstacles when natural barriers are circumvented in ART. The separation of human sperm from seminal plasma, non-germ cells and dead sperm is essential for clinical practice [12]. Viable sperm should be isolated as soon as possible. Although WHO guidelines recommend separation of sperm from seminal plasma within one hour after ejaculation [12], it was reported that fertilization ability will permanently decrease if separation is not done in 30 minutes [24]. It is also recommended to maintain a sterile environment and use sterile equipment. Samples should be examined for the presence of round cells (leucocytes or epithelial cells) or bacteria. Viscosity may constitute a common issue among all ART methods, which substantially decreases the efficiency of the techniques. It is a common procedure to allow samples to liquefy completely at 37 °C (usually 15-30 minutes are sufficient). In cases of highly viscous semen samples it is sometimes advised to perform an enzymatic digestion. According to the WHO guidelines, if the sample cannot be induced to liquefy by the addition of medium or by the repeated passage through a needle, the digestion with bromelain for 10 minutes is recommended [12]. Some authors also recommend to treat the sample with a trypsin solution, not more

than 5 minutes before performing the separating technique, as it will increase efficiency without any harmful effect to sperm [25]. Upon liquefaction, the samples are ready to be processed.

3.5 Sperm selection methods for ART

According to the clinical scenario, the appropriated sperm selection technique must be chosen. The sperm selection method should eliminate seminal plasma, dead sperm cells and other cells, while concentrating motile sperm and selecting morphologically normal sperm [26]. Conventional sperm selection methods, such as simple sperm wash, glass wool preparation, swim-up, or density gradient centrifugation are usually the first step on semen processing for ART (Table 3.2). These methods are characterized as simple, fast, safe and inexpensive, although with limited usefulness. [1]. Direct swim-up technique is still the gold standard for patient with normal sperm parameters, whereas in cases of oligozoospermia, teratozoospermia or asthenozoospermia, density gradient centrifugation is usually preferred due to the higher total number of motile sperm recovered [12]. In cases of pronounced or severe oligozoospermia the volume of gradient can be reduced or a simple wash can be chosen. Ejaculates from oligozoospermic or asthenozoospermic men, or ejaculates from patients with viral diseases have to be treated with different techniques, to achieve optimal efficiency. Advanced sperm selection methods are able to further improve conventional methods, with the downside of being more expensive and occasionally time-consuming (Table 3.3).

With these techniques, sperm cells are separated from other constituents present on the ejaculate such as seminal fluid, leukocytes, epithelial cells, or unviable sperm. A common ground between these techniques is that it is often possible to isolate a sperm subpopulation of higher fertility potential from an ejaculated sample. This subpopulation is characterized by higher motility, flawless morphology, and functional membrane and acrosome integrity [14]. Upon isolation of this subpopulation, it is possible for a low-quality semen sample to be suitable for a successful intrauterine insemination (IUI) or *in vitro* fertilization (IVF). In case of poor quality samples, the advanced sperm selection methods allow identification and selection of the most promising sperm for intracytoplasmic sperm injection (ICSI). In the following sections the most common conventional and advanced sperm selection methods will be discussed in detail, from the general method to their advantages and disadvantages.

Table 3.2 Comparison of various conventional sperm selection procedures.

Procedure	Advantages	Disadvantages
Simple sperm wash	Simple, fast and inexpensive	All cells present on the semen sample, including leukocytes, epithelial cells and non-viable sperm cells, are separated from the seminal fluid and pelleted with the viable sperm cells
Glass wool filtration	- Isolates viable motile sperm cells - Efficient in removing leukocytes from the sample - Efficient for viscous semen samples - Retrieve sperm with normal chromatin condensation - Select a higher percentage of sperm with intact acrosome than swim-up or density gradient centrifugation - Higher percentage of fertilization following IVF than swim-up	- More expensive - Originates some cellular debris
Swim-up	- Simple, fast and inexpensive - Isolates motile and morphologically normal sperm cells - Gentle method, reduces ROS production and DNA fragmentation - Reduces the proportion of sperm cells with chromosomal defects - Can be performed following density gradient centrifugation in order to further improve the quality of the retrieved sperm	- Low number of sperm cells is retrieved - Low efficiency in separating competent sperm cells from cellular debris, immature sperm cells or other cells when pelleting - Low efficiency in cases of high viscosity
Density gradient centrifugation	- Simple and fast - Isolates a higher number of motile and morphologically normal sperm cells - Easier to standardize or adapt to the clinical scenario - Efficient in reducing the number of sperm cells with aneuploidy and diploidy - Reduced ROS production and oxidative stress - The conjugation with swim-up is recommended for samples from HIV-infected men	- Low efficiency in cases of high viscosity - Overloading the gradient may cause the aggregation of sperm and other cells

Table 3.3 Comparison of advanced sperm selection procedures.

Procedure	Advantages	Disadvantages
Zeta potential	- Selects mature sperm cells based on the plasma membrane negative charge - Simple and inexpensive - Select sperm cells with reduced DNA fragmentation - Select a higher percentage of sperm with normal morphology and higher motility when compared with the density gradient centrifugation - Improved fertilization rate, percentage of top embryo quality and pregnancy outcome when compared to density gradient centrifugation	- There is no association between the zeta potential and total motility, requiring the prior use of other method - The time factor is essential, as zeta potential decreases with the onset of sperm capacitation - The number of retrieved sperm cells is low, leading to limited use for cases of oligozoospermia or testicular sperm - Potential bias for selecting X-bearing sperm cells
Electrophoretic sperm selection	- Retrieve viable, highly mobile, and morphologically normal sperm cells - Retrieved sperm cells present reduced DNA fragmentation - Can be successfully used for cryopreserved samples and testicular biopsies	- Requires an electrophoresis system - Potential bias for selecting X-bearing sperm cells - Contradictory results concerning the ART outcomes
MSOME/IMSI	- Improved selection based on sperm cells ultra-structure - Beneficial for severe cases (oligoasthenoteratozoospermia and teratozoospermia) - Beneficial for cases with repeated ICSI failures	- Expensive - Time-consuming - Requires a skilled embryologist - Absence guidelines and standardization - Contradictory results concerning the ART outcomes
PICSI and hyaluronan methods	- Hyaluronic acid receptors are only expressed on mature sperm cells - Retrieval of sperm cells with reduced aneuploidy, chromosomal disomies, and diploidy - Improved ART outcomes when associated with ICSI	Insufficient or inconclusive data concerning ART outcomes
MACS	- Select viable sperm cells with reduced DNA fragmentation - Retrieve sperm cells with higher motility and normal morphology	Insufficient or inconclusive data concerning ART outcomes
Microfluidic separation	- Select sperm cells based on the motility or morphology - The damage caused by centrifugation is eliminated	Insufficient or inconclusive data concerning ART outcomes

	• Decreased DNA fragmentation when compared with conventional methods or unprocessed sperm • Direct use of the sample	
HOST	• Simple and inexpensive • Evaluates the plasma membrane integrity without damaging sperm cells • Useful for the identification of viable but immotile sperm cells	• Insufficient or inconclusive data concerning ART outcomes
LAISS	• Considered as a safe method • Select viable but immotile sperm cells based on the curling of the sperm tail upon exposition to a laser shot • Useful for cryopreserved semen samples	• Expensive • Mechanism responsible for the tail curling is unknown • Insufficient or inconclusive data concerning ART outcomes
Polarization microscopy	• Allows to evaluate the nucleus structural organization and the state of the acrosome reaction • The pattern of the sperm head birefringence is correlated with sperm parameters	• Inconclusive data concerning DNA fragmentation • Time consuming • Requires a skilled embryologist • Insufficient or inconclusive data concerning ART outcomes

3.5.1 Conventional sperm selection methods

3.5.1.1 Simple sperm wash

The simple wash technique is a simple and straight-forward technique that should only be used when the semen sample has optimal parameters. This technique is performed to prepare sperm for IUI, or in cases of severe oligoasthenoteratozoospermia or cryptozoospermia [25]. With the simple wash technique, all cells present on the semen sample are separated from the seminal fluid. No more than one hour upon ejaculation and following complete liquefaction, culture medium or a washing sperm solution (e.g. a saline buffer) is added to the sample and centrifuged in order to pellet the cells. It is crucial to use low centrifugal forces (300 g are often described in protocols and always less than 500 g) and as few as possible centrifugation steps in order to minimize the production of reactive oxygen species (ROS) [25]. High ROS production results in DNA and organelles damage and initiates the apoptotic cascade leading to the loss of motility and viability, though low levels of ROS are essential for normal sperm function and for the promotion of signaling pathways associated with capacitation [27, 28]. The downsides of the simple wash technique are a direct consequence of its simplicity, as all cells are separated from the seminal fluid including leukocytes and epithelial cells present on the semen sample. Non-

viable sperm are also present, which are reported to compromise sperm ability to capacitate and fertilize the oocyte [29].

3.5.1.2 Glass wool filtration

The glass wool filtration is a separation method based on the filtration of the sample through a densely packed glass wool fibers placed in a column [1], which makes this method efficient for highly viscous samples [14]. Through this method, it is possible to separate motile from immotile spermatozoa, leukocytes and seminal fluid. In fact, Henkel et al. reported that this method is able to reduce the leukocytes in the sample by about 87.5% [30]. In addition, glass wool filtration allows the retrieval of motile sperm with normal chromatin condensation [31], and a higher percentage of sperm cells with intact acrosome than both the density gradient centrifugation and the simple wash method [32]. The filtered sperm is also reported to result in higher percentage of fertilization following IVF when compared with the spermatozoa retrieved with the swim-up procedure [33, 34]. Unfortunately, this method is rather expensive when compared with other conventional techniques and might result in some cellular debris [35].

3.5.1.3 Swim-up

The swim-up is a migration technique and one of the simplest, fastest, and commonly used methods for sperm preparation. With this method, a liquefied semen sample (direct swim-up) or a previously washed cell pellet (conventional swim-up) is placed in a tube with an overlapping layer of culture medium. The tubes are left on a 45° angle at 37 °C for 1 hour before the supernatant is collected. The swim-up method is based on the fact that spermatozoa are attracted and swim into the nourished environment of the culture medium, which is then collected. The collected fraction has a high probability of containing only motile and morphologically normal spermatozoa [35]. When the concentration and motility parameters are within the normal range, the swim-up is regarded as the preferred technique. However, in cases of oligozoospermia the direct swim-up might be recommended. This method is mainly applied for IVF or ICSI as it selects sperm for their motility and is convenient when the percentage of motile spermatozoa in the sample is low [12].

The swim-up is a very gentle method, leading to reduced production of ROS and DNA fragmentation [36, 37]. In addition, this technique mainly retrieves healthy and mature sperm cells [38]. On the other hand, the swim-up method also has some downsides. The first one is inherent to one of the advantages of the technique. Only a low percentage (5-

10%) of sperm are retrieved, although it is a subpopulation of highly mobile and morphologically normal sperm [35]. Another disadvantage is the fact that higher volumes or higher concentration requires further precautions. In cases of samples with high volume (more than 3 mL), it should be divided into several tubes in order to increase the area of contact with the culture medium. In cases of high concentration, pelleting the cells should be avoided as some spermatozoa might be trapped in the middle of the pellet and thus unable to migrate. In these cases, dilution and division into several tubes might be advised. The viscosity of the sample may also be an issue that needs to be addressed.

3.5.1.4 Density gradient centrifugation

Density gradient centrifugation is a method that separates sperm cells based on their density, through a colloidal suspension of silica particles stabilized with covalent bonded hydrophilic silane. Most commonly, there are two gradients layers: a lower phase (80-90%) and an upper phase (40-45%), whereas the sample is loaded as the top layer. The density gradient might also be continuous, gradually increasing from the top to the bottom [1]. The main principle of this method is the fact that normal and abnormal spermatozoa as well as other cells have distinct densities. It is reported that mature and morphologically normal spermatozoa are characterized by a density of more than 1.10 g/mL while immature or aberrant spermatozoa exhibit a density between 1.06 and 1.09 g/mL [39]. The outcome obtained upon density gradient centrifugation is a pellet containing the sperm subpopulation characterized by higher motility and normal morphology, whereas leukocytes, cell debris and aberrant sperm remain on the top layers [40].

One advantage is that it is easier to standardize or adapt the density gradient centrifugation to the clinical scenario than the swim-up method, leading to more consistent results [12]. The density gradient centrifugation is also very efficient in decreasing the number of sperm with aneuploidy and diploidy [41], selecting also motile sperm without enhancing oxidative stress [42-44]. When performing a density gradient centrifugation, it is still advised to use the lowest possible centrifugal force and time (usually 300 g and 20 minutes are the recommended settings by the density gradient manufacturers) in order to minimize the production of ROS and the consequent oxidative damage. If even upon the liquefaction time period the sample remains too viscous, it might be advised to increase the centrifugal force up to 600.g [35]. It is essential that the reagents are at the room temperature prior to use, which must be occur within one hour after performing the gradient in order to avoid the eventual blend of the two phases. It is also advised to avoid overloading the gradient since it may cause “rafting”, which is the aggregation of spermatozoa and other undesirable components present on the sample upon centrifugation [35].

It is possible to perform the swim-up method following the density gradient centrifugation in order to improve the isolated spermatozoa quality, although fewer cells are retrieved. Both swim-up and density gradient centrifugation, alone or in combination, are successful in isolating viable and morphologically normal spermatozoa with intact chromatin and DNA, thus improving the chances of achieving a viable pregnancy [45-48], as both are equally efficient in recovering sperm with longer telomeres [49, 50].

3.5.2 Advanced sperm selection methods

3.5.2.1 Zeta potential

Zeta potential is the electrokinetic potential on the surface of particles on a colloidal suspension. The membrane of mature human spermatozoa possesses a zeta potential between -16 to -20 mV, which is conferred by a coating of sialic acids during spermatogenesis and epididymal maturation [51, 52]. This negative potential can be used as a biomarker for the selection of mature sperm cells [53]. Based on this principle, Chan et al. developed a sperm selection method where mature sperm cells adhere to positively charged surfaces [54]. As there is no association between the zeta potential and total motility, the authors recommend that this method must be performed on selected motile spermatozoa obtained by a density gradient centrifugation. The zeta potential method should be performed immediately after liquefaction due to the decrease on zeta potential with the onset of sperm capacitation [55]. Briefly, new centrifugation tubes (preferably glass tubes) are placed inside a latex glove and rotated two-three times in order to obtain a positively charged tube. To allow adherence of mature sperm to the wall, the tube is kept 1 min at room temperature and then centrifugated and inverted in order to discard non-adhering sperm. Mature sperm cells are retrieved by neutralizing the charge on the tube with a serum-supplemented media [54, 56, 57].

The main advantages of zeta potential method rely on its simplicity and that it is inexpensive, as there is no need for electrophoresis equipment. This method has been reported to select spermatozoa with less DNA fragmentation and a higher percentage of sperm cells with normal morphology and hyperactive motility when compared with the conventional density gradient centrifugation [58-60]. It was also reported that the zeta potential method improved the fertilization rate, the percentage of top embryo quality and pregnancy outcomes when compared to performing only a density gradient centrifugation [58, 61]. The yield of retrieved spermatozoa is low, leading to its limited use in cases of oligozoospermia or testicular sperm retrieval [54]. Ishijima et al. reported that the net zeta potential on the surface of human X-bearing sperm is higher than Y-bearing sperm [51],

which led some authors to hypothesize that there might be a bias for the selection of X-bearing sperm [56]. More recently, Esfahani et al. compared the results of ICSI between the zeta potential method and the conventional density gradient centrifugation and reported that the sex ratio (XY/XX) at birth following the zeta potential method were lower despite the similar ratio of X/Y-bearing sperm obtained from both methods [61]. However, more studies are needed to clarify if the zeta potential method indeed changes the sex ratio.

3.5.2.2 Electrophoretic sperm selection

Similar to the Zeta potential method, the electrophoretic sperm selection is based on the fact that the plasma membrane of mature sperm is negatively charged. In the electrophoretic method semen samples are placed in an electrophoretic device and then a current is applied to select the negatively charged spermatozoa. In 2005, Ainsworth et al. introduced the first electrophoresis-based technology designed for sperm selection (Microflow®), which enabled the separation of spermatozoa by size and membrane charge [62]. This device consists of two inner chambers, for inoculation of sperm cells, two outer chambers, for collection of the selected sperm cells, two electrodes and two buffer pumps. The two chambers are separated by a polycarbonate membrane which allows mature spermatozoa to cross it while larger cells are blocked. Then, it was possible to retrieve viable, mobile, and morphologically normal spermatozoa with low levels of DNA damage [62]. Later in 2015, a novel device for electrophoretic sperm selection named micro-electrophoresis chamber was introduced. This device consists of a disposable sterile electrophoresis system, a power supply and connecting electrodes. It was reported that negatively charged spermatozoa was not correlated with any sperm parameters besides decreased DNA damage [63, 64].

Although the electrophoretic sperm selection seems to allow the selection of healthier sperm cells, there are few studies reporting the impact of this technology in ART. One advantage over the zeta potential is that this method was shown to be as effective when applied to cryopreserved samples and testicular biopsies (immature spermatozoa) as with fresh samples [65]. The electrophoretic sperm selection was reported to be positively associated with the fertilization, implantation, and clinical pregnancy rate [63], as it was also reported a successful normal birth following this method in an ART clinic [65]. On the other hand, Fleming et al. compared the electrophoretic method with the density gradient centrifugation and did not find any significant difference in fertilization and cleavage rates [66]. Furthermore, the net negative-charge on the cell surface of human X-bearing is higher than Y-bearing spermatozoa, which could lead to a lower sex ratio (XY/XX) [51]. However, Ainsworth et al. reported that the electrophoretic method did not result in a significant

skewing of the ratio of X- and Y-bearing sperm cells [67]. Despite some evidence pointing to an improvement in sperm parameters following the electrophoretic method, studies concerning the ART outcomes are not only limited but also provide contradictory results. Additional studies are required to determine the effectiveness of the method in clinical practice.

3.5.2.3 Motile sperm organelle morphology examination (MSOME)

The advent of the ICSI in 1992, by Gianpiero Palermo et al. [68], for the treatment of severe male infertility allowed improved understanding of sperm biology. As a result, more stricter criteria for sperm selection were adopted. While several parameters such as sperm concentration, motility, and DNA fragmentation are correlated with the reproductive outcome, several studies highlight that sperm morphology is decisive for the fertility potential [69-71]. In fact, some sperm cells considered as normal in a conventional morphological analysis may present ultra-structural defects that negatively affect the ART outcome [53]. A new method for detailed morphological assessment of motile spermatozoa in real time was developed in 2002 – motile sperm organelle morphology examination (MSOME). MSOME is a method that is performed using a magnification of 6000x, which is a much higher magnification than the used for conventional ICSI [72], that allows the assessment of sperm cells ultra-structure. MSOME analyzes six sperm organelles: acrosome, post-acrosome lamina, nucleus, neck, tail, and mitochondria. Among those, sperm nucleus appears to be the organelle that mostly influences ICSI outcome [72-74]. While the shape and size of the head is assessed, a normal nucleus should present a smooth, symmetric, and oval configuration. The presence of vacuoles is also evaluated. If the vacuoles occupy more than 4% of the head, the spermatozoon is labeled as abnormal [72, 75]. In fact, several studies highlight the detrimental effects of the presence of nuclear vacuoles in the ART outcomes [76, 77].

When MSOME is applied on an ICSI micromanipulation system it is known as intracytoplasmic morphologically selected sperm (IMSI) [75]. The IMSI is performed on a conventional inverted microscope attached to specific accessories. Besides the standard equipment necessary to perform ICSI (micromanipulation and microinjection system plus the 400x Hoffman optics), in IMSI it is also required a 100x oil-immersion objective and Nomarski optics attached to the microinjection system. In addition, the IMSI process uses a 6000x magnification that can only be obtain digitally [14, 72]. Taken together, all the necessary equipment renders this technique as expensive, time-consuming and requires a skilled embryologist to be correctly performed. This method also has an inherent subjectivity associated to intra and inter-observer variability. IMSI has proved to be a valid tool for sperm

selection in some specific group of patients. There is evidence that patients with severe alterations in sperm parameters (oligoasthenoteratozoospermia and teratozoospermia) could benefit from IMSI [78-81]. It has also been shown that if IMSI is applied to couples with repeated ICSI failures the pregnancy rates and the number of quality embryos can be improved, and miscarriages rates decreased [82, 83]. In addition, some studies demonstrate that IMSI improves the percentage of top-quality embryos, implantation and pregnancy rates and significantly reduces miscarriage rates compared with conventional ICSI [81, 84-86]. Even though some positive outcomes are highlighted in the literature, there is no consensus about the efficiency of the method or specific guidelines instructing how to perform IMSI. In fact, Teixeira et al. concluded that there is not enough evidence to support the clinical practice of IMSI and that further trials are necessary to elucidate its efficiency and outcomes [86].

3.5.2.4 Hyaluronic acid mediated sperm selection

The hyaluronic acid that surrounds the human oocyte act as a natural mechanism of sperm selection [87, 88]. Mature sperm express hyaluronic acid binding glycoproteins in the plasma membrane and in the acrosome membrane [89, 90], which are responsible to bind and digest hyaluronic acid in order to fertilize the oocyte. The binding to hyaluronic acid is reported to mediate some intracellular signaling pathways, leading to increased motility [91]. The expression of hyaluronic acid receptors can be used as a biomarker to select and isolate mature sperm for ART [53, 88]. There are two techniques based on hyaluronic acid mediated sperm selection, namely the physiological intracytoplasmic sperm injection (PICSI) and the hyaluronan (or hyaluronic acid)-rich medium. The PICSI technique is performed using a PICSI dish, which is similar to a petri dish with coated spots of hyaluronic acid in the base. Washed sperm sample is subsequently placed on this dish and after a period of incubation the hyaluronic acid-bounded sperm are selected for ICSI. The alternative technique uses a viscous solution of hyaluronan-rich medium, commercially available as SpermSlow™ (CooperSurgical, United States), that can be added to the conventional ICSI dish. In brief, a droplet of a washed sperm sample is placed near a droplet of SpermSlow™ so that there is a contact zone between the drops. The sperm cells in the interface of both droplets, which are bounded to hyaluronic acid, are selected for ICSI [88].

Although some advantages had been reported on the literature, the clinical efficiency of this method remains to be clarified. Interestingly, both PICSI and the hyaluronan-rich medium are reported to have the same efficiency in selecting hyaluronic acid-bound sperm [92], retrieving sperm with less DNA fragmentation and reduced aneuploidy rate [88, 93]. It was also reported that hyaluronic acid-bound sperm exhibit reduced frequencies of

chromosomal disomies and reduced diploidy [94, 95]. There is evidence for an improved embryo quality and implantation [88], higher fertilization and clinical pregnancy rate with the conjugation of hyaluronic acid mediated sperm selection and ICSI [93, 96]. On the other hand, there is contradictory data. For instance, Razavi et al. reported no difference in DNA fragmentation levels between these methods and conventional sperm selection procedures [97]. In a systematic review, McDowell et al. concluded that, although no adverse effects were observed, there is no sufficient evidence to validate that spermatozoa selected by hyaluronic acid improves ART outcomes [98]. Further studies are required to evaluate whether hyaluronic acid-based methods can be recommended for use in clinical practice.

3.5.2.5 Magnetic-activated cell sorting (MACS)

Magnetic activated cell sorting (MACS) is an advanced method for sperm selection that uses Annexin-V-coated magnetic beads in order to separate apoptotic from non-apoptotic spermatozoa. Annexin-V is a phospholipid binding protein with high affinity for phosphatidylserine. Phosphatidylserine is a negatively charged phospholipid that is present in the inner layer of the plasma membrane of both somatic and sperm cells [53, 99], which is externalized to the outer layer of the cell during the early phases of apoptosis [100]. Thus, phosphatidylserine is used as a biomarker for apoptotic sperm cells. In MACS, a washed sample is incubated with Annexin-V-coated magnetic beads which will bind to the externalized phosphatidylserine of apoptotic sperm cells. This suspension is placed inside a MACS column that produces an external magnetic field, retaining the apoptotic sperm cells that are bound to the Annexin-V-coated magnetic beads while the non-apoptotic sperm pass through and is collected [57, 101]. At the end of the MACS protocol two distinct fractions are obtained: Annexin-negative (non-apoptotic sperm with intact membrane) and Annexin-positive (apoptotic sperm with externalized phosphatidylserine) [99, 101]. As there are other components in semen that may decrease the efficiency of this technique, the association of this method with a standard sperm wash technique, such as density gradient centrifugation, is recommended [53]. As an alternative to the magnetic beads, glass wool can be coated with Annexin-V. This technique, commonly referred as Annexin-V glass wool or molecular glass wool, further improves the filtration process as apoptotic sperm are bounded to the Annexin-V. This technique was successfully used on both fresh and cryopreserved semen samples, although more studies are needed in order to validate its efficiency for clinical practice [102].

As the correlation between DNA damage and apoptosis with the consequent externalization of phosphatidylserine is well established [103], it is no surprise that several studies reported that the spermatozoa retrieved with MACS have reduced levels of DNA

fragmentation [104-108]. In addition, the retrieved spermatozoa exhibit higher motility [107, 109, 110] and higher percentage of sperm with normal morphology [104, 108]. Although with MACS protocol it is possible to obtain better spermatozoa, the published data concerning clinical outcomes in ART is still inconclusive. Romany et al. demonstrated, in a large-sample prospective randomized trial, that fertilization and implantation rates were not improved by MACS [109]. Moreover, Gil et al. conducted a systematic review and concluded that MACS did not provide a significant improvement in the implantation and miscarriage rates compared with conventional sperm selection, although a trend for higher pregnancy rates were reported [111]. Some studies showed a higher pregnancy and implantation rate with the use of MACS in patients with infertility attributed to the male factor, but no improvement in fertilization rate were observed [112, 113]. Although some advantages in sperm parameters have been reported, it is still inconclusive whether MACS procedure is valuable for clinical practice and in which patients should be applied.

3.5.2.6 Microfluidic separation of sperm

Microfluidics is a novel technology based on physical and chemical principles of fluid behaviour in a controlled environment at a sub-microliter level. This technology may be used in several scientific areas, from genetics to ART. Significant advances have been made in order to evaluate the potential utility of microfluidics for sperm selection, where it aims to mimic the selection process that occurs in *in vivo* fertilization. Currently the majority of these devices are mainly for research purposes and present low applicability for clinical practice [53, 114, 115]. Several microfluidic devices have been tested for sperm selection based on characteristics such as morphology, motility, the ability to swim up a chemical gradient (chemotaxis), thermal gradient (thermotaxis), or on their response to fluid flow variations (rheotaxis) [115-118]. The first reports that describe the potential use of microfluidics for human sperm selection for ART occurred in 2003 and the idealized device relied on the progressive motility of spermatozoa. This method was based on the capacity of motile sperm to pass through streamlines in a laminar fluid stream. A self-contained integrated microfluidic device that was able to isolate motile sperm cells from non-motile and other cells present on the semen sample was developed and reached commercialization after some promising results [119, 120].

As the microfluidic systems are still in a developing phase, limited data concerning the advantages, disadvantages or ART outcomes are available in the literature. One major advantage is the fact that microfluidic systems are a one-step technique that eliminates the damage that arises from the centrifugation steps of conventional techniques. This method is considered advantageous for samples that are difficult to handle, such as cases of severe

oligoasthenozoospermia, although it is unable to identify non-motile but viable sperm cells for ICSI [19, 64]. Some studies reported that some microfluidic systems selected sperm cells with a higher percentage of motility and lower DNA fragmentation when compared to conventional sperm methods or unprocessed sperm [43, 121-123]. Two studies have analysed the ART outcomes following the microfluidic technology. When Yildiz et al. compared the fertilization and pregnancy rates following sperm selection through density gradient centrifugation or microfluidics chip systems in couples with unexplained infertility and recurrent unsuccessful IVF treatments, some interesting results were obtained [124]. Although no difference was observed in the fertilization and pregnancy rates in patients with unexplained infertility, the use of a microfluidic system significantly improved the fertilization rate in couples who received at least two IVF treatments before the study, leading the authors to conclude that this method may be beneficial for patients with recurrent unsuccessful ART treatments. Yetkinel et al. demonstrated that the use of microfluidic systems improved the quality of embryos but did not alter the fertilization, clinical pregnancy, or life birth rates when compared to conventional swim-up in couples with unexplained infertility [125]. Further research is required to evaluate the potential of microfluidic systems and its clinical application in ART units.

3.5.3 Alternative advanced methods

3.5.3.1 Hypo-osmotic swelling test (HOST)

The hypo-osmotic swelling test (HOST) was initially developed as a diagnostic sperm test to assess the functional integrity of the sperm plasma membrane, which is essential for fertilization. In hypo-osmotic conditions, the tail of sperm cells with a normal functional membrane swell or curl due to water influx, which could be used as an indicative of sperm potential [126]. The HOST has already been established as a diagnostic test and is currently used as a reliable indicator of the sperm quality in clinical practice [14]. However, the possibility that the HOST could be applied as a sperm selection method for the ICSI procedure was first introduced in 1994. In ART labs a rescue-ICSI is used for oocytes that failed fertilization upon a conventional IVF. Desmet et al. used the HOST to identify viable immotile sperm in a rescue-ICSI protocol and reported a 30% fertilization rate [127]. In this method, sperm cells are incubated at 37 °C in a culture dish in a hypo-osmotic solution. Then, HOST-positive sperm (curled tails) are selected and washed prior to conventional ICSI [128]. Since then, some studies focused on the composition of the hypo-osmotic solution in order to establish a standard procedure [129, 130].

It is possible to obtain clinical pregnancies from viable but immotile sperm with the HOST, which confirms its practical applicability [128, 131-134]. It was also observed a tendency, with no statistical significance, for higher fertilization and implantation rates and more quality embryos in sperm selected with HOST before ICSI in cases of total absence of sperm motility [128, 132, 135, 136]. Despite being an easy and inexpensive method with positive results reported, novel methods as PICSI or laser assisted immotile sperm selection renders HOST as more useful for standard semen analyses than as a sperm selection method.

3.5.3.2 Laser-assisted immotile sperm selection (LAISS)

In 2000, Montag et al. tested the use of laser to immobilize sperm cells before ICSI and observed that motile spermatozoa curled the tail upon exposition to the laser shot [137]. The same group performed this technique in order to select viable but immotile sperm cells from ejaculates and testicular biopsies, introducing the laser-assisted immotile sperm selection (LAISS) method. They reported a higher fertilization and cleavage rates and a trend for a higher take-home-baby rate following LAISS, when compared to morphological criteria alone [138]. Although the mechanism responsible for the curling are unknown, the authors hypothesize that it must be related to the influx of medium into the sperm tail following the laser shot [138]. Ebner et al. compared LAISS with conventional methods and did not find any difference concerning fertilization and cleavage rate or blastocyst formation. However, it was highlighted that LAISS may be a potential alternative to conventional mechanical approach for spermatozoa immobilization as it is less time consuming [139]. The achievement of pregnancy following LAISS in immotile frozen-thawed spermatozoa has been described, which validates the use of this method for cryopreserved samples [140]. This fact became highly advantageous as testicular biopsies are, generally, cryopreserved before the IVF treatment. Although rather expensive, compared to HOST, LAISS is considered as a non-hazardous and safe method for the identification of viable spermatozoa from immotile samples [141]. More studies are required to clarify the potential of LAISS as a routine procedure for sperm immobilization before ICSI.

3.5.3.3 Polarization Microscopy

The polarization microscopy for sperm selection uses anisotropic properties of sperm cells to assess its birefringent patterns, which is based on the fact that the nucleus of mature spermatozoa has an intrinsic birefringence (double refraction) associated with longitudinally oriented nucleoprotein filaments [142]. The birefringence of the sperm head can be used as an indicator of the normal structural organization of its nucleus. In addition, the

birefringence can also assess the state of the acrosome reaction, due to the fact that mature sperm cells exhibit birefringence over the entire head, while reacted spermatozoa exhibit birefringence in the postacrosomal region [143]. The sperm head birefringence is correlated with some sperm parameters. Magli et al. described the correlation between sperm motility and normal morphology with the birefringence patterns, reporting that sperm cells with morphological abnormalities or with conditioned motility have an abnormal pattern when compared to motile and morphologically normal spermatozoa [144]. In another study, Crippa et al. reported that the proportion of spermatozoa to head-birefringent-sperm was inversely correlated to the incidence of fragmented DNA [145]. Contrastingly, Petersen et al. reported that spermatozoa with total birefringence had more DNA fragmentation compared with those with partial birefringence [146], rendering the association of sperm head birefringence with DNA damage questionable. In 2008, the assessment of sperm birefringence was successfully used by Gianaroli et al. to select sperm for ICSI. When compared with the conventional ICSI procedure based on morphological sperm selection, the spermatozoa selected by polarized microscopy led to a better embryo development and clinical outcome [142]. Two years later, the same group evaluated the birefringence patterns on the basis of the acrosome integrity (acrosome-reacted and total acrosome-non-reacted sperm) and its relation to ART outcomes. The study concluded that there was no effect on the fertilizing capacity and embryo development of either type of sperm, although implantation rate and clinical pregnancy were higher in oocytes injected with reacted sperm [147]. Despite these successful results, further studies are needed to validate and standardize the method. Similar to IMSI, the assessment of birefringence patterns of sperm head is based on morphology examination, requiring a skilled embryologist to correctly perform this technique.

3.5.4 Case-specific recommendations for the preparation of semen samples

Preparation of epididymal and testicular sperm samples

The most common indication for epididymal aspiration is obstructive azoospermia and, in general, this type of samples provides an adequate number of sperm cells. Depending on the quality of the sample, a simple sperm wash or a density gradient centrifugation can be applied [12]. In cases of non-obstructive azoospermia, sperm must be retrieved through a testicular biopsy. In these cases, the testicular sperm can be retrieved either by open biopsy or by percutaneous needle biopsy [12]. In cases of testicular biopsy, the testicular tissue is immediately placed in culture media following the excision. Then, the tissue is shredded and the centrifuged with culture media. The supernatant is removed and the pellet is resuspended in fresh culture media [148]. Due to the abundance of red blood cells in

testicular biopsy samples, the search for sperm cells for ICSI can be challenging. In these cases, a protocol for the lysis of red blood cells might be recommended. Nagy et al. described a protocol that allows the lysis of red blood cells without damaging the sperm cells or affecting the ART outcome [149]. In cases of severe testicular failure with very low amounts of testicular spermatozoa, sperm cell collection remains difficult and unsuccessful even after red blood cells lysis. For these cases, Crabbé et al. reported the additional use of an enzymatic treatment with collagenase type IV and DNase as a safe procedure, with an acceptable fertilization rate [150]. The procedures described above for testicular or epididymal sperm preparation are routinely performed in ART laboratories and are validated and described in detailed in the WHO guidelines for semen sample preparation methods [12].

Preparation of retrograde ejaculation samples

In cases of retrograde ejaculation, semen enters in the bladder upon ejaculation which results in aspermia or no apparent ejaculate. Post-ejaculatory urine sample should be examined for the presence of sperm cells to confirm this diagnosis. Sperm cells may be obtained from the post-ejaculatory urine sample. For this purpose, the alkalinisation of the urine by ingestion of sodium bicarbonate is recommended, as it will improve the motility of sperm cells found in the urine [151]. According to the WHO guidelines, both the ejaculate, if present, and urine samples should be analysed. Both can be successfully processed using the density gradient centrifugation method [12].

Preparation of samples with immotile spermatozoa

In cases of samples with no motile sperm cells, the use of pentoxifylline might be recommended. Pentoxifylline is a phosphodiesterase inhibitor that enhances sperm motility by increasing intracellular cAMP (Terriou et al., 2000). Despite the potential embryotoxicity of this compound, pentoxifylline has been used in sample preparation for ART (Tournaye et al., 1993). Some studies suggest that the use of pentoxifylline is beneficial for asthenozoospermic patients (Sikka and Hellstrom, 1991; Yovich et al., 1988), and its use was also reported to improve the fertilization and pregnancy rates using immotile epididymal and testicular sperm cells (Terriou et al., 2000). The pentoxifylline protocol can vary between labs. Pentoxifylline can be added to fresh sperm samples or washed spermatozoa and it is necessary incubate the sperm at a temperature of 37°C [152, 153].

Preparation of HIV-infected semen samples

A combination of density gradient centrifugation followed by swim-up is recommended for samples of human immunodeficiency virus (HIV)-infected men, as a way of preventing

infection of uninfected female partners [154, 155]. In those cases, HIV is present in the semen, as viral RNA and proviral RNA are found free in seminal plasma and in non-sperm cells. Since the first study describing the use of semen washing in cases of HIV-discordant couples [156], several studies have evaluated the effect of this method in conjunction with ART. Zafer et al. concluded in a systematic review and meta-analysis that in HIV-discordant couples semen washing successfully prevents HIV transmission [157]. The use of density gradient centrifugation permits the isolation of sperm cells, potentially eliminating the viral RNA found in the seminal plasma and non-sperm cells. After liquefaction, the sperm sample is diluted 1:1 (v/v) with culture medium and centrifuged in order to eliminate the seminal plasma and reduce the potential risk of contamination. The pellet is resuspended in culture media and layered on top of a triple density gradient (90%-70%-45%). After centrifugation for 20 min at 300g, the supernatant is discarded, and the pellet resuspended in culture media. Swim-up might be used in order to isolate motile sperm cells. The retrieved sperm cells are divided in two parts: one is used for post-wash HIV testing and the other is cryopreserved. Post-wash HIV samples are tested by reverse transcription polymerase chain reaction (RT-PCR) to verify if the protocol was effective and ensure that the samples to be used in ART are HIV-free [12, 158]. Once the PCR results are available, the cryopreserved sample can be used for ART treatment or discarded in case of positive results [159].

3.6 Conclusions and future perspectives

Sperm are highly complex cells influenced by several internal and external stimuli, which may compromise its fertilizing potential. Considering the disclosure of several new biomarkers for sperm quality and its fertility potential [4, 5, 160, 161], the established standard semen quality parameters are rendered as limited. Few parameters are evaluated, which might explain the high percentage of cases classified as idiopathic. For instance, several metabolic diseases, such as diabetes mellitus or obesity, affect the male reproductive potential at the molecular level [162, 163]. Based on novel data and a subsequent deeper understanding of the human sperm physiology, innovative advanced sperm selection methods that go beyond the conventional parameters and aim to address more complex and specific cases have been developed. Although the use of some of these advanced sperm selection methods have proven to be able to improve ART outcomes, the majority still are in an embryonic state and require further studies to validate their efficiency in clinical practice. It is also important to highlight that there is a lack of standardization regarding protocols and laboratories, which makes it difficult to compare studies and their results.

More recently, epigenetics and ROS signaling became a hot topic for human reproduction academics. There are some evidences that metabolic diseases lead to epigenetic alterations on sperm cells, which may compromise the pregnancy or even precondition the offspring for its development [11, 164]. Further studies are needed to elucidate the influence of the epigenetic signature for the fertilization process and pregnancy before novel advanced sperm selection methods are developed to address the issue.

In sum, ART have significantly improved for the past decades, leading to higher percentages of successful pregnancies. Although the conventional methods remain as the most common and efficient method for the majority of the cases, more advanced techniques have been developed in order to address more specific cases. No method, conventional or advanced, is the perfect solution. Depending on the nature of each case and based on previous experiences of other couples with the same infertility diagnose, the embryologist should select which is the best option to apply.

3.7 Take-Home Message

- The application of ART has revolutionized the treatment of human infertility, bringing hope to patients previously considered incapable for reproductive purposes.
- The swim-up procedure and density gradient centrifugation are the most popular and widely used conventional methods for sperm selection.
- Novel and more advanced methods are continuously being developed and improved, leading to better ART rates and outcomes.

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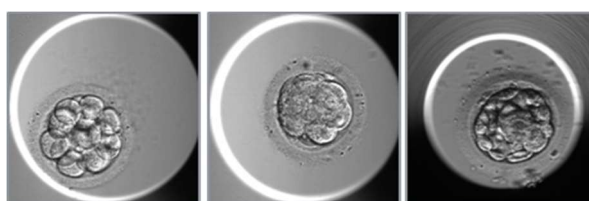
CAPÍTULO 4

Metabolomics integration in assisted reproductive technologies for enhanced embryo selection beyond morphokinetic analysis

Este capítulo foi adaptado integralmente do seguinte artigo publicado:

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O texto integral pode ser consultado no Anexo A desta Dissertação



4 Metabolomics Integration in Assisted Reproductive Technologies for Enhanced Embryo Selection Beyond Morphokinetic Analysis

4.1 Abstract

Embryo quality evaluation during *in vitro* development is a crucial factor for the success of assisted reproductive technologies (ARTs). However, the subjectivity inherent in the morphological evaluation by embryologists can introduce inconsistencies that impact the optimal embryo choice for transfer. To provide a more comprehensive evaluation of embryo quality, we undertook the integration of embryo metabolomics alongside standardized morphokinetic classification. The culture medium of 55 embryos (derived from 21 couples undergoing ICSI) was collected at two timepoints (days 3 and 5). Samples were split into Good (n = 26), Lagging (n = 19), and Bad (n = 10) according to embryo morphokinetic evaluation. Embryo metabolic performance was assessed by monitoring the variation in specific metabolites (pyruvate, lactate, alanine, glutamine, acetate, formate) using $^1\text{H-NMR}$. Adjusted metabolite differentials were observed during the first 3 days of culture and found to be discriminative of embryo quality at the end of day 5. Pyruvate, alanine, glutamine, and acetate were major contributors to this discrimination. Good and Lagging embryos were found to export and accumulate pyruvate and glutamine in the first 3 days of culture, while Bad embryos consumed them. This suggests that Bad embryos have less active metabolic activity than Good and Lagging embryos, and these two metabolites are putative biomarkers for embryo quality. This study provides a more comprehensive evaluation of embryo quality and can lead to improvements in ARTs by enabling the selection of the best embryos. By combining morphological assessment and metabolomics, the selection of high-quality embryos with the potential to result in successful pregnancies may become more accurate and consistent.

