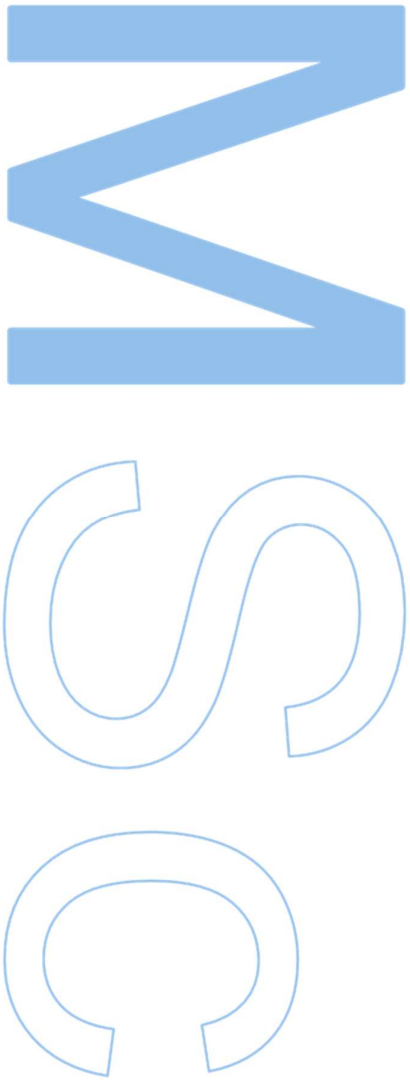
Unravelling biomarkers for Obesity-related inheritance of Prostate dysfunction and cancer



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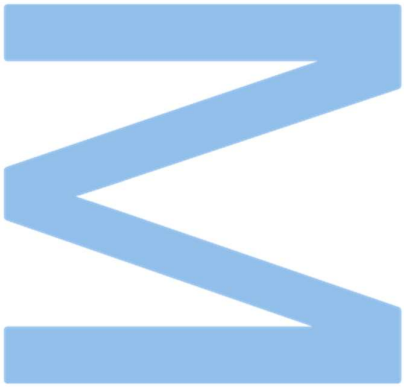

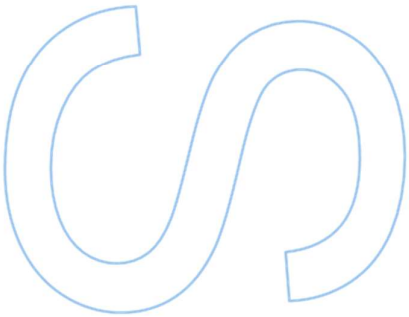

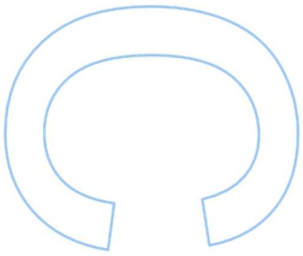

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Resumo

A obesidade promove desregulação hormonal, um estado crónico de inflamação e stress oxidativo (OS). Notavelmente, estes fatores também estão relacionados com o desenvolvimento do Cancro da próstata (PCa), um dos cancros mais comuns e fatais entre os homens. Fatores genéticos e epigenéticos associadas com a obesidade podem ser transmitidos transgeracionalmente. Tendo isto em conta, tem sido colocada a hipótese de estes fatores poderem ser associadas ao desenvolvimento de PCa em descendentes de pais com obesidade. Assim, o objetivo deste trabalho é estudar os efeitos da exposição a uma dieta rica em gordura (HFD), ao longo da vida ou apenas até à fase adulta, em biomarcadores distintos de PCa [nomeadamente Homeobox B13 (HOXB13) e o Recetor de Androgénio (AR)]. Pretendemos ainda perceber se existe uma correlação desses marcadores na próstata com a obesidade, estudando a expressão do gene de obesidade e massa de gordura associada (FTO) e do fator de necrose tumoral alfa (TNF-α). Por último, pretendemos averiguar se os efeitos na próstata, induzidos por mudanças na dieta, podem ser potencialmente transmitidos aos descendentes. Para testar esta hipótese, um modelo transgeracional com *Mus musculus* foi estabelecido, onde machos F0 foram expostos a diferentes dietas durante 200 dias: controlo, HFD e dieta transitória [HFD_t (60 dias de HFD, seguidos de 140 dias de dieta controlo)]. Biomarcadores de PCa foram avaliados por reação em cadeia da polimerase quantitativa. Mais ainda, os níveis de metilação do promotor do HOXB13 foram analisados, e o OS foi avaliado no tecido prostático através da nitração proteica e peroxidação lipídica. Por fim, a análise de dados foi efetuada utilizando a análise de variância, seguida do teste Tukey post-hoc para comparação múltipla. Os resultados revelaram que a expressão do *AR* na próstata não foi afetada pelos regimes alimentares nos progenitores nem nos seus descendentes. Por outro lado, a expressão do *HOXB13* apresentou um aumento na próstata de ratinhos alimentados com HFD até à fase adulta, enquanto os seus filhos apresentaram uma diminuição significativa, quando comparados com os filhos do grupo alimentado com HFD. Concordantemente, uma correlação positiva foi encontrada entre a expressão do *HOXB13* e do *AR* no tecido prostático de ratinhos de F0 e F1. Mais ainda, a expressão do *FTO* estava aumentada na próstata do grupo HFD_t, enquanto os seus descendentes apresentaram uma diminuição significativa em comparação com os filhos do grupo alimentado com HFD. Notavelmente uma correlação positiva foi encontrada entre a expressão do *FTO* e os biomarcadores de PCa. Enquanto isso, apesar dos níveis de metilação do promotor do *HOXB13* não serem afetados pelas alterações de dieta, os níveis de metilação encontravam-se correlacionados positivamente com a expressão do *FTO* e do *HOXB13*.

Ademais, a expressão do *TNF-α* apresentou uma alteração significativa em F1 nos grupos HFD e HFD_t, revelando uma mudança em direção à inflamação. Consideravelmente, a expressão do *TNF-α* encontrava-se correlacionada positivamente com a expressão do *FTO* e *HOXB13* em F0 e F1, e mesmo com a expressão do *AR* em F0. Finalmente, enquanto a nitração proteica não foi significativamente alterada, a peroxidação lipídica apresentou alterações ao longo de todas as gerações, nomeadamente nos grupos de HFD e HFD_t. Em conclusão, o nosso trabalho destaca a interação entre fatores relacionados com a obesidade, inflamação, e o OS, bem como a epigenética no contexto de biomarcadores do PCa. Mais ainda, os nossos resultados sugerem que poderá existir um risco de aumento da expressão de biomarcadores de PCa em filhos de pais com obesidade. Notavelmente, o nosso estudo aumenta a necessidade para um melhor conhecimento, não só dos impactos de fatores da dieta na saúde atual, bem como em efeitos duradouros em futuras gerações.

Palavras-chave: Obesidade, Cancro da próstata, Transgeracional, Epigenética

Abstract

Obesity promotes hormonal dysregulation, a chronic state of inflammation and oxidative stress (OS). Notably, these factors are also linked to the development of Prostate Cancer (PCa), one of the most common and deadly cancers among men. Genetic and epigenetic factors associated with obesity can be transgenerationally transmitted. Given that, it is hypothesized that these factors can also be related to the development of PCa in the offspring of fathers with obesity. Hence the goal of this work is to study the effects of a High-fat diet (HFD) exposure, throughout life or until adulthood, in distinct PCa biomarkers [namely the Homeobox B13 (HOXB13) and the Androgen Receptor (AR)]. We also intend to perceive if a correlation exists between these biomarkers and obesity in the prostate, by studying the expression of the Fat Mass and obesity associated gene (FTO) and the Tumor Necrosis Factor-Alpha (TNF-α). Lastly, we intend to determine if effects induced by dietary changes could be potentially transmitted to offspring. To test this hypothesis, a transgenerational *Mus musculus* model was established, with F0 male exposed to different diets for 200 days: standard, HFD, and transient diet [HFD_t (60 days of HFD, followed by 140 days of standard diet)]. PCa biomarkers were assessed by quantitative polymerase chain reaction. Moreover, HOXB13 promoter methylation levels were evaluated, and OS was examined via protein nitration and lipid peroxidation. Finally, data analysis was performed using analysis of variance, followed by a Tukey post-hoc test for multiple comparisons. Results revealed that *AR* expression in the prostate was no affected by the dietary regimens in the progenitors nor their offspring. On the other hand, *HOXB13* expression presented an increase in the prostate of mice fed with HFD until adulthood, whereas their offspring presented a significant decrease when compared to the offspring of the group fed with HFD. Concurrently, a positive correlation was found between the expression of *HOXB13* and *AR* in the mice prostate tissue of F0 and F1. Moreover, *FTO* expression was increased in the prostate of the HFD_t group, while their offspring presented a significant decrease in comparison to the sons of the group fed with HFD. Notably, a positive correlation was found between the expression of *FTO* and the PCa biomarkers. Meanwhile, even though *HOXB13* promoter methylation levels were not affected by the dietary changes, the methylation levels were positively correlated with *FTO* and *HOXB13* expression. Furthermore, *TNF-α* expression presented a significant alteration in F1 in the HFD and HFD_t groups, revealing a switch towards inflammation. Considerably, *TNF-α* expression was positively correlated with the expression of *FTO* and *HOXB13* in F0 and F1, and even with *AR* expression in F0. Finally, while protein nitration was not significantly altered, lipid peroxidation presented alterations across all generations, particularly in the HFD and HFD_t groups. In conclusion,

our work highlights the interaction between obesity-related factors, inflammation, and OS, as well as epigenetics within the context of PCa biomarkers. On top of that, our results suggest that there could exist a risk for increased expression of PCa biomarkers in sons of fathers with obesity. Notably, our study enhances the need for a thorough understanding not only of the impacts of dietary factors on present health but also of lasting effects on future generations.

Key-words: Obesity, Prostate cancer, Transgenerational, Epigenetic

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List of Abbreviations

17β-estradiol	E2
Adenosine Monophosphate-activated Protein Kinase	AMPK
Analysis of Variance	ANOVA
Androgen-Deprivation Therapy	ADT
Androgen Receptor	AR
Androgen-Resistant Prostate Cancer	ARPC
Antioxidant Response Elements	ARE
Benign Prostatic Hyperplasia	BPH
Body Mass Index	BMI
Castration-Resistant Prostate Cancer	CRPC
Control	CTRL
Digital Rectal Examination	DRE
DNA Methyltransferase 1	DNMT1
Estrogen Receptor	ER
Fat Mass and Obesity Gene	FTO
Fatty Acid Synthase	FASN
Follicle-Stimulating Hormone	FSH
Glutathione Peroxidase	GPx
Glutathione-S-transferase	GST
Glutathione-S-transferase-P1	GSTP1
Gonadotropin-Releasing Hormone	GnRH
Hepatocellular Carcinoma	HCC
High-Fat Diet	HFD
Homeobox B13	HOXB13

Hypothalamic-Pituitary-Gonadal	HPG
Insulin-like Growth Factors	IGFs
Insulin-like Growth Factor Binding Proteins	IGFBP
Interleukin-1	IL-1
Interleukin-6	IL-6
Luteinizing Hormone	LH
Mammalian Target of Rapamycin	mTOR
Methylation-Specific PCR	MSP
Mitogen-Activated Protein Kinase	MAPK
N6-methyladenosine	m6A
Nuclear Factor Kappa B	NF-kB
Oxidative Stress	OS
Periprostatic Adipose Tissue	PPAT
Phosphate Buffer Saline	PBS
Phosphatidylinositol 3-kinase	PI3K
Polymerase Chain Reaction	PCR
Prostate Cancer	PCa
Prostate-specific Antigen	PSA
Prostatic Intraepithelial Neoplasia	PIN
Quantitative Methylation-Specific PCR	qMSP
Reactive Oxygen Species	ROS
Real-Time Polymerase chain reaction	qPCR
Sex Hormone-binding Globulin	SHBG
Signal Transducer and Activator of Transcription 3	STAT3
Single-nucleotide Polymorphisms	SNPs
Sterol Regulatory Element-binding Proteins	SREBPs

Transfer-RNA Derived Small RNA	tsRNA
Transient High-fat Diet	HFD_t
Transmembrane Leptin Receptor	ObR
Tumor Necrosis Factor-Alpha	TNF-α
Type-2 Diabetes	T2D
World Health Organization	WHO

Chapter I

Introduction and Aims

1. Introduction

The prevalence of metabolic disorders, including obesity and overweight, has experienced a substantial surge in recent years, with projections indicating a continued rise in the forthcoming decades. Urbanization, population expansion, aging demographics, and the widespread adoption of Western lifestyle norms stand as the primary drivers behind the escalating occurrence of metabolic irregularities. Unquestionably, there exists a legitimate apprehension regarding the global public health trajectory moving forward. In accordance with the World Health Organization (WHO), obesity is identified by a body mass index (BMI) equal to or exceeding 30, while overweight is characterized by a BMI equal to or surpassing 25 [1, 2]. The global trends report a 50% and 80% increase in overweight and obesity incidence, respectively, in adult individuals (> 20 years old), from 1980 to 2015. In 2015, it was estimated that 39% of the global population was overweight and obese [3]. By 2030, it is predicted that 1.35 billion adults will be overweight, and 573 million individuals will be obese. However, if recent trends continue, the numbers will be higher [4]. The World Obesity Federation has predicted that by 2035, an increase of up to 24% will occur in the prevalence of obesity, expecting nearly 2 billion individuals with metabolic disorders [5]. These trends illustrate the globalization of the Western lifestyle. Obesity/overweight is a multifactorial disease that can be described as a dysregulation of the overall energy homeostasis of the body [6].

This metabolic disorder can emerge as a consequence of shifts in lifestyle patterns. These changes encompass fluctuations in physical activity levels, initially marked by a decline and subsequently, a surge in sedentary behaviours. Additionally, alterations in dietary practices play a pivotal role, exemplified by an escalated consumption of high-calorie diets or overindulgence. These dietary choices, often more affordable and congruent with contemporary living, contribute to the prevailing scenario. However, recent insights have underscored that the genesis of obesity is multifaceted. Beyond lifestyle influences, genetic predisposition, environmental pollutants, hormonal dynamics, pharmaceutical interactions, endocrine disruptors, and factors intricately linked to metabolism have surfaced as intertwined contributors to the onset of obesity [7-9]. In fact, monogenic obesity describes a disorder promoted by single-gene mutations, usually in genes associated with endocrine regulation, that results in an obese phenotype [10]. In parallel, epigenetic markers like DNA methylation and histone modifications exert their influence on genes intertwined with growth and metabolic processes. Studies have documented where anomalies in the imprinting of metabolic genes disrupt their normal

expression patterns, consequently triggering metabolic perturbations, including obesity [11-13]. While our understanding of the precise ramifications of these epigenetic alterations on the progression of metabolic disorders remains in its primary stages, a palpable unease has emerged regarding the metabolic well-being of successive generations. Additionally, obesity/overweight is associated with the development of several other comorbidities, such as type-2 diabetes (T2D), cardiovascular disease, hypertension, infertility, endocrine disruptors, and cancer [14-16]. It is suggested that obesity is responsible for approximately 20% of all cancer cases, and the cause of death of approximately 20% of women and 14% of men [17]. In fact, obesity has surpassed smoking as the most widespread high-risk for carcinogenesis in the United States of America [18].

Excess weight and obesity play a pivotal role in fostering tumour development through multiple molecular pathways. The state of being overweight or obese triggers hormonal dysregulation, culminating in elevated levels of circulating leptin and estrogens. This hormonal imbalance subsequently fuels cell proliferation across various carcinoma types (breast [19, 20], colon [21], pancreas [22], and others), contributing to the intricate network that promotes carcinogenesis. Meanwhile, chronic inflammation associated with obesity leads to the overexpression of inflammatory adipokines by activated macrophages, such as Interleukin-6 (IL-6) and Tumor Necrosis Factor-Alpha (TNF-α), both correlated to cell proliferation and carcinoma migration [23]. These alterations can also help cancerogenic cells to avoid death. Leptin and IL-6 stimuli are known to promote the expression of pro-survival factors such as the B-cell lymphoma-2 (BCL-2) in cancer cell lines [24-26]. Leptin was also found to promote telomerase activity [27]. The reactivation of telomerase stands as a defining trait of cancer cells, conferring upon them an unrestricted capacity for proliferation. In sum, obesity elevates the susceptibility to carcinogenesis by fostering unbounded cell proliferation and fortifying resistance against cell death mechanisms. Nonetheless, we are merely scratching the surface in unravelling these intricate mechanisms.

Among a myriad of other coexisting conditions, men afflicted by obesity exhibit an elevated susceptibility to the onset of Prostate cancer (PCa) [28]. PCa stands as the second most prevalent malignancy and ranks as the fifth primary contributor to male mortality. The global prevalence of PCa continues to surge, with escalating rates not only in its occurrence but also in mortality [29]. Individuals with excess weight and obesity are at higher risk of developing PCa. In addition to the aforementioned mechanisms, the prostate is abundant in insulin receptors. The insulin resistance linked to obesity potentially wields an influence on the progression of PCa. However, substantiating this

assertion necessitates further research and comprehensive studies [30]. Additionally, the hypoandrogenism found in individuals with obesity appears to be correlated with the development of androgen-independent PCa, which severely compromises the effectiveness of hormonal therapy and is associated with worse prognostics [31]. Interestingly, leptin was proposed to be a mediator of androgen-independent PCa proliferation [32, 33]. On the other hand, traditional PCa diagnosis tests have a higher probability of failing in individuals with obesity. This happens due to the increased serum volume found in these individuals, which decreases the serum concentration of Prostate-specific antigen (PSA) leading to a false negative test. Meanwhile, the enlargement of the prostate may affect the digital rectal examination (DRE), leading to a faulty diagnosis [34]. Overall, the increased risk for aggressive PCa development and later diagnosis is reflected in the high mortality rate found for individuals with obesity.

1.1. Deciphering how obesity fuels carcinogenesis - a role for inflammation, hormonal dysregulation, and oxidative stress

Obesity, as defined by the WHO, entails an abnormal and excessive buildup of fat. This atypical accumulation of fat profoundly affects numerous physiological processes, with the undeniable focal point being the extensive impact on adipose tissue. To start, excessive fat accumulation induces adipocyte hypertrophy and hyperplasia [35, 36]. Hyperplasia manifests as a rise in the adipocyte count, while hypertrophy facilitates substantial fat accumulation. Nevertheless, as adipocytes progressively enlarge, blood supply diminishes, precipitating a state of hypoxia [37, 38]. Hypoxia can be a stimulation cause for necrosis and macrophage infiltration into the adipose tissue, leading to an overproduction of pro-inflammatory factors, like inflammatory chemokines (Figure 1). This results in localized inflammation, which evolves into an overall systemic inflammation associated with the development of obesity-related comorbidities [39]. The progressive enlargement of adipocytes eventually results in their rupture and consequent death. This process leads to low-grade chronic inflammation, promoted by the abnormal production of cytokines by the fat-saturated and necrotic adipocytes, which promotes the infiltration and activation of macrophages into the adipose tissue (Figure 1) [40, 41]. IL-6 and TNF-α are pro-inflammatory cytokines associated with white adipose tissue expansion, and saturation [40, 42]. Moreover, IL-6 and TNF are also known to be tumour-promoting cytokines. Park EJ and colleagues explored the role of obesity in the development and expansion of hepatocellular carcinoma (HCC), a form of liver cancer. To explore this phenomenon, mice were subjected to a High-fat diet (HFD) to induce obesity, while HCC was induced through diethylnitrosamine administration. The study's findings unveiled that obesity indeed facilitated the progression of HCC, evidenced by

increased TNF and IL-6 levels detected within the hepatic tissue of these animals. The authors postulated that the escalated TNF and IL-6 concentrations observed in the obese mice acted to amplify the expression of the oncogenic transcription factor, signal transducer, and activator of transcription 3 (STAT3). This cascade of events, involving STAT3 activation, was identified as a pivotal prerequisite for the development of HCC [23]. In fact, tumour-promoting inflammation is one of the hallmarks recognized for cancer development. Before tumour initiation, inflammation can promote a favourable microenvironment in which cancer cells can thrive, stimulating cell proliferation and angiogenesis [40]. Additionally, the activation of the innate response by the damaged adipocytes also promotes a state of oxidative stress (OS) [43]. Pro-inflammatory cytokines produced by the damaged adipocytes, such as TNF-α, IL-6, and Interleukin-1 (IL-1), promote the production of reactive oxygen species (ROS) and reactive nitrogen species by the cells of the innate immune response, increasing lipid peroxidation levels (Figure 1). Furthermore, the production of ROS at sites of inflammation intensifies cellular damage, triggering apoptosis. This, in turn, augments the release of pro-inflammatory cytokines, instigating a self-perpetuating cycle of inflammation [43-45]. The protein, lipid, and DNA damage induced by OS is also associated with neoplastic activation and aberrant cellular proliferation [46-48]. Concurrently, the oxidation of DNA bases promotes the occurrence of mutations and altered DNA methylation patterns, which can promote the abnormal expression of oncogenes [49].

Among the several cytokines that are involved in the process of inflammation, TNF-α, IL-1, and IL-6 are known to carry out important roles in tumour initiation and progression [50]. Nuclear factor kappa B (NF-kB) is a transcription factor that is activated in response to the stimulus of TNF-α, IL-6, and IL-1. NF-kB regulates the expression of genes associated with cell proliferation, inflammation, and apoptosis, being altered in many tumours [51]. More specifically, TNF-α is also associated with the activation of caspase 3. Caspase 3 is proposed to interfere by cleaving the ribosomal protein RPS3, a subunit of the NF-kB complex. This modification retards the translocation of RPS3 into the nucleus, where it would exert its anti-apoptotic effects [52]. Additionally, the TNF-α stimuli promote the development of tissue architecture needed for tumour progression, while promoting the expression of other cytokines and factors leading to increased tumour growth and survival [53, 54]. While the intricate molecular pathways governed by TNF-α remain partially elucidated, targeting TNF-α inhibition emerges as a promising avenue in cancer therapy [55]. Notably, TNF-α and IL-6 exhibit elevated serum concentrations in cancer patients, with both cytokines displaying heightened systemic levels among individuals grappling with obesity [56]. This underscores inflammation as a critical

hallmark of cancer, which is further potentiated by obesity. Consequently, it becomes a strategic focal point for combatting the disease.

In addition to inflammation, obesity is known to promote severe hormonal dysregulation. The adipose tissue is a major player in energy homeostasis regulation and responds to both metabolic and endocrine cues [57]. In fact, adipokines play a crucial role in energy storage and immunity [58]. Adipokines can be released by adipocytes or macrophages infiltrated in the adipose tissue, as a response to fat mass expansion that occurs in obesity [59, 60]. Their profile is modified depending on the adipose tissue changes and their release results in metabolic alterations [61, 62]. Adipokines induce low-grade chronic inflammation, insulin resistance, and the development of obesity-related diseases. Moreover, adipokines have pro- and anti-inflammatory properties [59, 63]. Leptin, an adipokine and growth factor, is known to promote satiety, reduce food intake, and increase metabolic rate, preventing lipid accumulation [64]. Furthermore, its levels are positively correlated with BMI [65]. This adipokine is mainly produced by adipocytes and through hypothalamus stimulation controls weight [66]. Leptin is also involved in the immunity response, being a modulator of the pro-inflammatory T helper 1 immune response. Hence, leptin is a powerful monocyte/macrophage chemoattractant, promoting their migration [67] and through the stimulation of macrophage activation, leptin promotes the expression of inflammatory cytokines, such as TNF-α and IL-6 (Figure 1) [68, 69]. The elevated levels of leptin identified in individuals with obesity are posited to constitute a component of the chronic inflammation mechanism observed in this population, as we previously discussed. Additionally, leptin is a growth factor and a direct physiological agent in cancer growth. Studies have reported the role of leptin in cell proliferation, suppression of apoptosis, and tumour cell development [58, 70]. Furthermore, leptin has been shown to induce telomerase activity in cancer, promoting cell proliferation, and expressing survival factors, leading to chemotherapeutic resistance (Figure 1) [71]. Leptin can be produced by epithelia tumour cells and other cells within the tumour-stroma and can affect tumours in an endocrine, paracrine, and autocrine manner [72-74]. The actions of leptin are mediated by the transmembrane leptin receptor (ObR). When leptin binds to ObR, it induces the activation of several pathways involved in cell proliferation, survival and invasion [75]. Ishikawa and colleagues demonstrated the overexpression of leptin and its receptor in breast cancer [76]. The authors postulated that leptin could potentially foster carcinogenesis and metastasis, thereby considering the inhibition of leptin as a prospective treatment strategy [76]. In a study with a rat model of breast cancer related to induced obesity, tumour incidence and aggressiveness was followed with an increase in leptin and its receptor [77]. In fact, leptin acts on a

transcriptional pathway in order to promote cancer progression. Here, STAT3 along with a histone methyltransferase silenced miR-200c, an altered miRNA in neoplastic stem cells, promote cancer cell formation [77, 78]. Concurrently in PCa, leptin was shown to increase cell proliferation and suppression of apoptosis, thereby enhancing tumour growth [79]. In PCa cell lines, DU145 and PC-3, leptin induced expression of the vascular growth factor (VEGF), transforming growth factor-β1 (TGF-β1) and basic fibroblast growth factor (bFGF), which resulted in stimulation of cell survival pathways, proliferation and angiogenesis [80]. Leptin also exerts an impact on estrogen metabolism within human prostate cells. Research has revealed that it elevates the expression of estrogen receptor (ER)-α while simultaneously reducing ER-β expression, resulting in stimulated cell proliferation. Furthermore, leptin has been shown to upregulate aromatase expression [81]. Consequently, studies underscore the significance of leptin as a potential biomarker for assessing cancer risk, particularly in individuals who are overweight or obese [82, 83].

Adiponectin is another important adipokine and in contrast to leptin, its expression levels are decreased in individuals with obesity (Figure 1) [84, 85], therefore its levels are inversely correlated with BMI and body fat accumulation [86]. Adiponectin is responsible for the regulation of insulin sensitivity, glucose metabolism, and fatty acid breakdown [87-89]. TNF-α, which is increased in the adipose tissue of subjects with obesity, can inhibit adiponectin through the alteration of a disulfide bond modification in the endoplasmic reticulum, which could also explain the lower expression of adiponectin found in these individuals [90]. Adiponectin affects several pathways, having a crucial role in the activation of adenosine monophosphate-activated protein kinase (AMPK). Through the mammalian target of rapamycin (mTOR), AMPK interferes with cellular growth signalling and inhibits carcinogenesis promotion, tumour cell adhesion, and migration (Figure 1) [91]. Furthermore, *ex-vivo* cultivation of mouse adipocytes yielded insightful results. It was observed that OS orchestrated a downregulation in the expression of adiponectin, consequently leading to the dampening of both mRNA expression and the secretion of adiponectin [92]. A separate study conducted with non-diabetic human subjects unveiled a notable correlation between fat accumulation and systemic OS, with the latter inversely linked to plasma adiponectin levels [92]. This intricate connection underscores that the diminished adiponectin levels often observed in individuals with obesity pose a tangible risk, fostering an environment conducive to uncontrolled cell proliferation and thereby the progression of cancer [93, 94]. Thus, the interplay between obesity, OS, adiponectin levels, and their impact on cell proliferation

unveils a compelling framework that can potentially aid in understanding and addressing the complex link between obesity and cancer progression.

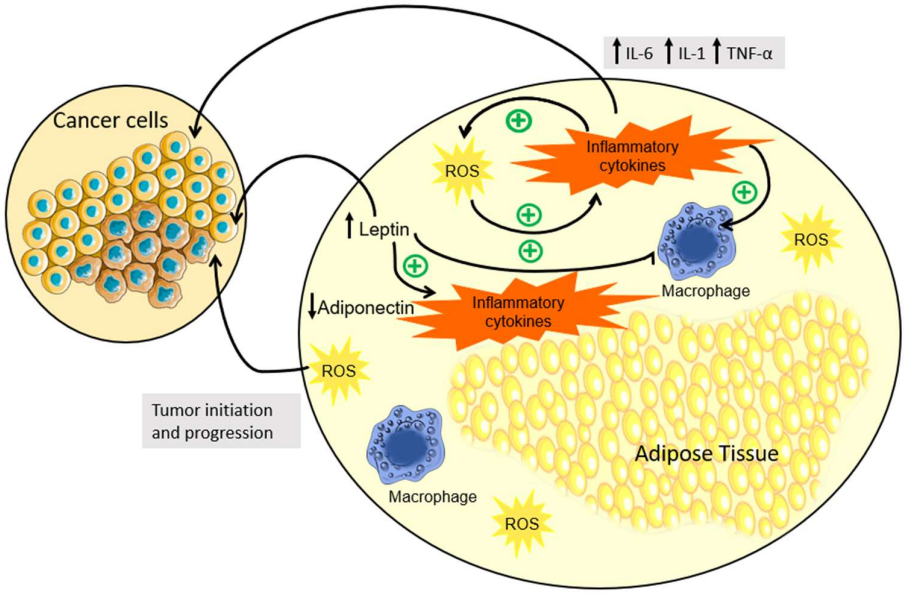

Figure 1: Schematic representation of inflammation, hormonal dysregulation, and OS in the adipose tissue as a result of obesity. In individuals with obesity, a notable expansion of adipose tissue triggers an aberrant production and secretion of cytokines, accompanied by the disruption of adipokine regulation. This cascade instigates a series of interconnected events: cytokines foster heightened ROS production, inciting apoptosis, which then exacerbates cytokine release, perpetuating a self-perpetuating cycle. This cytokine orchestration not only contributes to the perpetuation of low-grade chronic inflammation but also significantly augments the landscape for tumour development. Concurrently, elevated leptin levels in obesity correlate with heightened inflammatory cytokine levels, fostering an environment conducive to both the initiation and progression of tumours. In contrast, the diminished presence of adiponectin compounds the scenario, offering a conducive milieu for tumour development. In summary, the complex interplay between obesity, cytokine dynamics, and adipokine regulation unveils a multifaceted process that intricately contributes to chronic inflammation and the initiation and advancement of tumorigenesis.