4.2 Introduction

In recent years, the incidence of infertility has been increasing, which can be attributed to various social, biological, and biochemical factors. The World Health Organization (WHO) defines infertility as the inability of a couple to achieve pregnancy after one year of sexual intercourse without contraception [1]. It is estimated that infertility affects between 4 and 14% of the population globally, making it a significant public health burden. Assisted reproductive technologies (ARTs) have emerged as a solution to this problem, with the

WHO advocating for increased accessibility to such treatments. However, success rates of ARTs are still below expectations, indicating a need for further investigation into the biochemical processes involved in ART techniques, particularly during embryonic development, and the identification of biomarkers associated with the outcome of ART programs [2]. One of the significant challenges of ARTs is the optimization of embryo selection. The existing approaches to gauging the potential for embryo implantation primarily hinge on morphological assessment. However, this method does not consistently provide accurate predictions of successful implantation [3].

Metabolomics, a quantitative and non-invasive technology, has recently emerged as a promising tool for measuring metabolites present in cells, tissues, body fluids, and culture media. By quantifying the metabolites secreted by gametes and embryos during culture, metabolomics can provide insights into embryonic viability and help to identify embryonic biomarkers that may improve the outcomes of ART treatments [4,5]. Several studies have already established correlations between embryonic viability and carbohydrate, pyruvate, and amino acid metabolism during embryonic development [5–10]. Indeed, the metabolic performance of embryos post-compaction has emerged as a noteworthy biomarker indicative of superior-quality blastocysts [9,11]. Despite these promising findings, the lack of consensus in this field largely stems from the absence of standardization in the molecular assessment of embryo quality [12]. Therefore, this study aims to integrate metabolomics with the standardized morphological classification of embryos to identify biomarkers that can optimize the selection of the best embryo for transfer. By combining metabolomic and standardized morphological embryo evaluations, this study can provide valuable insights into the complex metabolic processes underlying embryonic development and quality. Ultimately, the integration of metabolomics in ARTs can offer a powerful tool for improving treatment outcomes and identifying biomarkers associated with embryonic viability and quality. The identification of such biomarkers can greatly enhance the efficiency and effectiveness of ART, leading to better pregnancy rates and improved clinical outcomes for infertile couples.

4.3 Results

4.3.1 Clinical Data and Embryo Characterization

As shown in Table 4.1, 21 intracytoplasmic sperm injection (ICSI) cycles were included in this study. All the treatments were made in accordance with clinical indication, and fertilization was achieved with the ICSI technique. The embryo culture was extended until day 5 (D5). The cycle characteristics are presented in Table 4.1.

Table 4.1 Characterization of the studied population and fertilization cycles.

Baseline Characteristics of Studied Population		Patient Cycle Characteristics	
Number of couples	21	Fertilization technique (%)	
Number of cycles	21	ICSI	100 (21/21)
Female age (years)	35.05 ± 6.59	Stimulation duration (days)	9.29 ± 1.38
Female body mass index (kg/m ²)	23.59 ± 3.49	FSH total dose (IU)	2827±1245
Male age (years)	40.71 ± 5.49	Serum E ₂ on trigger day (pg/mL)	2481 ±1486
Male body mass index (kg/m ²)	24.84 ± 5.82	Serum P on trigger day (ng/mL)	0.95 ± 0.46
Infertility duration (months)	49.86 ± 32.29	Oocytes retrieved (n)	8.48 ± 2.66
Previous IVF attempts (%)		Metaphase II oocytes (n)	7.43 ± 2.31
0	42.9 (9/21)	Maturation rate (%)	85.2 ± 14.4
1	23.8 (5/21)	Fertilized oocytes (n)	6.33 ±2.46
2	19.0 (4/21)	Fertilization rate (%)	85.20 ±14.38
≥ 3	14.3 (3/21)	Embryo stage	
Types of infertility (%)		Day 3 embryos (n)	6.14 ± 2.5
Primary infertility	66.7 (14/21)	Day 5 embryos (n)	4.95 ± 2.41
Secondary infertility	33.3 (7/21)	Cleavage rate (%)	96.7 ± 7.1
Anti-Müllerian hormone (ng/mL)	4.02 ± 4.34	Embryo utilization rate (%)	53.6 ± 21.5
Total number of analyzed embryos	55	Embryo transfers (%)	92.9 (13/14)
Good	26	1 embryo transferred	69.2 (9/13)
Lagging	19	2 embryos transferred	30.8 (4/13)
Bad	10	Freeze all cycles	7.1 (1/14)
		Biochemical pregnancy (%)	61.5 (8/13)
		Clinical pregnancy (%)	61.5 (8/13)

Data are shown as the mean ± standard deviation. Legend: E₂, estradiol; FSH, follicle-stimulating hormone; ICSI, intracytoplasmic sperm injection; P, progesterone.

In 7 cycles, the treatment involved Preimplantation Genetic Testing (PGT) without fresh embryo transfer. In this type of procedure, the embryos that develop into blastocysts with good quality are biopsied and then cryopreserved for deferred transfer after genetic results. Of the remaining 14 cycles, 5 were performed with oocyte donation and in 9 cycles, the ICSI protocol has no additional technique. Apart from the PGT cycles, a fresh embryo transfer had to be called off during a particular treatment due to elevated progesterone serum levels. Consequently, in this scenario, embryos of good quality were preserved through vitrification for a subsequent transfer. Across all 13 remaining cycles, the fresh embryo transfers were successfully carried out, aligning with the precise number of embryos transferred in accordance with the established guidelines of the Centro de Genética Prof. Alberto Barros (CGR) clinic. The surplus good-quality embryos were vitrified for later use. A total of 110

culture medium samples from 55 oocytes were collected during the study period, 55 samples of culture medium from embryos on day 3 (D3) and 55 samples of culture medium from embryos on D5. Degenerated embryos, non-cleavage embryos, abnormal fertilization, and parthenogenetic embryos were excluded from statistical analyses.

The embryos were classified as described below. Based on morphological assessment on D5, a blastocyst of good quality was considered as BL3BB or better (BL5—AA, AB, BA, BB; BL4—AA, AB, BA, BB; BL3—AA, AB, BA, BB), and a Bad embryo was considered if it had inner cell mass or/and trophectoderm grade C (BL5/BL4/BL3—AC, CA, CC). An embryo was classified as Lagging if the embryo development was delayed considering the standardized timing of embryo observations established in the Istanbul Consensus [13]. From the 55 observed embryos, 26 were classified as Good, 19 as Lagging, and 10 as Bad embryos.

4.3.2 Embryo Quality at Day 5 Correlates with Metabolite Consumption or Release

Metabolite consumption and release by the embryos were assessed by ^1H -NMR analysis of the embryo culture media on D3 (Figure 4.1 and Table 4.2) and on D5 (Figure 4.2 and Table 4.2). Alanine (Figure 4.1 A and Figure 4.2 A), acetate (Figure 4.1 B and Figure 4.2 B), pyruvate (Figure 4.1 C and Figure 4.2 C), glutamine (Figure 4.1 D and Figure 4.2 D), lactate (Figure 4.1 E and Figure 4.2 E), and formate (Figure 4.1 F and Figure 4.2 F) were the most relevant metabolites identified in the culture media of both D3 and D5. Embryos that were categorized into Good, Lagging, or Bad according to their quality at D5 were evaluated for metabolite consumption or production using ^1H -Nuclear Magnetic Resonance (NMR) spectroscopy, and differences were analyzed by one-way ANOVA, followed by Tukey's post hoc test.

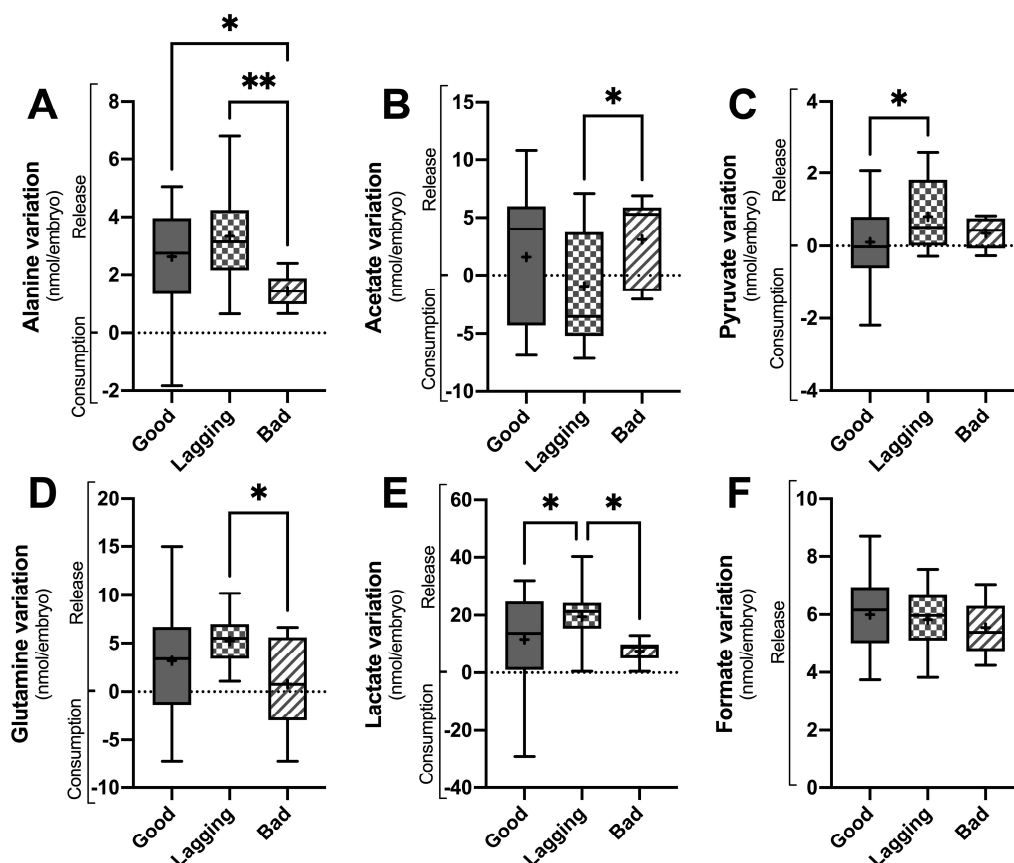


Fig 4.1 Metabolite variation in culture media of human embryos between day 3 (D3) and day 0 grouped according to their quality at day 5 (D5) (Good vs. Lagging vs. Bad). The figure shows the variation (Consumption or Release) in selected metabolites (Panel **A**-alanine; Panel **B**-acetate; Panel **C** -pyruvate; Panel **D**-glutamine; Panel **E**-lactate; Panel **F**-formate) quantified in the embryo culture media at the end of D3 (n = 55). Data are presented as mean ± standard deviation (S.D.). Normality and heteroscedasticity tests were performed, and univariate ANOVA was conducted on the selected metabolites identified in the embryo culture medium. Pairwise comparisons between groups were corrected for multiple hypotheses testing by Tukey's post hoc test. All p values < 0.05 were considered statistically significant (* p < 0.05; ** p < 0.005). + represents group average.

On D3 of embryo culture, there were significant differences in the levels of alanine detected in the culture media. Specifically, both Good and Lagging embryos showed a higher release of alanine (2.63 ± 1.68 nmol/embryo and 3.34 ± 1.65 nmol/embryo, respectively) compared to Bad embryos (1.42 ± 0.57 nmol/embryo). Similarly, glutamine release was significantly higher in Lagging embryos (5.24 ± 2.69 nmol/embryo) than in Bad embryos (0.80 ± 4.66 nmol/embryo). In Figure 4.1 D, it can be observed that the release of glutamine to the culture media by Good embryos was intermediate, with a mean value of 3.16 ± 4.94 nmol/embryo, compared to the values observed for Bad and Lagging embryos.

Lagging embryos consumed acetate (-0.94 ± 4.98 nmol/embryo), contrarily to Good (1.59 ± 5.23 nmol/embryo) and Bad (3.11 ± 3.66 nmol/embryo) embryos, which released it to the media (Figure 4.1 B). Pyruvate (Figure 4.1 C) and lactate (Figure 4.1 E) release was higher in Lagging (0.66 ± 0.99 and 19.44 ± 9.05 nmol/embryo) embryos than in Good (0.10 ± 1.23 and 11.55 ± 15.05 nmol/embryo) and Bad (0.35 ± 0.42 and 7.49 ± 3.99 nmol/embryo) embryos. No differences were observed regarding formate release between Good (6.00 ± 1.18 nmol/embryo), Lagging (5.82 ± 1.06 nmol/embryo), and Bad (5.54 ± 0.94 nmol/embryo) embryos (Figure 4.1 F).

Table 4.2 Metabolite variation in embryo culture media. Metabolite variation observed on the first 3 days (D0–3) and from day 3 to day 5 (D3–5) in culture media of human embryos grouped according to their quality (Good, Lagging, and Bad).

Metabolite (nmol/embryo)	Metabolite Variation in Embryo Culture Media					
	Day 0 to 3			Day 3 to 5		
	Good	Lagging	Bad	Good	Lagging	Bad
Alanine	2.63 ± 1.68	3.34 ± 1.65	$1.42 \pm 0.57^{**}$	4.92 ± 2.83	5.05 ± 2.62	5.27 ± 2.70
Acetate	1.59 ± 5.23	-0.94 ± 4.98	$3.11 \pm 3.66^{\#}$	-1.43 ± 4.44	1.04 ± 5.78	-1.24 ± 4.16
Pyruvate	0.10 ± 1.23	$0.81 \pm 0.94^{*}$	0.35 ± 0.42	0.23 ± 0.67	0.78 ± 0.38	0.33 ± 0.57
Glutamine	3.16 ± 4.94	5.24 ± 2.69	$0.80 \pm 4.66^{\#}$	3.10 ± 4.47	4.48 ± 5.65	4.90 ± 3.42
Lactate	11.55 ± 15.05	$19.44 \pm 9.05^{*}$	$7.49 \pm 3.99^{\#}$	16.60 ± 13.27	19.03 ± 10.39	15.55 ± 10.99
Formate	6.00 ± 1.18	5.82 ± 1.06	5.54 ± 0.94	5.85 ± 1.51	6.33 ± 1.21	6.43 ± 1.23

Data are shown as mean $\pm$ SD. Positive values represent release to the extracellular medium, while negative values mean consumption by the embryo. All p values < 0.05 were considered statistically significant (* vs. Good embryos, # vs. Lagging embryos).

Concerning D5 variation in metabolites (Figure 4.2), only pyruvate levels were significantly different between groups. Good embryos (0.23 ± 0.67 nmol/embryo, $p = 0.0112$) released significantly less pyruvate than Lagging embryos (0.78 ± 0.38 nmol/embryo), whereas Bad (0.33 ± 0.57 nmol/embryo) embryos' pyruvate release was intermediate (Figure 4.2 C). No differences were found concerning alanine (4.92 ± 2.83 ; 5.05 ± 2.62 and 5.27 ± 2.70 nmol/embryo) (Figure 4.2 A), glutamine (3.10 ± 4.47 ; 4.48 ± 5.65 and 4.90 ± 3.42 nmol/embryo) (Figure 4.2 D), lactate (16.60 ± 13.27 ; 19.03 ± 10.39 and 15.55 ± 10.99 nmol/embryo) (Figure 4.2 E), and formate (5.85 ± 1.51 ; 6.33 ± 1.21 and 6.43 ± 1.23 nmol/embryo) release (Figure 4.2 F) levels between Good, Lagging, and Bad embryos, respectively. Although no statistical difference was observed, Good (-1.43 ± 4.44 nmol/embryo) and Bad (-1.24 ± 4.16 nmol/embryo) embryos tended to consume acetate,

whereas Lagging embryos (1.04 ± 5.78 nmol/embryo) tended to release it to the media (Figure 4.2 B).

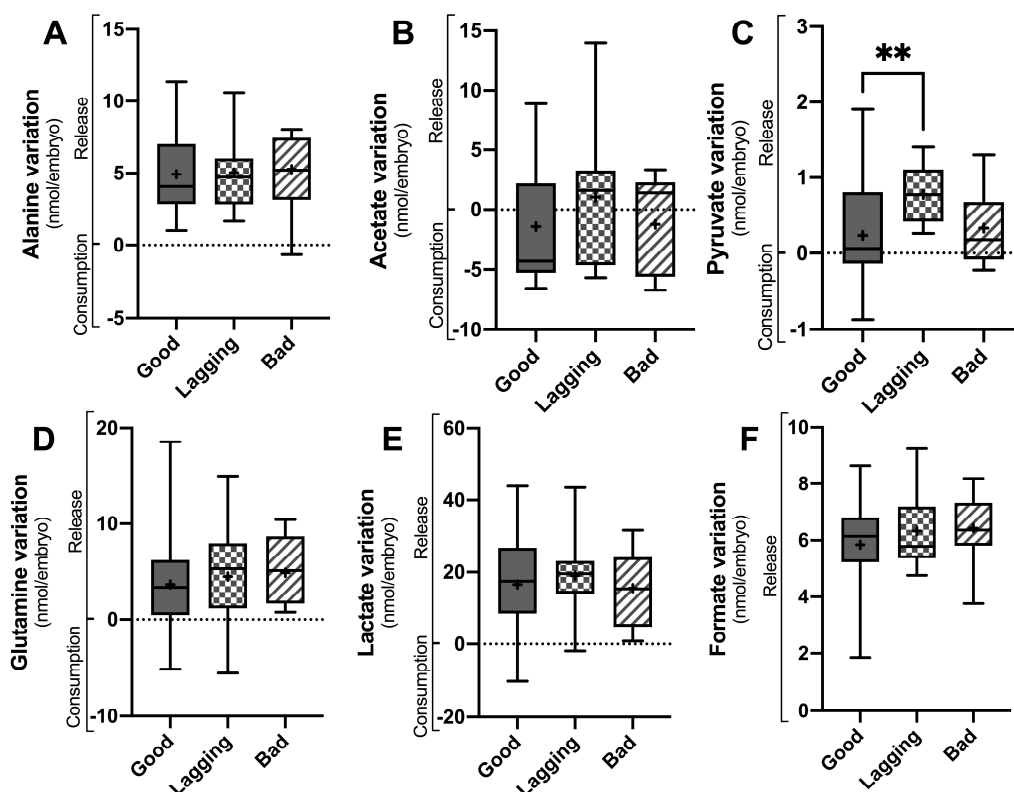


Fig 4.2 Metabolite variation in culture media of human embryos between day 3 (D3) and day 5 (D5) grouped according to their quality at D5 (Good vs. Lagging vs. Bad). The figure shows the variation (Consumption or Release) in selected metabolites (Panel A-alanine; Panel B-acetate; Panel C-pyruvate; Panel D-glutamine; Panel E-lactate; Panel F-formate) quantified in the embryo culture media at the end of D5 ($n = 55$). Data are presented as mean $\pm$ standard deviation (S.D.). Normality and heteroscedasticity tests were performed, and univariate ANOVA was conducted on the selected metabolites identified in the embryo culture medium. Pairwise comparisons between groups were corrected for multiple hypotheses testing by Tukey's post hoc test. All p values < 0.05 were considered statistically significant (** $p < 0.005$). + represents group average.

4.3.3 Metabolite Variation during the First 3 Days of Development Is a Strong Predictor of Embryo Quality at Day 5

Metabolite consumption or release by embryos was assessed in embryo culture medium on the first 3 days (D0–3) and from D3 to D5 (D3–5). Metabolite variations were compared between groups of embryos categorized according to their quality at D5 using MANCOVA, after adjusting for covariates. One of the assumptions to conduct MANCOVA was not met

when analyzing the D0–3 period: The homogeneity of covariance matrices across groups is not assumed (Box's $M = 120.66$, $F(56, 2017.579) = 1.584$, $p = 0.004$), but it is assumed in the D3–5 interval. Also, the homogeneity of variance of all dependent variables was confirmed with Levene's test in both intervals. Linearity and multicollinearity were graphically assessed and were considered acceptable in both intervals. Therefore, we considered that the MANCOVA model was sufficiently robust.

In addition to the mathematical evaluation of the methodology, we have conducted a thorough deliberation while selecting the covariates to be integrated into the model. Our choices have been driven by their intrinsic physiological significance and relevance. Recent studies have established a direct proportion between the age of the donor of the female gamete and embryo oxygen consumption rate (OCR) during the preimplantation phase [14–16]. Although OCR and maximum OCR are crucial and measurable markers of early embryo metabolism and development, other factors such as DNA integrity, antioxidant pool, and pre-collection hormonal levels further contribute to embryo development [17–19]. The same variables act on the male gamete and contribute to the fate of the embryo during early development [20,21]. Age and body mass index (BMI) have been reported to be correlated with metabolic, antioxidant, and DNA integrity in both female and male gametes, with direct influence over early embryo development [18–23]. Therefore, we selected the broader and easily measurable “age” and “BMI” of both male and female as covariates in our MANCOVA model.

In the D0–3 interval, metabolite differentials are discriminative of embryo quality category at D5 (Roy's Largest Root = 0.644, $F(7, 41) = 3.77$, $p = 0.003$, partial $\eta^2 = 0.392$), after controlling for covariates. The discriminant power of the model is supported by the differences in alanine ($F(2, 46) = 6.428$; $p = 0.003$), acetate ($F(2, 46) = 4.940$; $p = 0.011$), pyruvate ($F(2, 46) = 3.443$; $p = 0.040$), and glutamine ($F(2, 46) = 3.448$; $p = 0.040$). Pairwise comparisons were performed to identify which groups had different metabolite yields after incubation, corrected by Šidak's method (Figure 4.3 A).

After adjusting for confounding factors, alanine release during D0–3 was lower in Bad embryos (1.11 ± 1.48 nmol/embryo) than in Lagging embryos (3.25 ± 1.44 nmol/embryo, $p = 0.003$) and Good embryos (2.78 ± 1.45 nmol/embryo, $p = 0.018$). The remaining observed differences—acetate, pyruvate, and glutamine—were found comparing D0–3 differentials between Lagging and Bad embryos (Figure 4.3 A and Table 4.3). Interestingly, Lagging embryos display a consumption of acetate contrary to the other groups, which released it (Figure 3A). Bad embryos consumed pyruvate and glutamine, energetic substrates, contrary to the other groups. However, pyruvate differentials vary within limited ranges, suggesting that this metabolite is readily consumed and produced. In the D3–5 interval, metabolite differentials are not discriminative of embryo quality category at D5 (Roy's

Largest Root = 0.180, $F(7, 41) = 1.054$, $p = 0.410$, partial $\eta^2 = 0.153$, after controlling for covariates (Figure 3B). Detailed estimates after accounting for covariates are provided in Table 4.3.

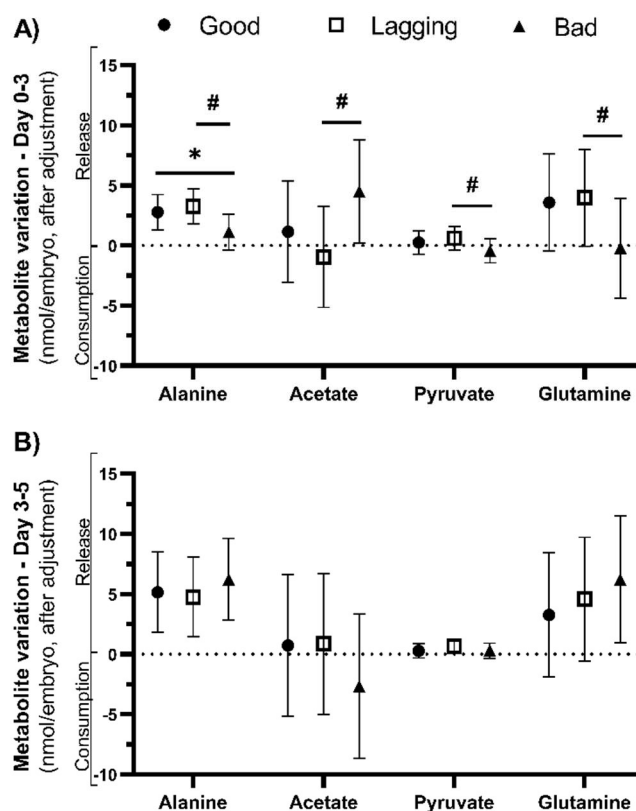


Fig 4.3 Metabolite variation observed on the first 3 days (D0–3) and from day 3 to day 5 (D3–5) in culture media of human embryos grouped according to their quality (Good vs. Lagging vs. Bad). The figure shows the metabolite differentials of relevant metabolites (alanine, acetate, pyruvate, and glutamine) observed on the first 3 days (Panel A) and from day 3 to day 5 (Panel B) ($n = 55$). Data are presented as mean $\pm$ standard deviation (S.D.). The impact of each metabolite differential on embryo quality at day 5 was assessed using a univariate F-test, after adjusting for the covariates. Pairwise comparisons between groups were corrected for multiple hypotheses testing by Šidak's method. All p values < 0.05 were considered statistically significant (□ vs. Good embryos; # vs. Lagging embryos).

Table 4.3 Adjusted metabolite variation in embryo culture media. Metabolite variation observed on the first 3 days (D0–3) and from day 3 to day 5 (D3–5) in culture media of human embryos grouped according to their quality (Good, Lagging, and Bad)

Metabolite (nmol/embryo)	Adjusted Metabolite Variation in Embryo Culture Media ^a					
	Day 0 to 3			Day 3 to 5		
	Good	Lagging	Bad	Good	Lagging	Bad
Alanine	2.78 ± 1.45	3.25 ± 1.44	1.11 ± 1.48 * [#]	5.15 ± 3.33	4.75 ± 3.32	6.22 ± 3.40
Acetate	1.15 ± 4.20	-0.95 ± 4.19	4.50 ± 4.29 [#]	0.72 ± 5.88	0.86 ± 5.86	-2.67 ± 6.00
Pyruvate	0.25 ± 0.97	0.61 ± 0.97	-0.45 ± 0.99 [#]	0.28 ± 0.61	0.66 ± 0.61	0.29 ± 0.62
Glutamine	3.59 ± 4.05	3.98 ± 4.03	-0.22 ± 4.14 [#]	3.27 ± 5.18	4.57 ± 5.15	6.22 ± 5.28
Lactate	13.07 ± 12.13	14.69 ± 12.08 *	4.09 ± 12.38 [#]	18.26 ± 17.04	15.00 ± 16.96	17.21 ± 17.38
Formate	5.10 ± 2.36	4.81 ± 2.34	5.39 ± 2.41	5.37 ± 2.54	5.26 ± 2.53	5.90 ± 2.59

Data are shown as mean ± SD. Positive values represent release to the extracellular medium, while negative values mean consumption by the embryo. ^a Covariates: Female Age = 34.52, Male Age = 39.43, Female BMI = 23.611934, Male BMI = 26.344065, technique = 2.80. All p values < 0.05 were considered statistically significant (* vs. Good embryos, [#] vs. Lagging embryos).

4.4 Discussion

The prevailing approaches employed to assess embryo quality throughout *in vitro* development exhibit certain limitations. These methods predominantly rely on the expertise of an experienced embryologist for morphological evaluation. Thus, these methodologies are subjective, and there can be inter- and intra-observer variability, which can affect the consistency of embryo grading and impact the selection of the best embryo for transfer. Moreover, morphological assessment is typically performed at specific time points during embryo development, which may not be optimal for evaluating embryo quality [12]. Hence, morphological assessment is not always effective in predicting implantation success. Even high-quality embryos can face challenges in terms of successful implantation or may not culminate in viable pregnancies. This inherent variability can lead to the potential misclassification of certain embryos, thereby contributing to a reduction in the overall success rates of ART treatments. In this sense, the integration of embryo metabolomics with standardized morphological classification can help address some of these limitations by providing a more comprehensive evaluation of embryo quality, including information on potential biomarkers for embryo selection [4].

In this work, we evaluate the metabolic performance of embryos, using ^1H -NMR spectroscopy, during *in vitro* development, which were monitored in a time-lapse incubator during a period of 5 days and subject to standardized morphokinetic categorization, according to their development. The application of ^1H -NMR in the analysis of human IVF culture media comes with both advantages and potential pitfalls. One limitation is the sensitivity of ^1H -NMR to sample composition. The complex nature of culture media, containing a myriad of compounds, can lead to signal overlap, making it challenging to discern individual metabolites accurately. Additionally, certain metabolites may exist in low concentrations, making their detection and quantification less reliable. Despite these challenges, it is important to highlight that ^1H -NMR remains a very valuable tool for metabolomic analysis, offering non-destructive, high-throughput, and quantitative insights into the metabolic profiles of biological samples. Researchers must navigate these limitations judiciously, considering them in the context of the specific goals and characteristics of their study, as we did in the present case. Indeed, after standardized morphokinetic categorization of embryos, media were collected to determine those embryos' metabolic performance. This allows us to search for a possible metabolic biomarker associated with the morphokinetic evaluation.

The definitive classification of embryos was determined through the utilization of Gardner's blastocyst grading scale [24], with adherence to the guidelines set by the Istanbul Consensus for the timing of observations and the identification of non-viable embryos [13]. Following this classification, they were divided into Good, Lagging, and Bad embryos, as described above. Our goal was to enhance the evaluation of embryo quality by integrating embryo metabolomics with standardized morphokinetic classification, in order to contribute to a more accurate and consistent selection of high-quality embryos with the potential for successful pregnancies. While recognizing that the inclusion of degenerated embryos, non-cleavage embryos, those with abnormal fertilization, and parthenogenetic embryos could yield data with more distinctive differences, we intentionally excluded them to maintain the focus, quality, and consistency of the data. Including embryos with abnormalities or arrested development could introduce confounding variables and detract from the study's objective, as these embryos are readily identifiable as low-quality with no potential for successful pregnancies.

During the 5-day culture, we chose to correlate the embryo development status with the variation, at D3 and D5, in six specific metabolites that are on the crossroad of major cellular pathways: pyruvate, lactate, alanine, glutamine, acetate, and formate. Indeed, with the exception of glucose (whose consumption remained unaffected across all our experimental conditions—supplemental data Figure S1), these metabolites stand as key protagonists within diverse metabolic pathways. Particularly during the intricate process of embryo

development, they assume a pivotal role, participating in energy generation, biosynthesis, and intricate signaling cascades [25,26]. For the aforementioned reasons, we hypothesized that one or more of these metabolites may serve as biomarkers for assessing the developmental competence of embryos, providing valuable insights into the underlying metabolic processes that contribute to successful embryogenesis. The identification of metabolites that are sufficiently abundant in the culture media to be easily quantified emphasizes their potential significance as key clinical indicators of the metabolic health and viability of developing embryos. This underscores the rationale for the inclusion of those metabolites in the study and their eventual future relevance in establishing a method that could more or less easily quantify them in the setting of the embryology laboratory.

Pyruvate is the end-product of the energy-yielding process of glycolysis and can be converted to acetyl-CoA, which enters the tricarboxylic acid cycle (TCA) to generate more ATP. Lactate and alanine are produced from pyruvate, particularly when the redox status (NAD⁺/NADH ratio) is altered. They can be converted back to pyruvate and generate ATP by being driven to the TCA [27]. Acetate is produced during fatty acid metabolism and can be used as an energy source by some cells [28]. Glutamine is an amino acid that is involved in several metabolic pathways, including the synthesis of nucleotides, proteins, and other amino acids, and is also an important source of energy for rapidly dividing cells such as embryos [29]. Formate can be derived from various pathways, including the breakdown of serine, being involved in one-carbon metabolism, which is important for the synthesis of nucleotides and other biomolecules [30]. Research findings have unequivocally shown that human preimplantation embryos actively engage in the consumption and secretion of these metabolites, changing their concentration in the surrounding culture media. This serves as a clear indicator of the embryo's robust metabolic activity [5,6,12].

Based on our analysis, the adjusted metabolite differentials (for covariates such as the male age, female age, male BMI, female BMI, and ART technique) observed in the first 3 days of embryo culture are discriminative of embryo quality category at the end of D5. The inclusion of covariates related to individual characteristics of the donors of the gametes is crucial to assess the impact of embryo culture medium metabolites in early embryo development. Although ICSI circumvents part of the individual variability, the age and BMI of the donors have been reported to influence the success rate of the ICSI [21,31,32], particularly in European cohorts [33,34]; therefore, we have considered these covariates to add another layer of embryo selection. The major contributors for discriminating Good, Lagging, and Bad embryos were pyruvate, alanine, glutamine, and acetate, after correcting for the confounders. Pyruvate, in the context of embryonic development, serves as an essential energy substrate during the preimplantation stages [27]. This metabolite has been linked to embryo development and increased embryo quality in some species, including

cattle and sheep [35]. Although its importance for early embryo development has been evidenced in some studies, the role of pyruvate in human embryonic development is still less clear when other metabolic sources are present [8,27]. In our study, adjusted pyruvate variation in the culture media of 3-day human embryos was quite distinct in Bad embryos from Good and Lagging embryos. While Bad embryos consumed pyruvate, Good and Lagging embryos were able to export and accumulate it in the culture media, suggesting an active metabolism. A similar trend was observed for glutamine, with Good and Lagging embryos being able to export and accumulate this amino acid in the culture media, while Bad embryos consumed it. Glutamine is an important metabolite for embryo development, as it serves as a precursor for various metabolic pathways [9]. It is a non-essential amino acid that can be synthesized by the embryo or taken up from the surrounding culture medium. During early embryonic development, glutamine is predominantly utilized for energy production through the TCA cycle [36,37]. Studies have shown that the addition of glutamine to the culture medium during *in vitro* embryo development can improve embryo quality and blastocyst formation rates [38]. This suggests that glutamine plays an important role in embryonic metabolism and development [29]. Human embryos have been shown to export glutamine during *in vitro* development [11]. *In vitro* studies have shown that the usage of glutamine by embryos increases during the blastocyst stage and that the export of glutamine is associated with increased cell proliferation and blastocyst formation [39], as observed in our experimental work.

Alanine is a non-essential amino acid that also plays an important role in the metabolism of developing embryos. During embryonic development, alanine is involved in the synthesis of proteins, the regulation of glucose metabolism, and the maintenance of cellular redox balance [40]. Studies have shown that alanine is an important nutrient for embryonic growth and development [40]. The availability of alanine can affect the developmental potential of embryos, with lower levels of alanine being associated with decreased embryo quality and lower rates of implantation [39]. In our work, up until D3, Bad embryos produce significantly lower amounts of alanine than Good or Lagging embryos. In fact, during development, the embryo has been shown to produce and export alanine to the surrounding environment, with embryos that produce and export higher levels of alanine having better developmental potential and being linked to higher pregnancy rates [5]. The export of alanine by human embryos is significant because it reflects the metabolic state of the embryo and its ability to maintain energy balance during early development. It has been suggested that the export of alanine may serve as a protective mechanism against stress and nutrient deprivation, allowing the embryo to maintain its viability and developmental potential [41]. Contrastingly, concerning acetate, both Bad and Lagging embryos showed a different trend from that of Good embryos in the variation of this short-chain fatty acid in culture media. While Bad

embryos produced higher amounts of acetate in our experimental conditions, Lagging embryos consumed it. Acetate has been shown to be an important energy source for embryo development [6,42]. As the embryo develops and the number of cells increases, the energy demand also increases, and the embryo begins to utilize other energy sources, including acetate [6]. It has been shown that acetate is critical for proper embryo development, as it enhances cell growth and division, regulates gene expression, and improves embryonic quality, particularly for embryos during the preimplantation period [35]. Overall, the higher amounts of acetate observed in the culture media of Bad embryos at the end of D3 should be due to lower utilization of this metabolite due to the poor development of these embryos. The significance of the consumption of acetate by Lagging embryos is unclear, but it may reflect the need to increase the metabolic rates of these cells to enhance the formation of blastocysts.

As a remark, and although no differences were observed in the variation of formate in any of the experimental conditions used, it is of note that our embryos consistently exported significant amounts of this monocarboxylic anion. It is known that mammalian cells generate formate as a byproduct of various metabolic reactions, including the breakdown of certain amino acids and the metabolism of folate. Formate is then either excreted from the cell or used in various biosynthetic pathways, including the synthesis of purines, pyrimidines, and amino acids. In addition, formate can also play a role in regulating cellular redox balance and oxidative stress. For example, formate has been shown to protect cells from oxidative damage by acting as an antioxidant. Overall, the cellular significance of formate in mammalian cells is diverse and multifaceted, with important roles in both metabolism and cellular defense mechanisms [30]. During early embryonic development, formate is produced by the mitochondria and is used as a source of one-carbon units for nucleotide synthesis and methylation reactions [7]. Formate is the main one-carbon donor for de novo purine biosynthesis in the developing embryo. It has been shown that the inhibition of formate metabolism in early embryos can lead to developmental defects and reduced viability [43]. In fact, some studies suggested formate levels as a biomarker for embryonic viability in ART, as the concentration of formate in the culture media can be linked to the metabolic activity of the embryo and in some conditions can be indicative of embryonic viability and quality.

4.5 Materials and Methods

4.5.1 Chemicals

Embryo and gamete culture media and reagents were purchased from Origio® (Malov, Denmark), Nidacon (Molndaln, Sweden), and Vitrolife (Gothenburg, Sweden). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

4.5.2 Patient Selection and Ovarian Stimulation

Twenty-one couples (who underwent a total of 21 cycles) were involved in this study. The treatments of ARTs were conducted at CGR, Porto, Portugal between June 2019 and February 2021. The procedures of the Centre for Reproductive Genetics Alberto Barros are covered by the provisions of the National Medically Assisted Procreation Act (2017) and overseen by the National Council for Medically Assisted Procreation (CNPMA-2018). According to these rules and guidelines, the clinical databases and patient biological material for diagnosis and research may be used without further ethical approval, under strict individual anonymity, and after patient written informed consent. Regarding the use of human samples for laboratory experimentation, the Ethics Committee authorization number is 2021/CE/P02 (P342/2021/CETI), approved on 26 February 2021. The inclusion criteria were couples who were referred to infertility treatment with ICSI technique due to the need to avoid the interference of cumulus cells or spermatozoa during metabolomics quantification. The couple was excluded from the study if they had a testicular biopsy performed (testicular sperm aspiration or testicular sperm extraction) or cryopreserved ejaculated spermatozoa. So, all the ICSI were performed with fresh ejaculated spermatozoa. Semen samples were collected on the day of oocyte retrieval by masturbation. The clinical procedures and laboratory protocols were performed following the CGR standard protocols. Controlled ovarian stimulation was performed using a gonadotropin-releasing hormone antagonist protocol (GnRH-ant). Recombinant follicle-stimulating hormone (rFSH—follitropin alfa: Bemfola®, Gedeon Richter, Budapest, Hungary and Gonal F®, Merck-Serono, Schiphol-Rijk, The Netherlands; follitropin beta: Puregon®, MSD Biotech B.V., Noord-Brabant, The Netherlands), recombinant follicle-stimulating hormone with recombinant luteinizing hormone (rFSH and rLH in a 2:1 ration—Pergoveris®, Schiphol-Rijk, Merck-Serono, The Netherlands), menotropin (highly purified human menopausal gonadotrophin, hMG—Menopur®, Ferring Pharmaceutical, Madrid, Spain), or corifollitropin alpha (Elonva®, N.V. Organon, Oss, The Netherlands) were used to stimulate ovaries with initial doses based on the individual characteristics of patients. Beyond day 6

and when the leading follicle reached 12 mm, treatment was continued with gonadotropin-releasing hormone antagonist ganirelix (Orgalutran®, N.V. Organon, Oss, The Netherlands) or cetrorelix acetate (Cetrotide®, Merck-Serono, The Netherlands) until final follicular maturation. Stimulation was prolonged until the observation by ultrasound of at least three dominant follicles of 17 mm or greater in diameter or in cases of a low number of growing follicles, at least one. The final oocyte maturation was triggered with 250 µg of recombinant human chorionic gonadotropin (rhCG—choriogonadotropin alfa; Ovitrelle®, Merck Serono, Schiphol-Rijk, The Netherlands) or 0.2 mg of GnRH agonist (Triptorelin acetate, Decapeptyl®, Ipsen Pharma, Barcelona, Spain) or both. The serum hormone levels of estradiol (E2) and progesterone (P) were evaluated on the day of the trigger. Transvaginal oocyte retrieval was performed 36 h after the final follicular maturation.

In the cases in which embryo transfer was performed, pregnancy was evaluated at two different stages. Women were classified as having a biochemical pregnancy when β -HCG concentration surpassed the value of 20 mIU/mL (positive serum β -HCG levels), 12 days after embryo transfer [24]. All women with negative serum β -HCG levels ($[\beta\text{-HCG}] < 20$ mIU/mL) were not considered pregnant. Biochemical pregnancies evolved into clinical pregnancies when a fetal heartbeat was detected.

4.5.3 Gamete Collection

After liquefaction, sperm concentration and progressive motility were assessed according to the WHO [44]. The semen preparation standard protocol included a discontinuous density gradient of 90–45% centrifugation (PureSperm®, Nidacon, Mölndal, Sweden) followed by a swim-up protocol with fertilization medium (Sequential Fert™, Origio, Ballerup, Denmark). In cases of severe oligoasthenoteratozoospermia, the sperm sample was only washed with a sperm preparation medium (Sperm Preparation Medium™, Origio, Ballerup, Denmark) followed by a swim-up protocol with fertilization medium (Sequential Fert™). The final sperm preparation was incubated at 35 °C with 6% O₂ at least 30 min before the beginning of the ICSI procedure. After the follicular aspiration, the oocyte–cumulus complexes (OCCs) were isolated and washed in a gamete preparation medium (SynVitro™ Flush, Origio, Ballerup, Denmark). The retrieved OCCs were placed in a 5-well dish with 250 µL of fertilization medium (Sequential Fert™) covered with oil (OVOIL™, Vitrolife, Barcelona, Spain) that had been previously equilibrated overnight (37 °C, 5% CO₂, and 6% O₂). OCCs were denuded to evaluate oocyte nuclear maturity. So, 2 h after egg retrieval, cumulus cells were removed using a combination of mechanic and enzymatic (ICSI Cumulase®, Origio, Ballerup, Denmark) denudation protocol.