Regardless, the hormonal dysregulation promoted by obesity goes beyond the adipose tissue. The Hypothalamic-pituitary-gonadal (HPG) axis, the main endocrine regulator of the reproductive system, is also severely affected. The axis starts with the secretion of the Gonadotropin-Releasing Hormone (GnRH) by the hypothalamus. In the pituitary, GnRH promotes the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Both neurohormones act on the male gonads, the testes. LH acts on Leydig cells, upregulating steroidogenesis and stimulating testosterone secretion. FSH acts on Sertoli cells, promoting spermatogenesis, while stimulating the synthesis of inhibin B, a growth-like factor [70, 95], which feeds the negative loop that inhibits the secretion of FSH by the pituitary and stimulates the secretion of testosterone in Leydig cells [70, 95, 96]. Men with obesity present an impaired reproductive hormonal profile, with lower serum levels of the sex hormone-binding globulin (SHBG), and testosterone

(Figure 2) [97]. This can occur through two main mechanisms. The first revolves around the conversion of testosterone into 17β-estradiol (E2) within the adipose tissue. The hypertrophy and hyperplasia of adipose tissue frequently observed in individuals with obesity subsequently trigger heightened levels of aromatase expression and activity. Consequently, individuals grappling with obesity experience elevated rates of testosterone conversion into E2. As E2 operates as an estrogen, it can perpetuate a negative loop within the HPG axis. By acting on the pituitary gland, it contributes to the reduction of neurohormone expression [98]. The second mechanism by which obesity impairs testosterone serum levels arises from the inadequate expression of neurohormones by the pituitary gland. This deficiency leads to a diminished stimulation of steroidogenesis, a process executed by Leydig cells. This cascading effect ultimately results in decreased testosterone synthesis (Figure 2) [70, 99]. Meanwhile, the chronic state of inflammation found in individuals with obesity also contributes to the decreased levels of serum testosterone [100]. In fact, TNF-α, which is increased in the serum of individuals with obesity, can inhibit Leydig cells' steroidogenesis by decreasing the expression levels of the steroidogenic acute regulatory protein (StAR), which is responsible for the transport of cholesterol from the outer to the inner mitochondrial membrane, an essential step of steroid hormone synthesis [101]. TNF-α also appears to induce the expression and activation of the dosage-sensitive sex reversal adrenal hypoplasia congenital critical region on X chromosome, gene 1 (DAX-1), a protein known to be involved in the inhibition of steroidogenesis by Leydig cells [102]. As a natural consequence, it is anticipated that men grappling with obesity would exhibit diminished fertility potential in comparison to their normal-weight counterparts, a notion substantiated by numerous studies [70, 95]. The epigenetic alterations observed in the sperm DNA of men with obesity, further compounded by an elevated risk of disease, metabolic irregularities, and diverse forms of cancer in their offspring, underscore the intricate interplay between paternal health status and its lasting impact on the generations to come [70]. In essence, these findings emphasize the far-reaching implications of obesity not only on an individual's health but also on the health trajectories of future generations.

Insulin-like Growth Factors (IGFs), signalling molecules synthesized by a wide array of body tissues, play a pivotal role in the landscape of carcinogenesis. Elevated IGF expression within cancerous or stromal cells aligns with the stimulation of cell cycle advancement and the impediment of apoptosis. This influence is enacted through both direct and indirect interactions with oncogenic pathways. Compellingly, research has unveiled a correlation between heightened serum IGF levels and an augmented risk of

developing cancer [103, 104]. Obesity is known for its increased inflammation status, release of pro-inflammatory cytokines, and modified patterns of adipokine secretion. These damages can trigger an escalation in insulin resistance, prompting an elevated requirement for insulin to maintain optimal glucose levels [105]. Moreover, there is growing evidence linking insulin to the process of carcinogenesis [106]. Both insulin and IGF play pivotal roles in instigating various mechanisms that foster tumour growth, encompassing proliferation, angiogenesis, and anti-apoptosis. These cascades of effects stem from intricate cellular processes initiated via the phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin (PI3K-AKT-mTOR) pathway, which governs cellular growth and differentiation. Simultaneously, the Ras-Raf-MEK-Mitogen-Activated Protein Kinase (MAPK) pathway augments proliferation. Stimulation of the insulin receptor or IGF-1 Receptor sets in motion the generation of lipid messengers by PI3K, culminating in the activation of the Akt murine thymoma viral oncogene homolog (Akt) cascade. This cascade culminates in the activation of mTOR, a critical regulator of cell growth, proliferation, and survival. Notably, mTOR stimulation is frequently observed within tumour environments [107, 108].

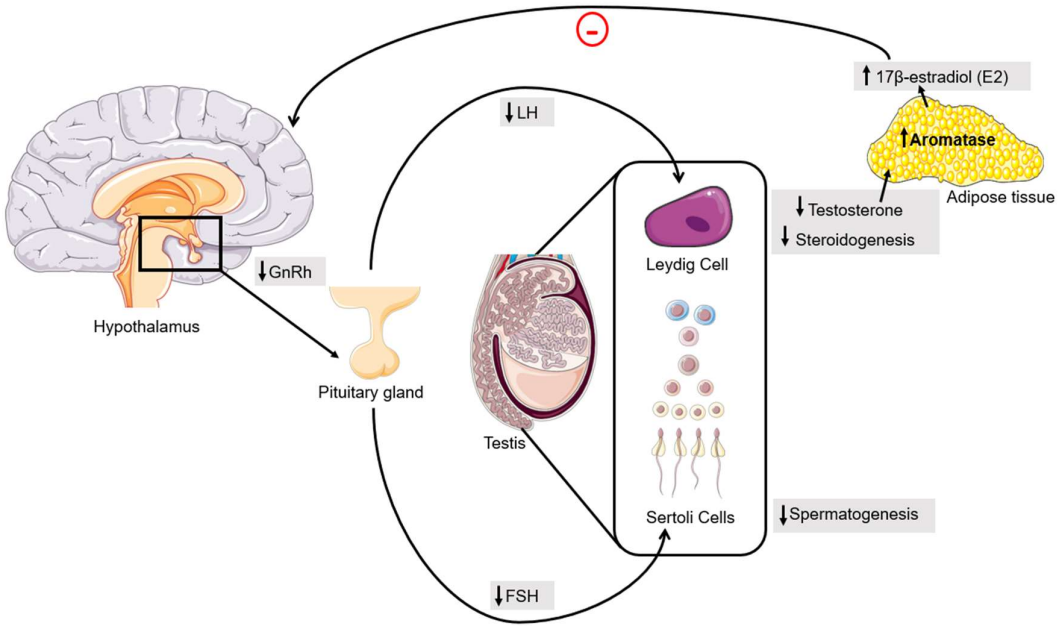

Figure 2: Hormonal dysregulation within the male reproductive system of individuals dealing with obesity. Two mechanisms can result in an impaired hormonal profile as a result of obesity. The first one corresponds to the conversion of testosterone into 17β-estradiol in the adipose tissue due to higher aromatase activity. This creates a negative loop in the HPG axis decreasing neurohormones expression. The second one is the decrease in testosterone serum levels due to lack of neurohormones expression, decreasing steroidogenesis stimulation by the Leydig cells.

In resume, excess weight and obesity can promote an ideal microenvironment for the development of tumours. This aligns with recent statistics indicating that obesity has now

surpassed smoking as the most prevalent high-risk factor for carcinogenesis in the United States of America [18]. In the upcoming section of this study, our attention will be directed towards exploring the contribution of excess weight and obesity to the progression of PCa.

1.2. Obesity is an amplifier factor for PCa development

The prostate, situated in the lower pelvis just below the urinary bladder, is a composite organ composed of glandular and muscular elements. As an integral part of the male reproductive system, it functions as an accessory gland [109]. This gland is divided into different histologic zones, the peripheral, transition, and central zones (Figure 3). The peripheral zone is the region of origin of most prostate cancers, consisting of 70% of tissue in normal cancers. The transition zone consists of about 5% of the prostate and is enlarged by benign prostatic hyperplasia (BPH), which is a common benign proliferation. Nevertheless, tumours that develop in the transition zone tend to stay in this area. The central zone, consisting of around 25%, is not a site of origin of any disease but still can be affected by cancer [110, 111]. The growth and differentiation of the prostate is mainly under androgen control, but also dependent on other steroid and peptide hormones. Both genetic and environmental factors can lead to the development of cancer cells, through various molecular mechanisms. Initiation of prostate carcinoma often involves the accumulation of cancerous cells, with changes in cell compartments. There are two possibilities for the cellular origin of PCa [112]. The first one is the transformation of final differentiated luminal cells, leading to partial dedifferentiation and immortality status. The other possibility consists of the oncogenic modification in prostate stem cells, believed to be located among basal cells [113]. These cells will originate proliferative cells with a luminal phenotype, consisting of the tumour [114]. In older individuals, BPH can occur due to prostate changes. BPH typical characteristics include basal layer expansion and hyperproliferation of the stromal. On the other hand, prostatic intraepithelial neoplasia (PIN) is an *in situ* carcinoma, considered a precursor to invasive prostate carcinoma. The transition from PIN lesions to adenocarcinomas involves various histological changes in the invasive epithelial cells with a cytokeratin profile, such as, excessive branching morphogenesis, basal cell layer lost, cytologic atypia with nuclei and nucleoli expansion [113, 115]. In early PCa stages, an important change occurring is the formation of proliferative inflammatory atrophy regions of epithelial cells, that are related to inflammation in the stromal compartment. These regions present a higher cell proliferation and are proposed as a precursor to PIN [116, 117].

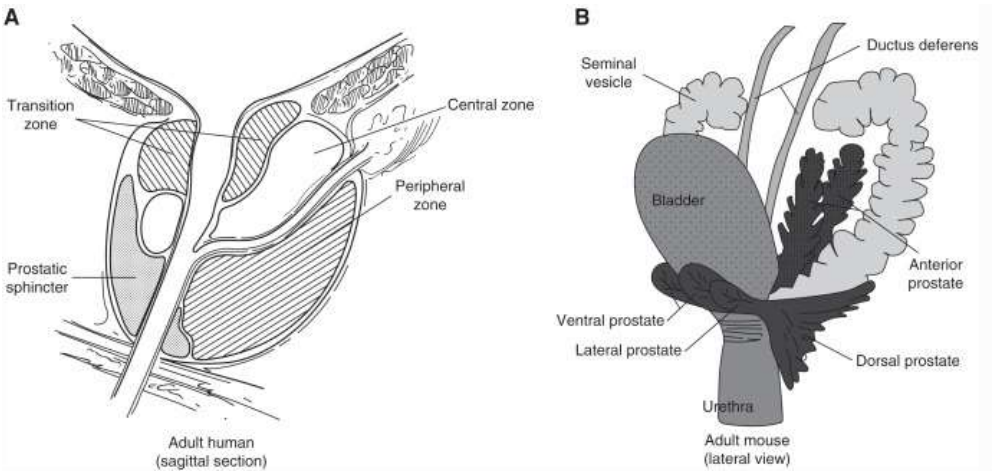

Figure 3: Schematic representation of the human and mouse prostate, and adjacent structures. (A) Human prostate representing the localization of three zones of the prostate (the peripheral, transition, and central zones). (B) Mouse prostate representing different lobes of the gland and their relation to adjacent structures. Image from a study by Ittmann, 2018 [110].

Hence the prostate is under androgen control and thus, the Androgen Receptor (AR) presents an important physiological role in its normal function. More notably, AR is crucial for PCa development and proliferation. In normal tissue, the AR is responsible for maintaining the differentiation of endogenous stromal cells, inhibiting organ growth. As previously mentioned, the majority of PCa cases manifest as adenocarcinomas, stemming from the epithelial cells. The prostate epithelium, comprising two key cell types - basal and luminal - both express AR. For decades, the prevailing belief was that elevated testosterone levels could stimulate the proliferation of PCa. Consequently, various therapeutic approaches targeting PCa, including castration and Androgen-Deprivation Therapy (ADT), have been centred around the primary goal of reducing androgen levels [118]. Additionally, lower androgen levels have been associated with decreased PCa progression, but recent evidence suggests that the correlation between PCa and androgen levels is not as simple as initially thought. To begin with, there was no correlation between PCa proliferation and testosterone levels. Witao and colleagues reported that physiological levels of testosterone could inhibit the proliferation of PCa cells *in vitro* while suggesting that lower testosterone levels were essential for PCa initiation [119, 120]. Meanwhile, supraphysiological testosterone levels were found to decrease the proliferation of PCa, as suggested by several authors [121-123]. Nowadays, the influence of testosterone on PCa development is recognized as having a dual nature. Lower testosterone levels are requisite for the initiation and progression of tumours, while supraphysiological levels hinder these processes. The impact of testosterone on PCa is intricately regulated by AR. The role of AR expression levels is

pivotal in mediating the effects of testosterone on PCa. The therapeutic efficacy of supraphysiological testosterone levels in treating PCa can be attributed to the elevated AR expression observed in PCa cases. This upregulation of AR expression in PCa is suggested to be a compensatory mechanism aimed at counterbalancing the reduced levels of testosterone necessary for initiating tumour growth [124].

Obesity is responsible for approximately 20% of cancer cases, promoting carcinogenesis in end-organs affected by adipose inflammation, OS, and hormonal dysregulation [17, 125]. Individuals with obesity present lower serum testosterone levels [126]. As it was mentioned earlier, lower levels of testosterone are important for PCa initiation and proliferation. Also, lower testosterone levels are associated with more aggressive phenotypes of PCa, largely due to the ineffectiveness of traditional treatments that aim to decrease androgen levels [34]. Unsurprisingly, a meta-analysis study has associated obesity with a higher risk of developing aggressive phenotypes of PCa, which are resistant to traditional treatment therapies [127]. Nevertheless, excess weight does not seem to be a key risk factor for PCa, unless it is associated with altered testosterone or other androgens levels [57]. Obesity is also associated with increased levels of E2. It has been suggested that the administration of E2 could be beneficial for the treatment of PCa. Steg A and Benoit G reported that the administration of percutaneous E2 to patients with untreated PCa promoted a significant decrease in serum testosterone levels while lowering LH and FSH levels as well, which the authors concluded to be beneficial for the treatment of PCa [128]. Montgomery B and colleagues used a castration-resistant PCa orchiectomized mice model to test the efficacy of E2 on the treatment of PCa. The authors reported that treatment with E2 was able to significantly suppress tumour testosterone and dihydrotestosterone levels while inhibiting tumour growth through the modulation of estrogen receptors [129]. The molecular mechanisms by which E2 exerts its effects on PCa are yet to be unveiled. Nevertheless, it has been suggested that the impact of E2 on PCa is primarily governed by the interplay between ER-α and ER-β receptors. It has come to light that PCa cell lines exhibiting a higher ratio of ER-α to ER-β tend to display heightened aggressiveness, with E2 seemingly exacerbating OS. Conversely, within PCa cell lines characterized by a lower ER-α/ER-β ratio, E2 encourages the upregulation of uncoupling proteins (UCPs) and other antioxidant enzymes [130]. As far as we know, no correlation has been established between obesity and high levels of serum E2 with the development, progression, or aggressiveness of PCa [131]. Nonetheless, a most cohesive correlation has been established between the increase expression of adipokines, in particular leptin, in individuals with obesity and the development and progression of PCa. Obesity can particularly affect the prostate's

physiology since prostate epithelial cells express adipokine receptors, making it more susceptible to the hormonal alterations imposed by this condition [132, 133]. As previously described, adipokines are known to be released during fat mass expansion occurring in obesity and cause metabolism alterations, inducing the development of obesity-related disease. Studies have indicated a potential role of adipokines and obesity in cancer progression, suggesting an important contribution to disease progression and risk, as reviewed by Booth and colleagues [134]. Leptin is associated with increased inflammatory cytokines [135], macrophage activation, and ROS production [136, 137]. This adipokine acts in GABA (ɣ-aminobutyric acid)-ergic neurons in order to prevent the effects caused by obesity [138]. The association of leptin, obesity, and PCa was briefly approached before. Leptin might be a predictor of advanced PCa in patients with obesity, hence higher leptin is associated with PCa risk [139]. On the other hand, adiponectin, another adipokine, helps to attenuate liver inflammation and fibrosis through AMPK activation and peroxisome proliferator-activated receptor-α pathways, increase free fatty acid oxidation and reduce inflammatory cytokine suppression, as well as anti-inflammatory cytokine induction [140, 141]. Most studies found lower levels of adiponectin in PCa, as reviewed by Angel and colleagues [142]. Dysregulated adiponectin may contribute to tumour initiation or progression due to its anti-inflammatory and antiangiogenic properties [143]. The clinical association between PCa and obesity might also be explained by the levels of adiponectin in individuals with obesity since adiponectin can inhibit OS in PCa. The mechanism underlying involves the induction of anti-oxidative enzymatic defence mechanisms [144].

As previously described, insulin resistance is a phenomenon occurring in the context of obesity. It takes place when there is a decrease in glucose transportation stimulated by insulin and metabolic processes within adipocytes. Such alterations can be attributed, in part, to compromised insulin signalling in adipocytes, leading to the downregulation of GLUT4, a pivotal glucose transporter responsive to insulin [145]. This decrease in insulin sensitivity is related to a normal concentration of insulin producing a less than normal biological response [146]. Insulin is important for the reduction of blood glucose through the induction of glucose uptake, stimulation of fatty acid synthesis, and induction of cell proliferation [147]. Notably, insulin has been associated with cancer development [148, 149]. Evidence has confirmed a possible insulin's role in stimulating PCa growth [150]. In this study, authors used a transplantable rat-derived cell line expressing PCa, PA-III, to test the effects of insulin and IGFs on cell proliferation. Two different IGFs were used, IGF-I and IGF-II, which are thought to be associated with cancer progression. Results revealed an increase in cells and DNA synthesis in the presence of insulin and

IGFs in a dose-dependent manner. Moreover, both IGF-I and IGF-II showed a binding on PA-III cells with affinity of receptor sites [150]. In the realm of human health, elevated insulin levels have been identified as a risk factor for both the development and recurrence of PCa [151, 152]. Furthermore, the convergence of abdominal obesity and heightened insulin levels has been linked to an escalated risk of PCa [151, 153]. A study conducted by Hsing and colleagues unveiled a direct association between insulin resistance and an increased PCa risk, while insulin sensitivity was correlated with a diminished risk of PCa occurrence among Chinese men [30]. In the context of PCa cell lines, a reduction in insulin and IGF-1 levels has been shown to yield diminished growth and increased apoptosis [154]. Cancer has been linked to insulin by different mechanisms. The first one is in cases of chronic hyperinsulinemia, hence insulin exerts oncogenic potential through abnormal stimulation of various cellular pathways. This enhances growth factor-dependent cell proliferation and/or directly affects cell metabolism. Secondly, since obesity causes a low-grade inflammatory state, a higher production of IL-6, adiponectin, leptin, or TNF-α is present. Therefore, a transformation and progression in cancer are seen, as previously described [155]. The third one involves the suppression of SHBG production by insulin, which increases testosterone levels. Lastly, insulin increases IGF-I production in the liver, while reducing production of the insulin-like growth factor binding proteins 1 (IGFBP-1) and 2 (IGFBP-2). Even though, insulin can induce direct tumour growth, its mitogenic and antiapoptotic effects also result in tumour cell growth [155, 156]. The last two have already been related with PCa.

Obesity-associated OS is proposed to be involved in the conversion of androgen-dependent PCa into androgen-resistant prostate cancer (ARPC), through the regulation of AR expression. Androgens can have pro-oxidative effects through the activation of pro-oxidative signalling pathways, such as fatty acid oxidation in the mitochondria. This pro-oxidation state is further promoted by the downregulation of ROS detoxifying enzymes, and upregulation of NADPH oxidases [47, 157, 158]. OS has been associated with prostate hyperplasia development [48]. It is known that it significantly contributes to both the initiation and progression of PCa, exerting its influence on pivotal molecules such as DNA, transcription factors, and cell cycle regulators [48]. Moreover, the interplay of antioxidants and protective molecules against OS has been demonstrated to play a preventive role in PCa. Within the realm of antioxidant systems, a network of enzymes and enhancer elements operates to shield cells from the ravages of OS. These components encompass enzymes found in the glutathione redox system, as well as enhancer elements such as antioxidant response elements (ARE). This intricate defensive response is triggered in reaction to escalated levels of ROS [159]. For

instance, within the realm of the glutathione redox system, enzymes like glutathione-s-transferases (GST) and glutathione peroxidase (GPx) take centre stage. GST is instrumental in the detoxification of activated procarcinogen metabolites, influencing the cell's sensitivity to various harmful chemicals [160]. Simultaneously, GPx functions as an electron donor, quelling peroxyl radicals and thus diminishing cellular OS, which in turn curbs the progression of PCa [161]. On another front, the activation of ARE leads to the transcription of genes responsible for enhancing antioxidant defences [162].

OS can also be further intensified by prostatic inflammation. Prostatic inflammation is commonly linked (though not universally) with pathological infections that induce swelling of the prostate tissue, leading to painful and challenging urination, as well as a general sense of discomfort. This inflammatory state within the prostate is posited to play a role in the development and advancement of PCa, serving as a factor not only in the initiation of carcinogenesis but also in causing cellular and genomic damage, while fostering heightened cellular turnover [48]. Prostatic inflammation may be the risk of high-grade PCa, which is a condition further aggravated by the inflammation promoted by obesity [163]. Additionally, both adipose and prostate tissue have their DNA integrity and epigenetic regulation disturbed due to excessive adiposity [164, 165].

1.3. Can paternal obesity be promoting PCa development in the offspring?

Epigenetic transgenerational inheritance is a highly conserved mechanism characterized by the transmission of phenotypes through generations, in the absence of exposure to the original trigger in the germ cells of the developing fetus [166-168]. Transgenerational inheritance through the paternal line is established in two generations, except for when the F0 female is pregnant during exposure to the effect [169-171]. Curiously, for several years it was thought that the spermatozoa could not carry epigenetic marking to the oocyte, and the epigenetic inheritance could only come from the maternal side. This concept has its roots in the protamination process that spermatozoa must go through to achieve chromatin condensation and protect DNA for the long voyage until it reaches the oocyte [172, 173]. It was thought that protamination would erase the epigenetic marks carried on the paternal genome. Due to this, for years the epigenetic inheritance of the embryo was only attributed to the mother. Nowadays, we know that several epigenetic marks survive the protamination process, including the ones present on the 5-15% of chromatin that remains nucleosome-bound [172-174]. In fact, gene ontology analysis has reported that several metabolic genes are present in these nucleosome-bound regions and are known to be hotspots for epigenetic modifications [175, 176]. The last

years have been characterized by the rise of evidence that obesity can be transgenerational inherited by the paternal line [177]. Fullston and colleagues conducted an intriguing study involving male mice that were subjected to an HFD. These male mice were then bred with females who were lean and metabolically healthy [178]. Remarkably, the offspring - comprising both males and females - exhibited metabolic irregularities stemming from this paternal obesity. The initial impact was observed in terms of increased adiposity in the offspring, and intriguingly, this effect was exclusive to the daughters. Nevertheless, both male and female descendants exhibited compromised glucose tolerance and reduced insulin sensitivity. Astonishingly, even in the subsequent F2 generation, these metabolic aberrations persisted when descendants - both sons and daughters - were mated with partners who were lean and metabolically healthy [178]. Notably, the researchers delved further into the mechanisms at play. They found that the content of small non-coding RNAs (sncRNAs) within the spermatozoa of the F0 generation was altered, alongside discernible changes in DNA methylation patterns [178]. Crisóstomo and colleagues have also demonstrated that the consequences of an HFD until adulthood can also impose several alterations on testis metabolism that are not reverted by a diet switch [179]. The spermatozoa derived from these animals exhibited notable changes in the expression of sncRNAs. Intriguingly, these alterations were also discernible in the spermatozoa of the offspring stemming from animals exposed to this transitional diet [179]. These sncRNAs, which were formerly considered to lack function, have now emerged as pivotal players in the regulation of various processes, including embryo development. Chen and his team revealed a significant shift in the expression of a specific subset of sncRNAs - transfer-RNA derived small RNA (tsRNA), primarily originating from the 5’ halves - in spermatozoa from obese mice subjected to an HFD [180]. In a follow-up, the researchers extracted this pool of tsRNAs from these spermatozoa and introduced them into zygotes derived from normal, metabolically healthy parents. While the resulting pups did not exhibit remarkable changes in body weight compared to those raised on standard diets, they did, however, display glucose and insulin intolerance - akin to the offspring of males fed HFDs [180]. In sum, this research has illuminated the multifaceted interplay of epigenetic mechanisms, sncRNAs, and obesity in shaping the health destinies of subsequent generations. It serves as a clarion call for further investigation into the complex landscape of transgenerational health impacts, shedding light on the enduring legacy of parental choices and metabolic status in the realm of health and disease.

Metabolic disorders can be a result of a single gene mutation or a complex interaction between the environment and genetic factors. Studies have reported the association

between epigenetic mechanisms, the development of obesity, and even diet modifications. Factors like hypoxia and OS, intricately linked with obesity, have also been implicated with epigenetic modifications [166, 169, 171]. Obesity, characterized as a metabolic disorder, is intricately linked to a spectrum of comorbidities. Notably, insulin resistance, T2D mellitus, and respiratory and cardiovascular ailments are among the prominent associations. In the cardiovascular domain, numerous conditions exhibit heightened risks in individuals grappling with obesity. This encompasses hypertension, dyslipidemia, disorders in lipoprotein metabolism, and the emergence of diabetic cardiomyopathy - all of which often coincide with diabetes [181, 182]. Recent studies have consistently highlighted the link between obesity, cardiovascular diseases, and the transmission of specific genetic markers, underscored by the intricate workings of epigenetic mechanisms. Investigations have demonstrated a robust connection between maternal obesity during pregnancy and subsequent metabolic tissue modifications in offspring, alongside consequential epigenetic changes. Ultimately, these factors contribute to the emergence of cardiovascular disorders intricately linked with obesity [183, 184].

On the other hand, cancer initiation and development are known to be also promoted by genetic causes [185]. Cancer is characterized by complex genome alterations, implicating the association of the disease with epigenetics, and possibly its contribution to the development or promotion of carcinogenesis. Moreover, modifications in methylation patterns might be related to the promotion of cancer cell growth or even the activation of tumour suppressors [168]. When it comes to PCa, first-degree relatives' subjects have a 2- to 3-fold higher risk of developing the disease, and an increased risk is presented in a patient with several relatives affected [186]. As of now, our understanding of the transgenerational transmission of PCa remains limited, largely due to the intricate nature of the inherited genetic predisposition to this condition. While the specifics of inherited susceptibility to PCa are not fully elucidated, certain sites seem to exhibit heightened vulnerability, and a robust genetic influence is notably evident within familial contexts. Nonetheless, despite this intricate genetic component, it does not appear to interfere with the process of genetic transmission [187]. Furthermore, PCa appears to be one of those cancers explained by autosomal dominant inheritance, and studies have associated PCa, not only with genetic factors but also with environmental ones, like excess adiposity. Nevertheless, research with families shows that hereditary PCa occurs at a younger age, in comparison with sporadic PCa, and it has a higher heritability in men than any other cancer [188, 189]. Additionally, even though several works have explored the role of obesity in the development of PCa, as it was reviewed

in this work, to our knowledge, no work has been performed regarding the impact of obesity and the inheritance of PCa to future generations. With the rise of the obesity pandemic, it is important to understand how obesity can modulate the transgenerational inheritance of other disorders, identify at-risk individuals, and propose a better diagnosis and treatment. The health of future generations is at risk.

1.3.1. Is it possible to determine a biomarker for the inheritance of PCa (and obesity)?

The most classical PCa biomarker used for the diagnosis is PSA, a member of the kallikrein family. Its higher levels are useful for the detection of PCa since an increase in PSA is often indicative of PCa. This biomarker is secreted by prostate epithelial cells, being present in the serum of patients with prostate disease, such as PCa, BPH, and acute prostatitis [190]. PSA detection has a low specificity and sensitivity for PCa since its levels can be affected by another prostatic disease, age, and depends on race [191]. On the other hand, a lower level of PSA has been associated with individuals with obesity, which can lead to a delay in the diagnosis of PCa. This association can be explained by hemodilution, a condition characterized by the increased blood volume found in individuals with obesity, which causes the dilution of tumour markers like PSA [192]. The diminished levels of testosterone typically observed in these individuals are concurrently linked to reduced PSA production. Nonetheless, the precise underlying mechanism that ties together obesity and the decline in PSA levels remains enigmatic [193-196]. Since obesity imposes an obstacle to cancer screening and early diagnosis, individuals with obesity are normally diagnosed with advanced stages of the disease. Taking this into consideration, it is imperative to find new biomarkers for PCa diagnosis in individuals with obesity [197, 198].

1.3.1.1. Androgen Receptor

As discussed, AR is present in all phases of PCa progression but is not singly responsible for the growth of PCa cells. AR is required for PCa initiation since the stimulation of low levels of testosterone is an important step in this process. Additionally, while AR is associated with cell differentiation in the normal prostate tissue, in PCa it promotes proliferation [199, 200]. Elevated expression levels of AR have been correlated with instances of treatment failure in androgen ablation therapy. This is due to the phenomenon wherein even in the presence of low testosterone levels, tumour progression can still be promoted [201, 202]. Furthermore, it is worth noting that antiandrogen therapies can induce DNA damage and heightened radiosensitivity. This

outcome is linked to the engagement of AR stimulation in specific DNA repair processes [203].