4.5.4 Intracytoplasmic Sperm Injection (ICSI) and Embryo Culture

Four hours after egg retrieval, oocytes were injected following the standard ICSI procedure. Injected oocytes were rinsed and cultivated individually in a 30 μ L drop of culture medium (25 μ L of culture medium plus 5 μ L embryo washed in the same culture medium) (Sequential CleavTM, Origio[®], Ballerup, Denmark) under 1500 μ L of oil (OVOILTM, Vitrolife, Sweden), which was previously equilibrated overnight in an incubator at 37°C, 5% CO₂, and 6% O₂. The culture dish (EmbryoSlide[®], Vitrolife, Barcelona, Spain) allows an individualized oocyte/embryo culture with no connection between the culture medium of adjacent oocytes/embryos. The culture dish was placed in a time-lapse incubator (EmbryoScope[®], Vitrolife, Barcelona, Spain) (Day 0) for the following assessment. Normal fertilization was confirmed 16-18 h after ICSI by the presence of two pronuclei and a second polar body (Day 1). Embryo culture was extended until the blastocyst stage (D5). The standard laboratory protocol applied uses a sequential culture media. The embryos were cultured in a cleavage medium (Sequential CleavTM, Origio, Ballerup, Denmark) until D3 and then the culture media were replaced with a blastocyst medium (Sequential BlastTM, Origio, Ballerup, Denmark) to support embryo culture until the blastocyst stage (D5). After embryo culture, in some cases, embryos were immediately transferred, while in other cases, embryos were vitrified for subsequent transfer. Thus, we did not evaluate the correlation of our metabolomics results with the success rates in the different types of fertility treatments as it would not be suitable to compare results, because in some treatments the embryo transfer was a fresh embryo transfer and in others, the transfer was deferred.

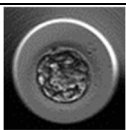
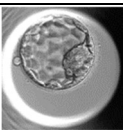
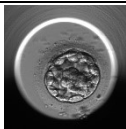
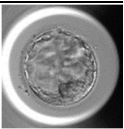
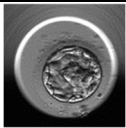
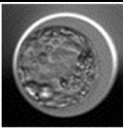
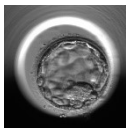
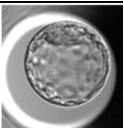
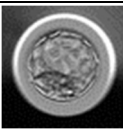
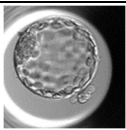
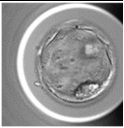
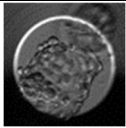
4.5.5 Embryo Morphology Assessment

The development of each embryo was evaluated from fertilization until D5 in a time-lapse system (Vitrolife, Barcelona, Spain). Routine observation of cleavage embryos (until day 4) mainly includes the number and symmetry of blastomeres, fragmentation percentage and presence of multinucleated blastomeres (day 2/3), and compaction grade (day 4). Cleavage embryos were scored according to four categories (A, B, C, and D), based solely on blastomeres number and fragmentation [45]. The blastocysts were graded according to Gardner Score System [24]. This scoring system uses a number associated with a letter, and this combination allows us to know the blastocyst stage and blastocyst quality. Initially, the classification is based on the degree of expansion and hatching status of blastocysts, and the numeric score from 1 to 6 reflects the blastocyst stage (Table 4.4).

For blastocysts scored 3–6, the classification system includes the development of the inner cell mass and trophectoderm (Table 4.4). A blastocyst of good quality was considered as BL3BB or better. The term Lagging was used to characterize those embryos that had

acceptable morphological classification, but the embryo development was delayed considering the standardized timing of embryo observations established in the Istanbul Consensus.

Table 4.4 Classification of blastocysts, inner cell mass and trophectoderm.

Blastocyst Classification	Stage	Definition	Examples	Inner Cell Mass Classification	Definition	Examples
BL1	Early blastocyst	The blastocoel being less than half the volume of the embryo		A	Tightly packed with many cells	
BL2	Blastocyst	The blastocoel being greater than half the volume of the embryo		B	Loosely grouped with several cells	
BL3	Full blastocyst	The blastocoel completely filling the embryo		C	Very few cells	
BL4	Expanded blastocyst	The blastocoel volume now being larger than that of the early embryo and the zona pellucida starting to thin		Trophectoderm Classification	Definition	Examples
				A	Many cells forming a cohesive epithelium	
				B	Few cells forming a loose epithelium	
BL5	Hatching blastocyst	The trophectoderm starting to herniate through the zona pellucida		C	Very few large cells	
BL6	Hatched blastocyst	The blastocyst having completely escaped from the zona pellucida				

Degree of expansion and hatching status of blastocysts. Gardner blastocysts grade system was assessed as indicated in the table. Inner cell mass and trophectoderm classification for onward full blastocyst stage (BL3 to BL6).

4.5.6 Sample Collection/Preparation

As mentioned before, the use of sequential media in an embryo culture implies that until D3 the embryos are cultured in a cleavage medium and afterwards there must be a change in culture medium to support embryo development until D5 (blastocyst stage). So, for each embryo used for the study, there will be two samples of culture medium: a sample of cleavage medium (day 0 until D3 of embryo development) and a sample of blastocyst medium (D3 until D5 of embryo development). Sample collection was carried out by pipetting 25 μ L from the 30 μ L drop of culture media into microcentrifuge tubes.

Each sample (25 μ L) was obtained from the spent culture media of embryos developed from day 0 (after ICSI) until D3 and from D3 until D5. To be used as controls, samples from the same corresponding batch of each medium used in embryo culture and with the same shelf-life were collected and analyzed by ^1H -NMR spectroscopy as described below and used to evaluate the content of the media at time 0. All samples were centrifuged ($1000\times g$; 10 min; 4 $^{\circ}\text{C}$) to fully separate any residues of oil. Twenty microliters of the bottom layer were carefully collected and stored at -80°C until metabolomic analysis.

4.5.7 Metabolomic Analysis by ^1H Nuclear Magnetic Resonance Spectroscopy

1D ^1H -NMR spectroscopy was applied to identify and quantify metabolites in the embryo culture media. NMR spectra were acquired using a 600 MHz VNMRs spectrometer (Varian, Inc. Palo Alto, CA, USA) equipped with a 3 mm indirect detection probe. About 140 μ L of deuterium oxide and 40 μ L of sodium fumarate solution (final concentration of 2 mM) were added to 20 μ L of each embryo culture media, and the final volume of 200 μ L was transferred into a 3 mm NMR tube for analysis. Sodium fumarate was used as an internal reference (6.50 ppm) to quantify the following metabolites (multiplicity, chemical shift (ppm)): alanine (doublet, 1.46), acetate (singlet, 1.90), pyruvate (singlet, 2.35), glutamine (multiplet, 2.45), lactate (quartet, 4.09), and formate (singlet, 8.44). NMR spectra were processed by applying exponential line broadening (0.2 Hz), baseline correction, and manual phasing, and the areas of the resonances were quantified using the NUTSproTM NMR Software (2D Professional Version-20070315, Acorn, Fremont, CA, USA). Blank control experiments were performed previously in which we replicated every step of the incubation except that there were no embryos being cultivated. With this procedure, we were able to assess the impact of the protein content (with HSA $\leq$ 0.05%) on the NMR spectra acquisition, ensuring that there were no signals generated that overshadowed the metabolites of interest. We were also able to perfect the separation of the oil from the spent culture (in order to obtain clear NMR spectra without disturbing overlapping or nearby signals derived from the oil), and we analyzed the impact of this incubation on the content

of the medium in water-soluble metabolites, as the ones we evaluated in the experiment. We saw no alteration in the content of these metabolites, in line with the results described by a previous study [46]. Moreover, fresh medium samples were collected for each distinct batch and within the same medium batch if different shelf times were used (21 control samples for D3 and 21 samples for D5). Subsequently, they were analyzed by $^1\text{H-NMR}$ together with correspondent spent media (same batch plus same shelf time) and used to calculate the initial metabolite content and subsequently the metabolite production or consumption.

4.5.8 Statistical Analysis

Univariate statistics were used to compare embryo culture medium metabolite differential in days 0–3 (D0–3) and 3–5 (D3–5) between embryos grouped by their assessed quality at D5 (Good vs. Lagging vs. Bad). Data are presented as mean $\pm$ standard deviation (S.D.). Embryo quality was assessed by the same trained and experienced embryologist. Normality and heteroscedasticity tests were performed, and univariate ANOVA was conducted on data collected concerning variation in selected metabolites identified in the embryo culture medium. Pairwise comparisons between groups were corrected for multiple hypotheses testing by Tukey's post hoc test. Multivariate analysis of metabolite consumption/production was performed using a linear model integrating corrections for confounding factors. Metabolite differentials in days 0–3 (D0–3) and 3–5 (D3–5) were compared between embryos grouped by their assessed quality at D5 (Good vs. Lagging vs. Bad) using multivariate analysis of covariance (MANCOVA), after controlling for covariates (male age, female age, male BMI, female BMI). These covariates were chosen based on multiple studies correlating maternal age [14,16] and BMI [18,19], and paternal age [20] and BMI [21,23] with changes in early embryo metabolism and viability [17,22]. The assumptions of equality of the variance–covariance matrices were evaluated using Levene's test of equality of error variances and Box's test of equality of covariance matrices (Box's M). The discriminant value of the independent variable (embryo quality at D5) considering the dependent variables (metabolite differential) was evaluated according to Roy's Largest Root criterion. This method was chosen as it is the most sensitive when a limited number of dependent variables is responsible for the differences between groups of the independent variable [47]. The impact of each metabolite differential on embryo quality at D5 was assessed using a univariate F-test, after adjusting for the covariates. Pairwise comparisons between groups were corrected for multiple hypotheses testing by Šidak's method. All statistical tests of hypotheses for all statistical methods were performed at the $\alpha = 0.05$ level using SPSS Statistics 28.0.0 for Windows 64-bit (IBM, Armonk, NY, US).

4.6 Conclusions

The goal of integrating metabolomics with standardized embryonic viability assessment could be crucial for non-invasive, real-time monitoring of embryo metabolic states during culture, offering valuable information on viability and quality. Current methods, such as morphological assessment and morphokinetic analyses, have limitations in predicting implantation success, making metabolomics an essential addition. Furthermore, this integration can lead to identifying biomarkers associated with embryonic viability and quality, potentially improving embryo selection for transfer and enhancing the chances of successful pregnancies. Still, our strategy does not address metabolic differences between inner cell mass (ICM) and trophoctoderm, noted in previous reports showing distinct metabolic rates between these cells. [48,49]. Moreover, the metabolic activity of ICM cells was suggested to play a role in embryo development [49,50]. Yet, the methods applied in these studies destroy or compromise the viability of the embryos. Existing methods compromising embryo viability highlight a need for non-destructive techniques. Alegre et al. proposed Raman spectroscopy, estimating the oxidant content of the culture medium, but its external variability and lower specificity compared to NMR or MS spectroscopy raise concerns [4]. Hence, NMR emerges as a potential compromise, as demonstrated in recent studies of early-stage mammalian embryos [51]. Despite evidence supporting metabolic screening's benefits for nearly a decade, routine implementation in ART clinics is lacking [52,53].

Overall, our results support that assessment of the exometabolome of *in vitro* cultured human embryos up to D3 can be discriminative of embryo quality category at D5. Notably, the metabolite profile at D3 is particularly selective between Lagging and Bad-quality embryos, whereas the metabolite profile of Good embryos is not different from Lagging embryos (except for alanine) at this stage. However, at D5, pyruvate is a potential predictor between Good and Lagging embryos. Therefore, the corrected variations (for interfering covariates, such as the male age, female age, male BMI, and female BMI) of metabolites such as alanine, pyruvate, acetate, and glutamine support that the integration of metabolomics with the standardized assessment of embryonic viability and quality has the potential to improve the efficiency and effectiveness of ARTs (Figure 4.4), leading to better pregnancy rates and improved clinical outcomes.

Metabolite variation in human embryo culture medium is a predictor of successful pregnancy in ARTs. Embryo metabolism is indirectly assessed by comparison of metabolite concentration in culture medium before and after incubation. We observed that Poor-quality embryos at post-fertilization day 5 displayed already anomalies in alanine, acetate, pyruvate, and glutamine metabolism, especially compared to embryos classified as Lagging at day 5. These differences were found after adjusting for covariates (male age, female age,

male BMI, female BMI). Considering unadjusted variables, lactate is another potential success predictor for ARTs at day 3. On day 5, considering unadjusted variables, net-zero pyruvate is a feature of good-quality embryos.

While the number of samples used in this study is low and may hamper some more definite conclusions, these findings suggest a potential advancement in refining embryo selection procedures. Incorporating metabolic assessments into protocols could improve ART outcomes by enhancing grading precision and potentially boosting implantation success. This study contributes to ongoing efforts to optimize ART procedures, offering a more comprehensive approach to embryo quality assessment beyond relying solely on morphological evaluation. The potential application of metabolic assessments as additional tools in embryo selection may represent progress in reproductive medicine.

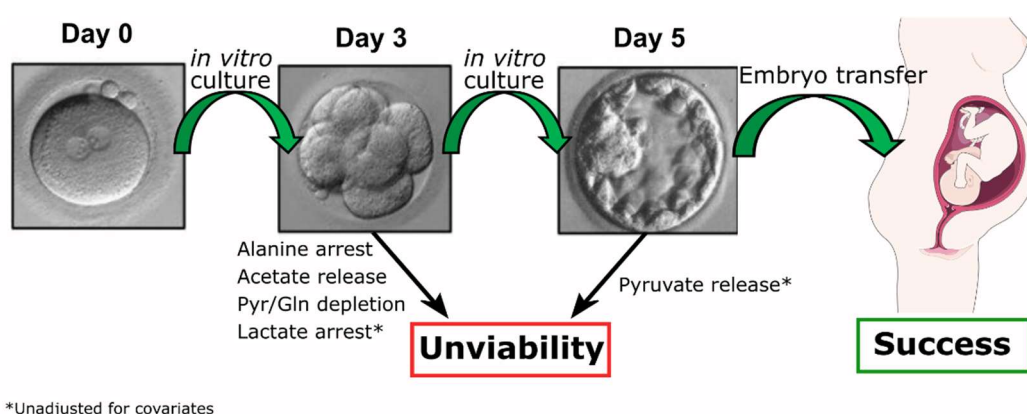


Fig 4.4 Metabolite variation in human embryo culture medium is a predictor of successful pregnancy in ARTs. Embryo metabolism is indirectly assessed by comparison of metabolite concentration in culture medium before and after incubation. We observed that Poor-quality embryos at post-fertilization day 5 displayed already anomalies in alanine, acetate, pyruvate, and glutamine metabolism, especially compared to embryos classified as Lagging at day 5. These differences were found after adjusting for covariates (male age, female age, male BMI, female BMI). Considering unadjusted variables, lactate is another potential success predictor for ARTs at day 3. On day 5, considering unadjusted variables, net-zero pyruvate is a feature of good-quality embryos.

Supplemental Figure S1.

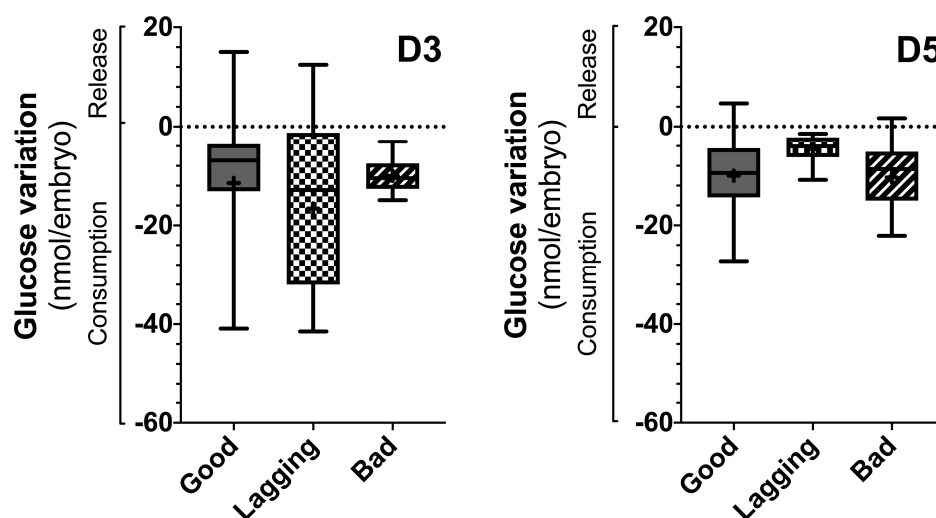


Fig 4.5 Supplemental Figure S1. Glucose variation in culture media of human embryos between day 3 and day 0 (D3) or day 5 day 3 (D5) grouped according to their quality at day 5 (Good vs. Lagging vs. Bad). The figure shows the consumption of glucose quantified in the embryo culture media at the end of day 3 or day (n=55). Data are presented as Mean $\pm$ Standard Deviation (S.D.). Normality and heteroscedasticity tests were performed, and Univariate ANOVA was conducted on the selected metabolites identified in the embryo culture medium. Pairwise comparisons between groups were corrected for multiple hypotheses testing by Tukey's post hoc test. All p values < 0.05 were considered statistically significant.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and the procedures were covered by the provisions of the National Medically Assisted Procreation Act (2017) and overseen by the National Council for Medically Assisted Procreation (CNPMA-2018). According to these rules and guidelines, the use of clinical databases and patient biological material for diagnosis and research may be used without further ethical approval, under strict individual anonymity, and after patient written informed consent. Regarding the use of human samples for laboratory experimentation, the Ethics Committee authorization number is 2021/CE/P02 (P342/2021/CETI), approved on 26 February 2021.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data is contained within the article and supplementary material.

Conflicts of Interest: The authors declare no conflicts of interest.

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CAPÍTULO 5

Sperm-borne miR-34c-5p and miR-191-3p as markers for sperm motility and embryo developmental competence

Este capítulo foi adaptado integralmente do seguinte artigo publicado:

Pinto S, Pereira SC, Rocha A, Barros A, Alves MG, Oliveira PF. Sperm-borne miR-34c-5p and miR-191-3p as markers for sperm motility and embryo developmental competence. *Andrology*. 2024; 1-12. <https://doi.org/10.1111/andr.13698>

O texto integral pode ser consultado no Anexo A desta Dissertação.



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5 Sperm-borne miR-34c-5p and miR-191-3p as markers for sperm motility and embryo developmental competence

5.1 Abstract

Background: Sperm-borne miRNAs play a pivotal role in influencing essential cellular processes during fertilization, impacting the quality of embryo development. Dysregulated miRNA profiles have been associated with compromised embryonic development and increased incidences of pregnancy loss.

Objective: This study aimed to investigate the potential associations between the abundance of miR-34c-5p and miR-191-3p in human spermatozoa with sperm quality, as well as with embryo quality and metabolic performance during *in vitro* development.

Material & Methods: Thirteen couples, who underwent a total of 13 cycles, participated in this study. Sperm quality was assessed using conventional methods following World Health Organization guidelines. Quantitative polymerase chain reaction was employed to measure miRNA abundance in spermatozoa. Embryos were categorized as Good, Lagging, or Bad based on morphokinetic evaluation. Evaluation of embryo metabolic performance involved tracking changes in specific metabolites within the cultured media using ¹H-NMR spectroscopy. Statistical analysis was conducted to explore the correlation between miRNA abundance in human spermatozoa and all other collected data.

Results: Our findings reveal a negative correlation between the abundance of miR-34c-5p (but not miR-191-3p) and total sperm motility, potentially mediated by the modulation of key signaling pathways. Additionally, higher levels of miR-34c-5p in sperm were strongly associated with the consumption or release of key metabolites by developing embryos, particularly those linked with lipid and glucose metabolism, suggesting enhanced metabolic performance, while miR-191-3p was mostly associated to glucose consumption. Concurrently, only miR-34c-5p content in spermatozoa correlated with higher embryo quality.

Discussion and Conclusion: This study provides evidence suggesting that the abundance of miR-34c-5p in spermatozoa is not only correlated with total sperm motility but also with markers of embryo developmental competence, highlighting the potential significance of this sperm miRNA content as a biomarker in assisted reproduction.

5.2 Introduction

MicroRNAs (miRNAs) are small, non-coding RNA molecules that play diverse and intricate roles in regulating gene expression across various biological processes. Research has uncovered multiple functions of miRNAs, including their involvement in cell proliferation, differentiation, apoptosis, and development [1]. miRNAs function by binding to target messenger RNA (mRNA) molecules, leading to mRNA degradation or translational repression, thereby influencing the expression of genes involved in numerous cellular pathways. Additionally, miRNAs have been implicated in the regulation of immune responses, metabolism, and stress responses, highlighting their broad impact on physiological functions [2, 3]. Altered miRNA expression has been linked to various diseases, including cancer, cardiovascular disorders, and neurological conditions, underscoring the importance of miRNAs in health and disease [4].

Recent research has shed light on the influential role of sperm miRNAs in shaping embryo quality [4, 5]. While it is widely recognized that sperm miRNAs play a role in modulating gene expression during embryonic development, uncertainties persist regarding the full extent of their impact. Moreover, the consequences of dysregulated sperm miRNAs on reproductive outcomes and offspring health remain ambiguous, with conflicting research findings regarding their correlation with infertility, suboptimal embryo quality, pregnancy complications and epigenetic inheritance. Further investigation is imperative to untangle the intricate relationship between sperm miRNAs, fertilization, and embryonic development, aiming to address controversies and deepen our comprehension of their biological significance [6-8]. Recent studies have demonstrated that sperm miRNAs are transmitted to the oocyte upon fertilization, where they have the potential to influence critical cellular processes essential for embryo development, including cell proliferation, differentiation, and apoptosis [5]. These miRNAs possess the capability to target and modulate the expression of specific genes involved in embryogenesis, thereby impacting the developmental quality of the resultant embryo. Additionally, abnormalities in sperm miRNA expression have been associated with diminished embryo quality and compromised developmental potential, contributing to impaired embryo development, increased rates of embryonic arrest, and elevated incidences of pregnancy loss [6, 9, 10].

miRNA-34c-5p and miRNA-191-3p are two miRNAs that have been associated to embryogenesis, although research on their specific action in embryo development is relatively limited compared to its extensively studied functions in other biological contexts [11, 12]. We chose these two sperm-borne miRNAs based on their distinct characteristics regarding their contribution to the embryo's miRNA pool [13, 14]. While miRNA-34c-5p is known to regulate critical cellular processes such as apoptosis and cell cycle progression, its involvement in embryonic development is scarcely addressed, with some studies

suggesting that it may play a role in modulating embryonic stem cell differentiation and lineage commitment, which could indirectly impact embryo development. Additionally, dysregulation of miRNA-34c-5p has been associated with various developmental disorders and pregnancy complications, indicating its potential relevance to embryo quality [7]. Similarly, miRNA-191-3p has been extensively studied in various biological contexts, including cancer and other diseases, while its involvement in embryonic development is not as well understood. However, some studies have suggested that miRNA-191-3p may play a role in early embryogenesis and may contribute to the regulation of genes involved in cell proliferation, differentiation, and apoptosis, which are critical processes for embryo development and quality [6]. Still, further research is needed to fully elucidate the role of these miRNAs (as of many others) in embryonic development and its potential implications for reproductive outcomes. Understanding the intricate role of sperm miRNAs in embryo quality holds promise for improving assisted reproductive techniques (ART) outcomes. By identifying specific miRNA signatures associated with optimal embryo development, researchers can aim to develop novel diagnostic tools and therapeutic interventions to enhance embryo quality and increase the success rates of ART procedures. Hence, in our work, we aimed to enlighten the putative correlation between miRNA-34c-5p and miRNA-191-3p spermatozoa content and several parameters of human sperm quality and seminal content, while at the same time with the outcomes of *in vitro* human embryo development, namely its quality and metabolic performance.

5.3 Materials and Methods

5.3.1 Chemicals

Embryo and gamete culture media and reagents were acquired from Origio® (Denmark), Nidacon (Sweden), and Vitrolife (Sweden). All remaining chemicals were procured from Sigma-Aldrich (St. Louis, MO, USA), unless specified otherwise.

5.3.2 Patient selection and ovarian stimulation

Thirteen caucasian couples, who underwent a total of 13 cycles, participated in this study. Assisted Reproductive Technologies (ART) treatments were administered at Centro de Genética Prof. Alberto Barros (CGR) in Porto, Portugal, between June 2019 and February 2021. The procedures at the Centre for Reproductive Genetics Alberto Barros adhere to the regulations of the National Medically Assisted Procreation Act (2017) and are supervised by the National Council for Medically Assisted Procreation (CNPMA-2018). In accordance with these regulations, clinical databases and patient biological material may be utilized for

diagnosis and research without further ethical approval, ensuring strict individual anonymity and obtaining written informed consent from patients. The Ethics Committee authorization number for laboratory experimentation with human samples is 2021/CE/P02 (P342/2021/CETI), approved on 26 February 2021. Couples eligible for inclusion were those referred for infertility treatment with the intracytoplasmic sperm injection (ICSI) technique, aiming to prevent interference from cumulus cells or spermatozoa during metabolomics quantification. Couples undergoing testicular biopsy (testicular sperm aspiration or testicular sperm extraction) or using cryopreserved ejaculated spermatozoa were excluded from the study, ensuring that all ICSI procedures were conducted using fresh ejaculated spermatozoa. Semen samples were collected via masturbation on the day of oocyte retrieval. Clinical procedures and laboratory protocols adhered to the CGR standard protocols. Controlled ovarian stimulation was carried out using a gonadotropin-releasing hormone antagonist protocol (GnRH-ant). Recombinant follicle-stimulating hormone (rFSH) from various sources, including follitropin alfa (Bemfola®, Gedeon Richter, Hungary and Gonal F®, Merck-Serono, Netherlands), follitropin beta (Puregon®, MSD Biotech B.V., Netherlands), recombinant follicle-stimulating hormone with recombinant luteinizing hormone (rFSH and rLH in a 2:1 ratio - Pergoveris®, Merck-Serono, Netherlands), menotropin (highly purified human menopausal gonadotrophin, hMG - Menopur®, Ferring Pharmaceutical, Spain), or corifollitropin alpha (Elonva®, N.V. Organon, Netherlands) were utilized to stimulate ovaries, with initial doses tailored to individual patient characteristics. Gonadotropin-releasing hormone antagonist ganirelix (Orgalutran®, N.V. Organon, The Netherlands) or cetrorelix acetate (Cetrotide®, Merck-Serono, The Netherlands) was introduced beyond day 6, once the leading follicle reached 12 mm, until final follicular maturation was achieved. Stimulation continued until ultrasound observation revealed at least three dominant follicles of 17 mm or greater in diameter, or, in cases of a low number of growing follicles, at least one dominant follicle. Final oocyte maturation was triggered using 250 µg of recombinant human chorionic gonadotropin (rhCG - choriogonadotropin alfa; Ovitrelle®, Merck Serono, The Netherlands) or 0.2 mg of GnRH agonist (Triptorelin acetate, Decapeptyl®, Ipsen Pharma, Spain), or a combination of both. Serum hormone levels of estradiol (E₂) and progesterone (P) were assessed on the day of trigger, and transvaginal oocyte retrieval was performed 36 hours after final follicular maturation.

5.3.3 Gamete collection

Following liquefaction, sperm concentration and progressive motility were evaluated in accordance with the World Health Organization guidelines [15]. Spermatozoa preparation protocol involved a discontinuous density gradient centrifugation (PureSperm®) ranging

from 90% to 45%, followed by a swim-up procedure utilizing fertilization medium (Sequential Fert™) in order to obtain highly motile spermatozoa, as described previously [16]. In cases of severe oligoasthenoteratozoospermia, the sperm sample underwent washing only with a sperm preparation medium (Sperm Preparation Medium™) before the swim-up process using fertilization medium (Sequential Fert™). The final sperm preparation was then incubated at 35°C with 6% O₂ for a minimum of 30 minutes before starting the ICSI procedure, with the remaining spermatozoa being used for evaluation of miRNAs content as described below. Subsequent to follicular aspiration, the oocyte-cumulus complexes (OCCs) were isolated and cleansed in gamete preparation medium (SynVibro™ Flush). These retrieved OCCs were then transferred into a 5-well dish containing 250 µL of fertilization medium (Sequential Fert™) and covered with oil (OVOIL™) that had been previously equilibrated overnight (37°C, 5% CO₂ and 6% O₂). The OCCs were denuded to assess oocyte nuclear maturity. Hence, 2 hours post egg retrieval, cumulus cells were removed using a denudation protocol involving a combination of mechanical and enzymatic methods (ICSI Cumulase®).

5.3.4 Intracytoplasmic sperm injection (ICSI) and embryo culture

Four hours post egg retrieval, oocytes underwent injection following the standard ICSI procedure. Injected oocytes were then rinsed and individually cultured in a 30 µL drop of culture medium (25 µL of culture medium plus 5 µL embryo washed in the same culture medium) (Sequential Cleav™) beneath 1500 µL of oil (OVOIL™) previously equilibrated overnight in an incubator at 37°C, 5% CO₂ and 6% O₂. The use of an EmbryoSlide® culture dish facilitated individualized oocyte/embryo culture without interconnection between the culture media of adjacent oocytes/embryos. This culture dish was then placed in a time-lapse incubator (EmbryoScope®) (Day 0) for subsequent assessment. Fertilization was confirmed 16-18 hours post ICSI by the presence of two pronuclei and a second polar body (Day 1). Embryo culture continued until the blastocyst stage (day 5). The standard laboratory protocol involved sequential culture media. Embryos were cultured in cleavage medium (Sequential Cleav™) until day 3, after which the culture media were replaced with blastocyst medium (Sequential Blast™) to support embryo culture until the blastocyst stage (day 5). Following embryo culture, some embryos were either immediately transferred or vitrified for subsequent transfer. Consequently, the correlation of our results with the success rates in different types of fertility treatments was not evaluated, as it would be inappropriate to compare results given the variation in embryo transfer methods (some treatments involved fresh embryo transfer, while others entailed deferred transfer).

5.3.5 Embryo morphology assessment

Each embryo's development was monitored from fertilization to day 5 using a time-lapse system (Vitrolife, Sweden). Standard observation of cleavage-stage embryos (up to day 4) primarily involved assessing the number and symmetry of blastomeres, percentage of fragmentation, and presence of multinucleated blastomeres (day 2/3), as well as compactation grade (day 4). Cleavage-stage embryos were categorized into four groups (A, B, C, and D) based solely on blastomere number and fragmentation [17]. Blastocysts were graded according to the Gardner Score System [18], which utilizes a combination of numbers and letters to indicate both the stage and quality of the blastocyst. Initially, the classification is based on the degree of expansion and hatching status, with a numeric score ranging from 1 to 6 reflecting the blastocyst stage. For blastocysts scored 3-6, the classification system further considers the development of the inner cell mass and trophoctoderm. A blastocyst was deemed of good quality if it scored 3BB or higher. The term "lagging" was used to describe embryos with acceptable morphological classification but whose development was delayed compared to the standardized timing of embryo observations established in the Istanbul Consensus.

5.3.6 Media Sample Collection/Preparation

As previously explained, utilizing sequential media in embryo culture involves culturing embryos in cleavage medium until day 3, followed by a change to blastocyst medium to support embryo development until day 5 (blastocyst stage). Therefore, for each embryo included in the study, two samples of culture medium were collected: one sample of cleavage medium (from Day 0 to day 3 of embryo development) and one sample of blastocyst medium (from day 3 to day 5 of embryo development). Sample collection was performed by pipetting 25 μ L from the 30 μ L drop of culture media into microcentrifuge tubes. Each sample (25 μ L) was obtained from the spent culture media of embryos developed from Day 0 (after ICSI) to day 3 and from day 3 to day 5. Control samples from the same corresponding batch of each medium used in embryo culture, with identical shelf-life, were collected and analyzed by ¹H-NMR Spectroscopy to assess the content of the media at time 0. All samples were centrifuged (1000xg; 10 minutes; 4°C) to thoroughly separate any residues of oil. Subsequently, 20 mL of the bottom layer were carefully collected and stored at –80°C until metabolomic analysis.

5.3.7 Reverse transcriptase cDNA synthesis

Spermatozoa RNA was isolated from a total of 13 samples using the miRNeasy Tissue/Cells Advanced Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, as described previously [19]. RNA concentrations and absorbance ratio A260/A280 were determined by a DeNovix DS-11 spectrophotometer system (Wilmington, USA). For cDNA synthesis, we adapted the method proposed by Ciera and Busk [20]. To each 50 ng of total RNA, it was added: 1 µL of 10x Reaction buffer E. coli poly (A) polymerase (New England Biolabs, Massachusetts, USA), 1 µL of 1 mM ATP (New England Biolabs, Massachusetts, USA), 1 µL of 10 µM reverse transcriptase primer (5'-CAGGTCCAGTTTTTTTTTTTTTTVN), 1 µL of dNTP mix (1mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, NZYTech, Lisbon, Portugal), 0.5 µL of M-MuLV reverse transcriptase (200 U/µL, NZYTech, Lisbon, Portugal) and 0.2 µL of E. coli poly (A) polymerase (5000 U/µL, New England Biolabs, Massachusetts, USA). Samples were then incubated at 42°C for 1h. Primers were designed by the miRprimer software [21] and can be consulted in Table 5.1.

5.3.8 Quantitative real time polymerase chain reaction (qPCR)

For the qPCR assay, to each 1 µL of cDNA sample it was added: 0.64 µL of primer forward (20 µM), 0.64 µL of primer reverse (20 µM), and 8.0 µL of Speedy Supreme NZYTaQ 2x Green Master Mix (NZYTech, Lisbon, Portugal). The volume was adjusted to 16 µL with RNase-free water. 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 60°C for primers annealing and extension. To ensure primer specificity, 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. The melting temperatures for each set of primers were the following: 78°C for has-RNU6B and miR-34c-5p, and 77.5°C for miR-191-3p. qPCR reactions were carried in duplicate, and the optical density was assessed by a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, California, USA). Amplification efficiencies were calculated for all primers and set between 90-110% as described previously [22]. Data was analyzed according to the 2-ΔCt method proposed by Pfaffl and the results are presented in arbitrary units (a.u.) [23].

Table 5.1 Primer sequences for homo sapiens (hsa) miRNAs and respective accession number. Primers were designed using the miRprimer software [21].

Target	Primer Sequence
<i>hsa-RNU6B</i> AN: NR_002752	Forward: ACTCAAGACAATGGTGATAATGGTT
	Reverse: ACAGAAAGGAATACGCAGAAAGG
<i>hsa-miR-191-3p</i> AN: MIMAT0001618	Forward: GCGGGCAACGGAATCCCAAAAGCA
	Reverse: GTCCAGTTTTTTTTTTTTTTTAGGCA
<i>hsa-miR-34c-5p</i> AN: MIMAT0000686	Forward: CTAGGCAGTGTAGTTAGCTGATTGCT
	Reverse: AGGTCCAGTTTTTTTTTTTTTTTAATCT

Legend: AN – accession number; hsa – homo sapiens; miR – microRNA

5.3.9 Metabolomic analysis by ¹H Nuclear Magnetic Resonance Spectroscopy (¹H-NMR)

¹H-NMR spectroscopy was applied to identify and quantify metabolite contents in embryo culture media, by methods described previously [16]. NMR spectra were acquired using a 600 MHz VNMRs spectrometer (Varian, Inc. Palo Alto, CA, USA) equipped with a 3 mm indirect detection probe. For culture media, 140 µL of deuterium oxide and 40 µL of sodium fumarate solution (final concentration of 2 mM) were added to 20 µL of each embryo culture media, and the final volume of 200 µL was transferred into a 3 mm NMR tube for analysis. Sodium fumarate was used as an internal reference (6.50 ppm) to quantify the following metabolites (multiplicity, ppm): alanine (doublet, 1.46), acetate (singlet, 1.90), pyruvate (singlet, 2.35), glutamine (multiplet, 2.45), lactate (quartet, 4.09), formate (singlet, 8.40). NMR spectra were processed by applying exponential line broadening (0.2 Hz), baseline correction, and manual phasing, and the areas of the resonances were quantified using the NUTSpro™ NMR Software (Acorn, Fremont, CA, USA).

Blank control experiments were conducted beforehand for the embryo culture media, wherein each step of the incubation process was replicated except for the absence of embryos being cultivated. Through this procedure, we could evaluate the influence of protein content (with HSA≤0.05%) on NMR spectra acquisition, ensuring that no signals were generated to obscure the metabolites of interest. Additionally, we refined the separation of oil from the spent culture to obtain clear NMR spectra without interference from overlapping or nearby oil-derived signals. We also examined the impact of this incubation on the medium's content of water-soluble metabolites, similar to those assessed in the experiment, and found no alterations in their content, consistent with findings from a prior study [16]. Furthermore, fresh medium samples were gathered for each unique batch

and within the same batch if varying shelf times were employed. These samples were then subjected to ¹H-NMR analysis alongside corresponding spent media (same batch and shelf time) to determine the initial metabolite content. Subsequently, metabolite production or consumption until day 5 (D5) was calculated based on these analyses.

5.3.10 Statistical Analysis

Embryo quality was assessed by the same trained and experienced embryologist. The association between miRNA abundance in spermatozoa with other data from male patients, such as age, BMI and sperm quality parameters (sperm concentration, sperm motility, sperm viability, sperm membrane integrity, semen volume, and high motility sperm concentration), as well as with embryo quality and embryo metabolite consumption or release, were evaluated by computing Pearson correlation coefficients (R) assuming a Gaussian distribution and a confidence interval of 95%. A P-value of less than 0.05 indicates statistical significance, while a P-value less than 0.10 suggests a notable tendency toward significance. The statistical analysis was performed using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA).

5.4 Results

5.4.1 Clinical reproductive data and embryo quality characterization

As depicted in Table 5.2, 13 ICSI cycles were analyzed in this study from a total of 13 sperm samples. Treatments adhered to clinical indications, and fertilization occurred via the ICSI technique to standardize sperm cell influence on metabolomic quantification. Embryo culture extended to day 5. Cycle characteristics and female partner data are detailed in our previous work [16] and relevant data for this study are summarized in Table 5.2. Although all investigations carried out in the male partner or embryo, maternal influence was minimized to the greatest extent possible, with no notable differences observed in age, the quantity of retrieved oocytes, or the number of MII (metaphase II stage) oocytes among the female partners.

Table 5.2 Supplemental Table. Characterization of studied male population, fertilization cycles, and embryo quality and metabolic performance.

Baseline data of studied population		Baseline data of embryos	
Number of couples	13	Total number of embryos	20
Number of cycles	13	Good	9
Infertility duration (months)	49.9 ± 32.3	Lagging	7
		Bad	4
Male Age (years)	41.4 ± 1.4		
Body mass index (kg/m ²)	25.4 ± 0.6	Metabolite consumption (nmol/embryo)	
		Glucose	14.70 ± 1.32
Semen volume (mL)	3.6 ± 0.3	Isoleucine	1.50 ± 0.17
Sperm concentration (10 ⁶ cells/mL)	67.2 ± 18.3	Valine	1.78 ± 0.24
Total sperm motility (%)	43.4 ± 4.5	Aspartate	3.00 ± 0.42
High-motility sperm concentration (10 ⁶ cells/mL)	2.6 ± 0.9	Metabolite release (nmol/embryo)	
Sperm vitality (%)	64.7 ± 4.6	Pyruvate	1.18 ± 0.14
Sperm membrane integrity (%)	55.4 ± 5.4	Acetate	8.80 ± 0.50
		Lactate	40.46 ± 3.08
		Formate	12.22 ± 0.83
		Alanine	9.54 ± 0.88
		Glutamine	10.76 ± 1.02

Data are shown as the mean ± standard error of the mean.

Culture medium samples from total 20 oocytes were analyzed, corresponding to the sequential culture of the embryos for 5 days. Excluded from statistical analyses were degenerated, non-cleavage, abnormal fertilization, and parthenogenetic embryos. Embryo classification followed predetermined criteria, as described: a blastocyst was deemed "Good" if BL3BB or better (BL5- AA, AB, BA, BB; BL4- AA, AB, BA, BB; BL3- AA, AB, BA, BB), while "Bad" if it had inner cell mass and/or trophectoderm grade C (BL5/BL4/BL3 – AC, CA, CC); embryos were labeled as "Lagging" if their development was delayed per Istanbul Consensus standards [24]. Hence, out of the 20 observed embryos, 9 of these were categorized as Good, 7 as Lagging, and 4 as Bad embryos. No statistically significant differences in age of the men or the women, sperm concentrations, total sperm motility and vitality, female and male baseline evaluation, and number of retrieved oocytes were noted among donors for the three established groups.

Table 5.3 Association between sperm hsa-miR-191-3p and hsa-miR-34c-5p content with male anthropometric data and semen quality parameters.

miRNAs vs anthropometric data, hormonal profile and semen quality parameters	Sperm content hsa-miR-191-3p		Sperm content hsa-miR-34c-5p	
	P	R	P	R
Sperm hsa-miR-191-3p content			0,817	0,071
Sperm hsa-miR-34c-5p content	0,817	0,071		
Male age	0,229	0,358	0,285	0,321
Male BMI	0,130	-0,443	0,487	0,212
Semen Volume	0,983	-0,007	0,459	-0,225
Total sperm concentration	0,337	0,290	0,412	0,249
Total sperm motility	0,549	-0,192	0,040	-0,600
Total sperm vitality	0,889	-0,045	0,859	0,057
Total sperm membrane integrity	0,514	-0,251	0,844	0,077
Highly motile sperm concentration	0,499	-0,206	0,063	0,529

Statistical analysis was performed by computing Pearson correlation coefficients (R) assuming a Gaussian distribution (confidence interval of 95%). The correlation coefficient (R) and the corresponding significance level (P-value) are provided (N=13). A P-value of less than 0.05 indicates statistical significance, while a P-value less than 0.10 suggests a notable tendency toward significance.

5.4.2 Content of miR-34c-5p in human spermatozoa exhibits a correlation with total sperm motility

The presence of miR-34c-5p and miR-191-3p was previously identified in human spermatozoa [6, 7]. In our work, we further confirmed that these microRNAs are present in human spermatozoa by a conventional PCR (data not shown). We then evaluated the association of sperm miR-34c-5p and miR-191-3p content with the age and BMI of the individual and we found no significant correlation of sperm miR-34c-5p and miR-191-3p content with any of the analysed anthropometric data of the male individuals (Figure 5.1 and Table 5.3).

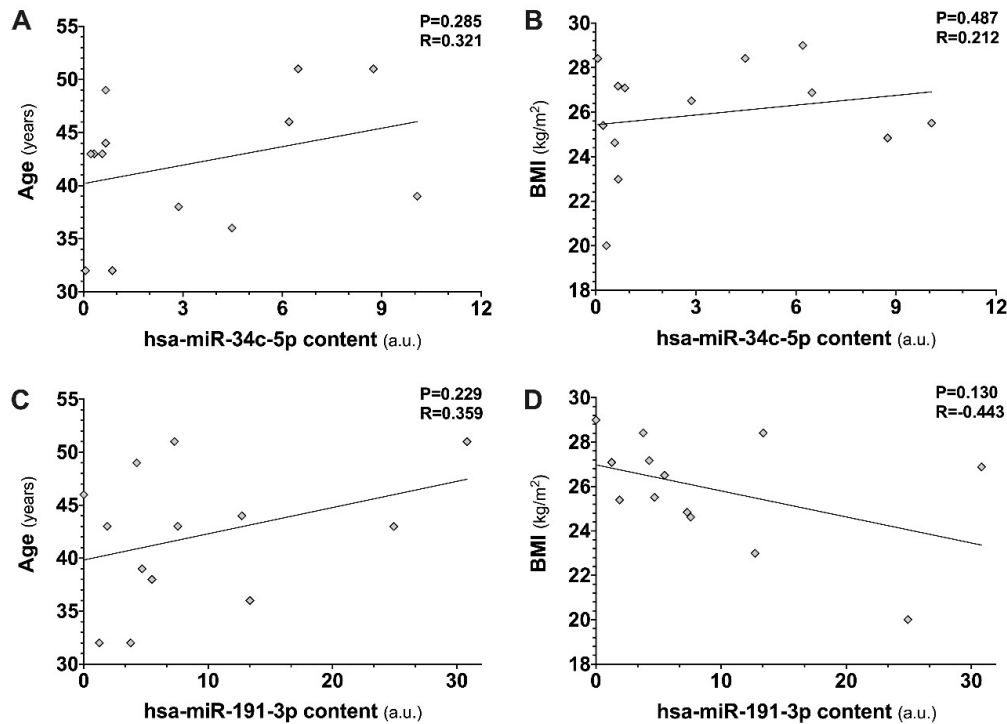


Fig 5.1 Association of hsa-miR-34c-5p or miR-191-3p content in human spermatozoa with the BMI and age of the male individuals. The figure shows the distribution of the hsa-miR-34c-5p or miR-191-3p abundance of spermatozoa with the individual's age (A or C) and BMI (B or D). Data were collected from 13 patients with an average age of 41 years (age range 31–51 years) and an average BMI of 25.5 (BMI range 20–29 kg/m²). The relationship between hsa-miR-34c-5p abundance and the different parameters was evaluated by computing the Pearson correlation coefficients (R), assuming a Gaussian distribution (confidence interval of 95%). The correlation coefficient (R) and the significance level (P-value) of the correlation are indicated. A P-value of less than 0.05 indicates statistical significance, while a P-value less than 0.10 suggests a notable tendency toward significance.

Given that sperm parameters serve as dependable markers for male fertility potential, we conducted an analysis to examine the relationship between the abundance of miR-34c-5p and miR-191-3p and various seminal parameters, including semen volume, total sperm concentration, total sperm motility, highly motile sperm concentration, sperm vitality and membrane integrity. Once more, we found no correlation of sperm miR-191-3p content with any of the seminal quality data (Table 5.3). Contrastingly, when we evaluated the association with miR-34c-5p, we could identify a significant negative correlation of this miRNA with total sperm motility ($P=0.040$; $R=-0.600$) and a positive tendency toward significance of its association with the concentration of highly motile sperm ($P=0.063$; $R=0.529$) (Figure 5.2).

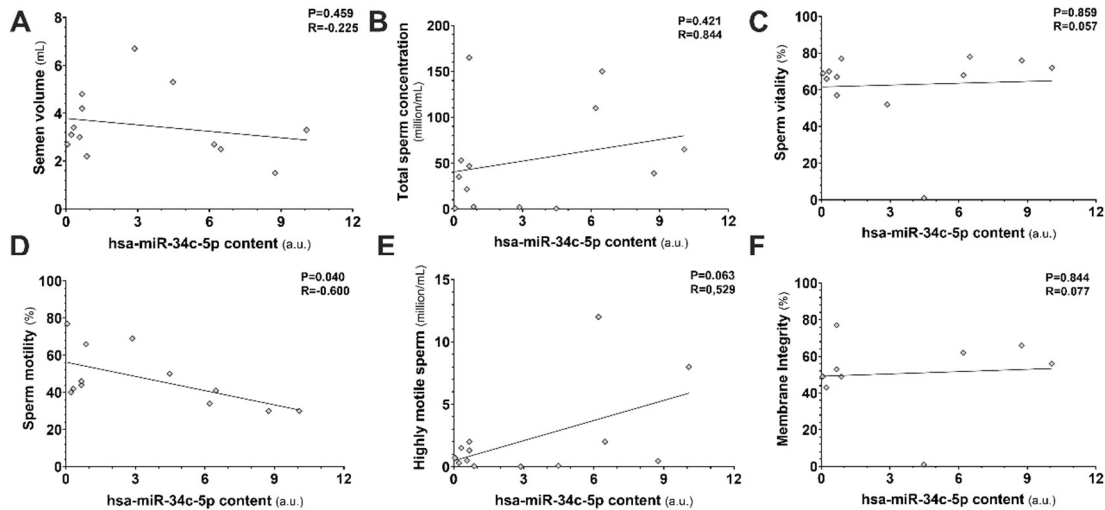


Fig 5.2 Association of hsa-miR-34c-5p content in human spermatozoa with seminal volume and sperm quality parameters. The figure shows the correlation of the hsa-miR-34c-5p abundance of spermatozoa with classic seminal parameters, including seminal volume (A), total sperm concentration (B), sperm vitality (C), sperm motility (D), concentration of highly motile sperm (E) and sperm membrane integrity (F). Sperm parameter data were collected from 13 patients with an average age of 41 years (age range 31–51 years) and an average BMI of 25.5 (BMI range 20–29 kg/m²). The relationship between hsa-miR-34c-5p abundance and the different parameters was evaluated by computing the Pearson correlation coefficients (R), assuming a Gaussian distribution (confidence interval of 95%). The correlation coefficient (R) and the corresponding significance level (P-value) are provided. A P-value of less than 0.05 indicates statistical significance, while a P-value less than 0.10 suggests a notable tendency toward significance.

5.4.3 Content of miR-34c-5p in human spermatozoa differentially correlates with the release of monocarboxylates by embryos during *in vitro* culture

Studies suggest that sperm miRNA content influences gene expression patterns in early embryos, potentially affecting embryo development [25]. Hence, we evaluated the correlation between the content of the selected miRNA and embryo metabolic performance, by assessing the exometabolome during *in vitro* culture.

When we evaluated the association of miR-34c-5p sperm content with metabolite variation in the embryo culture media, we could identify a significant correlation of this miRNA with the release of four monocarboxylates into the media by embryos during *in vitro* culture, with contrasting results (Figure 5.3). While the release of both pyruvate and acetate by embryo was positively correlated with miR-34c-5p sperm content (P=0.001; R=0.639 and P=0.021; R=0.458), the release of formate was negatively correlated (P=0.002; R=0.586) and that of lactate showed decrease tendency toward significance when associated with

the content of miR-34c-5p on human sperm ($P=0.063$; $R=0.529$) (Figure 5.3. Conversely, apart from aspartate and isoleucine, no significant correlation or tendency was observed on the consumption of metabolites by cultured embryos and miR-34c-5p sperm content (Table 5.4). Aspartate consumption showed a negative association with the content of this miRNA on spermatozoa ($P=0.048$; $R=-0.399$), while isoleucine consumption showed a decrease tendency toward significance when associated with the content of miR-34c-5p on human sperm ($P=0.067$; $R=-0.372$) (Figure 5.3).

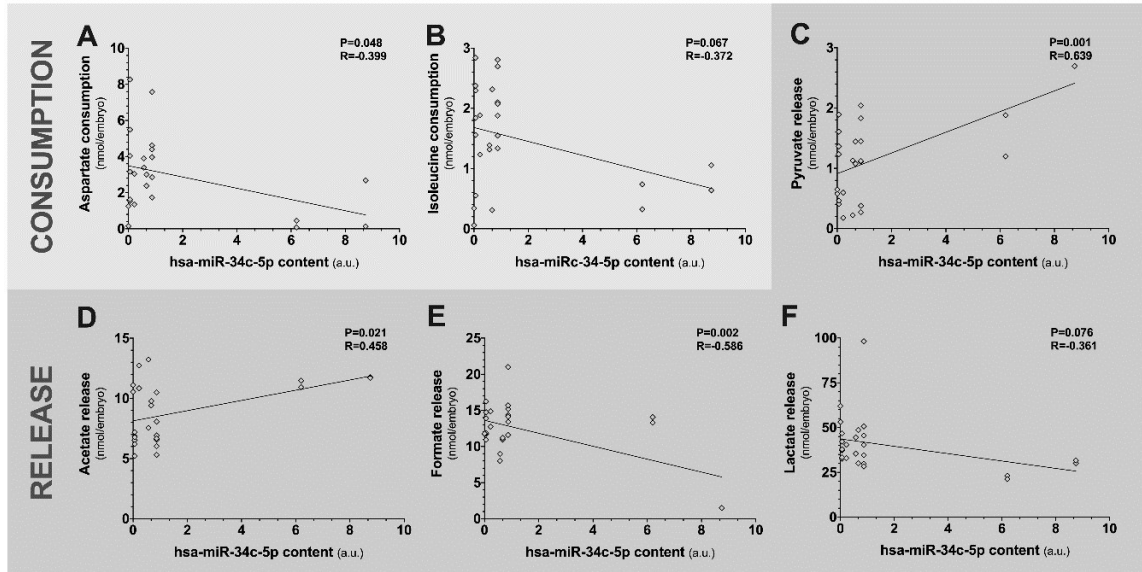


Fig 5.3 Association of hsa-miR-34c-5p content in human spermatozoa with metabolite variation in embryo culture media. The figure shows the correlation between the variation (Consumption or Release) of selected metabolites (Panel A-aspartate; Panel B-isoleucine; Panel C-pyruvate; Panel D-acetate; Panel E-formate; Panel F-lactate) quantified in the embryo culture media at the end of day 5 of *in vitro* culture. Sperm miRNA data were collected from 13 patients, which resulted in 20 embryos ($n=20$). The relationship between hsa-miR-34c-5p abundance and the variation of the different metabolite in embryo culture media was evaluated by computing the Pearson correlation coefficients (R), assuming a Gaussian distribution (confidence interval of 95%). The correlation coefficient (R) and the corresponding significance level (P -value) are provided. A P -value of less than 0.05 indicates statistical significance, while a P -value less than 0.10 suggests a notable tendency toward significance.

Table 5.4 Association between sperm hsa-miR-191-3p and hsa-miR-34c-5p content with embryo metabolic performance.

miRNAs vs Embryo metabolic performance	Sperm hsa-miR-191-3p content		Sperm hsa-miR-34c-5p content	
	P	R	P	R
Glucose consumption	0,023	0,453	0,268	0,230
Isoleucine consumption	0,012	-0,493	0,067	-0,372
Valine consumption	0,170	-0,283	0,240	-0,244
Aspartate consumption	0,133	-0,309	0,048	-0,399
Pyruvate release	0,457	-0,156	0,001	0,639
Lactate release	0,145	0,300	0,076	-0,361
Acetate release	0,138	0,305	0,021	0,458
Formate release	0,177	-0,279	0,002	-0,586
Alanine release	0,025	0,447	0,322	-0,207
Glutamine release	0,363	-0,190	0,597	-0,111

Statistical analysis was performed by computing Pearson correlation coefficients (R) assuming a Gaussian distribution (confidence interval of 95%). The correlation coefficient (R) and the corresponding significance level (P-value) are provided (N=25). A P-value of less than 0.05 indicates statistical significance, while a P-value less than 0.10 suggests a notable tendency toward significance.

5.4.4 miR-191-3p content in human spermatozoa shows a positive association with the consumption of glucose by embryos

We also evaluated the association of miR-191-3p sperm content with metabolite variation in the embryo culture media. Although we found no significant correlation with the variation of most metabolites in the media by embryos during *in vitro* culture (Supplemental Table S3), we could observe a significant positive correlation of the sperm content for this miRNA with the consumption of glucose (P=0.023; R=0.453) by embryos during *in vitro* culture, contrasting with the negative correlation observed for isoleucine consumption (P=0.012; R=-0.493). Concurrently, alanine release showed a positive association with the content of this miRNA on spermatozoa (P=0.025; R=0.305) (Figure 5.4).

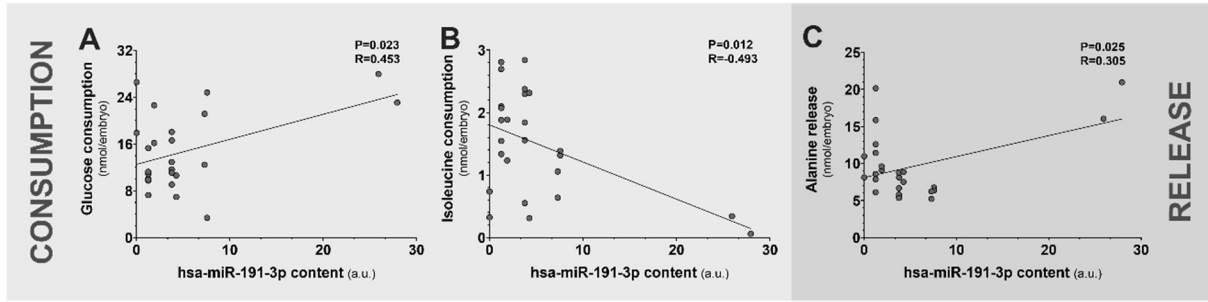


Fig 5.4 Association of hsa-miR-191-3p content in human spermatozoa with metabolite variation in embryo culture media. The figure shows the correlation between the variation (Consumption or Release) of selected metabolites (Panel A-glucose; Panel B-isoleucine; Panel C-alanine) quantified in the embryo culture media at the end of day 5 of *in vitro* culture. Sperm miRNA data were collected from 13 patients, which resulted in 20 embryos (n=20). The relationship between hsa-miR-191-3p abundance and the variation of the different metabolite in embryo culture media was evaluated by computing the Pearson correlation coefficients (R), assuming a Gaussian distribution (confidence interval of 95%). The correlation coefficient (R) and the corresponding significance level (P-value) are provided. A P-value of less than 0.05 indicates statistical significance, while a P-value less than 0.10 suggests a notable tendency toward significance.

5.4.5 Increased sperm miR-34c-5p content correlates with improved *in vitro* embryo development

We further evaluated if the content of human sperm miR-191-3p and miR-34c-5p was correlated with embryo development quality *in vitro* (Figure 5.5). This analysis revealed that among embryos categorized as Good (n=9), Lagging (n=7), or Bad (n=4), those derived from sperm with higher levels of miR-34c-5p exhibited improved quality. The content of miR-34c-5p in sperm samples that originated Good embryos was 2.99 ± 1.13 arbitrary units, while that that originated both lagging and bad embryos was significantly lower (0.25 ± 0.10 and 0.26 ± 0.07 arbitrary units, respectively). Conversely, no significant differences were observed in the content of sperm miR-191-3p that originated the different embryo quality categories (Good 4.17 ± 0.96 a.u., Lagging 3.39 ± 0.33 a.u., and Bad 3.64 ± 0.85 a.u.).

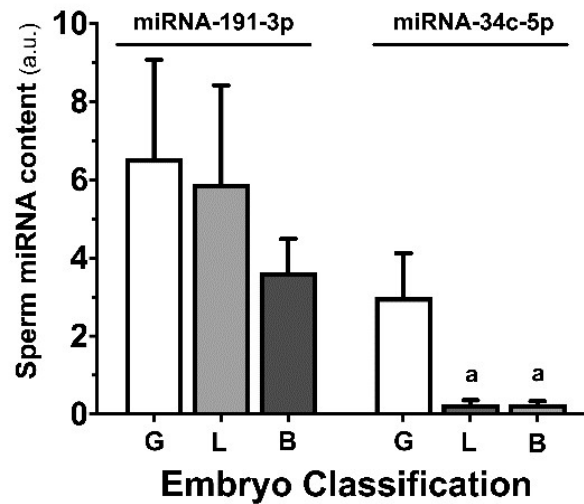


Fig 5.5 miRNA content in human spermatozoa used for obtaining embryos grouped according to their quality at day 5 (Good vs. Lagging vs. Bad). The figure shows the content of hsa-miR-191-3p and hsa-miR-34c-5p in human sperm that originated embryos categorized as Good (n=9), Lagging (n=7), or Bad (n=4) after 5 days of *in vitro* development. Data are presented as Mean $\pm$ Standard Error of the Mean (S.E.M.). Normality test was performed, and Univariate ANOVA was conducted. Pairwise comparisons between groups were corrected for multiple hypotheses testing by Tukey's post hoc test. A P-value of less than 0.05 indicates statistical significance, while a P-value less than 0.10 suggests a notable tendency toward significance (a-versus Good Embryos).

5.5 Discussion

Sperm selection techniques for assisted reproductive technologies aim to isolate the most competent spermatozoon which should be characterized by the highest fertilizing capacity. The prevailing approaches are based mostly on morphofunctional evaluation, offering a substantial degree of subjectivity. This inherent variability can lead to the potential misselection, thereby contributing to a reduction in the overall success rates of ART treatments, highlighting the relevance of finding new biomarkers for gamete quality and its fertility potential. Integrating these biomarkers into selection protocols may improve treatment outcomes by enhancing the accuracy of sperm selection and minimizing the risk of embryo implantation failure, miscarriage, and developmental abnormalities [26]. The relevance of the use of molecular biomarkers in sperm selection for assisted reproductive technologies has been debated in the last decades [26, 27] in the perspective of developing tools that allow the objective and evidence-based insights into sperm quality, allowing the identification of optimal sperm candidates for fertilization. Within this context, researchers have explored sperm miRNAs because of their regulatory roles in sperm function and early embryonic development [25, 28, 29], aiming to identify specific signatures associated with

sperm quality, fertilization potential, and embryo development competence. In this study, we focused on the association between sperm content in specific miRNAs, sperm quality parameters and embryo development (with a particular emphasis on embryo metabolic performance), in the expectation to offer valuable insights into potential clinical implications for couples using ART. We chose two sperm-borne miRNAs, miR-34c-5p and miR-191-3p, based on their distinct characteristics regarding their contribution to the embryo's miRNA pool. Although both miRNAs have been linked to embryogenesis [6, 9], miR-191-3p exhibits consistent expression [13], while miR-34c-5p has been reported to be absent in human oocytes [14]. We firstly evaluated the content of both miR-191-3p and miR-34c-5p in spermatozoa and explored their correlation with male anthropometric profiles (see Supplemental Table 1). While no significant results were obtained for the correlation of both age or BMI with the content of these two miRNAs in human spermatozoa, the content of miR-34c-5p was correlated with specific sperm quality parameters. In fact, it has been reported that several miRNAs have implications in sperm physiology, including sperm motility and morphology, although the exact function of those miRNA in sperm is yet to be fully understood. It has been proposed that they might represent residues from spermatogenesis, preserved for potential future involvement in fertilization, or they could be transferred to the spermatozoon (for instance, in the epididymis) to exert influence on early embryonic development [30]. In fact, our results highlighted a negative association for miRNA-34c-5p with total sperm motility. Data from Curry and colleagues highlighted a similar trend, establishing a link between increased seminal content of this miRNA and asthenozoospermia [30]. miRNA-34c-5p is a member of the miR-34 family, which is also composed of miR-34a and miR-34b, which have highly homologous sequences and share similar target genes and functions [31]. While data on their molecular targets in testicular cells are scarce, in other cellular systems these miRNAs were found to potentially target the expression of members NOTCH signaling pathway, inhibiting events such as cell proliferation, motility and invasion [32, 33]. Previous results have shown that NOTCH signaling blockade was associated with a reduction in sperm motility, prompting for a role of for this signaling pathway during sperm maturation in the epididymis [34]. Hence, although further studies are needed to validate our assumption, the negative association we observed between miRNA-34c-5p and total sperm motility may root its essence in the disruption of NOTCH signaling pathway.

As previously discussed, the two sperm-borne miRNAs we selected to study have been associated also to embryogenesis, although research on their molecular action in embryo development is relatively limited compared to its extensively studied functions in other biological contexts. While miRNA-34c-5p is known to regulate critical cellular processes such as cell metabolism, cell cycle progression and apoptosis, its involvement in embryonic

development is scarcely addressed, with some studies suggesting that members of the miR-34 family are associated with various developmental disorders and pregnancy complications, highlighting its potential relevance to embryo quality [35]. Additionally, dysregulation of the miRNAs of this family seems to play a role in modulating embryonic stem cell differentiation and lineage commitment, which could indirectly impact embryo development [36]. miR-34b/c have been reported in sperm, but not in oocytes, while miR-34c has been identified as crucial for the first embryo cleavage division *in vitro* [35]. Indeed, sperm-delivered miR-34c has been associated with preimplantation embryonic development, affecting diverse biological processes such as mRNA degradation and cell proliferation [37]. However, limited information is available regarding its impact on embryo metabolism.

In a recent study by Cui and colleagues [38], it was reported that microinjection of an miR-34c inhibitor resulted in impaired embryonic development, accompanied by reduced expression of genes related to cellular metabolism, particularly during the initial phases of embryo development. Specifically, these affected genes were primarily associated with lipid metabolism, including processes involved in very long-chain fatty acids, as well as with responses to carbohydrates, highlighting that the miR-34c inhibitor affected energy metabolism in these early-stage embryos [38]. Hence, those authors hinted that sperm-borne miR-34c-5p may have a role in embryonic metabolism, an event crucial for embryo development. In our study, we were able to follow the consumption (glucose, isoleucine, valine, and aspartate) or the release (pyruvate, lactate, acetate, formate alanine, and glutamine) into the culture medium of these water-soluble metabolites during the 5 days of embryo *in vitro* development and correlate this metabolic performance with the content of the selected sperm-borne miRNAs. We observed that the miRNA-34c-5p content was positively associated with the release of two key monocarboxylates, pyruvate and acetate. Acetate has been shown to be an important energy source for embryo development [39]. Mammalian cells can produce acetate from acetyl-CoA, a crossroad metabolite of lipid and carbohydrate metabolism [40]. An increased expression of genes linked to lipid metabolism prompted by miR-34c-5p, as suggested by Cui and colleagues [38], could explain the observed release of acetate by embryos. In fact, the promotion of lipid metabolism, with the concurrent increase of acetyl-CoA, which could result in the increased production/release of acetate. The increased production of acetyl CoA would also inhibit the conversion of pyruvate into this thioester, leading to the putative accumulation and export of pyruvate, as observed. In embryonic development, pyruvate serves as a vital energy substrate during preimplantation stages, associated with enhanced embryo quality in certain species like cattle and sheep [41]. While its significance in early human embryonic development remains less understood in the presence of other metabolic sources, exporting and accumulating

pyruvate in culture media is surely a sign that embryos exhibit active metabolism. On the other hand, formate (another monocarboxylate) release is negatively correlated with miR-34c-5p content. During early embryonic development, formate is produced by the mitochondria and some studies suggested formate levels as a biomarker for embryonic viability in ART, as its concentration in culture media correlates with embryo metabolic activity and may indicate embryonic quality and viability under certain conditions [42]. It is known that mammalian cells produce formate as a byproduct of several metabolic processes, particularly amino acid breakdown. Hence, the observed correlation for formate might be linked with the concurrent negative correlation observed between the sperm miR-34c-5p content and the consumption of the two amino acids aspartate and isoleucine (showing a notable tendency toward significance). Although there is still much progress to be made to enlighten the significance of amino acids such as aspartate on embryo metabolism, advancements have been achieved in elucidating the roles of these metabolites [43], that initially were viewed solely as biosynthetic precursors, but are now understood to possess multiple, interconnected functions, being intimately connected with embryo during *in vitro* development [16, 44].

Similarly to miR-34c-5p, the role miR-191-3p has been extensively studied in various biological contexts, including cancer and other diseases, while its involvement in embryonic development is not as well understood. However, some studies have suggested that miRNA-191-3p may play a role in early embryogenesis and may contribute to the regulation of genes involved in cell proliferation, differentiation, and apoptosis, which are critical processes for embryo development and quality [45]. In our work, sperm miR-191-3p content did not exhibit any correlation with sperm quality parameters, contrastingly to what we reported for miR-34c-5p. Nevertheless, we observed that sperm-borne miR-191-3p content was also associated with the metabolic performance of embryos. Embryos with higher content of this miRNA exhibited a higher consumption of glucose (and a lower consumption of isoleucine), concurrent with a lower release of alanine.

Glucose is known to be vital for human embryo development due to its role as a primary energy source. During early embryogenesis, glucose is the key provider of the necessary energy for various cellular processes, including cell proliferation and differentiation. Additionally, glucose metabolism fuels the rapid growth and development of the embryo as it progresses through different stages of development, emphasizing the importance of proper glucose availability and metabolism for supporting healthy embryo development in humans [46]. While to the best of our knowledge no data is available concerning the role of this miRNA on embryo glucose metabolism, MiR-191-3p seems to play a role in glucose metabolism, as it is mentioned as a key regulator of glucose homeostasis in various systems [47]. Our results suggest that this miRNA is involved in modulating glucose metabolism also

in developing embryos. Indeed, it also correlates (negatively) with the consumption of isoleucine and with the release of alanine, a byproduct of glucose metabolism. Alanine is a non-essential amino acid whose export by human embryos reflects its metabolic state and its ability to maintain energy balance during early development [16]. It has been suggested that the export of alanine may serve as a protective mechanism against stress and nutrient deprivation, allowing the embryo to maintain its viability and developmental potential [48], which might what is happening in our high-metabolism embryos.

Finally, when we evaluated the association of the sperm content for these two miRNAs with embryo developmental quality, as assessed by means of morphokinetic evaluation, we observed that Good embryos were derived from sperm with a higher content of miR-34c-5p (as compared with that of Lagged or Bad embryos). This association was not observed for sperm miR-191-3p content, contrasting with a study from Xu and colleagues [6]. This difference might be due to the studied populations which are very distinct, being that for instance this former study used only normozoospermic men, and to discrepancies in the method of evaluation of embryo quality. Beyond the inherent subjectivity of the evaluation of embryos, the 2020 study used a method described by Veeck and colleagues [49], rating the development of embryo on the third day, while we monitored the embryos from fertilization to day 5 using a time-lapse system and the Istanbul Consensus [24], as we described previously in detail [16]. These differences further highlight the need of the establishment of solid molecular biomarkers for gamete and embryo quality. In this sense, our results emphasize the potential significance of sperm miR-34c-5p content as a biomarker, not only of sperm quality, but also of embryo developmental potential.

5.6 Conclusion

This study explores how sperm-borne miRNAs may relate to male reproductive factors and the quality of embryo development. Firstly, it studied how sperm miRNA content affects sperm motility, a crucial aspect of male fertility. This study shows a significant negative link between miR-34c-5p levels and overall sperm motility, suggesting that irregular miRNA expression in sperm could impact sperm function and fertility outcomes. Additionally, it examines how sperm miRNA content influences embryo metabolism during *in vitro* culture. By studying the substances released by cultured embryos, the researchers identify connections between sperm miRNAs and specific metabolites released by embryos. Of particular interest is the link between miR-34c-5p levels in sperm and the release of pyruvate, acetate and formate, indicating a potential role for sperm miRNAs in shaping embryonic metabolic pathways. Furthermore, the study explores how sperm miRNA content relates to the quality of embryo development *in vitro*. It finds a correlation between higher

miR-34c-5p levels in sperm samples and better embryo morphokinetic grading, suggesting that analyzing sperm miRNAs could help predict embryo developmental competence.

Overall, these findings deepen our understanding of how sperm miRNAs influence male fertility and embryo development. They hint at the possibility of using sperm miRNA profiles as an additional tool to complete embryo assessment and improve *in vitro* fertilization success rates. Understanding the intricate role of sperm miRNAs in embryo quality holds promise for improving ART outcomes. By identifying specific miRNA signatures associated with optimal embryo development, one could aim at developing novel diagnostic tools and therapeutic interventions to enhance embryo quality and increase the success rates of ART procedures. However, more research is needed to confirm these findings in larger groups and understand the molecular mechanisms behind miRNA effects on reproductive outcomes.

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Disclosures

The authors have no conflict of interest to declare.

Author's contribution

Conceptualization, So.P., M.G.A., and P.F.O.; Methodology, So.P., Sa.P., M.G.A., and P.F.O.; Formal analysis, So.P., and Sa.P.; Investigation, So.P.; Writing—original draft preparation, So.P., and P.F.O.; Writing—review and editing, M.G.A., A.R., A.B., and P.F.O.; Supervision, A.R., A.B., and P.F.O.; Project administration, P.F.O.; Funding acquisition, M.G.A. and P.F.O. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki, and the procedures were covered by the provisions of the National Medically Assisted Procreation Act (2017) and overseen by the National Council for Medically Assisted Procreation (CNPMA-2018). According to these rules and guidelines, the use of clinical databases and patient biological material for diagnosis and research may be used without further ethical approval, under strict individual anonymity, and after patient written informed consent.

Regarding the use of human samples for laboratory experimentation, the Ethics Committee authorization number is 2021/CE/P02 (P342/2021/CETI), approved on 26 February 2021.

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CAPÍTULO 6

Conclusão Geral



6 Conclusão Geral

A jornada rumo à parentalidade através da procriação medicamente assistida oferece esperança a milhões de pessoas que sofrem de infertilidade. A área da biologia da reprodução tem sido um foco de interesse e investigação contínua porque, apesar dos avanços alcançados em PMA já permitirem a muitos casais a concretização do seu projecto procriativo, as taxas de sucesso continuam muito aquém do desejado.

A importância do espermatozóide no desenvolvimento embrionário tem sido uma das linhas de investigação que mais se evidenciou nos últimos anos. As técnicas de fertilização *in vitro*, ao ultrapassarem as barreiras naturais de selecção espermática que existem *in vivo*, podem promover o uso de espermatozoides de qualidade condicionada comprometendo o desenvolvimento embrionário e a descendência que daí resultar. O impacto da má qualidade espermática no desenvolvimento e qualidade do embrião realça a importância da selecção dos espermatozoides para os tratamentos de PMA.

Um dos nossos objectivos era a revisão bibliográfica dos métodos de preparação espermática (convencionais e avançados) e avaliar de que forma esses procedimentos poderiam contribuir para a melhoria das taxas de sucesso. Para isso, analisámos os diferentes procedimentos de preparação e selecção de espermatozoides - Capítulo 3: “Sperm selection strategies and their impact on assisted reproductive technology outcomes”. Concluiu-se que, apesar de alguns métodos avançados apresentarem melhores resultados, nomeadamente: na taxa de fertilização; na qualidade embrionária; na redução do uso espermatozoides com anomalias cromossómicas; na melhoria da taxa de gravidez e na obtenção de espermatozoides com melhores características morfológicas, a maioria ainda está em fase de validação e são necessários estudos adicionais para comprovar a sua aplicabilidade na rotina laboratorial. Nenhum dos métodos deve ser encarado como a solução ideal, e a escolha do método a aplicar depende do diagnóstico de infertilidade, da amostra espermática inicial e da capacidade laboratorial. Da análise dos diferentes trabalhos constatou-se que a falta de padronização dos protocolos utilizados pelos vários laboratórios dificultou a comparação dos estudos publicados. Contudo, concluímos que os métodos convencionais continuam a ser os mais utilizados e os mais eficientes na preparação espermática para a maioria das situações existentes na prática clínica, nomeadamente o *swim-up* e os gradientes de densidade.

Um dos pontos cruciais para o sucesso da PMA reside na identificação dos embriões com maior potencial de desenvolvimento e implantação. Tradicionalmente, a selecção embrionária baseia-se na avaliação morfocinética, partindo do pressuposto de que a qualidade morfológica traduz a competência embrionária. Esta extrapolação resulta da

correlação que existe entre a qualidade morfológica do embrião e as taxas de gravidez. O método apresenta algumas limitações, nomeadamente a subjectividade entre operadores (e actualmente também entre algoritmos na tecnologia time-lapse), é pouco consistente e é redutor na informação analisada. Na tentativa de hierarquizar parâmetros morfológicos e uniformizar critérios foram criados vários sistemas de classificação, o que é revelador da necessidade de desenvolver meios complementares para uma classificação embrionária mais eficiente e criteriosa. Nesse sentido, o foco das investigações mais recentes está na identificação de biomarcadores – biomoléculas quantificáveis que nos permitem compreender de forma mais profunda a “condição geral” do embrião e o seu potencial de desenvolvimento. O nosso trabalho direccionou-se para dois caminhos de investigação promissores: análise de metabolitos produzidos pelo embrião (exometaboloma) e análise de microRNAs (miRNAs) presentes nos espermatozóides.

Os metabolitos embrionários fornecem uma visão dos processos metabólicos em curso no embrião, refletindo a sua funcionalidade e viabilidade. Estas pequenas moléculas são produzidas por actividade celular e, portanto, podem ser associadas a processos e/ou alterações biológicas precisas. Ao analisar o exometaboloma específico presente no meio de cultura que circunda um embrião em desenvolvimento, os investigadores podem obter conhecimentos mais detalhados sobre a actividade metabólica e o seu potencial de implantação.

O nosso primeiro estudo investigou o potencial de seis metabolitos-chave – piruvato, lactato, alanina, glutamina, acetato e formato – no desenvolvimento e selecção embrionários. A técnica utilizada para a determinação dos perfis metabólicos – ^1H -NMR – demonstrou ser eficiente mesmo em volumes baixos (20 μl). Observámos níveis distintos de metabolitos entre embriões de diferentes qualidades morfológicas: “boa qualidade”, “atrasados” (desenvolvimento lento para o ideal estabelecido) e “má qualidade”. Analisando a variação metabolómica, concluímos que a avaliação do exometaboloma de embriões no terceiro dia de desenvolvimento pode ser preditiva da qualidade que os embriões apresentam em fase de blastocisto (5º dia de desenvolvimento). Esta constatação sugere que o perfil metabólico específico de um embrião pode ser um indicador válido do seu potencial de desenvolvimento.

Foi observado que os embriões considerados de “boa qualidade” e “atrasados”, exportam piruvato, alanina e glutamina para o meio circundante. Sugerimos que essa exportação signifique uma utilização eficiente destes metabolitos para a produção de energia e para os processos celulares essenciais. O facto de embriões de “boa qualidade” e “atrasados” apresentarem perfis semelhantes para estes metabolitos pode ser indicativo do carácter pouco preciso da avaliação morfocinética, uma vez que os embriões de boa

qualidade são caracterizados por um metabolismo mais eficiente e robusto que se deveria traduzir num perfil diferenciado.

Por outro lado, os embriões de “má qualidade” exibiram um perfil metabólico distinto, caracterizado pelo consumo de glutamina e piruvato. Isto sugere que a actividade metabólica pode estar comprometida e que o embrião pode estar num potencial esforço para manutenção das necessidades energéticas necessárias para um desenvolvimento adequado. O consumo de glutamina pode também ser um mecanismo compensatório para mitigar o stress oxidativo, induzido por condições externas ou intrínsecas do próprio embrião, o que pode traduzir um esforço adicional do embrião para manutenção da sua viabilidade.

Relativamente à alanina, nos embriões de “má qualidade” ela também é excretada para o meio, mas em menores quantidades comparativamente aos embriões de “boa qualidade” e “atrasados”. Portanto, a quantificação do piruvato, da glutamina e da alanina ao terceiro dia de cultura, apesar de neste trabalho não permitir de forma eficiente a separação entre as 3 classes morfológicas, permite identificar os embriões que apresentarão menor qualidade ao 5º dia de cultura embrionária distinguindo-os daqueles que terão uma qualidade aceitável para transferência e congelação (embriões utilizáveis).

O facto destes resultados se evidenciarem após ajuste das covariáveis (índice de massa corporal e idade dos progenitores) enfatiza a importância de considerar outras variáveis, para além das inerentes ao embrião, na identificação de perfis metabólicos de qualidade embrionária. Esta constatação enaltece a interacção complexa entre os vários factores que contribuem para o desenvolvimento embrionário. Uma abordagem integrativa que considere, não apenas as características intrínsecas do embrião, mas também os factores subjacentes associados à qualidade espermática, ovocitária e laboratorial, é crucial para maximizar a precisão da selecção embrionária.

O epigenoma define-se como o conjunto de modificações químicas que podem ocorrer no DNA (e/ou proteínas associadas), assim como o conteúdo em RNAs não codificantes (ncRNAs), e que podem activar ou inactivar genes específicos de uma determinada célula. Enquanto os metabolitos embrionários fornecem conhecimentos sobre o estado metabólico do embrião, os microRNAs (miRNAs) espermáticos fornecem uma visão na regulação genética embrionária. Estas pequenas moléculas são RNA não codificantes que desempenham um papel fundamental na regulação da expressão génica nas células, incluindo espermatozóides e embriões. Para além disso, actuam como reguladores-chave, ajustando a produção de proteínas essenciais a vários processos celulares. Estudos sugerem que a presença e a quantidade de miRNAs específicos no esperma podem fornecer informações importantes sobre a qualidade e o potencial de desenvolvimento do

embrião resultante, comprovando a importância que o epigenoma paterno tem nas primeiras fases do desenvolvimento embrionário.

Um desses miRNAs, o miR-34c-5p, ganhou significativa atenção no nosso trabalho, uma vez que detectámos uma correlação entre os níveis mais elevados deste miRNA nos espermatozóides e um melhor desempenho no desenvolvimento embrionário *in vitro* (avaliado pelo número de embriões com boa classificação morfológica). Ao cruzarmos as informações obtidas pela análise do perfil metabólico do embrião com a quantidade de miR-34c-5p dos espermatozóides, verificámos a existência de uma correlação positiva com os níveis de piruvato e acetato no exometaboloma. Estes resultados sugerem que o miR-34c-5p pode potencialmente servir como biomarcador do desenvolvimento embrionário adequado, comprovando a contribuição que o epigenoma do espermatozóide pode ter na formação de um embrião com melhor potencial de desenvolvimento e implantação.

Verificámos também que conteúdo de o miR-34c-5p no espermatozóide exibia uma correlação negativa com a motilidade total e que, apesar de não existir correlação estatística evidente, existe uma tendência para a sua associação com a reduzida percentagem de espermatozóides com motilidade progressiva rápida (parâmetro importante para o prognóstico/diagnóstico masculino e para a escolha da técnica de PMA a aplicar).

Neste trabalho foi também analisada a importância que o miR-191-3p, outro miRNA espermático, pode ter quer na espermatogénese quer na viabilidade embrionária. Curiosamente, apesar de nos nossos dados este miRNA não estar correlacionado com a qualidade espermática em si, parece influenciar a actividade metabólica do embrião em desenvolvimento. Embriões resultantes de espermatozóides com níveis altos de miR-191-3 apresentam um perfil metabólico caracterizado por um maior consumo de glucose, um menor consumo de isoleucina e uma baixa produção de alanina. Esta observação direciona para a possibilidade deste miRNA ter alguma influência na regulação do desenvolvimento embrionário, independentemente da qualidade espermática. Esta constatação destaca a interacção complexa entre os miRNAs de origem espermática e os próprios mecanismos de regulação intrínsecos do embrião.

A integração de informações obtidas, tanto do exometaboloma embrionário como do perfil de miRNAs do espermatozóide, pode ser uma perspectiva promissora para a PMA por contribuírem para a definição de biomarcadores de viabilidade embrionária. Ao combinarem estas duas ferramentas poderosas, investigadores, clínicos e embriologistas podem, potencialmente, obter um conhecimento mais abrangente do desenvolvimento embrionário e com isso otimizar a selecção dos embriões.

Os trabalhos realizados estabelecem algumas linhas de investigação. Contudo são necessárias pesquisas mais abrangentes para validar os resultados já descritos e

estabelecer protocolos de investigação robustos em ambiente clínico. Só dessa forma se poderão superar os obstáculos inerentes à implementação deste tipo de ferramentas na prática clínica. O objectivo é a melhoria significativa das taxas de sucesso na PMA aproximando o desejo da parentalidade à concretização dessa realidade em contexto de tratamento.