Obesity is known to alter both the volume and composition of the adipose tissue, which in turn will influence lipid availability and utilization [204]. Men with obesity have more aggressive disease and a higher recurrence rate following surgery [34]. Cancer cells are known to have high survival requirements such as the need for increased lipids, which are used for membrane production, energy, and intracellular signalling. Cancer cells can acquire lipids by the overexpression of enzymes for *de novo* lipid synthesis or by the uptake of exogenous lipids from circulation. PCa cells tend to utilize fatty acids as energetic substrates and lipids utilized by these cells can drive proliferation and invasiveness [205]. Androgens and AR are known to regulate lipid metabolism, an important link to carcinogenesis. AR mediates lipid biosynthesis, a highly conserved process able to influence the lipid profile of prostate cells. In more advanced and aggressive cases of PCa, intense lipid signals and droplets are present. When lipid biosynthesis is reactivated via AR, PCa progresses to Castration-Resistant Prostate Cancer (CRPC). In the context of ADT, the augmentation of lipid biosynthesis inhibition leads to a reduction in tumour growth [205-207]. Regardless, through the indirect activation and enhancing expression of sterol regulatory element-binding proteins (SREBPs), androgens can stimulate *de novo* lipogenesis and lipid uptake. Hence, to regulate this process, a reciprocal relationship between AR and SREBP is essential [205, 208]. LNCaP cells, an established human prostate carcinoma cell line, has been used in the study of PCa progression, during different stages, and in response to several therapies, specifically involving AR signalling, since LNCaP cells present the unique ability to express AR [209]. A noticeable effect of androgens on LNCaP cells is the accumulation of neutral lipids. Since these lipids are *de novo* synthesized, LNCaP cells are thought to express all the enzymes needed for endogenous lipogenesis. Some of these enzymes have their expression and/or activity affected by androgens. AR mediates androgens, which stimulate the expression and activity of the Fatty acid synthase (FASN). FASN stimulation represents part of the mechanism by which androgens induce neutral lipid accumulation [210]. In fact, abnormal FASN expression has been associated with PCa progression via lipid metabolism dysregulation [211]. A study by Huang and colleagues demonstrated that SREBP-1 can promote PCa growth and progression via AR/lipogenesis axis. The authors concluded that SREBP-1 induced the expression of AR and FASN, which resulted in fatty acid induction and lipid droplets in PCa cells. Moreover, SREBP-1 stimulated ROS levels, a mechanism known to contribute to carcinogenesis. Overall, SREBP-1 was shown to regulate PCa proliferation and

development in LNCaP cells [212]. Taking all of this into account, AR might be a link between obesity and PCa, making it a useful biomarker for individuals with obesity.

In some cases, a genetic component is associated with increased PCa risk. Researchers have been investigating the relationship between a hereditary component and the development of PCa. Common single-nucleotide polymorphisms (SNPs) have been associated with familial relative or development PCa risk in various populations, as reviewed by Benafif and Eeles [213]. AR dysregulation plays an important role in the onset and progression of PCa. These changes include point mutations (such as single-base substitution), AR overexpression, AR splice variants, androgen biosynthesis modifications, or AR cofactor alterations. Most of the mutations in AR variants identified in PCa tissue rarely occur within the germline. Mutated AR variants result in small changes in the AR protein. For example, the mutation AR^{RT877A} is the most frequent. This mutation consists of the substitution of an alanine by a threonine, leading to specificity loss for the agonist. Therefore, the mutated AR can be activated also by steroid hormones and lead to tumour growth [214-216]. Nevertheless, specific AR germline polymorphisms have been associated with an increased risk of developing PCa and therefore may have a potential transgenerational effect [199]. A groundbreaking research conducted by Hu and his team unveiled a pivotal mutation stemming from a germline substitution within the AR of three African American family members, all of whom had a documented history of early-onset PCa. This mutation has been conclusively linked to the advancement of the disease and exerts a profound influence on AR signalling. The modification in DNA-binding affinity induced by this mutation results in altered responses to androgens, anti-androgens, and non-androgenic steroids, illuminating a previously uncharted dimension of the disease's molecular intricacies [217]. Additionally, an article with Finnish PCa patients revealed that the R726L substitution in AR may increase PCa risk and contribute to its progression. Moreover, it contributed to familial and sporadic PCa in the Finnish population [218, 219]. On the other hand, few studies have analysed if *AR* expression can be transgenerationally affected. For example, grandmothers of F2 were exposed to a mixture of Polychlorinated biphenyls during late gestation. Preliminary data reported reduced *AR* mRNA expression in the F1 males in the preoptic area and increased expression in F2 [220]. Another study exposed pregnant female rats to two different doses of Bisphenol A (1.2 µg and 2.4 µg) during the perinatal period. The 1.2 µg dose revealed a decreased *AR* expression in the testicular tissue of rats from all three generations. The 2.4 µg dose only presented a decreased in the *AR* expression in the F3 generation when compared with the control (CTRL) groups [221].

1.3.1.2. Homeobox B13

Localized on chromosome 17, the Homeobox B13 (HOXB13) is a homeobox transcription factor with mutations being reported in 0,7% to 1,4% of PCa cases [222]. The homeobox genes encode for nuclear proteins and possess the main function of being transcription factors. This class of genes is highly conserved and crucial for correct embryonic development [223]. HOXB13 presents important roles in the regulation of the AR target gene, in the differentiation of the epithelial of the prostate gland, and the development of the prostate and its secretory functions [224]. The interaction between HOXB13 and AR is complex. It was reported that the suppression of the AR activity along with the overexpression of HOXB13 has been seen in androgen-independent tumours [225, 226]. HOXB13 exhibits a dual role in the regulation of prostate cell growth by effectively suppressing it through the inhibition of androgen-mediated signalling. Paradoxically, several studies have unveiled an intriguing association between elevated HOXB13 levels and the progression of tumour growth [223, 227-230]. This complex relationship makes the heightened expression of *HOXB13* a potential diagnostic marker for PCa. The predominant localization of HOXB13 within the nucleus, accompanied by sporadic presence in the cytoplasm, grants it remarkable sensitivity and specificity for PCa detection [231]. The hereditary occurrence of PCa is relatively rare, accounting for merely 0.6% to 6.25% of patients [189, 232]. Importantly, a significant correlation has been established between hereditary PCa and increased familial risk with aberrant *HOXB13* expression, as indicated by reference [233]. During the early stages of mouse embryogenesis, HOXB13 levels remain low and are concurrent with AR presence. Intriguingly, the knockdown of HOXB13 triggers noteworthy perturbations in pathways associated with the cell cycle, encompassing DNA replication, G1/S phase transition, and metaphase checkpoint. Notably, murine models with HOXB13 loss-of-function mutations exhibit discernible prostate abnormalities [234, 235]. Concomitantly, a notable germline mutation in HOXB13 has garnered attention due to its association with both PCa risk and hereditary PCa. Carriers of this mutation exhibit a heightened susceptibility to developing PCa [233, 236]. The G84E missense mutation, identified as a founder mutation in Nordic populations, displays carrier frequencies ranging from 0.2% to 1.4%. In Western European populations, these frequencies range between 0.1% and 0.5% [237]. This mutation is situated within the evolutionarily conserved functional domain of HOXB13, suggesting its involvement in promoting PCa. Despite this, the precise underlying mechanism remains elusive. However, as suggested by others, it is plausible that the interaction between the HOXB13 domain and members of the MEIS protein family contributes to this mechanism, though further investigation is warranted [238]. On

the other hand, HOXB13 activity and expression are related to lipogenesis. A study by Lu and colleagues found that HOXB13 directly suppresses lipogenic transcriptional programs by its interaction with HDAC3, a histone deacetylase. HDAC3, once activated and recruited to lipogenic enhancers, catalyses histone deacetylation of target genes and suppresses lipogenic programs. Hence, a captivating mechanism emerges wherein HOXB13 exerts a suppressive influence on *de novo* lipogenesis by directly engaging with lipogenic enhancers within PCa cells [239]. Notably, this effect is achieved through its direct interaction with HDAC3, a pivotal cofactor that contributes to the intricate process of epigenome remodelling. This collaborative partnership between HOXB13 and HDAC3 underpins the orchestration of regulatory events critical to cellular function [239, 240]. Furthermore, the attenuation of HOXB13 expression provides a window for the expression of pivotal lipogenic regulators, which orchestrates a cascade of events. This ultimately results in lipid accumulation within PCa cells - a hallmark of carcinogenesis - propelling heightened cell motility when examined *in vitro* and instigating the alarming phenomenon of tumour metastasis *in vivo* [239]. Intriguingly, the same investigation suggests that the expression of HOXB13 registers an upswing during the initial phases of PCa, solidifying its role as an indispensable AR cofactor that fuels the augmentation of PCa growth. However, the dynamics take a compelling twist in metastatic castration-resistant prostate cancer (mCRPC), where the levels of HOXB13 are observed to undergo a decline. This transformation in its expression profile is further mirrored by an increase in its methylation levels, underscoring a remarkable dichotomy that potentially contributes to the intricate landscape of CRPC [239]. *HOXB13* expression has also been associated with the activity of the Fat mass and obesity gene (FTO). FTO is associated with obesity, body weight, fat mass and BMI [241]. Despite the scarcity of comprehensive insights into its precise functional attributes, FTO stands as a genomic locus in the context of adiposity. It has been proposed to wield a substantial influence over the regulation of feeding behaviours and energy expenditure, thus positioning it as a key player in the intricate interplay governing these physiological processes [242]. Situated on chromosome 16q12.2, the FTO gene garners attention for its robust conservation across an array of vertebrate species, reinforcing its evolutionary significance as a genetic entity [243]. Unveiling its relevance in the realm of obesity, a noteworthy revelation emerges from bariatric surgery outcomes. An impressive 71.2% of individuals subjected to bariatric surgical interventions were found to harbour at least one risk allele associated with the FTO gene [244]. This intriguing finding suggests a potential modulatory role of FTO in influencing the efficacy and consequences of such surgical interventions, accentuating its potential impact on therapeutic outcomes. On top of that,

FTO acts as a demethylase enzyme and has the capacity to interfere with the alternative splicing patterns of genes associated with adipogenesis. An intriguing hypothesis, conceptualized by Zhao X and fellow researchers, establishes a compelling link between the demethylation of N6-methyladenosine (m6A) modification, the FTO gene, and the intricate process of adipogenesis. This hypothesis finds its basis in the realm of epitranscriptomic nucleotide modifications, which wield a profound influence over the destiny of mRNA molecules. These modifications, akin to methylation, endure within the nucleotide sequence, imparting them with an enduring impact on cellular processes. The focal point is the remarkable inverse correlation discovered between the expression of FTO and the levels of m6A during the course of adipogenesis. This discovery fuels the hypothesis, suggesting that FTO's influence on gene splicing could potentially underpin the metabolic shifts associated with obesity. This intriguing proposition points towards a multifaceted mechanism that connects these molecular players to the broader context of metabolic regulation and the development of obesity [245, 246]. Through this property, FTO can erase the m6A modification from mRNA, and the mature mRNA is transported to the cytoplasm. Hence, FTO indirectly regulates the mRNA metabolism. The m6A modification is known to participate in DNA repair and important cellular processes. This modification is very frequent in the RNA of eukaryotes and a predominant internal modification in the mRNA of mammals [247, 248]. Without FTO, the m6A modification is recognized by the YTHDF2 protein due to the higher binding capacity between them. Then, the protein will promote the transportation of mRNA into the p-body, accelerating mRNA degradation, inhibiting protein translation [249]. The m6A modification is located in the 3'UTR region of the HOXB13 mRNA. Therefore, if FTO is not present, the YTHDF2 protein can also bind to the HOXB13 mRNA and regulate it in the way described before. When FTO is present, its protein binds to the HOXB13 mRNA and regulates it through the m6A mechanism. FTO catalyses the demethylation modification in 3'UTR region of HOXB13 mRNA, erasing the m6A modification recognition with the YTHDF2 protein (Figure 4). Therefore, FTO can promote the expression of *HOXB13* and accelerate it. Furthermore, the 5' end of HOXB13 mRNA contains two CpG islands, indicating that its expression may be regulated by DNA methylation, as previously seen [239, 250, 251].

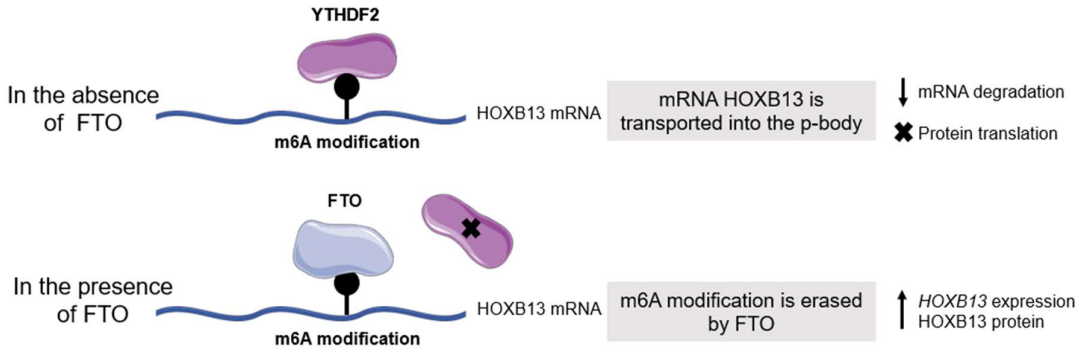

Figure 4: m6A modification in HOXB13 mRNA in the absence and presence of FTO. The dynamic landscape of m6A modifications within HOXB13 mRNA reveals an intriguing dichotomy that hinges on the presence or absence of FTO. In the absence of FTO, the m6A modification attracts the binding of the YTHDF2 protein. This interaction orchestrates the translocation of mRNA to the p-body, a process that expedites mRNA degradation while simultaneously impeding the translation of proteins. In a distinct scenario where FTO is a participant, its presence holds the key to a transformative change. Acting as a molecular recognition system, FTO identifies the m6A modification and initiates a catalytic cascade, ultimately leading to the demethylation of the modification. This orchestrated demethylation event serves as a trigger, ushering in an upsurge in the expression of *HOXB13*. This intricate regulatory mechanism highlights the pivotal role of FTO in orchestrating a finely tuned balance between mRNA degradation and the augmentation of protein synthesis within the context of *HOXB13* expression.

In an article related to endometrial cancer, the relationship between FTO, HOXB13, and the m6A mechanism was studied. It was found that FTO knockdown was associated with decreased *HOXB13* expression and higher m6A modification levels [251]. On one hand, FTO silencing promoted the peak in m6A modification, which was followed by a reduction in *HOXB13* mRNA expression. On the other hand, the silencing of the YTHDF2 expression was related to increasing *HOXB13* mRNA expression. Indeed, within the realm of endometrial cancer, a noteworthy observation emerges: the expression levels of *HOXB13* exhibit an elevation within metastatic tumours. This surge in *HOXB13* expression is intricately tied to the facilitation of tumour cell invasion and the subsequent orchestration of metastatic events when examined *in vitro*. Furthermore, a pivotal connection becomes apparent as *FTO* expression enters the equation. The presence of heightened *FTO* expression in endometrial cancer seems to be a driving force behind metastasis, with an even more pronounced effect catalysed by the concurrent activation of the Wnt signalling pathway. It is worth noting that *HOXB13* expression has a well-documented association with the Wnt signalling pathway, effectively amplifying the intricate web of regulatory interactions that lead to the augmentation of tumour invasion and metastasis [251]. This interplay between HOXB13, FTO, and the Wnt signalling pathway adds a new layer of complexity to our understanding of endometrial cancer progression, illuminating potential targets for therapeutic intervention and presenting a fascinating area for further investigation. In another study analysing the expression of

FTO, HOXB13, and the m6A mechanism in gastric cancer, a positive correlation between *FTO* and *HOXB13* was detected [250]. Elevated levels of both *FTO* and *HOXB13* expression have been observed in the context of gastric cancer. Intriguingly, the inhibition of their expression initiates a cascade of effects, ultimately leading to the suppression of crucial processes in gastric cancer cell biology: proliferation, migration, and invasion. Delving deeper into the molecular intricacies, the heightened expression of *FTO* seems to wield a powerful influence over HOXB13. By engaging in the demethylation of HOXB13's mRNA, FTO contributes to the upregulation of *HOXB13* expression. This orchestrated interplay contributes to the tumorigenic properties of gastric cancer cells. Furthermore, insights gleaned from inhibition experiments have highlighted HOXB13's pivotal role. Inhibition of HOXB13 manifests as a powerful deterrent against cell proliferation, migration, and invasion *in vitro*, underscoring its significance in the malignancy's progression [250]. Thus, augmented expression of *FTO* potentially orchestrates HOXB13's regulation by inducing a reduction in its methylation levels. This dynamic relationship further solidifies the intricate interplay between FTO, HOXB13, and the underlying mechanisms that drive gastric cancer's aggressive behaviour. These findings not only deepen our comprehension of gastric cancer biology but also offer potential avenues for therapeutic exploration.

Epigenetic changes, distinct from modifications to the nucleotide sequence, possess the remarkable capacity to endure across generations, shaping the genetic landscape for the descendants. One prime illustration of this phenomenon lies in the modifications of methylation patterns [252, 253]. This suggests that shifts observed in the expression levels of genes like *HOXB13* or modifications within its methylation patterns can be inherited, potentially spanning multiple generations in a transgenerational transmission fashion. Dysregulation of HOXB13 could be intricately linked to heightened PCa risk and the potential for its inheritance. A compelling study led by Legoff and colleagues embarked on an exploration, analysing the prostates of mice spanning both the F1 generation, directly exposed to chlordecone, and the F3 generation, which remained unexposed. Within this investigation, discernible alterations surfaced in the prostate structures, alongside the revelation of transgenerational inheritance of its epigenetic effects. This intriguing discovery underscores the profound impact of environmental exposures on the intricate interplay between genetics and epigenetics, presenting a pathway through which hereditary PCa risk might be influenced and transgenerationally transmitted [254]. In the F1 generation, a notable decline in *HOXB13* expression was distinctly evident. Conversely, when scrutinizing the third generation (F3), a trend of reduced expression persisted, though not achieving statistical significance. This

intriguing outcome postulates that the repercussions of chlordecone exposure observed in the first generation continue to reverberate through to the third, unveiling a compelling narrative of transgenerational inheritance in the realm of HOXB13 dysregulation. The authors postulated a hypothesis that gains remarkable significance within this context. Recognizing that alterations in *HOXB13* expression have been correlated with an elevated risk of PCa, the inquiry emerges: Could the exposure to chlordecone, a potential catalyst for prostate cell proliferation, be mediating its effects through the avenue of HOXB13 modification [254]? This hypothesis fuels the exploration of an intricate interplay between environmental factors, gene expression dysregulation, and the broader landscape of PCa susceptibility.

1.3.1.3. DNA Hypermethylation

The growth and development of tumours have been related to changes in DNA methylation. Moreover, it has been reported that hypermethylation associated with specific gene promoters can lead to the silencing of tumour suppressor genes [255]. One of the most frequently observed occurrences involves the heightened methylation of cytosines positioned at the 5' end within CpG islands situated within the regulatory regions of suppressor genes [256-259]. In renal cell carcinoma lines and primary tumours, HOXB13 methylation status is correlated with loss of *HOXB13* expression, which was also associated with tumour grade and microvessel invasion. Concurrently, HOXB13 inactivation may play an important role in carcinogenesis and its progression [258]. In gastric cancer, lower *HOXB13* expression was associated with tumour differentiation, lymph node metastasis, and depth invasion. Furthermore, a correlation between the methylation level and gene expression could be a potential predictor of malignancy degree for this type of cancer [260].

Events like DNA methylation, specially hypermethylation, have been seen in PCa, both in advanced and metastatic prostate tumours [261, 262]. Angulo and colleagues reported that 61 genes were significantly hypermethylated in 20% of PCa tumours analysed. These patterns of hypermethylation were later associated with metastasis and a negative response to ADT [263]. Events of specific somatic alterations have a higher rate of recurrence in DNA methylation, supporting the strong selective pressure for them to occur. DNA methylation can offer insights into the origin and evolution of a tumour hence it is a stable event [264]. These hypermethylation events can occur in cancer cells and have been associated with PCa [265]. Promoter methylation is affected in Glutathione-S-transferase-P1 (GSTP1), adenomatosis polyposis coli (APC), retinoic acid receptor β (RARB), and Ras-associated domain family 1 (RASSF1) in several PCa cases, making

it possible to consider these genes as PCa biomarkers [266-269]. GSTP1 hypermethylation is the most commonly identified epigenetic alteration found in PCa, which can be used as a molecular biomarker for its diagnosis due to the detection in PCa and PIN, not in normal tissues or BPH [256, 270]. GSTP1 encodes GST and is involved in key cellular functions, being responsible for the protection of cells from OS and chemical attacks [256]. Furthermore, the loss of function of GSTP1 could potentially predispose normal prostatic cells to undergo DNA damage, influenced by inflammation and/or dietary intake, which can lead to carcinogenesis [271].

Obesity possesses the capacity to exert its influence on methylation patterns and gene expression. This occurs due to the profound impact that dietary and nutritional alterations can have on DNA methylation within genes crucial to metabolic processes [272-274]. Notably, investigations have revealed that HFDs serve as promotors of epigenetic shifts, particularly within inherited genes intricately tied to metabolic pathways [275, 276]. However, the intricate linkages between the effects of obesity and the reprogramming of the epigenome remain largely shrouded in mystery. This enigma extends even further to encompass aspects of transgenerational inheritance, raising questions about the potential for these effects to cascade across generations, along with the underlying mechanisms that drive such phenomena.

As discussed earlier, obesity triggers the release of pro-inflammatory cytokines, which in turn can facilitate the elevation of DNA methyltransferase 1 (DNMT1) expression and its associated enzymatic activity. Remarkably, individuals with obesity often exhibit heightened levels of DNMT1 expression. Once DNMT1 becomes activated, it selectively engages in the methylation of the adiponectin promoter, thereby fostering a heterochromatin configuration. This molecular shift prompts a consequential reduction in gene expression, achieved through the inhibition of transcription. Consequently, the hypermethylation of the adiponectin promoter – an outcome observed in mice with obesity under the influence of HFD – inevitably culminates in the suppression of gene expression [277]. In an experimental mice model simulating basal-like breast cancer, the author tested if obesity could potentially drive epigenetic reprogramming. To address this, three distinct groups were established: the CTRL diet group, the diet-induced obesity (DIO) group, and the formerly obese (FOb) group. Notably, the FOb group initially experienced diet-induced obesity before transitioning to a CTRL diet. Upon scrutiny, it was apparent that heightened DNA methylation levels were notably present within both the DIO and FOb groups, as compared to the CTRL group. Furthermore, the outcomes seemed to suggest that altering the diet alone did not suffice to reverse the epigenetic

reprogramming effects induced by obesity [278]. This finding shed light on the lasting impact of obesity on epigenetic mechanisms, even when dietary conditions are altered.

Studies by Crisóstomo and colleagues, analysed the effects of exposure to an HFD and diet correction in a transgenerational *Mus musculus* model across three generations (F0, F1, and F2) through the division in three different diets: CTRL (standard chow), HFD (carbohydrate: 35.7%, protein: 20.5%, and fat: 36.0%) and transient diet [HFD_t (60 days HFD, plus 140 days standard diet)], ad libitum for 200 days post-weaning [279, 280]. In the first generation (F0), HFD exposure resulted in weight gain, while diet correction was able to reverse the effects induced until adulthood and lead to weight loss. In their sons (F1), the group exposed to HFD throughout life was significantly heavier and the one exposed until adulthood presented lower fat mass, while in the grandsons (F2) no differences were found. This indicates that the father's diet has an impact on their progeny's body composition. Interestingly, HFD exposure in F0 displayed an increase in insulin resistance and fasting glycemia. Meanwhile, their sons showed higher glucose levels, indicating altered insulin response. In the last generation, the CTRL and HFD_t groups had increased fasting glycemia. However, no differences were found in F1 and F2 for fasting insulinemia. Therefore, HFD exposure throughout life induces glucose intolerance, as well as insulin resistance, and reversion of diet might prevent these effects [280]. Consequently, paternal HFD exposure results in inter- and transgenerational sperm defects. F1 displayed differences in sperm morphology, mainly the HFD_t group presented an increased proportion of normal sperm. On the other hand, in F2, HFD and HFD_t groups, showed decreased sperm counts and viability, compared to their progenitors [279, 280]. Concurrently, testicular metabolism was affected by HFD, with effects persisting for generations. For example, leucine testicular levels were increased in HFD of F1, while in their offspring levels were decreased. A similar scenario succeeded with acetate, with low testicular levels in groups exposed to HFD throughout life or until adulthood in F0, and an overcompensation in their sons, even in grandsons. Likewise, in F0 HFD_t testicular glutamine levels were higher, but their grandsons presented decreased levels. In contrast, inosine testicular levels were lower in the HFD group in F0, as well as in the HFD_t group in F2, which might lead to a pro-inflammatory environment. Lastly, in F1, the HFD group presented increased glycine testicular levels, overcompensating the damage induced in their progenitors, and the HFD_t group displayed leucine testicular levels altered, which might be linked to insulin resistance [280]. Concurrently, the alterations induced by HFD in testicular metabolism were correlated with sperm defects across three generations [279, 280]. Even though no differences were found in F0, paternal HFD exposure induced alterations in the

reproductive axis of F1 and F2. While the HFD_t group in F1 showed reduced E2 levels, the HFD group in F2 presented increased levels. Additionally, in HFD_t of F2, higher levels of FSH and lower levels of LH were detected [279]. Further, paternal HFD exposure disrupts the antioxidant defences in the testis and the mitochondrial function of the offspring. This was indicated by the lower catalase activity present in the HFD group in F0, which persisted in their offspring, alongside reduced mitochondrial complex I and IV in this group. Followed by the lower glutathione-disulfide reductase activity in mice fed with HFD in F0. However, in the offspring of parents exposed until adulthood, it was higher [279]. Regardless, testicular fatty acids and lipid metabolites were also affected by HFD exposure, with effects persisting for two more generations. In F2, the HFD group presented reduced polyunsaturated fatty acids levels. Additionally, testicular content of choline was disrupted in the HFD group of F1. Grandsons of mice fed with HFD until adulthood presented increased levels of ethanolamine and lower levels of phosphocholine and phosphoethanolamine. Moreover, 3-hydroxyburate, associated with testicular insulin resistance, was highly expressed in the HFD and HFD_t groups in F2 [279]. Overall, HFD exposure resulted in inherited metabolic memory which caused inter- and transgenerational sperm defects and metabolic changes, further able to stimulate pro-inflammatory environment [279, 280].

Adiponectin, a hormone intricately linked with obesity, exhibits an intriguing inverse relationship with the disease. Notably, in recent times, significant attention has been directed toward studying adiponectin, primarily due to its recognized anti-proliferative attributes in the context of carcinogenesis. An inverse correlation is present between adiponectin levels and PCa/PCa risk. Even though little is known about the mechanisms underlying this relation, adiponectin has been suggested as a link between obesity and PCa [281, 282]. A study by Tan and colleagues demonstrated that endogenous adiponectin is down regulated through promoter hypermethylation in PCa. This was associated with increased tumour proliferation and invasion [282]. As previously discussed, DNA methylation of adiponectin promoter has been observed in association with obesity. Methylation of adiponectin promoter might be a potential PCa biomarker.

Periprostatic adipose tissue (PPAT) has garnered notable attention due to its correlation with PCa, with obesity resulting in an accumulation of excess fat within PPAT. This adipose tissue is known to secrete adipokines, experience a decline in adiponectin levels, and contribute to an environment conducive to PCa growth. In this context, Cheng and colleagues undertook a significant investigation into the methylation patterns of PPAT from PCa patients who were also overweight or obese. Their findings illuminated the impact of surplus adiposity on the DNA methylation of PPAT tissue within individuals

with PCa. Significantly, the majority of pathways featuring promoter hypermethylated genes were interconnected with metabolic disorders that are recognized contributors to tumour development in PCa. This compelling observation suggests that the methylation patterns altered by obesity extend their influence on the modulation of the tumour microenvironment, subsequently influencing PCa development [283]. It is important to note that promoter hypermethylation catalysed by obesity might potentially exert far-reaching effects on PCa development, with the intriguing possibility of transgenerational transmission. Nonetheless, it is worth noting that only a limited number of studies have explored the connection between obesity-induced methylation and PCa. In the broader panorama, epigenetics occupies a pivotal role in both the initiation and progression of PCa, a notion previously established. Furthermore, the intricate interplay of nutritional factors is recognized to exert an influence on the mechanisms encompassed within this process [284].