7 ANEXO A

Artigos publicados

Pinto S, Carrageta DF, Alves MG, Rocha A, Agarwal A, Barros A, Oliveira PF. *Sperm selection strategies and their impact on assisted reproductive technology outcomes. Andrologia.* 2021 Mar;53(2):e13725. doi: <https://doi.org/10.1111/and.13725>

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Sperm selection strategies and their impact on assisted reproductive technology outcomes

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Abstract

The application of assisted reproductive technologies (ART) has revolutionised the treatment of human infertility, giving hope to the patients previously considered incapable of establishing pregnancy. While semen analysis is performed to access whether a sample has an adequate number of viable, motile and morphologically normal sperm cells able to achieve fertilisation, sperm selection techniques for ART aim to isolate the most competent spermatozoon which is characterised by the highest fertilising potential. Based on the semen analysis results, the correct sperm selection technique must be chosen and applied. In this review, different sperm selection strategies for retrieving spermatozoa with the highest fertilising potential and their impact on ART outcomes are discussed. In addition, advantages and disadvantages of each method and the best suited techniques for each clinical scenario are described.

KEYWORDS

ART, ICSI, IUI, IVF, spermatozoa

1 | INTRODUCTION

Assisted reproductive technologies (ART) revolutionised human fertility by providing treatment to patients previously considered incapable for reproductive purposes (Henkel & Schill, 2003). In 2017,

it was estimated that approximately 6 million infants were born through ART since 1978, when the first baby produced by in vitro fertilisation (IVF) was born. It has been reported that about half of these births were in the past decade, highlighting the increasing use of these technologies, (Hunter, 2017).

Initially, ART research was mainly focused on comprehending the factors that increase the likelihood of a successful pregnancy and

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improving outcomes for mothers and their ART-conceived children. Currently, the fathers health and sperm selection have deserved a higher attention from clinicians and researchers (Messerlian & Gaskins, 2017). Spermatozoa are complex cells, whose fertilising potential can be jeopardised by small morphologic, physiological or even biochemical alterations (Bernardino, Carrageta, Sousa, Alves, & Oliveira, 2019; Carrageta et al., 2020). It is estimated that 40%–50% of the infertility cases are due to male factors, mainly caused by defective spermatogenesis, retrograde ejaculation, hypogonadism or erectile dysfunction (Agarwal, Mulgund, Hamada, & Chyatte, 2015). However, the majority of these cases are classified as idiopathic (Kothandaraman, Agarwal, Abu-Elmagd, & Al-Qahtani, 2016; Kumar & Singh, 2015). Some of these cases are associated with lifestyle (such as sedentarism and smoking) or metabolic disorders (as obesity or diabetes mellitus), although the molecular links remain to be disclosed (Carrageta, Oliveira, Alves, & Monteiro, 2019; Oliveira, Sousa, Silva, Monteiro, & Alves, 2017; Skakkebaek et al., 2016).

The selected methods for sperm preparation depend on the results on sperm analysis and fresh sample in the day of the ART treatment. Sperm selection techniques for ART aim to isolate sperm cells that are characterised by a high fertilising potential and, subsequently, may lead to a successful pregnancy. Over the past years, the understanding on sperm physiology has been improved and more sophisticated techniques were developed in order to effectively isolate physiologically normal spermatozoa from those that are immature, immotile or have poor fertilising potential. Conventional methods remain an effective tool for sperm selection; however, more advanced methods are employed to address specific and complex infertility cases to attain higher take-home-baby rate. In this review, sperm selection techniques for the isolation of the most competent spermatozoa and their impact on ART outcomes will be discussed. Furthermore, each method and technique will be briefly presented to discuss which are the best for each clinical scenario.

2 | STANDARD SEMEN ANALYSIS

The guidelines for human semen analysis are described in the 5th edition of the World Health Organization (WHO) Manual for the Examination and Processing of Human Semen (WHO, 2010), which provides reference values for semen volume, pH, liquefaction, sperm count, total and progressive motility, vitality, morphology and leucocytes concentration (reference values are summarised in Table 1). Upon analysis, the patient is diagnosed accordingly and the most appropriate sperm selection technique is chosen. The main goal of sperm selection techniques is to isolate the spermatozoa with the most fertilising potential for use in ART. Generally, the chosen technique depends on the characteristics of the sample and human semen analysis.

Although widely applied as a general indicator, the semen analysis has several limitations. Simon et al. reported that 80% of men seeking fertility treatment had idiopathic infertility beyond diagnosis confirmed by established standard testing protocols (Simon et al., 2013). Spermatozoa are complex cells influenced by a myriad of internal and

TABLE 1 Reference values for normal sperm parameters according to the 5th WHO Manual for the Examination and Processing of Human Semen (WHO, 2010)

Semen parameter	Reference value
Semen volume	≥1.5
Sperm concentration	≥15 (10 ⁶ /ml)
Total sperm number	≥39 (10 ⁶ /ejaculate)
Total motility	≥40 (%)
Progressive motility	≥32 (%)
Normal morphology	≥4 (%)
Viability	≥58 (%)
Leucocytes concentration	<1 (10 ⁶ /ml)

external stimuli (such as pH, ionic concentration or temperature), with several factors that may compromise its fertilising potential still remaining elusive to identify. In fact, new devices for diagnosis are developed based on the novel data produced by the scientific community, such as computer-assisted semen analysis (CASA) systems, analysis of the DNA damage, accessing sperm telomere length or the adoption of stricter criteria for sperm morphology evaluation (Jeyendran, Caroppo, Rouen, Anderson, & Puscheck, 2019; Lopes, Oliveira, & Sousa, 2019). In particular, assays for analysing sperm DNA integrity have been extensively investigated over the past decade (Majzoub, Arafa, Elbardisi, & Agarwal, 2020; Panner Selvam, Sengupta, & Agarwal, 2020). Sperm DNA integrity is essential for fertilisation and early stages of the embryo development (Agarwal & Said, 2003). Sperm cells do not express natural DNA repair mechanisms and are not able to repair DNA post-spermiogenesis (Gonzalez-Marin, Gosalvez, & Roy, 2012; Rappa et al., 2016). DNA damage is inevitable due to sperm physiology and the environment that they are exposed, but upon fertilisation it can be repaired to some extent. When the damage exceeds the oocyte's repair capacity, it may result in infertility (Majzoub, Agarwal, & Esteves, 2019). Clinical data have shown that the chance of both natural and artificial conceptions is reduced if DNA fragmentation index is more than 30% (Gonzalez-Marin et al., 2012; Oleszczuk, Augustinsson, Bayat, Giwerzman, & Bungum, 2013; Robinson et al., 2012). For this purpose, several techniques have been developed with the finality of analysing sperm chromatin and DNA integrity although standardisation or guidelines to uniformise results remain absent.

3 | PREPARATION OF SEMEN SAMPLES

Seminal plasma and its constituents are helpful for spermatozoa to achieve fertilisation in natural conditions; however, they are obstacles when natural barriers are circumvented in ART. The separation of human spermatozoa from seminal plasma, nongerm cells and dead spermatozoon is essential for clinical practice (WHO, 2010). Viable spermatozoa should be isolated as soon as possible. Although WHO guidelines recommend separation of spermatozoa from seminal plasma within 1 hr after ejaculation (WHO, 2010), it was reported

that fertilisation ability will permanently decrease if separation is not done in 30 min (Rogers et al., 1983). It is also recommended to maintain a sterile environment and use sterile equipment. Samples should be examined for the presence of round cells (leucocytes or epithelial cells) or bacteria. Viscosity may constitute a common issue among all ART methods, which substantially decreases the efficiency of the techniques. It is a common procedure to allow samples to liquefy completely at 37°C (usually 15–30 min are sufficient). In cases of highly viscous semen samples, it is sometimes advised to perform an enzymatic digestion. According to the WHO guidelines, if the sample cannot be induced to liquefy by the addition of medium or by the repeated passage through a needle, the digestion with bromelain for 10 min is recommended (WHO, 2010). Some authors also recommend to treat the sample with a trypsin solution, not more than 5 min before performing the separating technique, as it will increase efficiency without any harmful effect to spermatozoa (Agarwal, Gupta, & Sharma, 2016). Upon liquefaction, the samples are ready to be processed.

4 | SPERM SELECTION METHODS FOR ART

According to the clinical scenario, the appropriated sperm selection technique must be chosen. The sperm selection method should eliminate seminal plasma, dead sperm cells and other cells, while concentrating motile spermatozoa and selecting morphologically

normal spermatozoa (ESHRE Guideline Group on Good Practice in IVF Labs et al., 2016). Conventional sperm selection methods, such as simple sperm wash, glass wool preparation, swim-up or density gradient centrifugation, are usually the first step on semen processing for ART (Table 2). These methods are characterised as simple, fast, safe and inexpensive, although with limited usefulness (Henkel & Schill, 2003). Direct swim-up technique is still the gold standard for patient with normal sperm parameters, whereas in cases of oligozoospermia, teratozoospermia or asthenozoospermia, density gradient centrifugation is usually preferred due to the higher total number of motile spermatozoa recovered (WHO, 2010). In cases of pronounced or severe oligozoospermia, the volume of gradient can be reduced or a simple wash can be chosen. Ejaculates from oligozoospermic or asthenozoospermic men, or ejaculates from patients with viral diseases have to be treated with different techniques, to achieve optimal efficiency. Advanced sperm selection methods are able to further improve conventional methods, with the downside of being more expensive and occasionally time consuming (Table 3).

With these techniques, sperm cells are separated from other constituents present on the ejaculate such as seminal fluid, leucocytes, epithelial cells or unviable spermatozoa. A common ground between these techniques is that it is often possible to isolate a sperm subpopulation of higher fertility potential from an ejaculated sample. This subpopulation is characterised by higher motility, flawless morphology, and functional membrane and acrosome integrity (Jeyendran et al., 2019). Upon

TABLE 2 Comparison of various conventional sperm selection procedures

Procedure	Advantages	Disadvantages
Simple sperm wash	Simple, fast and inexpensive	All cells present on the semen sample, including leucocytes, epithelial cells and nonviable sperm cells, are separated from the seminal fluid and pelleted with the viable sperm cells
Glass wool filtration	- Isolates viable motile sperm cells - Efficient in removing leucocytes from the sample - Efficient for viscous semen samples - Retrieve spermatozoa with normal chromatin condensation - Select a higher percentage of spermatozoa with intact acrosome than swim-up or density gradient centrifugation - Higher percentage of fertilisation following IVF than swim-up	- More expensive - Originates some cellular debris
Swim-up	- Simple, fast and inexpensive - Isolates motile and morphologically normal sperm cells - Gentle method, reduces ROS production and DNA fragmentation - Reduces the proportion of sperm cells with chromosomal defects - Can be performed following density gradient centrifugation in order to further improve the quality of the retrieved spermatozoa	- Low number of sperm cells is retrieved - Low efficiency in separating competent sperm cells from cellular debris, immature sperm cells or other cells when pelleting - Low efficiency in cases of high viscosity
Density gradient centrifugation	- Simple and fast - Isolates a higher number of motile and morphologically normal sperm cells - Easier to standardise or adapt to the clinical scenario - Efficient in reducing the number of sperm cells with aneuploidy and diploidy - Reduced ROS production and oxidative stress - The conjugation with swim-up is recommended for samples from HIV-infected men	- Low efficiency in cases of high viscosity - Overloading the gradient may cause the aggregation of sperm and other cells

Abbreviations: IVF, in vitro fertilisation; ROS, reactive oxygen species.

TABLE 3 Comparison of advanced sperm selection procedures

Procedure	Advantages	Disadvantages
Zeta potential	- Selects mature sperm cells based on the plasma membrane negative charge - Simple and inexpensive - Select sperm cells with reduced DNA fragmentation - Improved fertilisation rate, percentage of top embryo quality and pregnancy outcome when compared to density gradient centrifugation	- There is no association between the zeta potential and total motility, requiring the prior use of other method - The time factor is essential, as zeta potential decreases with the onset of sperm capacitation - The number of retrieved sperm cells is low, leading to limited use for cases of oligozoospermia or testicular spermatozoa - Potential bias for selecting X-bearing sperm cells
Electrophoretic sperm selection	- Retrieve viable, highly mobile, and morphologically normal sperm cells - Retrieved sperm cells present reduced DNA fragmentation - Can be successfully used for cryopreserved samples and testicular biopsies	- Requires an electrophoresis system - Potential bias for selecting X-bearing sperm cells - Contradictory results concerning the ART outcomes
MSOME/IMSI	- Improved selection based on sperm cells ultra-structure - Beneficial for severe cases (oligoasthenoteratozoospermia and teratozoospermia) - Beneficial for cases with repeated ICSI failures	- Expensive - Time consuming - Requires a skilled embryologist - Absence guidelines and standardisation - Contradictory results concerning the ART outcomes
PICSI and hyaluronan methods	- Hyaluronic acid receptors are only expressed on mature sperm cells - Retrieval of sperm cells with reduced aneuploidy, chromosomal disomies, and diploidy	Insufficient or inconclusive data concerning ART outcomes
MACS	- Select viable sperm cells with reduced DNA fragmentation - Retrieve sperm cells with higher motility and normal morphology	Insufficient or inconclusive data concerning ART outcomes
Microfluidic separation	- Select sperm cells based on the motility or morphology - The damage caused by centrifugation is eliminated - Decreased DNA fragmentation when compared with conventional methods or unprocessed spermatozoa - Direct use of the sample	Insufficient or inconclusive data concerning ART outcomes
HOST	- Simple and inexpensive - Evaluates the plasma membrane integrity without damaging sperm cells - Useful for the identification of viable but immotile sperm cells	Insufficient or inconclusive data concerning ART outcomes
LAISS	- Considered as a safe method - Select viable but immotile sperm cells based on the curling of the sperm tail upon exposition to a laser shot - Useful for cryopreserved semen samples	- Expensive - Mechanism responsible for the tail curling is unknown - Insufficient or inconclusive data concerning ART outcomes
Polarisation microscopy	- Allows to evaluate the nucleus structural organisation and the state of the acrosome reaction - The pattern of the sperm head birefringence is correlated with sperm parameters	- Inconclusive data concerning DNA fragmentation - Time consuming - Requires a skilled embryologist - Insufficient or inconclusive data concerning ART outcomes

Abbreviations: ART, assisted reproductive technology; HOST, hypo-osmotic swelling test; ICSI, intracytoplasmic sperm injection; IMSI, intracytoplasmic morphologically selected spermatozoa; LAISS, laser-assisted immotile sperm selection; MACS, magnetic-activated cell sorting; MSOME, motile sperm organelle morphology examination; PICSI, physiological intracytoplasmic sperm injection.

isolation of this subpopulation, it is possible for a low-quality semen sample to be suitable for a successful intrauterine insemination (IUI) or in vitro fertilisation (IVF). In case of poor quality samples, the advanced sperm selection methods allow identification and selection of the most promising spermatozoa for intracytoplasmic sperm injection (ICSI). In the following sections, the most common conventional and advanced sperm selection methods will be discussed in detail, from the general method to their advantages and disadvantages.

4.1 | Conventional sperm selection methods

4.1.1 | Simple sperm wash

The simple wash technique is a simple and straight-forward technique that should only be used when the semen sample has optimal parameters. This technique is performed to prepare spermatozoa for IUI, or in cases of severe oligoasthenoteratozoospermia or

criptozoospermia (Agarwal et al., 2016). With the simple wash technique, all cells present on the semen sample are separated from the seminal fluid. No more than 1 hr upon ejaculation and following complete liquefaction, culture medium or a washing sperm solution (e.g. a saline buffer) is added to the sample and centrifuged in order to pellet the cells. It is crucial to use low centrifugal forces (300 g is often described in protocols and always <500 g) and as few as possible centrifugation steps in order to minimise the production of reactive oxygen species (ROS) (Agarwal et al., 2016). High ROS production results in DNA and organelles damage and initiates the apoptotic cascade leading to the loss of motility and viability, though low levels of ROS are essential for normal sperm function and for the promotion of signalling pathways associated with capacitation (Aitken, Jones, & Robertson, 2012; Martin-Hidalgo, Bragado, Batista, Oliveira, & Alves, 2019). The downsides of the simple wash technique are a direct consequence of its simplicity, as all cells are separated from the seminal fluid including leucocytes and epithelial cells present on the semen sample. Nonviable spermatozoa are also present, which are reported to compromise sperm ability to capacitate and fertilise the oocyte (Rana, Jeyendran, Holmgren, Rotman, & Zaneveld, 1989).

4.1.2 | Glass wool filtration

The glass wool filtration is a separation method based on the filtration of the sample through a densely packed glass wool fibres placed in a column (Henkel & Schill, 2003), which makes this method efficient for highly viscous samples (Jeyendran et al., 2019). Through this method, it is possible to separate motile from immotile spermatozoa, leucocytes and seminal fluid. In fact, Henkel et al. reported that this method is able to reduce the leucocytes in the sample by about 87.5% (Henkel et al., 1997). In addition, glass wool filtration allows the retrieval of motile spermatozoa with normal chromatin condensation (Sauer, Coulam, & Jeyendran, 2012), and a higher percentage of sperm cells with intact acrosome than both the density gradient centrifugation and the simple wash method (Sterzik, De Santo, Uhlich, Gagsteiger, & Strehler, 1998). The filtered spermatozoon is also reported to result in higher percentage of fertilisation following IVF when compared with the spermatozoa retrieved with the swim-up procedure (Katayama, Stehlik, & Jeyendran, 1989; van der Ven et al., 1988). Unfortunately, this method is rather expensive when compared with other conventional techniques and might result in some cellular debris (Beydola, Sharma, Lee, & Agarwal, 2013).

4.1.3 | Swim-up

The swim-up is a migration technique and one of the simplest, fastest and commonly used methods for sperm preparation. With this method, a liquefied semen sample (direct swim-up) or a previously washed cell pellet (conventional swim-up) is placed in a tube with an overlapping layer of culture medium. The tubes are left on a

45° angle at 37°C for 1 hr before the supernatant is collected. The swim-up method is based on the fact that spermatozoa are attracted and swim into the nourished environment of the culture medium, which is then collected. The collected fraction has a high probability of containing only motile and morphologically normal spermatozoa (Beydola et al., 2013). When the concentration and motility parameters are within the normal range, the swim-up is regarded as the preferred technique. However, in cases of oligozoospermia the direct swim-up might be recommended. This method is mainly applied for IVF or ICSI as it selects spermatozoa for their motility and is convenient when the percentage of motile spermatozoa in the sample is low (WHO, 2010).

The swim-up is a very gentle method, leading to reduced production of ROS and DNA fragmentation (Oguz et al., 2018; Younglai, Holt, Brown, Jurisicova, & Casper, 2001). In addition, this technique mainly retrieves healthy and mature sperm cells (Jakab et al., 2003). On the other hand, the swim-up method also has some downsides. The first one is inherent to one of the advantages of the technique. Only a low percentage (5%–10%) of spermatozoa are retrieved, although it is a subpopulation of highly mobile and morphologically normal spermatozoa (Beydola et al., 2013). Another disadvantage is the fact that higher volumes or higher concentration requires further precautions. In cases of samples with high volume (more than 3 ml), it should be divided into several tubes in order to increase the area of contact with the culture medium. In cases of high concentration, pelleting the cells should be avoided as some spermatozoa might be trapped in the middle of the pellet and thus unable to migrate. In these cases, dilution and division into several tubes might be advised. The viscosity of the sample may also be an issue that needs to be addressed.

4.1.4 | Density gradient centrifugation

Density gradient centrifugation is a method that separates sperm cells based on their density, through a colloidal suspension of silica particles stabilised with covalent-bonded hydrophilic silane. Most commonly, there are two gradients layers: a lower phase (80%–90%) and an upper phase (40%–45%), whereas the sample is loaded as the top layer. The density gradient might also be continuous, gradually increasing from the top to the bottom (Henkel & Schill, 2003). The main principle of this method is the fact that normal and abnormal spermatozoa and other cells have distinct densities. It is reported that mature and morphologically normal spermatozoa are characterised by a density of more than 1.10 g/ml while immature or aberrant spermatozoa exhibit a density between 1.06 and 1.09 g/ml (Oshio, Kaneko, Iizuka, & Mohri, 1987). The outcome obtained upon density gradient centrifugation is a pellet containing the sperm subpopulation characterised by higher motility and normal morphology, whereas leucocytes, cell debris and aberrant spermatozoa remain on the top layers (Malvezzi, Sharma, Agarwal, Abuzenadah, & Abu-Elmagd, 2014).

One advantage is that it is easier to standardise or adapt the density gradient centrifugation to the clinical scenario than the swim-up

method, leading to more consistent results (WHO, 2010). The density gradient centrifugation is also very efficient in decreasing the number of spermatozoa with aneuploidy and diploidy (Brahem, Letaief, Ben Ali, Saad, & Mehdi, 2013), selecting also motile spermatozoa without enhancing oxidative stress (Quinn et al., 2018; Takeshima et al., 2017; Wang et al., 2014). When performing a density gradient centrifugation, it is still advised to use the lowest possible centrifugal force and time (usually 300 g and 20 min are the recommended settings by the density gradient manufacturers) in order to minimise the production of ROS and the consequent oxidative damage. If even upon the liquefaction time period the sample remains too viscous, it might be advised to increase the centrifugal force up to 600 g (Beydola et al., 2013). It is essential that the reagents are at the room temperature prior to use, which must be occur within 1 hr after performing the gradient in order to avoid the eventual blend of the two phases. It is also advised to avoid overloading the gradient since it may cause 'rafting', which is the aggregation of spermatozoa and other undesirable components present on the sample upon centrifugation (Beydola et al., 2013).

It is possible to perform the swim-up method following the density gradient centrifugation in order to improve the isolated spermatozoa quality, although fewer cells are retrieved. Both swim-up and density gradient centrifugation, alone or in combination, are successful in isolating viable and morphologically normal spermatozoa with intact chromatin and DNA, thus improving the chances of achieving a viable pregnancy (Brahem, Mehdi, Elghezal, & Saad, 2011; Jayaraman, Upadhyay, Narayan, & Adiga, 2012; Ricci et al., 2009; Yamanaka et al., 2016), as both are equally efficient in recovering spermatozoa with longer telomeres (Yang et al., 2015; Zhao, Yang, Shi, Luo, & Sun, 2016).

4.2 | Advanced sperm selection methods

4.2.1 | Zeta potential

Zeta potential is the electrokinetic potential on the surface of particles on a colloidal suspension. The membrane of mature human spermatozoa possesses a zeta potential between -16 and -20 mV, which is conferred by a coating of sialic acids during spermatogenesis and epididymal maturation (Ishijima, Okuno, & Mohri, 1991; Kirchhoff & Hale, 1996). This negative potential can be used as a biomarker for the selection of mature sperm cells (Simon, Shamsi, & Carrell, 2016). Based on this principle, Chan et al. developed a sperm selection method where mature sperm cells adhere to positively charged surfaces (Chan, Jacobson, Corselli, & Patton, 2006). As there is no association between the zeta potential and the total motility, the authors recommend that this method must be performed on selected motile spermatozoa obtained by a density gradient centrifugation. The zeta potential method should be performed immediately after liquefaction due to the decrease on zeta potential with the onset of sperm capacitation (Della Giovampaola et al., 2001). Briefly, new centrifugation tubes (preferably glass tubes) are placed inside a latex glove

and rotated two–three times in order to obtain a positively charged tube. To allow adherence of mature spermatozoa to the wall, the tube is kept 1 min at room temperature and then centrifugated and inverted in order to discard nonadhering spermatozoa. Mature sperm cells are retrieved by neutralising the charge on the tube with a serum-supplemented media (Chan et al., 2006; Henkel, 2012; Said & Land, 2011).

The main advantages of zeta potential method rely on its simplicity and that it is inexpensive, as there is no need for electrophoresis equipment. This method has been reported to select spermatozoa with less DNA fragmentation and a higher percentage of sperm cells with normal morphology and hyperactive motility when compared with the conventional density gradient centrifugation (Khajavi et al., 2009; Kheirollahi-Kouhestani et al., 2009; Zarei-Kheirabadi et al., 2012). It was also reported that the zeta potential method improved the fertilisation rate, the percentage of top embryo quality and pregnancy outcomes when compared to performing only a density gradient centrifugation (Kheirollahi-Kouhestani et al., 2009; Nasr Esfahani, Deemeh, Tavalae, Sekhavati, & Gourabi, 2016). The yield of retrieved spermatozoa is low, leading to its limited use in cases of oligozoospermia or testicular sperm retrieval (Chan et al., 2006). Ishijima et al. reported that the net zeta potential on the surface of human X-bearing spermatozoa is higher than Y-bearing spermatozoa (Ishijima et al., 1991), which led some authors to hypothesise that there might be a bias for the selection of X-bearing spermatozoa (Henkel, 2012). More recently, Esfahani et al. compared the results of ICSI between the zeta potential method and the conventional density gradient centrifugation and reported that the sex ratio (XY/XX) at birth following the zeta potential method was lower despite the similar ratio of X/Y-bearing spermatozoa obtained from both methods (Nasr Esfahani et al., 2016). However, more studies are needed to clarify whether the zeta potential method indeed changes the sex ratio.

4.2.2 | Electrophoretic sperm selection

Similar to the zeta potential method, the electrophoretic sperm selection is based on the fact that the plasma membrane of mature spermatozoa is negatively charged. In the electrophoretic method, semen samples are placed in an electrophoretic device, and then, a current is applied to select the negatively charged spermatozoa. In 2005, Ainsworth et al. introduced the first electrophoresis-based technology designed for sperm selection (Microflow[®]), which enabled the separation of spermatozoa by size and membrane charge (Ainsworth, Nixon, & Aitken, 2005). This device consists of two inner chambers, for inoculation of sperm cells, two outer chambers, for collection of the selected sperm cells, two electrodes and two buffer pumps. The two chambers are separated by a polycarbonate membrane which allows mature spermatozoa to cross it while larger cells are blocked. Then, it was possible to retrieve viable, mobile and morphologically normal spermatozoa with low levels of DNA damage (Ainsworth et al., 2005). Later in 2015, a

novel device for electrophoretic sperm selection named micro-electrophoresis chamber was introduced. This device consists of a disposable sterile electrophoresis system, a power supply and connecting electrodes. It was reported that negatively charged spermatozoa were not correlated with any sperm parameters besides decreased DNA damage (Simon et al., 2015; Simon, Murphy, et al., 2016).

Although the electrophoretic sperm selection seems to allow the selection of healthier sperm cells, there are few studies reporting the impact of this technology in ART. One advantage over the zeta potential is that this method was shown to be as effective when applied to cryopreserved samples and testicular biopsies (immature spermatozoa) as with fresh samples (Ainsworth, Nixon, Jansen, & Aitken, 2007). The electrophoretic sperm selection was reported to be positively associated with the fertilisation, implantation and clinical pregnancy rate (Simon et al., 2015), as it was also reported a successful normal birth following this method in an ART clinic (Ainsworth et al., 2007). On the other hand, Fleming et al. compared the electrophoretic method with the density gradient centrifugation and did not find any significant difference in fertilisation and cleavage rates (Fleming et al., 2008). Furthermore, the net negative charge on the cell surface of human X-bearing is higher than Y-bearing spermatozoa, which could lead to a lower sex ratio (XY/XX) (Ishijima et al., 1991). However, Ainsworth et al. reported that the electrophoretic method did not result in a significant skewing of the ratio of X- and Y-bearing sperm cells (Ainsworth, Nixon, & Aitken, 2011). Despite some evidence pointing to an improvement in sperm parameters following the electrophoretic method, studies concerning the ART outcomes are not only limited but also provide contradictory results. Additional studies are required to determine the effectiveness of the method in clinical practice.

4.2.3 | Motile sperm organelle morphology examination

The advent of the ICSI in 1992, by Palermo, Joris, Devroey, and Van Steirteghem (1992), for the treatment of severe male infertility allowed improved understanding of sperm biology. As a result, more stricter criteria for sperm selection were adopted. While several parameters such as sperm concentration, motility and DNA fragmentation are correlated with the reproductive outcome, several studies highlight that sperm morphology is decisive for the fertility potential (Kruger & Coetzee, 1999; Li et al., 2014; Mangoli & Khalili, 2020). In fact, some sperm cells considered as normal in a conventional morphological analysis may present ultra-structural defects that negatively affect the ART outcome (Simon, Shamsi, et al., 2016). A new method for detailed morphological assessment of motile spermatozoa in real time was developed in 2002—motile sperm organelle morphology examination (MSOME). MSOME is a method that is performed using a magnification of 6000×, which is a much higher magnification than the use for conventional ICSI (Bartoov et al., 2002) that allows the assessment of sperm cells ultra-structure. MSOME

analyses six sperm organelles: acrosome, post-acrosome lamina, nucleus, neck, tail and mitochondria. Among those, sperm nucleus appears to be the organelle that mostly influences ICSI outcome (Bartoov et al., 2002; Berkovitz et al., 2005; Lo Monte, Murisier, Piva, Germond, & Marci, 2013). While the shape and size of the head is assessed, a normal nucleus should present a smooth, symmetric and oval configuration. The presence of vacuoles is also evaluated. If the vacuoles occupy more than 4% of the head, the spermatozoon is labelled as abnormal (Bartoov et al., 2002, 2003). In fact, several studies highlight the detrimental effects of the presence of nuclear vacuoles in the ART outcomes (Ghazali, Talebi, Khalili, Aflatoonian, & Esfandiari, 2015; Vanderzwalmen et al., 2008).

When MSOME is applied on an ICSI micromanipulation system, it is known as intracytoplasmic morphologically selected spermatozoa (IMSI; Bartoov et al., 2003). The IMSI is performed on a conventional inverted microscope attached to specific accessories. Besides the standard equipment necessary to perform ICSI (micromanipulation and microinjection system plus the 400× Hoffman optics), in IMSI it is also required a 100× oil-immersion objective and Nomarski optics attached to the microinjection system. In addition, the IMSI process uses a 6000× magnification that can only be obtained digitally (Bartoov et al., 2002; Jeyendran et al., 2019). Taken together, all the necessary equipment renders this technique as expensive, time consuming and requires a skilled embryologist to be correctly performed. This method also has an inherent subjectivity associated with intra- and inter-observer variability. IMSI has proved to be a valid tool for sperm selection in some specific group of patients. There is evidence that patients with severe alterations in sperm parameters (oligoasthenoteratozoospermia and teratozoospermia) could benefit from IMSI (Balaban et al., 2011; Goswami, Sharma, Jugga, & Gouri, 2018; Kim et al., 2014; Mangoli et al., 2019). It has also been shown that if IMSI is applied to couple with repeated ICSI failures, the pregnancy rates and the number of quality embryos can be improved, and miscarriages rates decreased (Antinori et al., 2008; Delaroche et al., 2013). In addition, some studies demonstrate that IMSI improves the percentage of top-quality embryos, implantation and pregnancy rates and significantly reduces miscarriage rates compared with conventional ICSI (Balaban et al., 2011; Setti et al., 2010; Shalom-Paz et al., 2015; Teixeira et al., 2013). Even though some positive outcomes are highlighted in the literature, there is no consensus about the efficiency of the method or specific guidelines instructing how to perform IMSI. In fact, Teixeira et al. concluded that there is not enough evidence to support the clinical practice of IMSI and that further trials are necessary to elucidate its efficiency and outcomes (Teixeira et al., 2013).

4.2.4 | Hyaluronic acid-mediated sperm selection

The hyaluronic acid that surrounds the human oocyte acts as a natural mechanism of sperm selection (Parmegiani et al., 2010; Zhuo & Kimata, 2001). Mature spermatozoa express hyaluronic acid-binding glycoproteins in the plasma membrane and in the

acrosome membrane (Huszar et al., 2003; Ranganathan, Ganguly, & Datta, 1994), which are responsible to bind and digest hyaluronic acid in order to fertilise the oocyte. The binding to hyaluronic acid is reported to mediate some intracellular signalling pathways, leading to increased motility (Bains, Miles, Carson, & Adeghe, 2001). The expression of hyaluronic acid receptors can be used as a biomarker to select and isolate mature spermatozoa for ART (Parmegiani et al., 2010; Simon, Shamsi, et al., 2016). There are two techniques based on hyaluronic acid-mediated sperm selection, namely the physiological intracytoplasmic sperm injection (PICS) and the hyaluronan (or hyaluronic acid)-rich medium. The PICS technique is performed using a PICS dish, which is similar to a petri dish with coated spots of hyaluronic acid in the base. Washed sperm sample is subsequently placed on this dish, and after a period of incubation, the hyaluronic acid-bounded spermatozoa are selected for ICSI. The alternative technique uses a viscous solution of hyaluronan-rich medium, commercially available as SpermSlow™ (CooperSurgical), that can be added to the conventional ICSI dish. In brief, a droplet of a washed sperm sample is placed near a droplet of SpermSlow™ so that there is a contact zone between the drops. The sperm cells in the interface of both droplets, which are bounded to hyaluronic acid, are selected for ICSI (Parmegiani et al., 2010).

Although some advantages had been reported on the literature, the clinical efficiency of this method remains to be clarified. Interestingly, both PICS and the hyaluronan-rich medium are reported to have the same efficiency in selecting hyaluronic acid-bound spermatozoa (Parmegiani et al., 2012), retrieving spermatozoa with less DNA fragmentation and reduced aneuploidy rate (Mongkolchaipak & Vutyavanich, 2013; Parmegiani et al., 2010). It was also reported that hyaluronic acid-bound spermatozoa exhibit reduced frequencies of chromosomal disomies and reduced diploidy (Cayli et al., 2003; Jakab et al., 2005). There is evidence for an improved embryo quality and implantation (Parmegiani et al., 2010), higher fertilisation and clinical pregnancy rate with the conjugation of hyaluronic acid-mediated sperm selection and ICSI (Erberelli, Salgado, Pereira, & Wolff, 2017; Mongkolchaipak & Vutyavanich, 2013). On the other hand, there are contradictory data. For instance, Razavi et al. reported no difference in DNA fragmentation levels between these methods and conventional sperm selection procedures (Razavi, Nasr-Esfahani, Deemeh, Shayesteh, & Tavalae, 2010). In a systematic review, McDowell et al. concluded that, although no adverse effects were observed, there is no sufficient evidence to validate that spermatozoa selected by hyaluronic acid improve ART outcomes (McDowell et al., 2014). Further studies are required to evaluate whether hyaluronic acid-based methods can be recommended for use in clinical practice.

4.2.5 | Magnetic-activated cell sorting

Magnetic-activated cell sorting (MACS) is an advanced method for sperm selection that uses Annexin-V-coated magnetic beads in order

to separate apoptotic from nonapoptotic spermatozoa. Annexin V is a phospholipid-binding protein with high affinity for phosphatidylserine. Phosphatidylserine is a negatively charged phospholipid that is present in the inner layer of the plasma membrane of both somatic and sperm cells (Glander & Schaller, 1999; Simon, Shamsi, et al., 2016), which is externalised to the outer layer of the cell during the early phases of apoptosis (Vermes, Haanen, Steffens-Nakken, & Reutelingsperger, 1995). Thus, phosphatidylserine is used as a biomarker for apoptotic sperm cells. In MACS, a washed sample is incubated with Annexin-V-coated magnetic beads which will bind to the externalised phosphatidylserine of apoptotic sperm cells. This suspension is placed inside a MACS column that produces an external magnetic field, retaining the apoptotic sperm cells that are bound to the Annexin-V-coated magnetic beads while the nonapoptotic sperm pass through and is collected (Grunewald, Paasch, & Glander, 2001; Said & Land, 2011). At the end of the MACS protocol, two distinct fractions are obtained: Annexin-negative (nonapoptotic spermatozoa with intact membrane) and Annexin-positive (apoptotic spermatozoa with externalised phosphatidylserine; Glander & Schaller, 1999; Grunewald et al., 2001). As there are other components in semen that may decrease the efficiency of this technique, the association of this method with a standard sperm wash technique, such as density gradient centrifugation, is recommended (Simon, Shamsi, et al., 2016). As an alternative to the magnetic beads, glass wool can be coated with Annexin V. This technique, commonly referred as Annexin-V glass wool or molecular glass wool, further improves the filtration process as apoptotic spermatozoa are bounded to the Annexin V. This technique was successfully used on both fresh and cryopreserved semen samples, although more studies are needed in order to validate its efficiency for clinical practice (Grunewald et al., 2007).

As the correlation between DNA damage and apoptosis with the consequent externalisation of phosphatidylserine is well established (Muratori et al., 2003), it is no surprise that several studies reported that the spermatozoa retrieved with MACS have reduced levels of DNA fragmentation (Berteli, Da Broi, Martins, Ferriani, & Navarro, 2017; Lee et al., 2010; Rawe et al., 2009; Said et al., 2006; Zhang et al., 2018). In addition, the retrieved spermatozoa exhibit higher motility (Romany et al., 2014; Said et al., 2005, 2006) and higher percentage of spermatozoa with normal morphology (Berteli et al., 2017; Zhang et al., 2018). Although with MACS protocol it is possible to obtain better spermatozoa, the published data concerning clinical outcomes in ART are still inconclusive. Romany et al. demonstrated, in a large-sample prospective randomised trial, that fertilisation and implantation rates were not improved by MACS (Romany et al., 2014). Moreover, Gil et al. conducted a systematic review and concluded that MACS did not provide a significant improvement in the implantation and miscarriage rates compared with conventional sperm selection, although a trend for higher pregnancy rates was reported (Gil, Sar-Shalom, Melendez Sivira, Carreras, & Checa, 2013). Some studies showed a higher pregnancy and implantation rate with the use of MACS in patients with infertility attributed to the male factor, but no improvement in fertilisation rate

was observed (Dirican et al., 2008; Ziarati, Tavalaei, Bahadorani, & Nasr Esfahani, 2019). Although some advantages in sperm parameters have been reported, it is still inconclusive whether MACS procedure is valuable for clinical practice and in which patients should be applied.

4.2.6 | Microfluidic separation of spermatozoa

Microfluidics is a novel technology based on physical and chemical principles of fluid behaviour in a controlled environment at a sub-microlitre level. This technology may be used in several scientific areas, from genetics to ART. Significant advances have been made in order to evaluate the potential utility of microfluidics for sperm selection, where it aims to mimic the selection process that occurs in *in vivo* fertilisation. Currently, the majority of these devices are mainly for research purposes and present low applicability for clinical practice (Huang, Huang, & Yao, 2015; Simon, Shamsi, et al., 2016; Smith & Takayama, 2017). Several microfluidic devices have been tested for sperm selection based on characteristics such as morphology, motility, the ability to swim up a chemical gradient (chemotaxis), thermal gradient (thermotaxis) or on their response to fluid flow variations (rheotaxis; Marzano et al., 2020; Oseguera-Lopez, Ruiz-Diaz, Ramos-Ibeas, & Perez-Cerezales, 2019; Samuel et al., 2018; Smith & Takayama, 2017). The first reports that describe the potential use of microfluidics for human sperm selection for ART occurred in 2003, and the idealised device relied on the progressive motility of spermatozoa. This method was based on the capacity of motile spermatozoa to pass through streamlines in a laminar fluid stream. A self-contained integrated microfluidic device that was able to isolate motile sperm cells from nonmotile and other cells present on the semen sample was developed and reached commercialisation after some promising results (Cho et al., 2003; Schuster, Cho, Keller, Takayama, & Smith, 2003).

As the microfluidic systems are still in a developing phase, limited data concerning the advantages, disadvantages or ART outcomes are available in the literature. One major advantage is the fact that microfluidic systems are a one-step technique that eliminates the damage that arises from the centrifugation steps of conventional techniques. This method is considered advantageous for samples that are difficult to handle, such as cases of severe oligoasthenozoospermia, although it is unable to identify nonmotile but viable sperm cells for ICSI (Rappa et al., 2016; Simon, Murphy, et al., 2016). Some studies reported that some microfluidic systems selected sperm cells with a higher percentage of motility and lower DNA fragmentation when compared to conventional sperm methods or unprocessed spermatozoa (Kishi et al., 2015; Nosrati et al., 2014; Quinn et al., 2018; Shirota et al., 2016). Two studies have analysed the ART outcomes following the microfluidic technology. When Yildiz and Yuksel compared the fertilisation and pregnancy rates following sperm selection through density gradient centrifugation or microfluidics chip systems in couples with unexplained infertility and recurrent unsuccessful IVF treatments, some interesting results

were obtained (Yildiz & Yuksel, 2019). Although no difference was observed in the fertilisation and pregnancy rates in patients with unexplained infertility, the use of a microfluidic system significantly improved the fertilisation rate in couples who received at least two IVF treatments before the study, leading the authors to conclude that this method may be beneficial for patients with recurrent unsuccessful ART treatments. Yetkinel et al. demonstrated that the use of microfluidic systems improved the quality of embryos but did not alter the fertilisation, clinical pregnancy or live birth rates when compared to conventional swim-up in couples with unexplained infertility (Yetkinel et al., 2019). Further research is required to evaluate the potential of microfluidic systems and its clinical application in ART units.

4.3 | Alternative advanced methods

4.3.1 | Hypo-osmotic swelling test

The hypo-osmotic swelling test (HOST) was initially developed as a diagnostic sperm test to assess the functional integrity of the sperm plasma membrane, which is essential for fertilisation. In hypo-osmotic conditions, the tail of sperm cells with a normal functional membrane swell or curl due to water influx, which could be used as an indicative of sperm potential (Jeyendran, Van der Ven, Perez-Pelaez, Crabo, & Zaneveld, 1984). The HOST has already been established as a diagnostic test and is currently used as a reliable indicator of the sperm quality in clinical practice (Jeyendran et al., 2019). However, the possibility that the HOST could be applied as a sperm selection method for the ICSI procedure was first introduced in 1994. In ART laboratories, a rescue ICSI is used for oocytes that failed fertilisation upon a conventional IVF. Desmet et al. used the HOST to identify viable immotile spermatozoa in a rescue ICSI protocol and reported a 30% fertilisation rate (Desmet et al., 1994). In this method, sperm cells are incubated at 37 °C in a culture dish in a hypo-osmotic solution. Then, HOST-positive spermatozoa (curled tails) are selected and washed prior to conventional ICSI (Casper, Meriano, Jarvi, Cowan, & Lucato, 1996). Since then, some studies focused on the composition of the hypo-osmotic solution in order to establish a standard procedure (Tsai et al., 1997; Verheyen et al., 1997).