2. Objectives

Over the past decades, metabolic disorders have emerged as a significant public health concern due to their rapid escalation. Obesity is typified by hormonal dysregulation, OS, and chronic low-grade inflammation, with the same inflammatory biomarkers often associated with PCa. Notably, obesity has been implicated in impacting prostate health and potentially fostering cancer initiation, therefore obesity stands as a risk factor in the trajectory of carcinogenesis. The ethology of obesity carries a substantial genetic component, consequently propagating possible epigenetic modifications linked to the metabolic disorder into subsequent generations. Inherited obesity-related factors passed to offspring propel obesity's perpetuation, leading to growing apprehension about the inheritance of these factors that could potentially culminate in diverse comorbidities, notably PCa, and its underlying mechanistic factors. The hypothesis would be that the same genetic and epigenetic imprints correlated with a father with obesity might also underlie the development of PCa in the progeny. To explore this premise, the current study evaluates the expression of PCa biomarkers in lyophilized prostate tissue from a transgenerational mice model exposed to an HFD throughout their life or only until adulthood. The analysis extends to assessing inflammation levels, OS, and promoter methylation of a PCa biomarker within the prostate tissue. The principal objective of the research is to pinpoint specific biomarkers tied to PCa development, particularly in the context of obesity and hereditary elements. The ensuing specific objectives are:

1. Appraise the expression of PCa biomarkers, *AR* and *HOXB13*, in the prostates of animals (F0) subjected to diverse diets (chow, high-fat, and reversion diets), along with their offspring (F1 and F2) fed with standard chow diet;

2. Determine the expression levels of *FTO* in the prostates of animals (F0) exposed to diverse diets (chow, high-fat, and reversion diets), along with their offspring (F1 and F2) fed with standard diet;

3. Uncover a possible correlation between the expression of PCa biomarkers (*AR* and *HOXB13*) and *FTO*;

4. Evaluate the methylation level of the *HOXB13* promoter in the prostates of animals (F0) subjected to different diets (chow, high-fat, and reversion diets), as well as their offspring (F1 and F2) fed with CTRL diet and correlate it with *HOXB13* and *FTO* expression;

5. Investigate the inflammatory state of prostates of animals (F0) exposed to different diets (chow, high-fat, and reversion diets), along with their offspring (F1 and F2) fed with chow diet, gauging the expression of *TNF-α*;

6. Ascertain a correlation between the expression of PCa biomarkers and *TNF-α* in the prostates of animals (F0) exposed to diverse diets (chow, high-fat, and reversion diets), as well as with their offspring (F1 and F2) fed with standard diet;

7. Analyse OS levels in the prostates of animals (F0) subjected to different diets (chow, high-fat, and reversion diets), along with their offspring (F1 and F2) fed with a CTRL diet;

8. Establish a correlation between the expression of PCa biomarkers and OS in the prostates of animals (F0) exposed to diverse diets (chow, high-fat, and reversion diets), as well as their offspring (F1 and F2) fed with chow diet;

Hence, the overarching aim of the project is to study distinct PCa biomarkers (*AR*, *HOXB13*) within mice prostate tissue and discern any link with obesity's onset (*FTO*, *TNF-α*). Additionally, the study aspires to show whether changes resulting from dietary alterations could be potentially transmitted to offspring, heightening the risk of PCa onset among descendants.

Chapter II

Material and Methods

3. Materials and Methods

3.1. Animal model

For this study, we used the mouse animal model developed by Crisóstomo L, and colleagues [280], performed in three generations of *Mus musculus* C57BL6/J mice. Mice were kept in 12 hours dark: light cycles, in a climate room with constant temperature (20-24 º C), and 45-65% humidity. All animals had water *ad libitum*. The first generation (F0), originated from normoponderal progenitors was fed with standard chow (#F4031, BioServ, USA—Carbohydrate: 61.6%, Protein: 20.5%, Fat: 7.2–16.3% Kcals). After weaning (21-23 days), F0 was randomly allocated into 3 groups (12 animals per group, 4 animals per cage): CTRL, HFD, and HFD_t. The CTRL group was fed with a standard chow. The HFD group received an HFD (#F3282, BioServ, USA—Carbohydrate: 35.7%, Protein: 20.5%, Fat: 36.0–59.0% Kcals). The HFD_t group was fed with an HFD for 60 days (#F3282, BioServ, New Jersey, USA) and the rest of the period received a standard chow (#F4031, BioServ, New Jersey, USA). After 120 days on diet, F0 mice were mated with normoponderal, chow-fed, arbitrarily selected females of the same age. Mating lasted for 8 days and involved the placement of a male and female in the same cage for 6 hours each day, without food or water supply. After mating, males continued their diet until the 200th day, when mice were sacrificed by cervical dislocation, and tissues were collected for further analysis. F1 male mice were assigned to the same experimental group as their progenitors (F0). F1 was fed with standard chow (#F4031, BioServ), and water *ad libitum*. Mating followed the same conditions as F0. F2 mice were attributed to the same experimental groups as their progenitors (F1) and were fed *ad libitum* with standard chow (#F4031, BioServ), after the weaning period. Both F1 and F2 were sacrificed on the 200th day. From weaning until sacrifice, total body weight, water, and food intake were monitored weekly. A schematic representation of the transgenerational animal model can be found in Figure 5. After sacrifice, several organs and tissues were collected and immediately frozen in liquid nitrogen. The tissue samples were kept at -80 º C until they were lyophilized to better preserve sample integrity. After lyophilization, samples were kept on a desiccator until further use. The animal model is compliant with the ARRIVE guidelines, was internally reviewed by the Organization for Animal Welfare (ORBEA) and was approved and licensed by the Portuguese Veterinarian and Food Department (DGAV) with the registration number 0421/000/000/2016. All animal experiments were performed according to the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH Publication No. 85–23,

revised 1996) and European rules for the care and handling of laboratory animals (Directive 86/609/EEC).

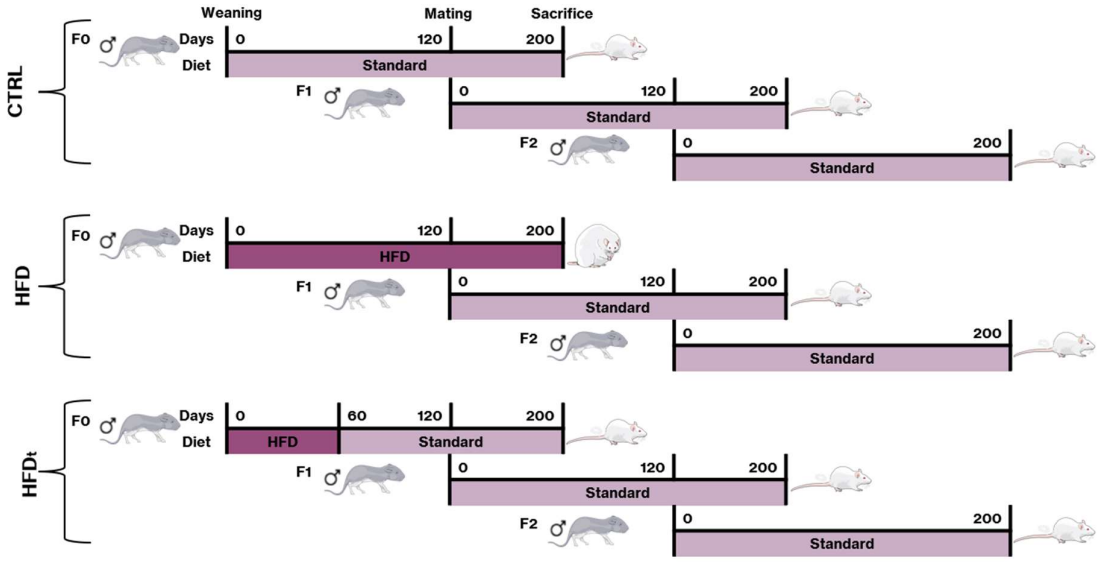

Figure 5: Schematic representation of the transgenerational animal model.

3.2. RNA extraction from prostate tissue

Prostate tissues from the CTRL group (n=8), HFD group (n=8), and HFD_t group (n=8) were selected. RNA extraction was performed using the NZYTech Total RNA isolation kit (NZYTech, Lisbon, Portugal), as indicated by the manufacturer. For this procedure, 7.0 mg of prostate tissue was used for each sample. First, 350 µL of buffer NR and 3.5 µL of β-mercaptoethanol were added to the tissue and mixed with a Tissue Rupture. The lysate was transferred into a NZYSpin Homogenization column placed in a 2 mL collection tube and centrifuged for 1 min at 11,000 x *g*, being the flow-through saved and transferred into a new 1.5 mL centrifuge tube. Then, 350 µL of 70% ethanol was added and mixed by pipetting up and down. The lysate was passed to a NYZSpin Binding column and centrifuged at 11,000 x *g* for 30 s. The flow-through was discarded. Previously, the digestion mix was prepared with 90 µL of Digestion Buffer and 10 µL of DNaseI for each isolation, being added 95 µL of the mix directly into the centre of the silica membrane column and incubated at room temperature for 15 min. Next, 200 µL of Buffer NWR1 was added, being centrifuged for 1 min at 11,000 x *g*, and the flow-through discarded. From the Buffer NWR2, 600 µL was added and centrifuged at 11,000 x *g* for 1 min, once again the flow-through was discarded, and the wash was repeated with 250 µL of Buffer NWR2 and centrifuged at 11,000 x *g* for 2 x 1 min to dry the column membrane. The column was placed in a clean 1.5 mL RNase-free microcentrifuge tube. After, 40 µL of RNase-free water was added directly to the column membrane and

centrifuged at 11,000 x *g* for 1 min to elute the RNA. RNA concentrations and absorbance ratios (A260/A280 and A260/A230) were determined by NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). The RNA samples were stored at -80º C until use. For this technique, liver tissue was used as a positive control for the identification of PCa biomarkers.

3.3. Complementary DNA (cDNA) synthesis

Total RNA was reverse transcribed in a final volume of 20 µL by M-MuLV Reverse Transcriptase (NYZTech, Lisbon, Portugal) according to manufacturer instructions. First, 2.5 µL of Random hexamer primers (50 ng/µL) (NYZTech, Lisbon, Portugal) and 1 µL of deoxynucleotides (dNTPs) (NYZTech, Lisbon, Portugal) were added to each sample, containing 1 µg of RNA. The volume was adjusted with RNase-free water up to 17 µL. Then, samples were incubated at 65º C for 5 min for RNA denaturation. After, M-MuLV Reverse Transcriptase and the respective enzyme buffer were added to each sample, and the samples were incubated in accordance with the manufacture protocol: 10 min at 25º C for the reagent activation, 50 min at 37º C for conversion, and 15 min at 70º C for enzyme inactivation. The cDNA samples were stored at -20º C until further use.

3.4. Polymerase chain reaction (PCR)

To identify the PCa biomarkers transcripts in the prostate tissues, PCR assays were performed according to standard methods. Previous, exon-exon spanning primers were designed for the mouse genes: AR and HOXB13. For the amplification of the chosen genes, NYZTaq II 2x Green Master Mix (NYZTech, Lisbon, Portugal) was used, being premixed ready to use a solution containing a Taq-derived DNA Polymerase, dNTPS, reaction buffer, and other additives to optimize the reaction. cDNA was amplified in a final volume of 12.5 µL. For the reaction, 1 µg of cDNA, 0.1 µL of primer forward (50 µM), 0.1 µL of primer reverse (50 µM), and 6.5 µL of Master Mix were added to each sample. The volume was then adjusted to 12.5 µL with RNase-free water. The conditions of PCR cycles were adjusted to better fit optimal temperatures of enzyme activation, DNA denaturation, annealing, and dissociation. The protocol for the reaction was the following: 5 min at 95º C for enzyme activation, 30 s at 95º C for DNA denaturation, 1 min at 72º C for extension, and 7 min at 72º C for final denaturation. The annealing temperatures were adjusted for each pair of primers, which can be consulted in Table 1. The samples were run in a desaturating 3% agarose gel and stained with GreenSafe (NYZTech, Lisbon, Portugal). The results were revealed in the ChemiDoc™ XRS+ System (Bio-Rad, California, USA).

3.5. Real-time polymerase chain reaction (qPCR)

To perform the qPCR assays, qPCR Green Master Mix (2x) (NZYTech, Lisbon, Portugal) containing DNA polymerase enzyme, buffer, and SYBR green nucleic acid stain, was used. The cDNA was amplified in a final volume of 16 µL. In each sample was added 0.64 µL of primer forward (10 µM), 0.64 µL of primer reverse (10 µM), and 8.0 µL of Master Mix. The cDNA quantity added to the mix was adjusted for each set of primers to ensure a reaction efficiency of 90-110%. The final volume was adjusted to 16 µL with RNase-free water. To better fit optimal temperatures of enzyme activation, DNA denaturation, annealing, and dissociation, the conditions of qPCR cycles were adjusted. The protocol for qPCR was the following: 10 min at 95º C for polymerase activation, 15 s at 95º C for DNA denaturation, and 1 min at 72º C for extension. The number of cycles and annealing temperatures needed for the exponential phase of amplification were optimized for each set of primers. The primer sequences, annealing temperatures, and the number of cycles can be consulted in Table 1. The melting curve was determined by an additional step: 1 min at 95º C, 30 s at 55º C and 30 s at 95º C. qPCR reactions were carried out in duplicate, and the optical density was assessed by a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, California, USA).

Table 1. Primer sequences and PCR and qPCR conditions were used to assess gene expression and mRNA abundance in mouse prostate tissue. *β*2-microglobulin was used as a housekeeping control.

Gene	GenBank	Primer sequence (5'-3')	Amplicon Length	Annealing Temperature	Cycles
mAR	NM_013476.4	Sense: GACTACTCTGCCTCCGAAGTG Anti-sense: AGTTCTCCATCCAAGGTCCCA	106 bp	58º C	33
mHOXB13	NM_008267.4	Sense: GGAGGGGGTCGGAATCTAGT Anti-sense: GTTGACAGTTGGCATCAGCG	81 bp	58º C	38
mFTO	NM_011936.2	Sense: GTGTCTCGCATCCTCATCGG Anti-sense: AGCCTCTGTGTACTTGACCG	111 bp	60º C	40
mTNF-α	NM_001278601.1	Sense: ACCCTCACACTCACAAACCAC Anti-sense: ACAAGGTACAACCCATCGGC	134 bp	60º C	37

mHOXB13 methylated	Sense: TTTTTTTAGGTTATAGTTAATT AGCGT Anti-sense: AAAAAATCCTAAACGTTTTAA ATCG	127 bp	52º C	35
mHOXB13 unmethylated	Sense: TTTTTTAGGTTATAGTTAATTA GTGT Anti-sense: AAAAATCCTAAACATTTTAAAT CACT	125 bp	52º C	35
mβ2-MGB	Sense: ACGTAACACAGTTCCACCCG Anti-sense: TCTCGATCCCAGTAGACGGT	217 bp	58º C (PCR) 60º C (qPCR)	35

3.6. DNA extraction from prostate tissue

DNA extraction of prostate tissue was performed using the NZYTech Tissue gDNA Isolation kit, as indicated by the manufacturer. For this procedure, 5.0 mg of prostate tissue was used for each sample. First, 180 µL of buffer NT1 and 25 µL of Proteinase K solution were added to the tissue and mixed by vortex, followed by incubation at 56º C for 2 hours, with occasional vortex. Then, 10 µL of RNase A solution was added to each sample and incubated for 5 min at room temperature. After the incubation, the samples were mixed by vortex, and then 200 µL of Buffer NL was added, being mixed by vortex. Then, 210 µL of 100% ethanol was added and mixed by vortex. The samples were passed to an NYZSpin Tissue column, placed in a 2 mL collection tube, and centrifuged at 11,000 x *g* for 1 min, being the flow-through discarded and transferred into a new collection tube. Next, 500 µL of Buffer NW1 was added, being centrifuged for 1 min at 11,000 x *g*, and the flow-through discarded. From the Buffer NW2, 600 µL was added and centrifuged at 11,000 x *g* for 1 min, once again the flow-through was discarded. The tissue column was placed in a new collection tube and centrifuged for 2 min at 11,000 x *g*. Once again, the column was placed in a clean microcentrifuge tube, and added 100 µL of water, previously pre-heated at 70º C, to the membrane column. The samples were incubated for 1 min at room temperature and then centrifuged at 11,000 x *g* for 2 min to elute DNA. DNA concentrations and absorbance ratios (A260/A280 and A260/A230) were determined by NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher

Scientific, Massachusetts, USA). For longer storage, the DNA samples were stored at -80º C.