It is possible to obtain clinical pregnancies from viable but immotile spermatozoa with the HOST, which confirms its practical applicability (Barros, Sousa, Angelopoulos, & Tesarik, 1997; Casper et al., 1996; El-Nour, Al Mayman, Jaroudi, & Coskun, 2001; Sallam et al., 2001; Ved et al., 1997). It was also observed a tendency, with no statistical significance, for higher fertilisation and implantation rates and more quality embryos in spermatozoa selected with HOST before ICSI in cases of total absence of sperm motility (Casper et al., 1996; El-Nour et al., 2001; Liu et al., 1997; Sallam, Farrag, Agameya, El-Garem, & Ezzeldin, 2005). Despite being an easy and inexpensive method with positive results reported, novel methods as PICSI or laser-assisted immotile sperm selection (LAISS) render HOST as more useful for standard semen analyses than as a sperm selection method.

4.3.2 | Laser-assisted immotile sperm selection

In 2000, Montag et al. tested the use of laser to immobilise sperm cells before ICSI and observed that motile spermatozoa curled the tail upon exposition to the laser shot (Montag, Rink, Delacrétaiz, & van der Ven, 2000). The same group performed this technique in order to select viable but immotile sperm cells from ejaculates and testicular biopsies, introducing the LAISS method. They reported a higher fertilisation and cleavage rates and a trend for a higher take-home-baby rate following LAISS, when compared to morphological criteria alone (Aktan et al., 2004). Although the mechanism responsible for the curling are unknown, the authors hypothesise that it must be related to the influx of medium into the sperm tail following the laser shot (Aktan et al., 2004). Ebner et al. compared LAISS with conventional methods and did not find any difference concerning fertilisation and cleavage rate or blastocyst formation. However, it was highlighted that LAISS may be a potential alternative to conventional mechanical approach for spermatozoa immobilisation as it is less time consuming (Ebner et al., 2001). The achievement of pregnancy following LAISS in immotile frozen-thawed spermatozoa has been described, which validates the use of this method for cryopreserved samples (Chen et al., 2017). This fact became highly advantageous as testicular biopsies are, generally, cryopreserved before the IVF treatment. Although rather expensive, compared to HOST, LAISS is considered as a nonhazardous and safe method for the identification of viable spermatozoa from immotile samples (Nordhoff et al., 2013). More studies are required to clarify the potential of LAISS as a routine procedure for sperm immobilisation before ICSI.

4.3.3 | Polarisation microscopy

The polarisation microscopy for sperm selection uses anisotropic properties of sperm cells to assess its birefringent patterns, which are based on the fact that the nucleus of mature spermatozoa has an intrinsic birefringence (double refraction) associated with longitudinally oriented nucleoprotein filaments (Gianaroli et al., 2008). The birefringence of the sperm head can be used as an indicator of the normal structural organisation of its nucleus. In addition, the birefringence can also assess the state of the acrosome reaction, due to the fact that mature sperm cells exhibit birefringence over the entire head, while reacted spermatozoa exhibit birefringence in the post-acrosomal region (Baccetti, 2004). The sperm head birefringence is correlated with some sperm parameters. Magli et al. described the correlation between sperm motility and normal morphology with the birefringence patterns, reporting that sperm cells with morphological abnormalities or with conditioned motility have an abnormal pattern when compared to motile and morphologically normal spermatozoa (Magli et al., 2012). In another study, Crippa et al. reported that the proportion of spermatozoa to head-birefringent spermatozoa was inversely correlated with the incidence of fragmented DNA (Crippa et al., 2009). Contrastingly, Petersen et al. reported that spermatozoa with total birefringence had more DNA fragmentation compared

with those with partial birefringence (Petersen et al., 2011), rendering the association of sperm head birefringence with DNA damage questionable. In 2008, the assessment of sperm birefringence was successfully used by Gianaroli et al. to select spermatozoa for ICSI. When compared with the conventional ICSI procedure based on morphological sperm selection, the spermatozoa selected by polarised microscopy led to a better embryo development and clinical outcome (Gianaroli et al., 2008). Two years later, the same group evaluated the birefringence patterns on the basis of the acrosome integrity (acrosome-reacted and total acrosome-nonreacted spermatozoa) and its relation to ART outcomes. The study concluded that there was no effect on the fertilising capacity and embryo development of either type of spermatozoa, although implantation rate and clinical pregnancy were higher in oocytes injected with reacted spermatozoa (Gianaroli et al., 2010). Despite these successful results, further studies are needed to validate and standardise the method. Similar to IMSI, the assessment of birefringence patterns of sperm head is based on morphology examination, requiring a skilled embryologist to correctly perform this technique.

5 | CASE-SPECIFIC RECOMMENDATIONS FOR THE PREPARATION OF SEMEN SAMPLES

5.1 | Preparation of epididymal and testicular sperm samples

The most common indication for epididymal aspiration is obstructive azoospermia, and in general, this type of samples provides an adequate number of sperm cells. Depending on the quality of the sample, a simple sperm wash or a density gradient centrifugation can be applied (WHO, 2010). In cases of nonobstructive azoospermia, spermatozoa must be retrieved through a testicular biopsy. In these cases, the testicular spermatozoa can be retrieved either by open biopsy or by percutaneous needle biopsy (WHO, 2010). In cases of testicular biopsy, the testicular tissue is immediately placed in culture media following the excision. Then, the tissue is shredded and centrifuged with culture media. The supernatant is removed, and the pellet is resuspended in fresh culture media (Popal & Nagy, 2013). Due to the abundance of red blood cells in testicular biopsy samples, the search for sperm cells for ICSI can be challenging. In these cases, a protocol for the lysis of red blood cells might be recommended. Nagy et al. described a protocol that allows the lysis of red blood cells without damaging the sperm cells or affecting the ART outcome (Nagy, Verheyen, Tournaye, Devroey, & Van Steirteghem, 1997). In cases of severe testicular failure with very low amounts of testicular spermatozoa, sperm cell collection remains difficult and unsuccessful even after red blood cells lysis. For these cases, Crabbé et al. reported the additional use of an enzymatic treatment with collagenase type IV and DNase as a safe procedure, with an acceptable fertilisation rate (Crabbe et al., 1998). The procedures described above

for testicular or epididymal sperm preparation are routinely performed in ART laboratories and are validated and described in detailed in the WHO guidelines for semen sample preparation methods (WHO, 2010).

5.2 | Preparation of retrograde ejaculation samples

In cases of retrograde ejaculation, semen enters in the bladder upon ejaculation which results in aspermia or no apparent ejaculate. Post-ejaculatory urine sample should be examined for the presence of sperm cells to confirm this diagnosis. Sperm cells may be obtained from the post-ejaculatory urine sample. For this purpose, the alkalisation of the urine by ingestion of sodium bicarbonate is recommended, as it will improve the motility of sperm cells found in the urine (Mahadevan, Leeton, & Trounson, 1981). According to the WHO guidelines, both the ejaculate, if present, and urine samples should be analysed. Both can be successfully processed using the density gradient centrifugation method (WHO, 2010).

5.3 | Preparation of samples with immotile spermatozoa

In cases of samples with no motile sperm cells, the use of pentoxifylline might be recommended. Pentoxifylline is a phosphodiesterase inhibitor that enhances sperm motility by increasing intracellular cAMP (Terriou et al., 2000). Despite the potential embryotoxicity of this compound, pentoxifylline has been used in sample preparation for ART (Tournaye, Van der Linden, Van den Abbeel, Devroey, & Van Steirteghem, 1993). Some studies suggest that the use of pentoxifylline is beneficial for asthenozoospermic patients (Sikka and Hellstrom, 1991; Yovich, Edirisinghe, Cummins, & Yovich, 1988), and its use was also reported to improve the fertilisation and pregnancy rates using immotile epididymal and testicular sperm cells (Terriou et al., 2000). The pentoxifylline protocol can vary between laboratories. Pentoxifylline can be added to fresh sperm samples or washed spermatozoa, and it is necessary incubate the spermatozoa at a temperature of 37°C (Tesarik, Mendoza, & Carreras, 1992; Tesarik, Thébault, & Testart, 1992).

5.4 | Preparation of HIV-infected semen samples

A combination of density gradient centrifugation followed by swim-up is recommended for samples of human immunodeficiency virus (HIV)-infected men, as a way of preventing infection of uninfected female partners (Gilling-Smith, Emiliani, Almeida, Liesnard, & Englert, 2005; Savasi et al., 2007). In those cases, HIV is present in the semen, as viral RNA and proviral RNA are found free in seminal plasma and in nonsperm cells. Since the first study describing the use of semen washing in cases of HIV-discordant couples (Semprini et al., 1992), several studies have evaluated the effect of this method

in conjunction with ART. Zafer et al. concluded in a systematic review and meta-analysis that in HIV-discordant couples semen washing successfully prevents HIV transmission (Zafer et al., 2016). The use of density gradient centrifugation permits the isolation of sperm cells, potentially eliminating the viral RNA found in the seminal plasma and nonsperm cells. After liquefaction, the sperm sample is diluted 1:1 (v/v) with culture medium and centrifuged in order to eliminate the seminal plasma and reduce the potential risk of contamination. The pellet is resuspended in culture media and layered on top of a triple density gradient (90%-70%-45%). After centrifugation for 20 min at 300 g, the supernatant is discarded, and the pellet resuspended in culture media. Swim-up might be used in order to isolate motile sperm cells. The retrieved sperm cells are divided in two parts: one is used for post-wash HIV testing, and the other is cryopreserved. Post-wash HIV samples are tested by reverse transcription polymerase chain reaction (RT-PCR) to verify whether the protocol was effective and ensure that the samples to be used in ART are HIV-free (Gilling-Smith, Nicopoulos, Semprini, & Frodsham, 2006; WHO, 2010). Once the PCR results are available, the cryopreserved sample can be used for ART treatment or discarded in case of positive results (Zamora, Obradors, Woodward, Vernaee, & Vassena, 2016).

6 | CONCLUSIONS AND FUTURE PERSPECTIVES

Spermatozoa are highly complex cells influenced by several internal and external stimuli, which may compromise its fertilising potential. Considering the disclosure of several new biomarkers for sperm quality and its fertility potential (Bernardino et al., 2019; Carrageta et al., 2020; Moreira, Monteiro, Sousa, Oliveira, & Alves, 2018; Moreira, Oliveira, & Alves, 2019), the established standard semen quality parameters are rendered as limited. Few parameters are evaluated, which might explain the high percentage of cases classified as idiopathic. For instance, several metabolic diseases, such as diabetes mellitus or obesity, affect the male reproductive potential at the molecular level (Crisostomo, Alves, Calamita, Sousa, & Oliveira, 2017; Crisostomo et al., 2019). Based on novel data and a subsequent deeper understanding of the human sperm physiology, innovative advanced sperm selection methods that go beyond the conventional parameters and aim to address more complex and specific cases have been developed. Although the use of some of these advanced sperm selection methods has proven to be able to improve ART outcomes, the majority still are in an embryonic state and require further studies to validate their efficiency in clinical practice. It is also important to highlight that there is a lack of standardisation regarding protocols and laboratories, which makes it difficult to compare studies and their results.

More recently, epigenetics and ROS signalling became a hot topic for human reproduction academics. There are some evidence that metabolic diseases lead to epigenetic alterations on sperm cells, which may compromise the pregnancy or even pre-condition the

offspring for its development (Oliveira et al., 2017; Sales, Ferguson-Smith, & Patti, 2017). Further studies are needed to elucidate the influence of the epigenetic signature for the fertilisation process and pregnancy before novel advanced sperm selection methods are developed to address the issue.

In sum, ART have significantly improved for the past decades, leading to higher percentages of successful pregnancies. Although the conventional methods remain as the most common and efficient method for the majority of the cases, more advanced techniques have been developed in order to address more specific cases. No method, conventional or advanced, is the perfect solution. Depending on the nature of each case and based on previous experiences of other couples with the same infertility diagnose, the embryologist should select which is the best option to apply.

7 | TAKE-HOME MESSAGE

- The application of ART has revolutionised the treatment of human infertility, bringing hope to patients previously considered incapable for reproductive purposes.
- The swim-up procedure and density gradient centrifugation are the most popular and widely used conventional methods for sperm selection.
- Novel and more advanced methods are continuously being developed and improved, leading to better ART rates and outcomes.

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Article

Metabolomics Integration in Assisted Reproductive Technologies for Enhanced Embryo Selection beyond Morphokinetic Analysis

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Abstract: Embryo quality evaluation during in vitro development is a crucial factor for the success of assisted reproductive technologies (ARTs). However, the subjectivity inherent in the morphological evaluation by embryologists can introduce inconsistencies that impact the optimal embryo choice for transfer. To provide a more comprehensive evaluation of embryo quality, we undertook the integration of embryo metabolomics alongside standardized morphokinetic classification. The culture medium of 55 embryos (derived from 21 couples undergoing ICSI) was collected at two timepoints (days 3 and 5). Samples were split into Good (n = 29), Lagging (n = 19), and Bad (n = 10) according to embryo morphokinetic evaluation. Embryo metabolic performance was assessed by monitoring the variation in specific metabolites (pyruvate, lactate, alanine, glutamine, acetate, formate) using ¹H-NMR. Adjusted metabolite differentials were observed during the first 3 days of culture and found to be discriminative of embryo quality at the end of day 5. Pyruvate, alanine, glutamine, and acetate were major contributors to this discrimination. Good and Lagging embryos were found to export and accumulate pyruvate and glutamine in the first 3 days of culture, while Bad embryos consumed them. This suggests that Bad embryos have less active metabolic activity than Good and Lagging embryos, and these two metabolites are putative biomarkers for embryo quality. This study provides a more comprehensive evaluation of embryo quality and can lead to improvements in ARTs by enabling the selection of the best embryos. By combining morphological assessment and metabolomics, the selection of high-quality embryos with the potential to result in successful pregnancies may become more accurate and consistent.

Keywords: embryo quality evaluation; morphokinetic assessment; embryo metabolomics; pyruvate; glutamine



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1. Introduction

In recent years, the incidence of infertility has been increasing, which can be attributed to various social, biological, and biochemical factors. The World Health Organization (WHO) defines infertility as the inability of a couple to achieve pregnancy after one year of sexual intercourse without contraception [1]. It is estimated that infertility affects between 4 and 14% of the population globally, making it a significant public health burden. Assisted reproductive technologies (ARTs) have emerged as a solution to this problem,

with the WHO advocating for increased accessibility to such treatments. However, success rates of ARTs are still below expectations, indicating a need for further investigation into the biochemical processes involved in ART techniques, particularly during embryonic development, and the identification of biomarkers associated with the outcome of ART programs [2]. One of the significant challenges of ARTs is the optimization of embryo selection. The existing approaches to gauging the potential for embryo implantation primarily hinge on morphological assessment. However, this method does not consistently provide accurate predictions of successful implantation [3].

Metabolomics, a quantitative and non-invasive technology, has recently emerged as a promising tool for measuring metabolites present in cells, tissues, body fluids, and culture media. By quantifying the metabolites secreted by gametes and embryos during culture, metabolomics can provide insights into embryonic viability and help to identify embryonic biomarkers that may improve the outcomes of ART treatments [4,5]. Several studies have already established correlations between embryonic viability and carbohydrate, pyruvate, and amino acid metabolism during embryonic development [5–10]. Indeed, the metabolic performance of embryos post-compaction has emerged as a noteworthy biomarker indicative of superior-quality blastocysts [9,11]. Despite these promising findings, the lack of consensus in this field largely stems from the absence of standardization in the molecular assessment of embryo quality [12]. Therefore, this study aims to integrate metabolomics with the standardized morphological classification of embryos to identify biomarkers that can optimize the selection of the best embryo for transfer. By combining metabolomic and standardized morphological embryo evaluations, this study can provide valuable insights into the complex metabolic processes underlying embryonic development and quality. Ultimately, the integration of metabolomics in ARTs can offer a powerful tool for improving treatment outcomes and identifying biomarkers associated with embryonic viability and quality. The identification of such biomarkers can greatly enhance the efficiency and effectiveness of ART, leading to better pregnancy rates and improved clinical outcomes for infertile couples.

2. Results

2.1. Clinical Data and Embryo Characterization

As shown in Table 1, 21 intracytoplasmic sperm injection (ICSI) cycles were included in this study. All the treatments were made in accordance with clinical indication, and fertilization was achieved with the ICSI technique. The embryo culture was extended until day 5 (D5). The cycle characteristics are presented in Table 1. In 7 cycles, the treatment involved Preimplantation Genetic Testing (PGT) without fresh embryo transfer. In this type of procedure, the embryos that develop into blastocysts with good quality are biopsied and then cryopreserved for deferred transfer after genetic results. Of the remaining 14 cycles, 5 were performed with oocyte donation and in 9 cycles, the ICSI protocol has no additional technique. Apart from the PGT cycles, a fresh embryo transfer had to be called off during a particular treatment due to elevated progesterone serum levels. Consequently, in this scenario, embryos of good quality were preserved through vitrification for a subsequent transfer. Across all 13 remaining cycles, the fresh embryo transfers were successfully carried out, aligning with the precise number of embryos transferred in accordance with the established guidelines of the Centro de Genética Prof. Alberto Barros (CGR) clinic. The surplus good-quality embryos were vitrified for later use. A total of 110 culture medium samples from 55 oocytes were collected during the study period, 55 samples of culture medium from embryos on day 3 (D3) and 55 samples of culture medium from embryos on D5. Degenerated embryos, non-cleavage embryos, abnormal fertilization, and parthenogenetic embryos were excluded from statistical analyses.

Table 1. Characterization of the studied population and fertilization cycles.

Baseline Characteristics of Studied Population		Patient Cycle Characteristics	
Number of couples	21	Fertilization technique (%)	
Number of cycles	21	ICSI	100 (21/21)
Female age (years)	35.05 ± 6.59	Stimulation duration (days)	9.29 ± 1.38
Female body mass index (kg/m ²)	23.59 ± 3.49	FSH total dose (IU)	2827 ± 1245
Male age (years)	40.71 ± 5.49	Serum E2 on trigger day (pg/mL)	2481 ± 1486
Male body mass index (kg/m ²)	24.84 ± 5.82	Serum Pr on trigger day (ng/mL)	0.95 ± 0.46
Infertility duration (months)	49.86 ± 32.29	Oocytes retrieved (n)	8.48 ± 2.66
Previous IVF attempts (%)		Metaphase II oocytes (n)	7.43 ± 2.31
0	42.9 (9/21)	Maturation rate (%)	85.2 ± 14.4
1	23.8 (5/21)	Fertilized oocytes (n)	6.33 ± 2.46
2	19.0 (4/21)	Fertilization rate (%)	85.20 ± 14.38
≥3	14.3 (3/21)	Embryo stage	
Types of infertility (%)		Day 3 embryos (n)	6.14 ± 2.5
Primary infertility	66.7 (14/21)	Day 5 embryos (n)	4.95 ± 2.41
Secondary infertility	33.3 (7/21)	Cleavage rate (%)	96.7 ± 7.1
Anti-Müllerian hormone (ng/mL)	4.02 ± 4.34	Embryo utilization rate (%)	53.6 ± 21.5
Total number of analyzed embryos	55	Embryo transfers (%)	92.9 (13/14)
Good	26	1 embryo transferred	69.2 (9/13)
Lagging	19	2 embryos transferred	30.8 (4/13)
Bad	10	Freeze all cycles	7.1 (1/14)
		Biochemical pregnancy (%)	61.5 (8/13)
		Clinical pregnancy (%)	61.5 (8/13)

Data are shown as the mean ± standard deviation or %. Legend: E2, estradiol; FSH, follicle-stimulating hormone; ICSI, intracytoplasmic sperm injection; Pr, progesterone.

The embryos were classified as described below. Based on morphological assessment on D5, a blastocyst of good quality was considered as BL3BB or better (BL5—AA, AB, BA, BB; BL4—AA, AB, BA, BB; BL3—AA, AB, BA, BB), and a Bad embryo was considered if it had inner cell mass or/and trophectoderm grade C (BL5/BL4/BL3—AC, CA, CC). An embryo was classified as Lagging if the embryo development was delayed considering the standardized timing of embryo observations established in the Istanbul Consensus [13]. From the 55 observed embryos, 26 were classified as Good, 19 as Lagging, and 10 as Bad embryos.

2.2. Embryo Quality at Day 5 Correlates with Metabolite Consumption or Release

Metabolite consumption and release by the embryos were assessed by ¹H-NMR analysis of the embryo culture media on D3 (Figure 1 and Table 2) and on D5 (Figure 2 and Table 2). Alanine (Figures 1A and 2A), acetate (Figures 1B and 2B), pyruvate (Figures 1C and 2C), glutamine (Figures 1D and 2D), lactate (Figures 1E and 2E), and formate (Figures 1F and 2F) were the most relevant metabolites identified in the culture media of both D3 and D5. Embryos that were categorized into Good, Lagging, or Bad according to their quality at D5 were evaluated for metabolite consumption or production using ¹H-Nuclear Magnetic Resonance (NMR) spectroscopy, and differences were analyzed by one-way ANOVA, followed by Tukey's post hoc test.

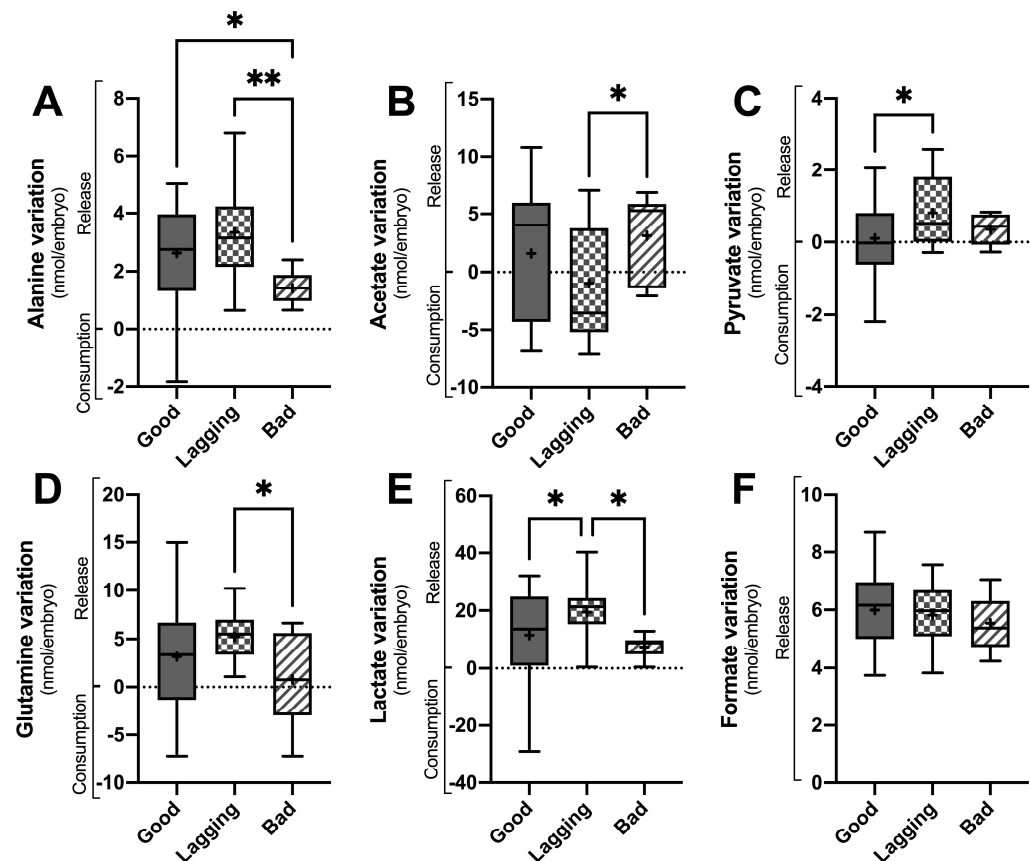


Figure 1. Metabolite variation in culture media of human embryos between day 3 (D3) and day 0 grouped according to their quality at day 5 (D5) (Good vs. Lagging vs. Bad). The figure shows the variation (Consumption or Release) in selected metabolites (Panel (A)-alanine; Panel (B)-acetate; Panel (C)-pyruvate; Panel (D)-glutamine; Panel (E)-lactate; Panel (F)-formate) quantified in the embryo culture media at the end of D3 (n = 55). Data are presented as mean $\pm$ standard deviation (S.D.). Normality and heteroscedasticity tests were performed, and univariate ANOVA was conducted on the selected metabolites identified in the embryo culture medium. Pairwise comparisons between groups were corrected for multiple hypotheses testing by Tukey's post hoc test. All *p* values < 0.05 were considered statistically significant (* *p* < 0.05; ** *p* < 0.005). + represents group average.

Table 2. Metabolite variation in embryo culture media. Metabolite variation observed on the first 3 days (D0–3) and from day 3 to day 5 (D3–5) in culture media of human embryos grouped according to their quality (Good, Lagging, and Bad).

Metabolite (nmol/Embryo)	Metabolite Variation in Embryo Culture Media					
	Day 0 to 3			Day 3 to 5		
	Good	Lagging	Bad	Good	Lagging	Bad
Alanine	2.63 $\pm$ 1.68	3.34 $\pm$ 1.65	1.42 $\pm$ 0.57 *,#	4.92 $\pm$ 2.83	5.05 $\pm$ 2.62	5.27 $\pm$ 2.70
Acetate	1.59 $\pm$ 5.23	−0.94 $\pm$ 4.98	3.11 $\pm$ 3.66 #	−1.43 $\pm$ 4.44	1.04 $\pm$ 5.78	−1.24 $\pm$ 4.16
Pyruvate	0.10 $\pm$ 1.23	0.81 $\pm$ 0.94 *	0.35 $\pm$ 0.42	0.23 $\pm$ 0.67	0.78 $\pm$ 0.38	0.33 $\pm$ 0.57
Glutamine	3.16 $\pm$ 4.94	5.24 $\pm$ 2.69	0.80 $\pm$ 4.66 #	3.10 $\pm$ 4.47	4.48 $\pm$ 5.65	4.90 $\pm$ 3.42
Lactate	11.55 $\pm$ 15.05	19.44 $\pm$ 9.05 *	7.49 $\pm$ 3.99 #	16.60 $\pm$ 13.27	19.03 $\pm$ 10.39	15.55 $\pm$ 10.99
Formate	6.00 $\pm$ 1.18	5.82 $\pm$ 1.06	5.54 $\pm$ 0.94	5.85 $\pm$ 1.51	6.33 $\pm$ 1.21	6.43 $\pm$ 1.23

Data are shown as mean $\pm$ SD. Positive values represent release to the extracellular medium, while negative values mean consumption by the embryo. All *p* values < 0.05 were considered statistically significant (* vs. Good embryos, # vs. Lagging embryos).

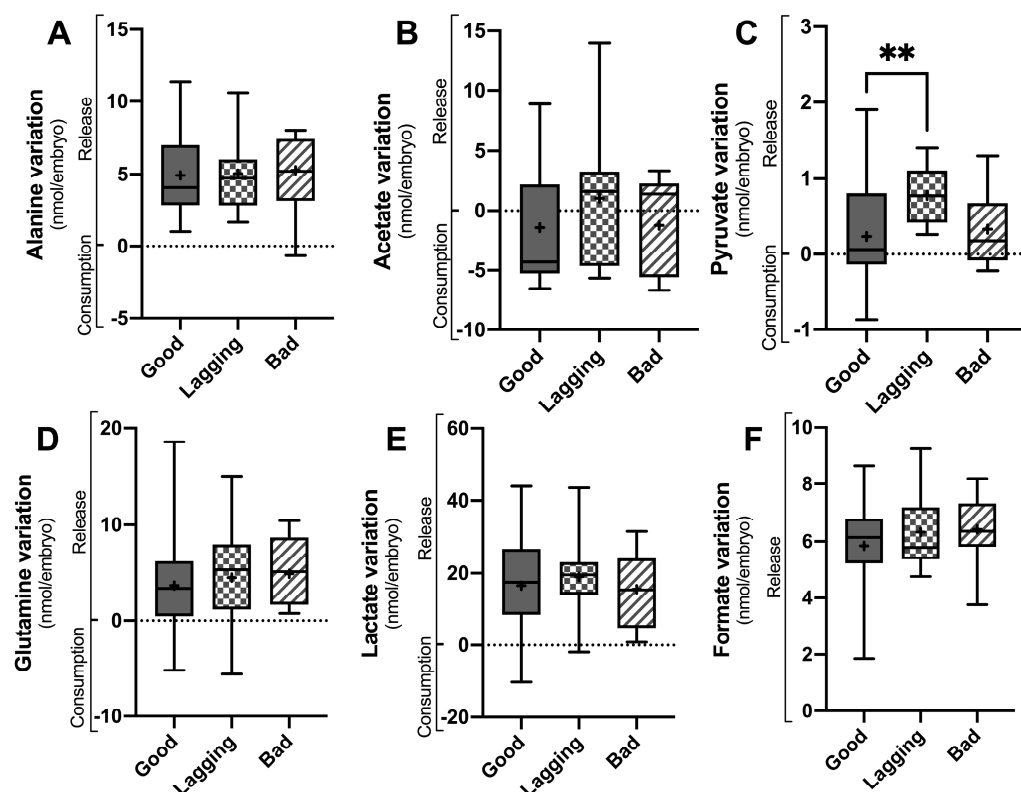


Figure 2. Metabolite variation in culture media of human embryos between day 3 (D3) and day 5 (D5) grouped according to their quality at D5 (Good vs. Lagging vs. Bad). The figure shows the variation (Consumption or Release) in selected metabolites (Panel (A)-alanine; Panel (B)-acetate; Panel (C)-pyruvate; Panel (D)-glutamine; Panel (E)-lactate; Panel (F)-formate) quantified in the embryo culture media at the end of D5 ($n = 55$). Data are presented as mean $\pm$ standard deviation (S.D.). Normality and heteroscedasticity tests were performed, and univariate ANOVA was conducted on the selected metabolites identified in the embryo culture medium. Pairwise comparisons between groups were corrected for multiple hypotheses testing by Tukey's post hoc test. All p values < 0.05 were considered statistically significant (** $p < 0.005$). + represents group average.

On D3 of embryo culture, there were significant differences in the levels of alanine detected in the culture media. Specifically, both Good and Lagging embryos showed a higher release of alanine (2.63 ± 1.68 nmol/embryo and 3.34 ± 1.65 nmol/embryo, respectively) compared to Bad embryos (1.42 ± 0.57 nmol/embryo). Similarly, glutamine release was significantly higher in Lagging embryos (5.24 ± 2.69 nmol/embryo) than in Bad embryos (0.80 ± 4.66 nmol/embryo). In Figure 1D, it can be observed that the release of glutamine to the culture media by Good embryos was intermediate, with a mean value of 3.16 ± 4.94 nmol/embryo, compared to the values observed for Bad and Lagging embryos.

Lagging embryos consumed acetate (-0.94 ± 4.98 nmol/embryo), contrarily to Good (1.59 ± 5.23 nmol/embryo) and Bad (3.11 ± 3.66 nmol/embryo) embryos, which released it to the media (Figure 1B). Pyruvate (Figure 1C) and lactate (Figure 1E) release was higher in Lagging (0.66 ± 0.99 and 19.44 ± 9.05 nmol/embryo) embryos than in Good (0.10 ± 1.23 and 11.55 ± 15.05 nmol/embryo) and Bad (0.35 ± 0.42 and 7.49 ± 3.99 nmol/embryo) embryos. No differences were observed regarding formate release between Good (6.00 ± 1.18 nmol/embryo), Lagging (5.82 ± 1.06 nmol/embryo), and Bad (5.54 ± 0.94 nmol/embryo) embryos (Figure 1F).

Concerning D5 variation in metabolites (Figure 2), only pyruvate levels were significantly different between groups. Good embryos (0.23 ± 0.67 nmol/embryo, $p = 0.0112$) released significantly less pyruvate than Lagging embryos (0.78 ± 0.38 nmol/embryo), whereas Bad (0.33 ± 0.57 nmol/embryo) embryos' pyruvate release was intermediate (Figure 2C). No differences were found concerning alanine (4.92 ± 2.83 ; 5.05 ± 2.62 and 5.27 ± 2.70 nmol/embryo)

(Figure 2A), glutamine (3.10 ± 4.47 ; 4.48 ± 5.65 and 4.90 ± 3.42 nmol/embryo) (Figure 2D), lactate (16.60 ± 13.27 ; 19.03 ± 10.39 and 15.55 ± 10.99 nmol/embryo) (Figure 2E), and formate (5.85 ± 1.51 ; 6.33 ± 1.21 and 6.43 ± 1.23 nmol/embryo) release (Figure 2F) levels between Good, Lagging, and Bad embryos, respectively. Although no statistical difference was observed, Good (-1.43 ± 4.44 nmol/embryo) and Bad (-1.24 ± 4.16 nmol/embryo) embryos tended to consume acetate, whereas Lagging embryos (1.04 ± 5.78 nmol/embryo) tended to release it to the media (Figure 2B).

2.3. Metabolite Variation during the First 3 Days of Development Is a Strong Predictor of Embryo Quality at Day 5

Metabolite consumption or release by embryos was assessed in embryo culture medium on the first 3 days (D0–3) and from D3 to D5 (D3–5). Metabolite variations were compared between groups of embryos categorized according to their quality at D5 using MANCOVA, after adjusting for covariates. One of the assumptions to conduct MANCOVA was not met when analyzing the D0–3 period: The homogeneity of covariance matrices across groups is not assumed (Box's $M = 120.66$, $F(56, 2017.579) = 1.584$, $p = 0.004$), but it is assumed in the D3–5 interval. Also, the homogeneity of variance of all dependent variables was confirmed with Levene's test in both intervals. Linearity and multicollinearity were graphically assessed and were considered acceptable in both intervals. Therefore, we considered that the MANCOVA model was sufficiently robust.

In addition to the mathematical evaluation of the methodology, we have conducted a thorough deliberation while selecting the covariates to be integrated into the model. Our choices have been driven by their intrinsic physiological significance and relevance. Recent studies have established a direct proportion between the age of the donor of the female gamete and embryo oxygen consumption rate (OCR) during the preimplantation phase [14–16]. Although OCR and maximum OCR are crucial and measurable markers of early embryo metabolism and development, other factors such as DNA integrity, antioxidant pool, and pre-collection hormonal levels further contribute to embryo development [17–19]. The same variables act on the male gamete and contribute to the fate of the embryo during early development [20,21]. Age and body mass index (BMI) have been reported to be correlated with metabolic, antioxidant, and DNA integrity in both female and male gametes, with direct influence over early embryo development [18–23]. Therefore, we selected the broader and easily measurable “age” and “BMI” of both male and female as covariates in our MANCOVA model.

In the D0–3 interval, metabolite differentials are discriminative of embryo quality category at D5 (Roy's Largest Root = 0.644, $F(7, 41) = 3.77$, $p = 0.003$, partial $\eta^2 = 0.392$), after controlling for covariates. The discriminant power of the model is supported by the differences in alanine ($F(2, 46) = 6.428$; $p = 0.003$), acetate ($F(2, 46) = 4.940$; $p = 0.011$), pyruvate ($F(2, 46) = 3.443$; $p = 0.040$), and glutamine ($F(2, 46) = 3.448$; $p = 0.040$). Pairwise comparisons were performed to identify which groups had different metabolite yields after incubation, corrected by Šidak's method (Figure 3A).

After adjusting for confounding factors, alanine release during D0–3 was lower in Bad embryos (1.11 ± 1.48 nmol/embryo) than in Lagging embryos (3.25 ± 1.44 nmol/embryo, $p = 0.003$) and Good embryos (2.78 ± 1.45 nmol/embryo, $p = 0.018$). The remaining observed differences—acetate, pyruvate, and glutamine—were found comparing D0–3 differentials between Lagging and Bad embryos (Figure 3A and Table 3). Interestingly, Lagging embryos display a consumption of acetate contrary to the other groups, which released it (Figure 3A). Bad embryos consumed pyruvate and glutamine, energetic substrates, contrary to the other groups. However, pyruvate differentials vary within limited ranges, suggesting that this metabolite is readily consumed and produced. In the D3–5 interval, metabolite differentials are not discriminative of embryo quality category at D5 (Roy's Largest Root = 0.180, $F(7, 41) = 1.054$, $p = 0.410$, partial $\eta^2 = 0.153$), after controlling for covariates (Figure 3B). Detailed estimates after accounting for covariates are provided in Table 3.

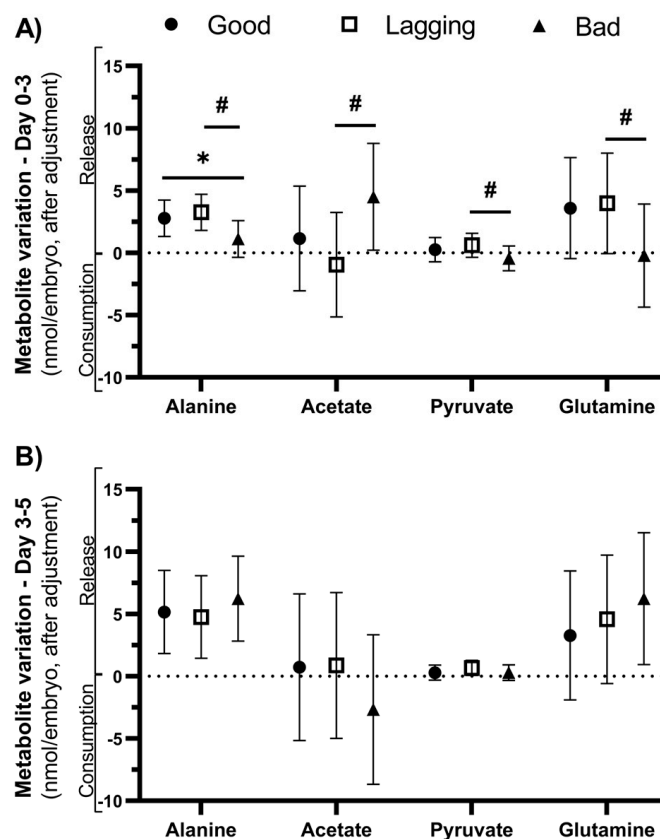


Figure 3. Metabolite variation observed on the first 3 days (D0–3) and from day 3 to day 5 (D3–5) in culture media of human embryos grouped according to their quality (Good vs. Lagging vs. Bad). The figure shows the metabolite differentials of relevant metabolites (alanine, acetate, pyruvate, and glutamine) observed on the first 3 days (Panel (A)) and from day 3 to day 5 (Panel (B)) ($n = 55$). Data are presented as mean $\pm$ standard deviation (S.D.). The impact of each metabolite differential on embryo quality at day 5 was assessed using a univariate F-test, after adjusting for the covariates. Pairwise comparisons between groups were corrected for multiple hypotheses testing by Šidak's method. All p values < 0.05 were considered statistically significant (* vs. Good embryos; # vs. Lagging embryos).

Table 3. Adjusted metabolite variation in embryo culture media. Metabolite variation observed on the first 3 days (D0–3) and from day 3 to day 5 (D3–5) in culture media of human embryos grouped according to their quality (Good, Lagging, and Bad).

Metabolite (nmol/Embryo)	Adjusted Metabolite Variation in Embryo Culture Media ^a					
	Day 0 to 3			Day 3 to 5		
	Good	Lagging	Bad	Good	Lagging	Bad
Alanine	2.78 $\pm$ 1.45	3.25 $\pm$ 1.44	1.11 $\pm$ 1.48 *,#	5.15 $\pm$ 3.33	4.75 $\pm$ 3.32	6.22 $\pm$ 3.40
Acetate	1.15 $\pm$ 4.20	−0.95 $\pm$ 4.19	4.50 $\pm$ 4.29 #	0.72 $\pm$ 5.88	0.86 $\pm$ 5.86	−2.67 $\pm$ 6.00
Pyruvate	0.25 $\pm$ 0.97	0.61 $\pm$ 0.97	−0.45 $\pm$ 0.99 #	0.28 $\pm$ 0.61	0.66 $\pm$ 0.61	0.29 $\pm$ 0.62
Glutamine	3.59 $\pm$ 4.05	3.98 $\pm$ 4.03	−0.22 $\pm$ 4.14 #	3.27 $\pm$ 5.18	4.57 $\pm$ 5.15	6.22 $\pm$ 5.28
Lactate	13.07 $\pm$ 12.13	14.69 $\pm$ 12.08 *	4.09 $\pm$ 12.38 #	18.26 $\pm$ 17.04	15.00 $\pm$ 16.96	17.21 $\pm$ 17.38
Formate	5.10 $\pm$ 2.36	4.81 $\pm$ 2.34	5.39 $\pm$ 2.41	5.37 $\pm$ 2.54	5.26 $\pm$ 2.53	5.90 $\pm$ 2.59

Data are shown as mean $\pm$ SD. Positive values represent release to the extracellular medium, while negative values mean consumption by the embryo. ^a Covariates: Female Age = 34.52, Male Age = 39.43, Female BMI = 23.611934, Male BMI = 26.344065, technique = 2.80. All p values < 0.05 were considered statistically significant (* vs. Good embryos, # vs. Lagging embryos).

3. Discussion

The prevailing approaches employed to assess embryo quality throughout in vitro development exhibit certain limitations. These methods predominantly rely on the expertise of an experienced embryologist for morphological evaluation. Thus, these methodologies are subjective, and there can be inter- and intra-observer variability, which can affect the consistency of embryo grading and impact the selection of the best embryo for transfer. Moreover, morphological assessment is typically performed at specific time points during embryo development, which may not be optimal for evaluating embryo quality [12]. Hence, morphological assessment is not always effective in predicting implantation success. Even high-quality embryos can face challenges in terms of successful implantation or may not culminate in viable pregnancies. This inherent variability can lead to the potential misclassification of certain embryos, thereby contributing to a reduction in the overall success rates of ART treatments. In this sense, the integration of embryo metabolomics with standardized morphological classification can help address some of these limitations by providing a more comprehensive evaluation of embryo quality, including information on potential biomarkers for embryo selection [4].

In this work, we evaluate the metabolic performance of embryos, using ^1H -NMR spectroscopy, during in vitro development, which were monitored in a time-lapse incubator during a period of 5 days and subject to standardized morphokinetic categorization, according to their development. The application of ^1H -NMR in the analysis of human IVF culture media comes with both advantages and potential pitfalls. One limitation is the sensitivity of ^1H -NMR to sample composition. The complex nature of culture media, containing a myriad of compounds, can lead to signal overlap, making it challenging to discern individual metabolites accurately. Additionally, certain metabolites may exist in low concentrations, making their detection and quantification less reliable. Despite these challenges, it is important to highlight that ^1H -NMR remains a very valuable tool for metabolomic analysis, offering non-destructive, high-throughput, and quantitative insights into the metabolic profiles of biological samples. Researchers must navigate these limitations judiciously, considering them in the context of the specific goals and characteristics of their study, as we did in the present case. Indeed, after standardized morphokinetic categorization of embryos, media were collected to determine those embryos' metabolic performance. This allows us to search for a possible metabolic biomarker associated with the morphokinetic evaluation.

The definitive classification of embryos was determined through the utilization of Gardner's blastocyst grading scale [24], with adherence to the guidelines set by the Istanbul Consensus for the timing of observations and the identification of non-viable embryos [13]. Following this classification, they were divided into Good, Lagging, and Bad embryos, as described above. Our goal was to enhance the evaluation of embryo quality by integrating embryo metabolomics with standardized morphokinetic classification, in order to contribute to a more accurate and consistent selection of high-quality embryos with the potential for successful pregnancies. While recognizing that the inclusion of degenerated embryos, non-cleavage embryos, those with abnormal fertilization, and parthenogenetic embryos could yield data with more distinctive differences, we intentionally excluded them to maintain the focus, quality, and consistency of the data. Including embryos with abnormalities or arrested development could introduce confounding variables and detract from the study's objective, as these embryos are readily identifiable as low-quality with no potential for successful pregnancies.

During the 5-day culture, we chose to correlate the embryo development status with the variation, at D3 and D5, in six specific metabolites that are on the crossroad of major cellular pathways: pyruvate, lactate, alanine, glutamine, acetate, and formate. Indeed, with the exception of glucose (whose consumption remained unaffected across all our experimental conditions—Supplemental Data Figure S1), these metabolites stand as key protagonists within diverse metabolic pathways. Particularly during the intricate process of embryo development, they assume a pivotal role, participating in energy generation,

biosynthesis, and intricate signaling cascades [25,26]. For the aforementioned reasons, we hypothesized that one or more of these metabolites may serve as biomarkers for assessing the developmental competence of embryos, providing valuable insights into the underlying metabolic processes that contribute to successful embryogenesis. The identification of metabolites that are sufficiently abundant in the culture media to be easily quantified emphasizes their potential significance as key clinical indicators of the metabolic health and viability of developing embryos. This underscores the rationale for the inclusion of those metabolites in the study and their eventual future relevance in establishing a method that could more or less easily quantify them in the setting of the embryology laboratory.

Pyruvate is the end-product of the energy-yielding process of glycolysis and can be converted to acetyl-CoA, which enters the tricarboxylic acid cycle (TCA) to generate more ATP. Lactate and alanine are produced from pyruvate, particularly when the redox status (NAD⁺/NADH ratio) is altered. They can be converted back to pyruvate and generate ATP by being driven to the TCA [27]. Acetate is produced during fatty acid metabolism and can be used as an energy source by some cells [28]. Glutamine is an amino acid that is involved in several metabolic pathways, including the synthesis of nucleotides, proteins, and other amino acids, and is also an important source of energy for rapidly dividing cells such as embryos [29]. Formate can be derived from various pathways, including the breakdown of serine, being involved in one-carbon metabolism, which is important for the synthesis of nucleotides and other biomolecules [30]. Research findings have unequivocally shown that human preimplantation embryos actively engage in the consumption and secretion of these metabolites, changing their concentration in the surrounding culture media. This serves as a clear indicator of the embryo's robust metabolic activity [5,6,12].

Based on our analysis, the adjusted metabolite differentials (for covariates such as the male age, female age, male BMI, female BMI, and ART technique) observed in the first 3 days of embryo culture are discriminative of embryo quality category at the end of D5. The inclusion of covariates related to individual characteristics of the donors of the gametes is crucial to assess the impact of embryo culture medium metabolites in early embryo development. Although ICSI circumvents part of the individual variability, the age and BMI of the donors have been reported to influence the success rate of the ICSI [21,31,32], particularly in European cohorts [33,34]; therefore, we have considered these covariates to add another layer of embryo selection. The major contributors for discriminating Good, Lagging, and Bad embryos were pyruvate, alanine, glutamine, and acetate, after correcting for the confounders. Pyruvate, in the context of embryonic development, serves as an essential energy substrate during the preimplantation stages [27]. This metabolite has been linked to embryo development and increased embryo quality in some species, including cattle and sheep [35]. Although its importance for early embryo development has been evidenced in some studies, the role of pyruvate in human embryonic development is still less clear when other metabolic sources are present [8,27]. In our study, adjusted pyruvate variation in the culture media of 3-day human embryos was quite distinct in Bad embryos from Good and Lagging embryos. While Bad embryos consumed pyruvate, Good and Lagging embryos were able to export and accumulate it in the culture media, suggesting an active metabolism. A similar trend was observed for glutamine, with Good and Lagging embryos being able to export and accumulate this amino acid in the culture media, while Bad embryos consumed it. Glutamine is an important metabolite for embryo development, as it serves as a precursor for various metabolic pathways [9]. It is a non-essential amino acid that can be synthesized by the embryo or taken up from the surrounding culture medium. During early embryonic development, glutamine is predominantly utilized for energy production through the TCA cycle [36,37]. Studies have shown that the addition of glutamine to the culture medium during in vitro embryo development can improve embryo quality and blastocyst formation rates [38]. This suggests that glutamine plays an important role in embryonic metabolism and development [29]. Human embryos have been shown to export glutamine during in vitro development [11]. In vitro studies have

shown that the usage of glutamine by embryos increases during the blastocyst stage and that the export of glutamine is associated with increased cell proliferation and blastocyst formation [39], as observed in our experimental work.

Alanine is a non-essential amino acid that also plays an important role in the metabolism of developing embryos. During embryonic development, alanine is involved in the synthesis of proteins, the regulation of glucose metabolism, and the maintenance of cellular redox balance [40]. Studies have shown that alanine is an important nutrient for embryonic growth and development [40]. The availability of alanine can affect the developmental potential of embryos, with lower levels of alanine being associated with decreased embryo quality and lower rates of implantation [39]. In our work, up until D3, Bad embryos produce significantly lower amounts of alanine than Good or Lagging embryos. In fact, during development, the embryo has been shown to produce and export alanine to the surrounding environment, with embryos that produce and export higher levels of alanine having better developmental potential and being linked to higher pregnancy rates [5]. The export of alanine by human embryos is significant because it reflects the metabolic state of the embryo and its ability to maintain energy balance during early development. It has been suggested that the export of alanine may serve as a protective mechanism against stress and nutrient deprivation, allowing the embryo to maintain its viability and developmental potential [41]. Contrastingly, concerning acetate, both Bad and Lagging embryos showed a different trend from that of Good embryos in the variation of this short-chain fatty acid in culture media. While Bad embryos produced higher amounts of acetate in our experimental conditions, Lagging embryos consumed it. Acetate has been shown to be an important energy source for embryo development [6,42]. As the embryo develops and the number of cells increases, the energy demand also increases, and the embryo begins to utilize other energy sources, including acetate [6]. It has been shown that acetate is critical for proper embryo development, as it enhances cell growth and division, regulates gene expression, and improves embryonic quality, particularly for embryos during the preimplantation period [35]. Overall, the higher amounts of acetate observed in the culture media of Bad embryos at the end of D3 should be due to lower utilization of this metabolite due to the poor development of these embryos. The significance of the consumption of acetate by Lagging embryos is unclear, but it may reflect the need to increase the metabolic rates of these cells to enhance the formation of blastocysts.

As a remark, and although no differences were observed in the variation of formate in any of the experimental conditions used, it is of note that our embryos consistently exported significant amounts of this monocarboxylic anion. It is known that mammalian cells generate formate as a byproduct of various metabolic reactions, including the breakdown of certain amino acids and the metabolism of folate. Formate is then either excreted from the cell or used in various biosynthetic pathways, including the synthesis of purines, pyrimidines, and amino acids. In addition, formate can also play a role in regulating cellular redox balance and oxidative stress. For example, formate has been shown to protect cells from oxidative damage by acting as an antioxidant. Overall, the cellular significance of formate in mammalian cells is diverse and multifaceted, with important roles in both metabolism and cellular defense mechanisms [30]. During early embryonic development, formate is produced by the mitochondria and is used as a source of one-carbon units for nucleotide synthesis and methylation reactions [7]. Formate is the main one-carbon donor for de novo purine biosynthesis in the developing embryo. It has been shown that the inhibition of formate metabolism in early embryos can lead to developmental defects and reduced viability [43]. In fact, some studies suggested formate levels as a biomarker for embryonic viability in ART, as the concentration of formate in the culture media can be linked to the metabolic activity of the embryo and in some conditions can be indicative of embryonic viability and quality.

4. Materials and Methods

4.1. Chemicals

Embryo and gamete culture media and reagents were purchased from Origio® (Malov, Denmark), Nidacon (Molndaln, Sweden), and Vitrolife (Gothenburg, Sweden). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

4.2. Patient Selection and Ovarian Stimulation

Twenty-one couples (who underwent a total of 21 cycles) were involved in this study. The treatments of ARTs were conducted at CGR, Porto, Portugal between June 2019 and February 2021. The procedures of the Centre for Reproductive Genetics Alberto Barros are covered by the provisions of the National Medically Assisted Procreation Act (2017) and overseen by the National Council for Medically Assisted Procreation (CNPMA-2018). According to these rules and guidelines, the clinical databases and patient biological material for diagnosis and research may be used without further ethical approval, under strict individual anonymity, and after patient written informed consent. Regarding the use of human samples for laboratory experimentation, the Ethics Committee authorization number is 2021/CE/P02 (P342/2021/CETI), approved on 26 February 2021. The inclusion criteria were couples who were referred to infertility treatment with ICSI technique due to the need to avoid the interference of cumulus cells or spermatozoa during metabolomics quantification. The couple was excluded from the study if they had a testicular biopsy performed (testicular sperm aspiration or testicular sperm extraction) or cryopreserved ejaculated spermatozoa. So, all the ICSI were performed with fresh ejaculated spermatozoa. Semen samples were collected on the day of oocyte retrieval by masturbation. The clinical procedures and laboratory protocols were performed following the CGR standard protocols. Controlled ovarian stimulation was performed using a gonadotropin-releasing hormone antagonist protocol (GnRH-ant). Recombinant follicle-stimulating hormone (rFSH—follicle-stimulating hormone: Bemfola®, Gedeon Richter, Budapest, Hungary and Gonal F®, Merck-Serono, Schiphol-Rijk, The Netherlands; follicle-stimulating hormone: Puregon®, MSD Biotech B.V., Noord-Brabant, The Netherlands), recombinant follicle-stimulating hormone with recombinant luteinizing hormone (rFSH and rLH in a 2:1 ration—Pergoveris®, Schiphol-Rijk, Merck-Serono, The Netherlands), menotropin (highly purified human menopausal gonadotrophin, hMG—Menopur®, Ferring Pharmaceutical, Madrid, Spain), or corifollicle-stimulating hormone (Elonva®, N.V. Organon, Oss, The Netherlands) were used to stimulate ovaries with initial doses based on the individual characteristics of patients. Beyond day 6 and when the leading follicle reached 12 mm, treatment was continued with gonadotropin-releasing hormone antagonist ganirelix (Orgalutran®, N.V. Organon, Oss, The Netherlands) or cetrorelix acetate (Cetrotide®, Merck-Serono, The Netherlands) until final follicular maturation. Stimulation was prolonged until the observation by ultrasound of at least three dominant follicles of 17 mm or greater in diameter or in cases of a low number of growing follicles, at least one. The final oocyte maturation was triggered with 250 µg of recombinant human chorionic gonadotropin (rhCG—choriogonadotropin alfa; Ovitrelle®, Merck Serono, Schiphol-Rijk, The Netherlands) or 0.2 mg of GnRH agonist (Triptorelin acetate, Decapeptyl®, Ipsen Pharma, Barcelona, Spain) or both. The serum hormone levels of estradiol (E2) and progesterone (P) were evaluated on the day of the trigger. Transvaginal oocyte retrieval was performed 36 h after the final follicular maturation.

In the cases in which embryo transfer was performed, pregnancy was evaluated at two different stages. Women were classified as having a biochemical pregnancy when β -HCG concentration surpassed the value of 20 mIU/mL (positive serum β -HCG levels), 12 days after embryo transfer [24]. All women with negative serum β -HCG levels ($[\beta\text{-HCG}] < 20 \text{ mIU/mL}$) were not considered pregnant. Biochemical pregnancies evolved into clinical pregnancies when a fetal heartbeat was detected.

4.3. Gamete Collection

After liquefaction, sperm concentration and progressive motility were assessed according to the WHO [44]. The semen preparation standard protocol included a discontinuous density gradient of 90–45% centrifugation (PureSperm[®], Nidacon, Mölndal, Sweden) followed by a swim-up protocol with fertilization medium (Sequential FertTM, Origio, Ballerup, Denmark). In cases of severe oligoasthenoteratozoospermia, the sperm sample was only washed with a sperm preparation medium (Sperm Preparation MediumTM, Origio, Ballerup, Denmark) followed by a swim-up protocol with fertilization medium (Sequential FertTM). The final sperm preparation was incubated at 35 °C with 6% O₂ at least 30 min before the beginning of the ICSI procedure. After the follicular aspiration, the oocyte–cumulus complexes (OCCs) were isolated and washed in a gamete preparation medium (SynVibroTM Flush, Origio, Ballerup, Denmark). The retrieved OCCs were placed in a 5-well dish with 250 µL of fertilization medium (Sequential FertTM) covered with oil (OVOILTM, Vitrolife, Barcelona, Spain) that had been previously equilibrated overnight (37 °C, 5% CO₂, and 6% O₂). OCCs were denuded to evaluate oocyte nuclear maturity. So, 2 h after egg retrieval, cumulus cells were removed using a combination of mechanic and enzymatic (ICSI Cumulase[®], Origio, Ballerup, Denmark) denudation protocol.

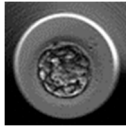
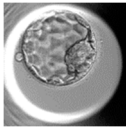
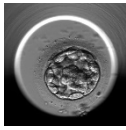
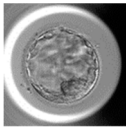
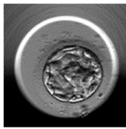
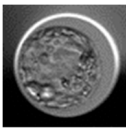
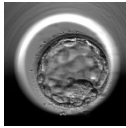
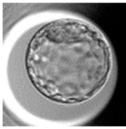
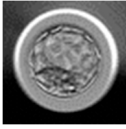
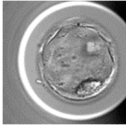
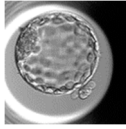
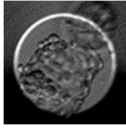
4.4. Intracytoplasmic Sperm Injection (ICSI) and Embryo Culture

Four hours after egg retrieval, oocytes were injected following the standard ICSI procedure. Injected oocytes were rinsed and cultivated individually in a 30 µL drop of culture medium (25 µL of culture medium plus 5 µL embryo washed in the same culture medium) (Sequential CleavTM, Origio[®], Ballerup, Denmark) under 1500 µL of oil (OVOILTM, Vitrolife, Sweden), which was previously equilibrated overnight in an incubator at 37 °C, 5% CO₂, and 6% O₂. The culture dish (EmbryoSlide[®], Vitrolife, Barcelona, Spain) allows an individualized oocyte/embryo culture with no connection between the culture medium of adjacent oocytes/embryos. The culture dish was placed in a time-lapse incubator (EmbryoScope[®], Vitrolife, Barcelona, Spain) (Day 0) for the following assessment. Normal fertilization was confirmed 16–18 h after ICSI by the presence of two pronuclei and a second polar body (Day 1). Embryo culture was extended until the blastocyst stage (D5). The standard laboratory protocol applied uses a sequential culture media. The embryos were cultured in a cleavage medium (Sequential CleavTM, Origio, Ballerup, Denmark) until D3 and then the culture media were replaced with a blastocyst medium (Sequential BlastTM, Origio, Ballerup, Denmark) to support embryo culture until the blastocyst stage (D5). After embryo culture, in some cases, embryos were immediately transferred, while in other cases, embryos were vitrified for subsequent transfer. Thus, we did not evaluate the correlation of our metabolomics results with the success rates in the different types of fertility treatments as it would not be suitable to compare results, because in some treatments the embryo transfer was a fresh embryo transfer and in others, the transfer was deferred.

4.5. Embryo Morphology Assessment

The development of each embryo was evaluated from fertilization until D5 in a time-lapse system (Vitrolife, Barcelona, Spain). Routine observation of cleavage embryos (until day 4) mainly includes the number and symmetry of blastomeres, fragmentation percentage and presence of multinucleated blastomeres (day 2/3), and compaction grade (day 4). Cleavage embryos were scored according to four categories (A, B, C, and D), based solely on blastomeres number and fragmentation [45]. The blastocysts were graded according to Gardner Score System [24]. This scoring system uses a number associated with a letter, and this combination allows us to know the blastocyst stage and blastocyst quality. Initially, the classification is based on the degree of expansion and hatching status of blastocysts, and the numeric score from 1 to 6 reflects the blastocyst stage (Table 4).

Table 4. Classification of blastocysts, inner cell mass, and trophectoderm. Degree of expansion and hatching status of blastocysts. Gardner blastocysts grade system was assessed as indicated in the table. Inner cell mass and trophectoderm classification for onward full blastocyst stage (BL3 to BL6).

Blastocyst Classification	Stage	Definition	Examples	Inner Cell Mass Classification	Definition	Examples
BL1	Early blastocyst	The blastocoel being less than half the volume of the embryo		A	Tightly packed with many cells	
BL2	Blastocyst	The blastocoel being greater than half the volume of the embryo		B	Loosely grouped with several cells	
BL3	Full blastocyst	The blastocoel completely filling the embryo		C	Very few cells	
BL4	Expanded blastocyst	The blastocoel volume now being larger than that of the early embryo and the zona pellucida starting to thin		Trophectoderm Classification	Definition	Examples
				A	Many cells forming a cohesive epithelium	
				B	Few cells forming a loose epithelium	
				C	Very few large cells	
BL5	Hatching blastocyst	The trophectoderm starting to herniate though the zona pellucida				
BL6	Hatched blastocyst	The blastocyst having completely escaped from the zona pellucida				

For blastocysts scored 3–6, the classification system includes the development of the inner cell mass and trophectoderm (Table 4). A blastocyst of good quality was considered as BL3BB or better. The term Lagging was used to characterize those embryos that had acceptable morphological classification, but the embryo development was delayed considering the standardized timing of embryo observations established in the Istanbul Consensus.

4.6. Sample Collection/Preparation

As mentioned before, the use of sequential media in an embryo culture implies that until D3 the embryos are cultured in a cleavage medium and afterwards there must be a change in culture medium to support embryo development until D5 (blastocyst stage). So, for each embryo used for the study, there will be two samples of culture medium: a sample of cleavage medium (day 0 until D3 of embryo development) and a sample of blastocyst medium (D3 until D5 of embryo development). Sample collection was carried out by pipetting 25 µL from the 30 µL drop of culture media into microcentrifuge tubes.

Each sample (25 μ L) was obtained from the spent culture media of embryos developed from day 0 (after ICSI) until D3 and from D3 until D5. To be used as controls, samples from the same corresponding batch of each medium used in embryo culture and with the same shelf-life were collected and analyzed by ^1H -NMR spectroscopy as described below and used to evaluate the content of the media at time 0. All samples were centrifuged ($1000\times g$; 10 min; 4°C) to fully separate any residues of oil. Twenty microliters of the bottom layer were carefully collected and stored at -80°C until metabolomic analysis.

4.7. Metabolomic Analysis by ^1H Nuclear Magnetic Resonance Spectroscopy

1D ^1H -NMR spectroscopy was applied to identify and quantify metabolites in the embryo culture media. NMR spectra were acquired using a 600 MHz VNMRs spectrometer (Varian, Inc. Palo Alto, CA, USA) equipped with a 3 mm indirect detection probe. About 140 μ L of deuterium oxide and 40 μ L of sodium fumarate solution (final concentration of 2 mM) were added to 20 μ L of each embryo culture media, and the final volume of 200 μ L was transferred into a 3 mm NMR tube for analysis. Sodium fumarate was used as an internal reference (6.50 ppm) to quantify the following metabolites (multiplicity, chemical shift (ppm)): alanine (doublet, 1.46), acetate (singlet, 1.90), pyruvate (singlet, 2.35), glutamine (multiplet, 2.45), lactate (quartet, 4.09), and formate (singlet, 8.44). NMR spectra were processed by applying exponential line broadening (0.2 Hz), baseline correction, and manual phasing, and the areas of the resonances were quantified using the NUTSproTM NMR Software (2D Professional Version-20070315, Acorn, Fremont, CA, USA). Blank control experiments were performed previously in which we replicated every step of the incubation except that there were no embryos being cultivated. With this procedure, we were able to assess the impact of the protein content (with HSA $\leq 0.05\%$) on the NMR spectra acquisition, ensuring that there were no signals generated that overshadowed the metabolites of interest. We were also able to perfect the separation of the oil from the spent culture (in order to obtain clear NMR spectra without disturbing overlapping or nearby signals derived from the oil), and we analyzed the impact of this incubation on the content of the medium in water-soluble metabolites, as the ones we evaluated in the experiment. We saw no alteration in the content of these metabolites, in line with the results described by a previous study [46]. Moreover, fresh medium samples were collected for each distinct batch and within the same medium batch if different shelf times were used (21 control samples for D3 and 21 samples for D5). Subsequently, they were analyzed by ^1H -NMR together with correspondent spent media (same batch plus same shelf time) and used to calculate the initial metabolite content and subsequently the metabolite production or consumption.

4.8. Statistical Analysis

Univariate statistics were used to compare embryo culture medium metabolite differential in days 0–3 (D0–3) and 3–5 (D3–5) between embryos grouped by their assessed quality at D5 (Good vs. Lagging vs. Bad). Data are presented as mean $\pm$ standard deviation (S.D.). Embryo quality was assessed by the same trained and experienced embryologist. Normality and heteroscedasticity tests were performed, and univariate ANOVA was conducted on data collected concerning variation in selected metabolites identified in the embryo culture medium. Pairwise comparisons between groups were corrected for multiple hypotheses testing by Tukey's post hoc test. Multivariate analysis of metabolite consumption/production was performed using a linear model integrating corrections for confounding factors. Metabolite differentials in days 0–3 (D0–3) and 3–5 (D3–5) were compared between embryos grouped by their assessed quality at D5 (Good vs. Lagging vs. Bad) using multivariate analysis of covariance (MANCOVA), after controlling for covariates (male age, female age, male BMI, female BMI). These covariates were chosen based on multiple studies correlating maternal age [14,16] and BMI [18,19], and paternal age [20] and BMI [21,23] with changes in early embryo metabolism and viability [17,22]. The assumptions of equality of the variance–covariance matrices were evaluated using

Levene's test of equality of error variances and Box's test of equality of covariance matrices (Box's M). The discriminant value of the independent variable (embryo quality at D5) considering the dependent variables (metabolite differential) was evaluated according to Roy's Largest Root criterion. This method was chosen as it is the most sensitive when a limited number of dependent variables is responsible for the differences between groups of the independent variable [47]. The impact of each metabolite differential on embryo quality at D5 was assessed using a univariate F-test, after adjusting for the covariates. Pairwise comparisons between groups were corrected for multiple hypotheses testing by Šidak's method. All statistical tests of hypotheses for all statistical methods were performed at the $\alpha = 0.05$ level using SPSS Statistics 28.0.0 for Windows 64-bit (IBM, Armonk, NY, USA).

5. Conclusions

The goal of integrating metabolomics with standardized embryonic viability assessment could be crucial for non-invasive, real-time monitoring of embryo metabolic states during culture, offering valuable information on viability and quality. Current methods, such as morphological assessment and morphokinetic analyses, have limitations in predicting implantation success, making metabolomics an essential addition. Furthermore, this integration can lead to identifying biomarkers associated with embryonic viability and quality, potentially improving embryo selection for transfer and enhancing the chances of successful pregnancies. Still, our strategy does not address metabolic differences between inner cell mass (ICM) and trophoctoderm, noted in previous reports showing distinct metabolic rates between these cells [48,49]. Moreover, the metabolic activity of ICM cells was suggested to play a role in embryo development [49,50]. Yet, the methods applied in these studies destroy or compromise the viability of the embryos. Existing methods compromising embryo viability highlight a need for non-destructive techniques. Alegre et al. proposed Raman spectroscopy, estimating the oxidant content of the culture medium, but its external variability and lower specificity compared to NMR or MS spectroscopy raise concerns [4]. Hence, NMR emerges as a potential compromise, as demonstrated in recent studies of early-stage mammalian embryos [51]. Despite evidence supporting metabolic screening's benefits for nearly a decade, routine implementation in ART clinics is lacking [52,53].

Overall, our results support that assessment of the exometabolome of in vitro cultured human embryos up to D3 can be discriminative of embryo quality category at D5. Notably, the metabolite profile at D3 is particularly selective between Lagging and Bad-quality embryos, whereas the metabolite profile of Good embryos is not different from Lagging embryos (except for alanine) at this stage. However, at D5, lactate is a potential predictor between Good and Lagging embryos. Therefore, the corrected variations (for interfering covariates, such as the male age, female age, male BMI, and female BMI) of metabolites such as alanine, pyruvate, acetate, and glutamine support that the integration of metabolomics with the standardized assessment of embryonic viability and quality has the potential to improve the efficiency and effectiveness of ARTs (Figure 4), leading to better pregnancy rates and improved clinical outcomes.

While the number of samples used in this study is low and may hamper some more definite conclusions, these findings suggest a potential advancement in refining embryo selection procedures. Incorporating metabolic assessments into protocols could improve ART outcomes by enhancing grading precision and potentially boosting implantation success. This study contributes to ongoing efforts to optimize ART procedures, offering a more comprehensive approach to embryo quality assessment beyond relying solely on morphological evaluation. The potential application of metabolic assessments as additional tools in embryo selection may represent progress in reproductive medicine.

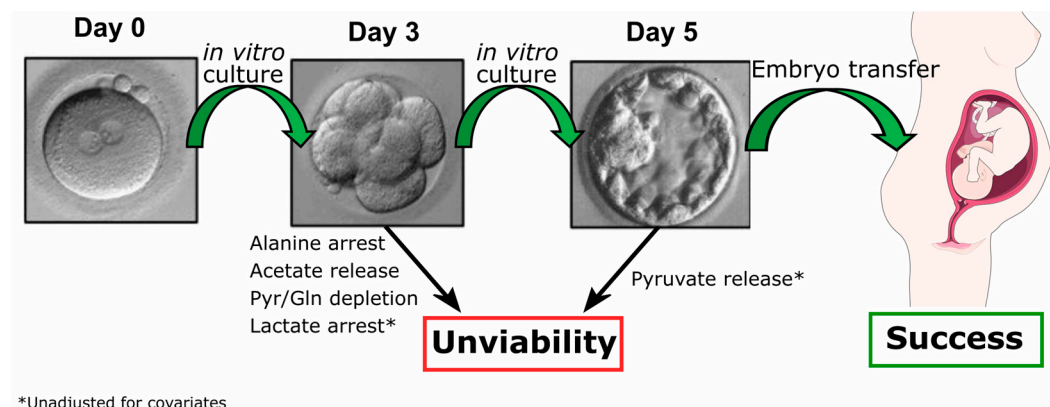


Figure 4. Metabolite variation in human embryo culture medium is a predictor of successful pregnancy in ARTs. Embryo metabolism is indirectly assessed by comparison of metabolite concentration in culture medium before and after incubation. We observed that Poor-quality embryos at post-fertilization day 5 displayed already anomalies in alanine, acetate, pyruvate, and glutamine metabolism, especially compared to embryos classified as Lagging at day 5. These differences were found after adjusting for covariates (male age, female age, male BMI, female BMI). Considering unadjusted variables, lactate is another potential success predictor for ARTs at day 3. On day 5, considering unadjusted variables, net-zero pyruvate is a feature of good-quality embryos.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and the procedures were covered by the provisions of the National Medically Assisted Procreation Act (2017) and overseen by the National Council for Medically Assisted Procreation (CNPMA-2018). According to these rules and guidelines, the use of clinical databases and patient biological material for diagnosis and research may be used without further ethical approval, under strict individual anonymity, and after patient written informed consent. Regarding the use of human samples for laboratory experimentation, the Ethics Committee authorization number is 2021/CE/P02 (P342/2021/CETI), approved on 26 February 2021.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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Sperm-borne miR-34c-5p and miR-191-3p as markers for sperm motility and embryo developmental competence

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Abstract

Background: Sperm-borne microRNAs play a pivotal role in influencing essential cellular processes during fertilization, impacting the quality of embryo development. Dysregulated microRNA profiles have been associated with compromised embryonic development and increased incidences of pregnancy loss.

Objective: This study aimed to investigate the potential associations between the abundance of miR-34c-5p and miR-191-3p in human spermatozoa with sperm quality, as well as with embryo quality and metabolic performance during in vitro development.

Materials and methods: Thirteen couples who underwent a total of 13 cycles participated in this study. The sperm quality was assessed using conventional methods following World Health Organization guidelines. Quantitative polymerase chain reaction was employed to measure microRNA abundance in spermatozoa. Embryos were categorized as good, lagging, or bad based on morphokinetic evaluation. Evaluation of embryo metabolic performance involved tracking changes in specific metabolites within the cultured media using nuclear magnetic resonance spectroscopy. Statistical analysis was conducted to explore the correlation between microRNA abundance in human spermatozoa and all other collected data.

Results: Our findings revealed a negative correlation between the abundance of miR-34c-5p (but not miR-191-3p) and total sperm motility, potentially mediated by the modulation of key signaling pathways. Additionally, higher levels of miR-34c-5p in spermatozoa were strongly associated with the consumption or release of key metabolites by developing embryos, particularly those linked with lipid and glucose metabolism, suggesting enhanced metabolic performance, while miR-191-3p was mostly associated with glucose consumption. Concurrently, only miR-34c-5p content in spermatozoa correlated with higher embryo quality.

Discussion and conclusion: This study provides evidence suggesting that the abundance of miR-34c-5p in spermatozoa is correlated not only with total sperm motility but also with markers of embryo developmental competence, highlighting the potential significance of this sperm microRNA content as a biomarker in assisted reproduction.

KEYWORDS

embryo development, miR-191-3p, miR-34c-5p, sperm miRNAs, sperm quality biomarker

1 | INTRODUCTION

MicroRNAs (miRNAs) are small, non-coding RNA molecules that play diverse and intricate roles in regulating gene expression across various biological processes. Research has uncovered multiple functions of miRNAs, including their involvement in cell proliferation, differentiation, apoptosis, and development.¹ miRNAs function by binding to target messenger RNA (mRNA) molecules, leading to mRNA degradation or translational repression, thereby influencing the expression of genes involved in numerous cellular pathways. Additionally, miRNAs have been implicated in the regulation of immune responses, metabolism, and stress responses, highlighting their broad impact on physiological functions.^{2,3} Altered miRNA expression has been linked to various diseases, including cancer, cardiovascular disorders, and neurological conditions, underscoring the importance of miRNAs in health and disease.⁴

Recent research has shed light on the influential role of sperm miRNAs in shaping embryo quality.^{4,5} Although it is widely recognized that sperm miRNAs play a role in modulating gene expression during embryonic development, uncertainties persist regarding the full extent of their impact. Moreover, the consequences of dysregulated sperm miRNAs on reproductive outcomes and offspring health remain ambiguous, with conflicting research findings regarding their correlation with infertility, suboptimal embryo quality, pregnancy complications, and epigenetic inheritance. Further investigation is imperative to untangle the intricate relationship between sperm miRNAs, fertilization, and embryonic development, aiming to address controversies and deepen our comprehension of their biological significance.^{6–8} Recent studies have demonstrated that sperm miRNAs are transmitted to the oocyte upon fertilization, where they have the potential to influence critical cellular processes essential for embryo development, including cell proliferation, differentiation, and apoptosis.⁵ These miRNAs possess the capability to target and modulate the expression of specific genes involved in embryogenesis, thereby impacting the developmental quality of the resultant embryo. Additionally, abnormalities in sperm miRNA expression have been associated with diminished embryo quality and compromised developmental potential, contributing to impaired embryo development, increased rates of embryonic arrest, and elevated incidences of pregnancy loss.^{6,9,10}

miRNA-34c-5p and miRNA-191-3p are two miRNAs linked to embryogenesis. However, research on their precise roles in embryo development is relatively limited compared to their extensively studied functions in other biological contexts.^{11,12} We chose these two sperm-borne miRNAs based on their distinct characteristics regarding their contribution to the embryo's miRNA pool.^{13,14} While miRNA-34c-5p is known to regulate essential cellular processes such as apoptosis and cell cycle progression, its involvement in embryonic development

has scarcely been addressed. Some studies have suggested that it may play a role in modulating embryonic stem cell differentiation and lineage commitment, which could indirectly impact embryo development. Additionally, dysregulation of miRNA-34c-5p has been associated with various developmental disorders and pregnancy complications, indicating its potential relevance to embryo quality.⁷ Similarly, miRNA-191-3p has been extensively studied in various biological contexts, including cancer and other diseases, but its involvement in embryonic development is not well understood. However, some studies suggest that miRNA-191-3p may play a role in early embryogenesis and contribute to the regulation of genes involved in cell proliferation, differentiation, and apoptosis, which are critical processes for embryo development and quality.⁶ Still, further research is needed to fully elucidate the role of these miRNAs (as of many others) in embryonic development and their potential implications for reproductive outcomes. Understanding the intricate role of sperm miRNAs in embryo quality holds promise for improving assisted reproductive technique (ART) outcomes. By identifying specific miRNA signatures associated with optimal embryo development, researchers can aim to develop novel diagnostic tools and therapeutic interventions to enhance embryo quality and increase the success rates of ART procedures. Hence, in our work, we aimed to enlighten the putative correlation between miRNA-34c-5p and miRNA-191-3p sperm content and several parameters of human sperm quality and seminal content, while at the same time with the outcomes of in vitro human embryo development, namely, quality and metabolic performance.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Embryo and gamete culture media and reagents were acquired from Origio, Nidacon, and Vitrolife. All remaining chemicals were procured from Sigma–Aldrich, unless otherwise specified.

2.2 | Patient selection and ovarian stimulation

Thirteen Caucasian couples who underwent a total of 13 cycles participated in this study. ART treatments were administered at Centro de Genética Prof. Alberto Barros (CGR) in Porto, Portugal, between June 2019 and February 2021. The procedures at the Centre for Reproductive Genetics Alberto Barros adhere to the regulations of the National Medically Assisted Procreation Act (2017) and are supervised by the National Council for Medically Assisted Procreation (CNPMA-2018). In accordance with these regulations, clinical databases and patient

biological material may be utilized for diagnosis and research without further ethical approval, ensuring strict individual anonymity and obtaining written informed consent from patients. The Ethics Committee authorization number for laboratory experimentation with human samples is 2021/CE/P02 (P342/2021/CETI), approved on 26 February 2021. Couples eligible for inclusion were those referred for infertility treatment with the intracytoplasmic sperm injection (ICSI) technique, aiming to prevent interference from cumulus cells or spermatozoa during metabolomics quantification. Couples undergoing testicular biopsy (testicular sperm aspiration or testicular sperm extraction) or using cryopreserved ejaculated spermatozoa were excluded from the study, ensuring that all ICSI procedures were conducted using fresh ejaculated spermatozoa. Semen samples were collected via masturbation on the day of oocyte retrieval. Clinical procedures and laboratory protocols adhered to the CGR standard protocols. Controlled ovarian stimulation was carried out using a gonadotropin-releasing hormone antagonist protocol (GnRH-ant). Recombinant follicle-stimulating hormone (rFSH) from various sources, including follitropin alfa (Bemfola, Gedeon Richter, and Gonal F, Merck-Serono), follitropin beta (Puregon, MSD Biotech B.V.), rFSH with recombinant luteinizing hormone (rFSH and rLH in a 2:1 ratio—Pergoveris, Merck-Serono), menotropin (highly purified human menopausal gonadotrophin, hMG—Menopur, Ferring Pharmaceutical), or corifollitropin alpha (Elonva, N.V. Organon), were utilized to stimulate ovaries, with initial doses tailored to individual patient characteristics. GnRH-ant ganirelix (Orgalutran, N.V. Organon) or cetrorelix acetate (Cetrotide, Merck-Serono) was introduced beyond day 6, once the leading follicle reached 12 mm, until final follicular maturation was achieved. Stimulation continued until ultrasound observation revealed at least three dominant follicles of 17 mm or greater in diameter or, in cases of a low number of growing follicles, at least one dominant follicle. Final oocyte maturation was triggered using 250 µg of recombinant human chorionic gonadotropin (rhCG—choriogonadotropin alfa; Ovitrelle, Merck-Serono), 0.2 mg of GnRH agonist (triptorelin acetate, decapeptyl, Ipsen Pharma), or a combination of both. Serum hormone levels of estradiol (E2) and progesterone (P) were assessed on the day of trigger, and transvaginal oocyte retrieval was performed 36 h after final follicular maturation.

2.3 | Gamete collection

Following liquefaction, sperm concentration, total and progressive motility, as well as sperm membrane integrity were evaluated in accordance with the World Health Organization guidelines.¹⁵ Sperm preparation protocol involved a discontinuous density gradient centrifugation (PureSperm) ranging from 90% to 45%, followed by a swim-up procedure utilizing fertilization medium (Sequential Fert) in order to obtain highly motile spermatozoa, allowing also the separation of spermatozoa from non-gamete cells, as described previously.¹⁶ In cases of severe oligoasthenoteratozoospermia, the sperm sample underwent washing only with a sperm preparation medium (Sperm Preparation Medium) before the swim-up process using fertilization medium (Sequential Fert). The final sperm preparation was then incu-

bated at 33°C with 6% CO₂ for a minimum of 30 min before starting the ICSI procedure, with the remaining spermatozoa being used for evaluation of miRNA content as described below. Subsequent to follicular aspiration, the oocyte–cumulus complexes (OCCs) were isolated and cleansed in gamete preparation medium (SynVibro Flush). These retrieved OCCs were then transferred into a five-well dish containing 250 µL of fertilization medium (Sequential Fert) and covered with oil (OVOIL) that had been previously equilibrated overnight (37°C, 5% CO₂, and 6% O₂). The OCCs were denuded to assess oocyte nuclear maturity. Hence, 2 h post-egg retrieval, cumulus cells were removed using a denudation protocol involving a combination of mechanical and enzymatic methods (ICSI Cumulase).

2.4 | Intracytoplasmic sperm injection and embryo culture

Four hours post-egg retrieval, oocytes underwent injection following the standard ICSI procedure. Injected oocytes were then rinsed and individually cultured in a 30 µL drop of culture medium (25 µL of culture medium plus 5 µL embryo washed in the same culture medium) (Sequential Cleav) beneath 1500 µL of oil (OVOIL) previously equilibrated overnight in an incubator at 37°C, 5% CO₂, and 6% O₂. The use of an EmbryoSlide culture dish facilitated individualized oocyte/embryo culture without interconnection between the culture media of adjacent oocytes/embryos. This culture dish was then placed in a time-lapse incubator (EmbryoScope) (day 0) for subsequent assessment. Fertilization was confirmed 16–18 h post-ICSI by the presence of two pronuclei and a second polar body (day 1). Embryo culture continued until the blastocyst stage (day 5). The standard laboratory protocol involved sequential culture media. Embryos were cultured in cleavage medium (Sequential Cleav) until day 3, after which the culture media were replaced with blastocyst medium (Sequential Blast) to support embryo culture until the blastocyst stage (day 5). Following embryo culture, some embryos were either immediately transferred or vitrified for subsequent transfer. Consequently, the correlation of our results with the success rates in different types of fertility treatments was not evaluated, as it would be inappropriate to compare results given the variation in embryo transfer methods (some treatments involved fresh embryo transfer, while others entailed deferred transfer).

2.5 | Embryo morphology assessment

Each embryo's development was monitored from fertilization to day 5 using a time-lapse system (Vitrolife). Standard observation of cleavage-stage embryos (up to day 4) primarily involved assessing the number and symmetry of blastomeres, percentage of fragmentation, presence of multinucleated blastomeres (day 2/3), and compactation grade (day 4). Cleavage-stage embryos were categorized into four groups (A, B, C, and D) based solely on blastomere number and fragmentation.¹⁷ Blastocysts were graded according to the Gardner Score System,¹⁸ which utilizes a combination of numbers and letters to indicate both the stage

and quality of the blastocyst. Initially, the classification is based on the degree of expansion and hatching status, with a numeric score ranging from 1 to 6 reflecting the blastocyst stage. For blastocysts scoring 3–6, the classification system further considers the development of the inner cell mass and trophectoderm. A blastocyst was deemed to be of good quality if it scored 3BB or higher. The term “lagging” was used to describe embryos with acceptable morphological classification but whose development was delayed compared to the standardized timing of embryo observations established in the Istanbul Consensus.

2.6 | Media sample collection/preparation

As previously explained, utilizing sequential media in embryo culture involves culturing embryos in cleavage medium until day 3, followed by a change to blastocyst medium to support embryo development until day 5 (blastocyst stage). Therefore, for each embryo included in the study, two samples of culture medium were collected: one sample of cleavage medium (from days 0 to 3 of embryo development) and one sample of blastocyst medium (from days 3 to 5 of embryo development). Sample collection was performed by pipetting 25 μ L from the 30 μ L drop of culture media into microcentrifuge tubes. Each sample (25 μ L) was obtained from the spent culture media of embryos that developed from days 0 (after ICSI) to 3 and from days 3 to 5. Control samples from the same corresponding batch of each medium used in embryo culture with identical shelf-life were collected and analyzed by ^1H nuclear magnetic resonance (^1H -NMR) spectroscopy to assess the content of the media at time 0. All samples were centrifuged (1000 $\times$ g; 10 min; 4°C) to thoroughly separate any residues of oil. Subsequently, 20 mL of the bottom layer was carefully collected and stored at -80°C until metabolomic analysis.

2.7 | Reverse transcriptase cDNA synthesis

Spermatozoa RNA was isolated from a total of 13 samples using the miRNeasy Tissue/Cells Advanced Kit (Qiagen) according to the manufacturer's instructions, as described previously.¹⁹ In resume, for each sample with approximately 0.5×10^6 spermatozoa, an adequate volume of RLT buffer (280 μ L) was added. The cells were disrupted via vortex and by passing the lysate through a 20-gauge needle several times. Next, 80 μ L of AL buffer was added to each sample, which were left to incubate at room temperature for at least 3 min. The lysates were transferred to a gDNA Eliminator spin column and centrifuged for 30 s at 8000 $\times$ g. To the flow-through, 20 μ L of RPP buffer was added. The tube caps were closed and samples were mixed vigorously by vortexing for at least 20 s and then left to incubate at room temperature for 3 min. The sample was centrifuged at 12,000 $\times$ g for 3 min at room temperature, and 380 μ L of isopropanol was added to the supernatants. The samples were then transferred to the RNeasy Mini column and centrifuged for 15 s at 8000 $\times$ g (the RNA was retained at the column). The column was washed with 700 μ L of RWT buffer, 500 μ L of RPE buffer, and 500 μ L of 80% ethanol (a centrifugation for

TABLE 1 Primer sequences for homo sapiens (hsa) microRNAs (miR) and respective accession number (AN).

Target	Primer sequence
<i>hsa-RNU6B</i> AN: NR_002752	Forward: ACTCAAGACAATGGTGATAATGGTT Reverse: ACAGAAAGGAATACGCAGAAAGG
<i>hsa-miR-191-3p</i> AN: MIMAT0001618	Forward: GCGGGCAACGGAATCCCAAAAGCA Reverse: GTCCAGTTTTTTTTTTTTTTAGGCA
<i>hsa-miR-34c-5p</i> AN: MIMAT0000686	Forward: CTAGGCAGTGTAGTTAGCTGATTGCT Reverse: AGGTCCAGTTTTTTTTTTTTTAAATCT

Note: Primers were designed using the miRprimer software.²¹

2 min at 8000 $\times$ g was carried out between every washing step). Finally, total RNA was eluted from the column with 30 μ L of RNase-free water. RNA concentrations and absorbance ratio A_{260}/A_{280} were determined by a DeNovix DS-11 spectrophotometer system. For cDNA synthesis, we adapted the method proposed by Ciera and Busk.²⁰ To each 50 ng of total RNA, 1 μ L of 10 $\times$ reaction buffer containing *Escherichia coli* poly (A) polymerase (New England Biolabs), 1 μ L of 1 mM ATP (New England Biolabs), 1 μ L of 10 μ M reverse transcriptase primer (5'-CAGGTCCAGTTTTTTTTTTTTTTVN), 1 μ L of dNTP mix (1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, NZYTech), 0.5 μ L of M-MuLV reverse transcriptase (200 U/ μ L, NZYTech), and 0.2 μ L of *E. coli* poly (A) polymerase (5000 U/ μ L, New England Biolabs) were added. The samples were then incubated at 42°C for 1 h. The primers used were designed by the miRprimer software²¹ and can be found in Table 1.

2.8 | Quantitative real-time polymerase chain reaction

For the quantitative real-time polymerase chain reaction (qPCR) assay, 0.64 μ L of primer forward (20 μ M), 0.64 μ L of primer reverse (20 μ M), and 8.0 μ L of Speedy Supreme NZYTaq 2 $\times$ Green Master Mix (NZYTech) were added to each 1 μ L of cDNA sample. The volume was adjusted to 16 μ L with RNase-free water. The protocol for qPCR was as follows: 2 min at 95°C for polymerase activation, 5 s at 95°C for DNA denaturation, and 30 s at 60°C for primers annealing and extension. To ensure primer specificity, 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. The melting temperatures for each set of primers were 78°C for *hsa-RNU6B* and *miR-34c-5p*, and 77.5°C for *miR-191-3p*. qPCR reactions were carried in duplicate, and the optical density was assessed by a CFX Connect Real-Time PCR Detection System (Bio-Rad). Amplification efficiencies were calculated for all primers and set between 90% and 110% as described previously.²² The data were analyzed according to the $2^{-\Delta\text{Ct}}$ method proposed by Pfaffl and the results are presented in arbitrary units (a.u.).²³ The results were normalized according to the expression of *hsa-RNU6B*, which has been previously described as a good housekeeping miRNA for human reproductive tissues and spermatozoa.²⁴

2.9 | Metabolomic analysis by ^1H -NMR spectroscopy

One-dimensional ^1H -NMR spectroscopy was applied to identify and quantify metabolite contents in embryo culture media, by methods described previously.¹⁶ NMR spectra were acquired using a 600 MHz VNMR spectrometer (Varian, Inc.) equipped with a 3 mm indirect detection probe. For culture media, 140 μL of deuterium oxide and 40 μL of sodium fumarate solution (final concentration of 2 mM) were added to 20 μL of each embryo culture media, and the final volume of 200 μL was transferred into a 3 mm NMR tube for analysis. Sodium fumarate was used as an internal reference (6.50 ppm) to quantify the following metabolites (multiplicity, ppm): alanine (doublet, 1.46), acetate (singlet, 1.90), pyruvate (singlet, 2.35), glutamine (multiplet, 2.45), lactate (quartet, 4.09), and formate (singlet, 8.40). NMR spectra were processed by applying exponential line broadening (0.2 Hz), baseline correction, and manual phasing, and the areas of the resonances were quantified using NUTSpro NMR Software (Acorn).

Blank control experiments were conducted beforehand for the embryo culture media, wherein each step of the incubation process was replicated except for the absence of embryos being cultivated. Through this procedure, we could evaluate the influence of protein content (with HSA $\leq 0.05\%$) on NMR spectra acquisition, ensuring that no signals were generated to obscure the metabolites of interest. Additionally, we refined the separation of oil from the spent culture to obtain clear NMR spectra without interference from overlapping or nearby oil-derived signals. We also examined the impact of this incubation on the medium's content of water-soluble metabolites, similar to that assessed in the experiment, and found no alterations in their content, consistent with findings from a prior study.¹⁶ Furthermore, fresh medium samples were gathered for each unique batch and within the same batch if varying shelf times were employed. These samples were then subjected to ^1H -NMR analysis alongside corresponding spent media (same batch and shelf time) to determine the initial metabolite content. Subsequently, metabolite production or consumption until day 5 was calculated based on these analyses.

2.10 | Statistical analysis

Embryo quality was assessed by the same trained and experienced embryologist. The association between miRNA abundance in spermatozoa and other data from male patients, such as age, body mass index (BMI) and sperm quality parameters (sperm concentration, sperm motility, sperm viability, sperm membrane integrity, semen volume, and high motility sperm concentration), as well as with embryo quality and embryo metabolite consumption or release, were evaluated by computing Pearson correlation coefficients (R) assuming a Gaussian distribution and a confidence interval of 95%. A p -value of less than 0.05 indicates statistical significance, while a p -value less than 0.10 suggests a notable tendency toward significance. The statistical analysis was performed using GraphPad Prism 9 (GraphPad Software Inc.).

3 | RESULTS

3.1 | Clinical reproductive data and embryo quality characterization

As depicted in Table S1, 13 ICSI cycles were analyzed in this study from a total of 13 sperm samples. Treatments adhered to clinical indications, and fertilization occurred via the ICSI technique to standardize sperm cell influence on metabolomic quantification. The embryo culture extended to day 5. The cycle characteristics and female partner data are detailed in our previous work,¹⁶ and relevant data for this study are summarized in Table S1. Despite all investigations carried out in the male partner or embryo, maternal influence was minimized to the greatest extent possible, with no notable differences observed in age, the quantity of retrieved oocytes, or the number of metaphase II (MII) stage oocytes among the female partners.

Culture medium samples from a total of 20 oocytes were analyzed, corresponding to the sequential culture of the embryos for 5 days. Degenerated, non-cleavage, abnormal fertilization, and parthenogenetic embryos were excluded from the statistical analyses. Embryo classification followed pre-determined criteria, as follows: a blastocyst was deemed "good" if BL3BB or better (BL5-AA, AB, BA, BB; BL4-AA, AB, BA, BB; BL3-AA, AB, BA, BB), while "bad" if it had inner cell mass and/or trophectoderm grade C (BL5/BL4/BL3-AC, CA, CC); embryos were labeled as "lagging" if their development was delayed per Istanbul Consensus standards.²⁵ Hence, out of the 20 observed embryos, nine of these were categorized as good, seven as lagging, and four as bad embryos. No statistically significant differences in age of the men or the women, sperm concentrations, total sperm motility and vitality, female and male baseline evaluation, and number of retrieved oocytes were noted among donors for the three established groups.

3.2 | Content of miR-34c-5p in human spermatozoa exhibits a correlation with total sperm motility

The presence of *miR-34c-5p* and *miR-191-3p* was previously identified in human spermatozoa.^{6,7} In our work, we further confirmed that these miRNAs are present in human spermatozoa by a conventional PCR (data not shown). We then evaluated the association of sperm *miR-34c-5p* and *miR-191-3p* content with the age and BMI of the individual and we found no significant correlation of sperm *miR-34c-5p* and *miR-191-3p* content with any of the analyzed anthropometric data of the male individuals (Figure 1 and Table S2).

Given that sperm parameters serve as dependable markers for male fertility potential, we conducted an analysis to examine the relationship between the abundance of *miR-34c-5p* and *miR-191-3p* and various seminal parameters, including semen volume, total sperm concentration, total sperm motility, highly motile sperm concentration, sperm vitality, and membrane integrity. Moreover, we found no correlation of sperm *miR-191-3p* content with any of the seminal quality

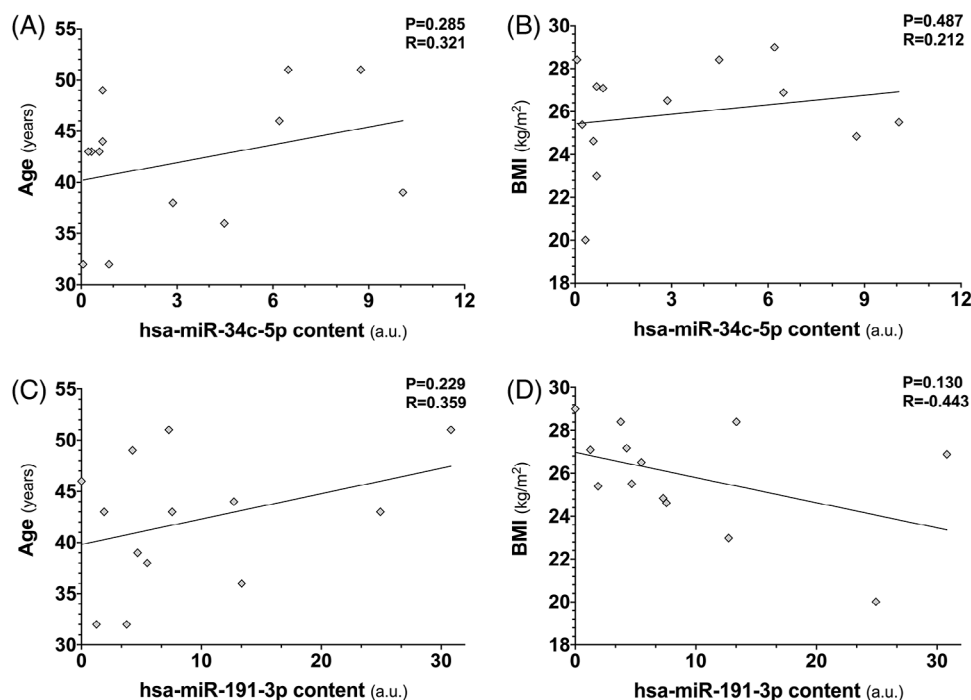


FIGURE 1 Association of hsa-miR-34c-5p or miR-191-3p content in human spermatozoa with the body mass index (BMI) and age of the male individuals. The figure shows the distribution of the hsa-miR-34c-5p or miR-191-3p abundance of spermatozoa with the individual's age (A or C) and BMI (B or D). Data were collected from 13 patients with an average age of 41 years (range 31–51 years) and an average BMI of 25.5 (range 20–29 kg/m²). The relationship between hsa-miR-34c-5p abundance and the different parameters was evaluated by computing the Pearson correlation coefficients (*R*), assuming a Gaussian distribution (confidence interval of 95%). The correlation coefficient (*R*) and the significance level (*p*-value) of the correlation are indicated. A *p*-value of less than 0.05 indicates statistical significance, while a *p*-value less than 0.10 suggests a notable tendency toward significance.

data (Table S2). In contrast, when we evaluated the association with *miR-34c-5p*, we could identify a significant negative correlation of this miRNA with total sperm motility ($p = 0.040$; $R = -0.600$) and a positive tendency toward significance of its association with the concentration of highly motile spermatozoa ($p = 0.063$; $R = 0.529$) (Figure 2).

3.3 | Content of *miR-34c-5p* in human spermatozoa differentially correlates with the release of monocarboxylates by embryos during in vitro culture

Studies suggest that sperm miRNA content influences gene expression patterns in early embryos, potentially affecting embryo development.²⁶ Hence, we evaluated the correlation between the content of the selected miRNA and embryo metabolic performance by assessing the exometabolome during in vitro culture.

When we evaluated the association of *miR-34c-5p* sperm content with metabolite variation in the embryo culture media, we could identify a significant correlation of this miRNA with the release of four monocarboxylates into the media by embryos during in vitro culture, with contrasting results (Figure 3). While the release of both pyruvate and acetate by embryo was positively correlated with *miR-34c-5p* sperm content ($p = 0.001$; $R = 0.639$ and $p = 0.021$; $R = 0.458$), the

release of formate was negatively correlated ($p = 0.002$; $R = 0.586$) and that of lactate showed decrease tendency toward significance when associated with the content of *miR-34c-5p* on human spermatozoa ($p = 0.063$; $R = 0.529$) (Figure 3). Conversely, apart from aspartate and isoleucine, no significant correlation or tendency was observed on the consumption of metabolites by cultured embryos and *miR-34c-5p* sperm content (Table S3). Aspartate consumption showed a negative association with the content of this miRNA on spermatozoa ($p = 0.048$; $R = -0.399$), while isoleucine consumption showed a decrease tendency toward significance when associated with the content of *miR-34c-5p* on human spermatozoa ($p = 0.067$; $R = -0.372$) (Figure 3).

3.4 | *miR-191-3p* content in human spermatozoa shows a positive association with the consumption of glucose by embryos

We also evaluated the association of *miR-191-3p* sperm content with metabolite variation in the embryo culture media. Although we found no significant correlation with the variation of most metabolites in the media by embryos during in vitro culture (Table S3), we could observe a significant positive correlation of the sperm content for this miRNA with the consumption of glucose ($p = 0.023$; $R = 0.453$) by embryos

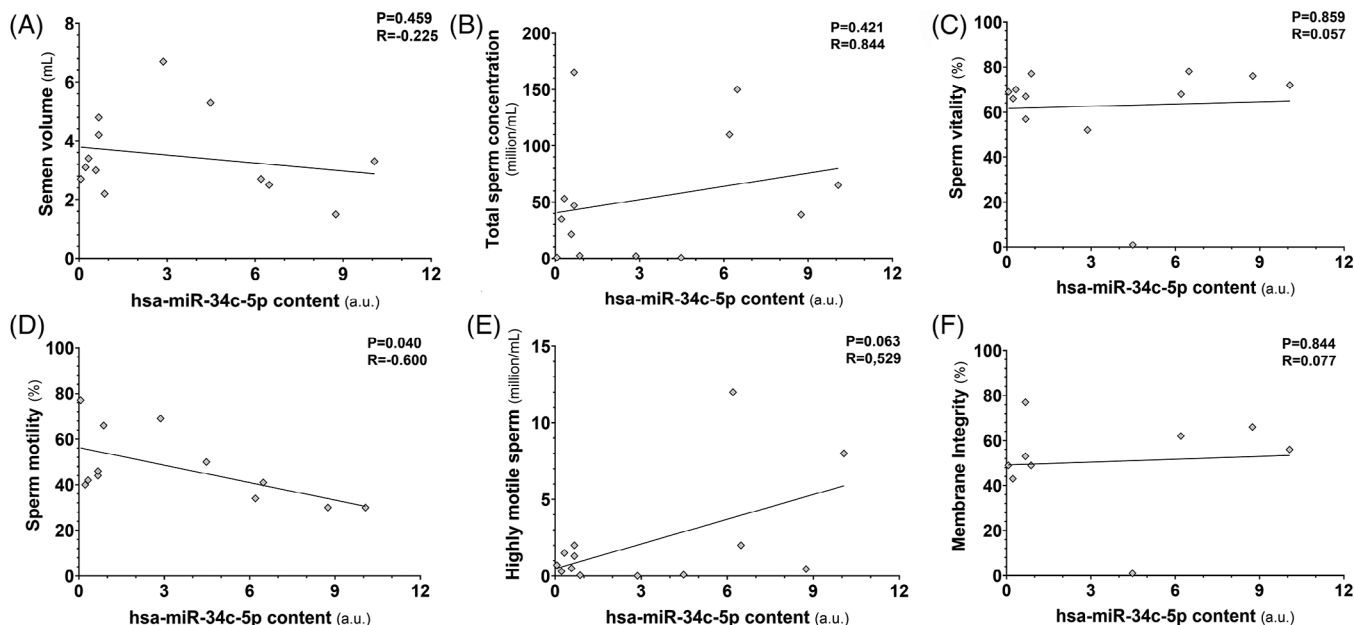


FIGURE 2 Association of hsa-miR-34c-5p content in human spermatozoa with seminal volume and sperm quality parameters. The figure shows the correlation of the hsa-miR-34c-5p abundance of spermatozoa with classic seminal parameters, including seminal volume (A), total sperm concentration (B), sperm vitality (C), sperm motility (D), concentration of highly motile spermatozoa (E), and sperm membrane integrity (F). Sperm parameter data were collected from 13 patients with an average age of 41 years (age range 31–51 years) and an average body mass index (BMI) of 25.5 (range 20–29 kg/m²). The relationship between hsa-miR-34c-5p abundance and the different parameters was evaluated by computing the Pearson correlation coefficients (*R*), assuming a Gaussian distribution (confidence interval of 95%). The correlation coefficient (*R*) and the corresponding significance level (*p*-value) are provided. A *p*-value of less than 0.05 indicates statistical significance, while a *p*-value less than 0.10 suggests a notable tendency toward significance.

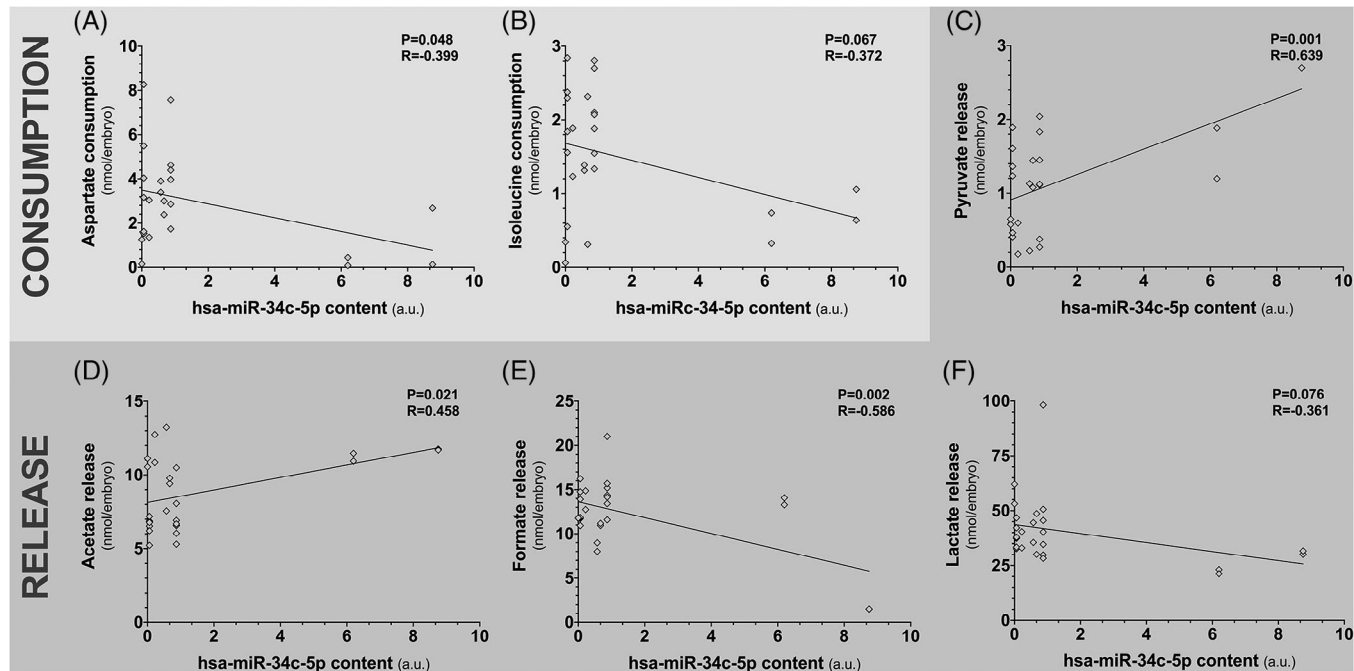


FIGURE 3 Association of hsa-miR-34c-5p content in human spermatozoa with metabolite variation in embryo culture media. The figure shows the correlation between the variation (consumption or release) of selected metabolites (panel A: aspartate; panel B: isoleucine; panel C: pyruvate; panel D: acetate; panel E: formate; panel F: lactate) quantified in the embryo culture media at the end of day 5 of *in vitro* culture. Sperm miRNA data were collected from 13 patients, which resulted in 20 embryos (*n* = 20). The relationship between hsa-miR-34c-5p abundance and the variation of the different metabolite in embryo culture media was evaluated by computing the Pearson correlation coefficients (*R*), assuming a Gaussian distribution (confidence interval of 95%). The correlation coefficient (*R*) and the corresponding significance level (*p*-value) are provided. A *p*-value of less than 0.05 indicates statistical significance, while a *p*-value less than 0.10 suggests a notable tendency toward significance.

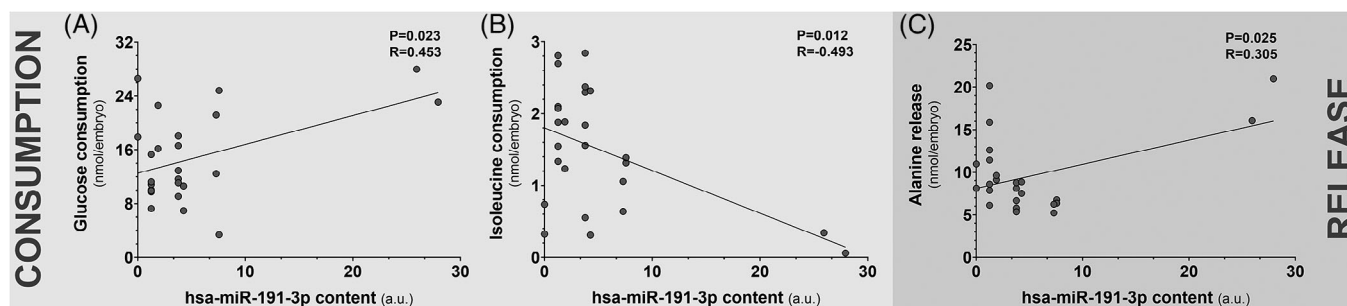


FIGURE 4 Association of hsa-miR-191-3p content in human spermatozoa with metabolite variation in embryo culture media. The figure shows the correlation between the variation (consumption or release) of selected metabolites (panel A: glucose; panel B: isoleucine; panel C: alanine) quantified in the embryo culture media at the end of day 5 of in vitro culture. Sperm miRNA data were collected from 13 patients, which resulted in 20 embryos ($n = 20$). The relationship between hsa-miR-191-3p abundance and the variation of the different metabolite in embryo culture media was evaluated by computing the Pearson correlation coefficients (R), assuming a Gaussian distribution (confidence interval of 95%). The correlation coefficient (R) and the corresponding significance level (p -value) are provided. A p -value of less than 0.05 indicates statistical significance, while a p -value less than 0.10 suggests a notable tendency toward significance.

during in vitro culture, contrasting with the negative correlation observed for isoleucine consumption ($p = 0.012$; $R = -0.493$). Concurrently, alanine release showed a positive association with the content of this miRNA on spermatozoa ($p = 0.025$; $R = 0.305$) (Figure 4).

3.5 | Increased sperm miR-34c-5p content correlates with improved in vitro embryo development

We further evaluated if the content of human sperm miR-191-3p and miR-34c-5p was correlated with embryo development quality in vitro (Figure 5). This analysis revealed that among embryos categorized as good ($n = 9$), lagging ($n = 7$), or bad ($n = 4$), those derived from spermatozoa with higher levels of miR-34c-5p exhibited improved quality. The content of miR-34c-5p in sperm samples that originated good embryos was 2.99 ± 1.13 a.u., while that that originated both lagging and bad embryos was significantly lower (0.25 ± 0.10 and 0.26 ± 0.07 a.u., respectively). Conversely, no significant differences were observed in the content of sperm miR-191-3p that originated the different embryo quality categories (good, 4.17 ± 0.96 a.u.; lagging, 3.39 ± 0.33 a.u.; and bad, 3.64 ± 0.85 a.u.).

4 | DISCUSSION

Sperm selection techniques for assisted reproductive technologies aim to isolate the most competent spermatozoon, which should be characterized by the highest fertilizing capacity. The prevailing approaches are based mostly on morphofunctional evaluation, which offers a substantial degree of subjectivity. This inherent variability can lead to the potential misselection, thereby contributing to a reduction in the overall success rates of ART treatments, highlighting the relevance of finding new biomarkers for gamete quality and its fertility potential. Integrating these biomarkers into selection protocols may improve treatment outcomes by enhancing the accuracy of sperm selection and

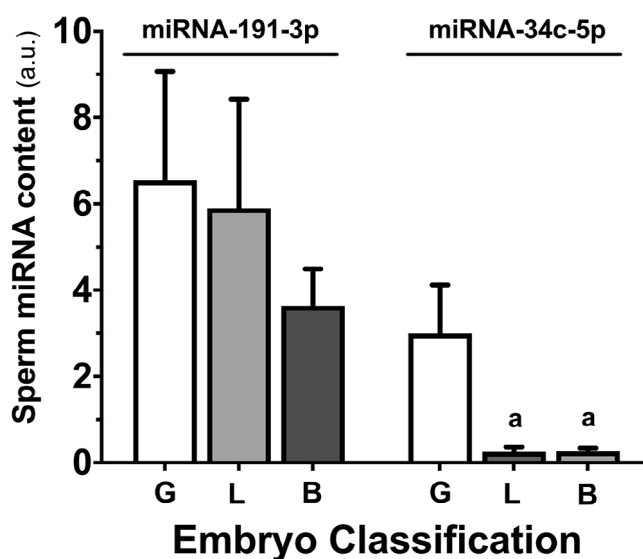


FIGURE 5 MicroRNA (miRNA) content in human spermatozoa used for obtaining embryos grouped according to their quality at day 5 (good [G] vs. lagging [L] vs. bad [B]). The figure shows the content of hsa-miR-191-3p and hsa-miR-34c-5p in human spermatozoa that originated embryos categorized as G ($n = 9$), L ($n = 7$), or B ($n = 4$) after 5 days of in vitro development. Data are presented as mean $\pm$ standard error of the mean (SEM). Normality test was performed, and univariate ANOVA was conducted. Pairwise comparisons between groups were corrected for multiple hypotheses testing by Tukey's post hoc test. A p -value of less than 0.05 indicates statistical significance, while a p -value less than 0.10 suggests a notable tendency toward significance (a: vs. good embryos).

minimizing the risk of embryo implantation failure, miscarriage, and developmental abnormalities.²⁷ The relevance of the use of molecular biomarkers in sperm selection for assisted reproductive technologies has been debated in the last decades^{27,28} in the perspective of developing tools that allow the objective and evidence-based insights into sperm quality, allowing the identification of optimal sperm candidates for fertilization. Within this context, researchers have explored

sperm miRNAs because of their regulatory roles in sperm function and early embryonic development,^{26,29,30} aiming to identify specific signatures associated with sperm quality, fertilization potential, and embryo development competence. In this study, we focused on the association between sperm content in specific miRNAs, sperm quality parameters, and embryo development (with a particular emphasis on embryo metabolic performance), in the expectation to offer valuable insights into potential clinical implications for couples using ART. We chose two sperm-borne miRNAs, miR-34c-5p and miR-191-3p, based on their distinct characteristics regarding their contribution to the embryo's miRNA pool. Although both miRNAs have been linked to embryogenesis,^{6,9} miR-191-3p exhibits consistent expression,¹³ while miR-34c-5p has been reported to be absent in human oocytes.¹⁴ We first evaluated the content of both miR-191-3p and miR-34c-5p in spermatozoa and explored their correlation with male anthropometric profiles (see Table S1). Although no significant results were obtained for the correlation of both age or BMI with the content of these two miRNAs in human spermatozoa, the content of miR-34c-5p was correlated with specific sperm quality parameters. In fact, it has been reported that several miRNAs have implications in sperm physiology, including sperm motility and morphology, although the exact function of these miRNAs in spermatozoa is yet to be fully understood. It has been proposed that they might represent residues from spermatogenesis, preserved for potential future involvement in fertilization, or they could be transferred to the spermatozoon (for instance, in the epididymis) to exert influence on early embryonic development.³¹ In fact, our results highlighted a negative association for miRNA-34c-5p with total sperm motility. Data from Curry et al. highlighted a similar trend, establishing a link between increased seminal content of this miRNA and asthenozoospermia,³¹ while others found that higher levels of this sperm-borne miRNA are positively associated with other sperm quality parameters. Specifically, low levels of miR-34c-5p are linked with reduced sperm count, reduced motility, and increased abnormal forms.³² miRNA-34c-5p is a member of the miR-34 family, which is also composed of miR-34a and miR-34b, which have highly homologous sequences and share similar target genes and functions.³³ While data on their molecular targets in testicular cells are scarce, in other cellular systems these miRNAs were found to potentially target the expression of members of the NOTCH signaling pathway, inhibiting events such as cell proliferation, motility, and invasion.^{34,35} Previous results have shown that NOTCH signaling blockade was associated with a reduction in sperm motility, prompting for a role of for this signaling pathway during sperm maturation in the epididymis.³⁶ Hence, although further studies are needed to validate our assumption, the negative association we observed between miRNA-34c-5p and total sperm motility may root its essence in the disruption of NOTCH signaling pathway.

As previously discussed, the two sperm-borne miRNAs we selected for study have also been associated with embryogenesis, although research on their molecular action in embryo development is relatively limited compared to their extensively studied functions in other biological contexts. While miRNA-34c-5p is known to regulate critical cellular processes such as cell metabolism, cell cycle progression, and apoptosis, its involvement in embryonic development has scarcely

been addressed, with some studies suggesting that members of the miR-34 family are associated with various developmental disorders and pregnancy complications, highlighting its potential relevance to embryo quality.³⁷ Additionally, dysregulation of the miRNAs of this family seems to play a role in modulating embryonic stem cell differentiation and lineage commitment, which could indirectly impact embryo development.³⁸ miR-34b/c have been reported in spermatozoa, but not in oocytes, while miR-34c has been identified as crucial for the first embryo cleavage division *in vitro*.³⁷ Indeed, spermatozoa-delivered miR-34c has been associated with preimplantation embryonic development, affecting diverse biological processes such as mRNA degradation and cell proliferation.³⁹ Higher levels of miR-34c-5p (when compared with miR-449b-5p) in spermatozoa were associated with an increase in the probability of obtaining viable embryos.³² However, limited information is available regarding its impact on embryo metabolism.

In a recent study by Cui et al.,⁴⁰ it was reported that microinjection of an miR-34c inhibitor resulted in impaired embryonic development, accompanied by reduced expression of genes related to cellular metabolism, particularly during the initial phases of embryo development. Specifically, these affected genes were primarily associated with lipid metabolism, including processes involved in very long-chain fatty acids, as well as with responses to carbohydrates, highlighting that the miR-34c inhibitor affected energy metabolism in these early-stage embryos.⁴⁰ Hence, these authors hinted that sperm-borne miR-34c-5p may have a role in embryonic metabolism, an event crucial for embryo development. In our study, we were able to follow the consumption (glucose, isoleucine, valine, and aspartate) or the release (pyruvate, lactate, acetate, formate alanine, and glutamine) of these water-soluble metabolites into the culture medium during the 5 days of embryo *in vitro* development and correlate this metabolic performance with the content of the selected sperm-borne miRNAs. We observed that the miRNA-34c-5p content was positively associated with the release of two key monocarboxylates, pyruvate and acetate. Acetate has been shown to be an important energy source for embryo development.⁴¹ Mammalian cells can produce acetate from acetyl-CoA, a crossroad metabolite of lipid and carbohydrate metabolism.⁴² An increased expression of genes linked to lipid metabolism prompted by miR-34c-5p, as suggested by Cui et al.,⁴⁰ could explain the observed release of acetate by embryos. In fact, the promotion of lipid metabolism, with the concurrent increase in acetyl-CoA could result in the increased production/release of acetate. The increased production of acetyl-CoA would also inhibit the conversion of pyruvate into this thioester, leading to the putative accumulation and export of pyruvate, as observed. In embryonic development, pyruvate serves as a vital energy substrate during preimplantation stages, associated with enhanced embryo quality in certain species such as cattle and sheep.⁴³ Although its significance in early human embryonic development remains less understood in the presence of other metabolic sources, exporting and accumulating pyruvate in culture media is surely a sign that embryos exhibit active metabolism. On the other hand, the release of formate (another monocarboxylate) is negatively correlated with miR-34c-5p content. During early embryonic development, formate is produced by the mitochondria and some studies have suggested that formate levels are

a biomarker for embryonic viability in ART, as its concentration in culture media correlates with embryo metabolic activity and may indicate embryonic quality and viability under certain conditions.⁴⁴ It is known that mammalian cells produce formate as a byproduct of several metabolic processes, particularly amino acid breakdown. Hence, the observed correlation for formate might be linked with the concurrent negative correlation observed between the sperm miR-34c-5p content and the consumption of the two amino acids aspartate and isoleucine (showing a notable tendency toward significance). Although there is still much progress to be made to enlighten the significance of amino acids such as aspartate on embryo metabolism, advancements have been achieved in elucidating the roles of these metabolites,⁴⁵ which were initially viewed solely as biosynthetic precursors but are now understood to possess multiple, interconnected functions that are intimately connected with embryo during in vitro development.^{16,46}

Similar to that of miR-34c-5p, the role of miR-191-3p has been extensively studied in various biological contexts, including cancer and other diseases, but its involvement in embryonic development is not as well understood. However, some studies have suggested that miRNA-191-3p may play a role in early embryogenesis and may contribute to the regulation of genes involved in cell proliferation, differentiation, and apoptosis, which are critical processes for embryo development and quality.⁴⁷ In our work, sperm miR-191-3p content did not exhibit any correlation with sperm quality parameters, in contrast to what we reported for miR-34c-5p. Nevertheless, we observed that sperm-borne miR-191-3p content was also associated with the metabolic performance of embryos. Embryos with higher content of this miRNA exhibited a higher consumption of glucose (and a lower consumption of isoleucine), concurrent with a lower release of alanine.

Glucose is known to be vital for human embryo development because of its role as a primary energy source. During early embryogenesis, glucose is the key provider of the necessary energy for various cellular processes, including cell proliferation and differentiation. Additionally, glucose metabolism fuels the rapid growth and development of the embryo as it progresses through different stages of development, emphasizing the importance of proper glucose availability and metabolism for supporting healthy embryo development in humans.⁴⁸ To the best of our knowledge, no data are available concerning the role of this miRNA on embryo glucose metabolism; however, miR-191-3p seems to play a role in glucose metabolism, as it is known to be a key regulator of glucose homeostasis in various systems.⁴⁹ Our results suggest that this miRNA is involved in modulating glucose metabolism also in developing embryos. Indeed, it also correlates (negatively) with the consumption of isoleucine and with the release of alanine, a byproduct of glucose metabolism. Alanine is a non-essential amino acid whose export by human embryos reflects its metabolic state and its ability to maintain energy balance during early development.¹⁶ It has been suggested that the export of alanine may serve as a protective mechanism against stress and nutrient deprivation, allowing the embryo to maintain its viability and developmental potential,⁵⁰ which might what is happening in our high-metabolism embryos.

Hence, when we evaluated the association of the sperm content for these two miRNAs with embryo developmental quality, as assessed by

means of morphokinetic evaluation, we observed that good embryos were derived from spermatozoa with a higher content of miR-34c-5p (as compared with that of lagged or bad embryos). This association was not observed for sperm miR-191-3p content, in contrast to the study by Xu et al.⁶ This difference might be because the studied populations are very distinct because, for instance, this former study used only normozoospermic men and because of discrepancies in the method of evaluation of embryo quality. Beyond the inherent subjectivity of the evaluation of embryos, the 2020 study used a method described by Veeck and Zaninovic,⁵¹ which rated the development of embryo on the third day, while we monitored the embryos from fertilization to day 5 using a time-lapse system and the Istanbul Consensus,²⁵ as we described previously in detail.¹⁶ These differences further highlight the need for the establishment of solid molecular biomarkers for gamete and embryo quality. In this sense, our results emphasize the potential significance of sperm miR-34c-5p content as a biomarker of not only sperm quality but also embryo developmental potential. We must note that our study did not account for different ovarian stimulation treatments and their impact on oocyte quality, which could be seen as a limitation. Controlled ovarian stimulation protocols vary widely, and patient profile heterogeneity complicates establishing an ideal protocol,⁵² as can be seen in our study. However, evidence suggests no significant differences in ART outcomes between rFSH and urinary gonadotrophins,⁵³ as well as between long-acting FSH and daily injections.⁵⁴ Furthermore, no clear differences in live birth rates were found between rLH combined with rFSH and rFSH alone.⁵⁵ This supports our decision not to include these variables in our evaluation.

5 | CONCLUSION

This study explored how sperm-borne microRNAs may be related to male reproductive factors and the quality of embryo development. First, how the sperm microRNA content affects sperm motility, a crucial aspect of male fertility, was studied. This study showed a significant negative link between miR-34c-5p levels and overall sperm motility, suggesting that irregular microRNA expression in spermatozoa could impact sperm function and fertility outcomes. Additionally, we examined how sperm microRNA content influences embryo metabolism during in vitro culture. By studying the substances released by cultured embryos, the researchers have identified connections between sperm microRNAs and specific metabolites released by embryos. Of particular interest is the link between miR-34c-5p levels in spermatozoa and the release of pyruvate, acetate, and formate, indicating a potential role for sperm microRNAs in shaping embryonic metabolic pathways. Furthermore, this study explored how sperm microRNA content is related to the quality of embryo development in vitro. A correlation was found between higher miR-34c-5p levels in sperm samples and better embryo morphokinetic grading, suggesting that analyzing sperm microRNAs could help predict embryo developmental competence.

Overall, these findings deepen our understanding of how sperm microRNAs influence male fertility and embryo development. These findings suggest the possibility of using sperm microRNA profiles as an

additional tool to complete embryo assessment and improve in vitro fertilization success rates. Understanding the intricate role of sperm microRNAs in embryo quality holds promise for improving assisted reproductive technique outcomes. By identifying specific microRNA signatures associated with optimal embryo development, one could aim at developing novel diagnostic tools and therapeutic interventions to enhance embryo quality and increase the success rates of assisted reproductive technique procedures. However, more research is needed to confirm these findings in larger groups and understand the molecular mechanisms behind microRNA effects on reproductive outcomes.

AUTHOR CONTRIBUTIONS

Conceptualization: Soraia Pinto, Marco G. Alves, and Pedro F. Oliveira. **Methodology:** Soraia Pinto, Sara C. Pereira, Marco G. Alves, and Pedro F. Oliveira. **Formal analysis:** Soraia Pinto and Sara C. Pereira. **Investigation:** Soraia Pinto. **Writing—original draft preparation:** Soraia Pinto and Pedro F. Oliveira. **Writing—review and editing:** Marco G. Alves, António Rocha, Alberto Barros, and Pedro F. Oliveira. **Supervision:** António Rocha, Alberto Barros, and Pedro F. Oliveira. **Project administration:** Pedro F. Oliveira. **Funding acquisition:** Marco G. Alves and Pedro F. Oliveira. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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2. Lin, S.-K. *Editorial: Non-Open Access and Its Adverse Impact on Molecules*. *Molecules* **2007**, 12, 1436-1437 (PDF format 16 K, HTML format).

3. A research paper of 30 pages has been published: ***Molecules* 2008, 13(5), 1081-1110 (/1420-3049/13/5/1081)**.

4. Some well written papers have been peer reviewed and published in less than two weeks from manuscript submission, see e.g.: [**Molecules** 2006, 11\(4\), 212-218 \(/1420-3049/11/4/212\)](#).

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