3.7. *HOXB13* promoter methylation evaluation

For the conversion of total DNA, the Zymo Research EZ DNA Methylation™ Kit was used. First, CT Conversion Reagent was prepared by adding 750 µL of water and 210 µL of M-Dilution Buffer to a tube of CT Conversion Reagent, mixed by vortex at room temperature. To the total DNA samples (1 ng), 5 µL of M-Dilution Buffer was added and the total volume was adjusted to 50 µL with water and mixed by pipetting up and down. The samples were incubated at 37º C for 15 min. Then, 100 µL of the CT Conversion Reagent was added to each sample and mixed. The samples were incubated in the dark at 50º C for 16 hours. After, samples were incubated in ice for 10 min. Meanwhile, 400 µL of M-Binding Buffer was added to a Zymo-Spin IC Column placed in a Collection tube. After the incubation, samples were added to the column and mixed by inverting the column several times. Then, samples were centrifuged at 10,000 x *g* for 30 s and the flow-through was discarded. Next, 100 µL of M-Wash Buffer was added to the column and centrifuged at 10,000 x *g* for 30 s. To the samples, 200 µL of M-Desulphonation Buffer was added and incubated for 15 min at room temperature, followed by being centrifuged at 10,000 x *g* for 30 s. Then, 200 µL of M-Wash Buffer was added to the column and centrifuged at 10,000 x *g* for 30 s. This step was repeated one more time. The column was then replaced into a 1.5 mL microcentrifuge tube and added 10 µL of M-Elution Buffer directly into the matrix, being then centrifuged at 10,000 x *g* for 30 s. DNA-converted samples were stored at -20º C until use.

3.8. Slot-Blot

Total proteins were extracted from lyophilized prostate tissue using RIPA buffer [1% Noridet P-40, 0.5% Sodium deoxycholate, 0.1% Sodium dodecyl sulfate 10% in phosphate buffer saline (PBS), 0.1% Phenylmethanesulfonyl fluoride 100 mM, 0.1% cocktail mix of protease and phosphatase inhibitors, 0.1% sodium orthovanadate 100 mM] and homogenized with microtubule piston. The samples were left to rest for 40 min at 4º C and then centrifuged at 14,000 x *g*, 4º C, 1 min. Protein concentration was determined by Pierce Bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Massachusetts, USA). Lipid peroxidation and protein nitration were evaluated by Slot-Blot. Protein samples (5 µg) were diluted in PBS in a final volume of 100 µL. The samples were transferred to activated polyvinylidene difluoride membranes by a Hybri-slot manifold system (Biometra, Göttingen, Germany). For total protein normalization and loading control, a Ponceau S staining solution (Merck, Millipore, Massachusetts, USA)

was used. The membranes were then incubated with a blocking solution of washing buffer (Tris-buffered saline solution with 0.05% Tween 20) with 5% Bovine Serum Albumin (BSA) for 1 hour. The membranes were washed 3 times for 5 min with washing buffer. Then, the membranes were incubated overnight (4º C) with the primary antibody diluted in washing buffer with 1% BSA. The list of antibodies and their concentrations used in this project can be consulted in Table 2. After incubation with primary antibodies, the membranes were washed with the washing buffer 3 times for 5 min and exposed to the secondary antibody diluted in washing buffer with 1% BSA for 1 hour, at room temperature. Afterward, the membranes were washed and reacted with ethyl chloroformate fluorescent (ECF) substrate (GE Healthcare, Buckinghamshire, UK). Results were visualized by ChemiDoc^{Tm} MP Imaging system (Bio-Rad, California, USA). Densities from each band were quantified by BioRad ImageLab Software.

Table 2. List of antibodies, and respective concentrations, used for Slot-Blot.

Antibody	Company	Catalog Number	Concentration for SB
Rabbit Anti-nitro-tyrosine	Cell Signaling Technology	9691S	1:1000
Rabbit Anti-4-hydroxynonenal	Abcam	Ab46545	1:1000
Anti-Rabbit IgC (whole molecule) – Alkaline Phosphatase antibody produced in goat	Sigma Aldrich	A3687 – 1 ML	1:5000

3.9. Statistical analysis

Statistical analysis of the expression of the PCa biomarkers, *FTO*, *TNF-α*, methylation levels, and OS was performed using analysis of variance (ANOVA) followed by a Tukey post-hoc test for multiple comparisons. Population normality was evaluated by the Shapiro-Wilk test and Kolmogorov-Smirnov test. Correlations were evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution with a confidence interval of 95%. Values of $P<0.05$ were considered statistically significant. The statistical analysis of this work was performed using GraphPad Prism 8 (GraphPad Software Inc., SanDiego, CA, USA).

Chapter III

Results and Discussion

4. Results

4.1. A diet rich in fats triggers weight gain in mice, which can be reversed through dietary adjustments

Obesity elevates the risk of various health complications and even mortality. Notably, metabolic disorders are intertwined with the emergence of several comorbidities, including cancer. Exposure to an HFD induces notable effects within the body, manifesting as an increased count of adipocytes, heightened fat mass, weight gain, alterations in body size, and shifts in body composition. In light of these considerations, several physiological parameters were assessed during the experiment. This physiological data can be consulted in the published work of Crisóstomo and colleagues [179]. In Figure 6, we undertook an analysis of body weight at sacrifice (200th day) across three generations in three distinct groups: the CTRL group, the HFD group, and the HFD_t group in the F0 and then in their offspring, through F1 and F2, always fed with standard chow.

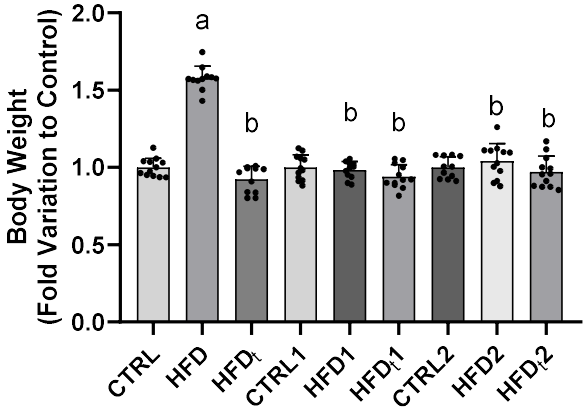

Figure 6: Body weight at sacrifice (200th day) of mice fed with a standard chow (CTRL) or high-fat diet throughout life (HFD) or only until adulthood (HFD_t) of F0, F1, and F2. Results are expressed as mean ± SD (n=12 for each graph bar), with the exception of the groups HFD and HFD_t in F0, HFD in F1 and CTRL in F2 (n=11). Outliers were identified by ROUT (Q = 10%). Significantly different results ($p < 0.05$) are indicated as (a) relative to CTRL F0 (CTRL) and (b) relative to HFD F0 (HFD).

As expected, exposure to an HFD resulted in an increased body weight (1.58 ± 0.08 g) when compared to the CTRL in F0 (1.00 ± 0.06 g). The reversion of the diet (HFD_t) resulted in a significantly lower weight of the mice (0.922 ± 0.09 g) when compared with those fed with HFD (1.58 ± 0.08 g). In fact, reversion of the diet after 60 days (from HFD to standard chow) led to a similar weight (0.922 ± 0.09 g) of the mice always fed with standard chow (1.00 ± 0.06 g) (Figure 6). This demonstrates that the swift of an HFD to a standard diet was able to reverse the gain of weight. Furthermore, F1 mice of those

fed with HFD (0.98 ± 0.06 g) and F2 (1.04 ± 0.11 g) presented a significantly lower weight when compared with their progenitor (1.58 ± 0.08 g). Similarly, HFD_t in F1 (0.94 ± 0.08 g) and F2 (0.97 ± 0.10 g) showed a significantly decreased body weight when compared to HFD from F0 (Figure 6). This demonstrates that, independently of the diet administrated to the F0 generation, no changes in the body weight of the F1 and F2 generations were identified.

4.2. Identification of the PCa biomarkers transcripts AR and HOXB13 in prostate tissue of mice

Obesity is recognized for its correlation with the onset of metabolic and hormonal imbalances, a persistent state of inflammation, and OS. Simultaneously, being overweight or obese induces genetic and epigenetic imprints that possess the potential to be transmitted across generations. All these factors collectively contribute to fostering the development of PCa. Consequently, the prostate emerges as a particularly responsive gland to the perturbations incited by obesity. Given the intimate interplay between the emergence of PCa and obesity, and considering the mounting body of evidence on the hereditary transmission of obesity-associated factors, an intriguing hypothesis arises: Can the inheritance of obesity-linked changes potentially serve as a trigger for the development of PCa, particularly in the offspring of individuals grappling with obesity? The initial phase of our study entailed the identification of PCa biomarkers mRNA, *AR* and *HOXB13*, within mice's prostate tissue (as depicted in Figure 7).

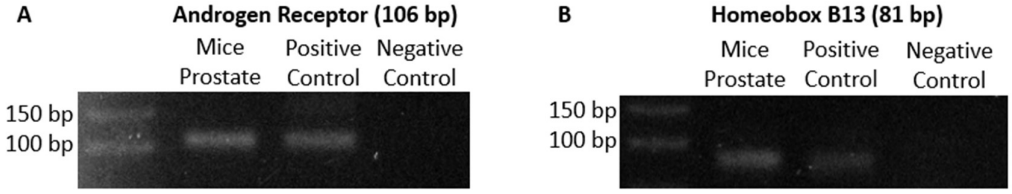

Figure 7: Identification of PCa biomarkers mRNA in mice prostate tissue subjected to standard chow by conventional PCR. Conventional PCR was used to identify the presence of *Androgen Receptor* (A) and *Homeobox B13* (B) transcripts in mice prostate tissue. A cDNA-free sample was used as negative control. A mice liver tissue sample was used as positive control.

Through conventional PCR, the presence of *AR* and *HOXB13* transcripts was confirmed in mice prostate tissue. The presence and expression of *AR* and *HOXB13* mRNA in the prostate of mice has been previously reported by other authors [285, 286]. For this technique, the positive control used was mice liver tissue, since both biomarkers are expressed in this tissue as well. The liver presents high AR levels and loss of its expression results in less OS and DNA damage. In HCC, AR promotes the proliferation

of cancer cells, as well as the suppression of apoptosis [287-289]. On the other hand, HOXB13 is overexpressed in tumour cells, contributing to carcinogenesis and its progression. In the liver, higher expression is shown in patients with tumour angiogenesis and HCC, with staining mainly in the cytoplasm [290, 291].

4.3. Transgenerational expression of PCa biomarkers in mouse prostate tissue following various dietary regimens

Metabolic disorders, including obesity, disrupt the delicate balance of overall body homeostasis. Obesity, in particular, exerts discernible effects on the prostate, owing to the gland's heightened sensitivity to external influences. These effects encompass disruptions in the proper operation of hormonal and metabolic pathways. The prostate becomes particularly susceptible to changes driven by the secretion and signalling of adipokines and cytokines connected to the inflammatory milieu. Importantly, these very factors also play a role in the progression of PCa, fostering an environment conducive to tumour growth and advancement [292, 293]. We hypothesized that the different dietary regimens applied in the animal model (HFD until adulthood and HFD throughout life) may have an effect on the expression of the PCa biomarkers in the prostate tissue in the progenitors but also in the offspring even if these consumed a standard chow. RNA from mice prostate tissue was extracted and subsequently cDNA synthesized. Expression of PCa biomarkers was determined by qPCR.

4.3.1. Dietary regimens did not elicit any discernible impact on *AR* expression within the prostate in the progenitors nor the offspring

The role of AR is pivotal in ensuring the proper development and functioning of the prostate. Additionally, AR is linked to PCa progression, consistently being present across all stages of its development. Moreover, AR demonstrates associations with obesity, primarily through its influence on food intake suppression, reduction in body weight, and augmentation of energy expenditure [294]. Recognizing the well-established connections between AR, PCa, and obesity, we developed a hypothesis centred on the notion that divergent dietary regimens could conceivably impact *AR* expression. This influence was anticipated not only within the parental generation but also cascading down to the subsequent generations, irrespective of their consumption of standard chow. Interestingly, the expression of *AR* remained consistent across various groups and generations (as depicted in Figure 8). However, it is worth noting a discernible trend toward a decrease in the prostate of the HFD group (0.53 ± 0.51) among the F0 generation. In F0, the prostate of the HFD_t (1.06 ± 1.13) presented a similar *AR* expression of the mice fed with standard chow (1.00 ± 0.85). Offspring of F0 did not

present a significant difference between expression of the prostate in HFD (0.71 ± 0.55) and HFD_t (0.21 ± 0.28) groups, when compared to expression in the prostate of mice fed with standard chow (1.00 ± 0.92), even though a decrease in expression in both groups is present. A similar scenario was present in F2 between the CTRL group (1.00 ± 0.45) with HFD (0.93 ± 0.50) and HFD_t (1.12 ± 0.57) (Figure 8). These findings collectively indicate that the dietary regimen in the parental generation did not exert any observable influence on the expression of this specific PCa biomarker in F0 nor throughout the offspring (F1 and F2).

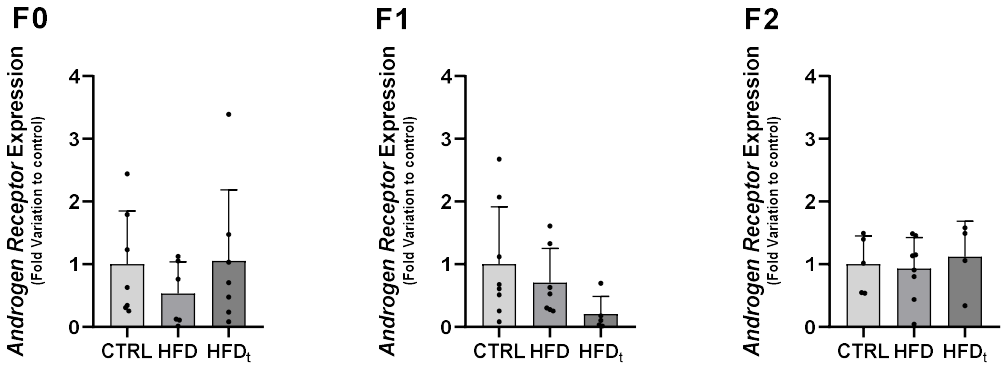

Figure 8: Expression of *AR* in the prostate of mice fed with a standard chow (CTRL) or high-fat diet throughout life (HFD) or only until adulthood (HFD_t) of F0, F1, and F2. Data shows the expression of *AR* in the prostate of mice of F0, F1, and F2. The expression was determined by qPCR. Results are expressed as mean ± SD (n=8 for each graph bar), with the exception of the groups CTRL (n=7), HFD (n=6), and HFD_t (n=7) in F0, HFD (n=7) and HFD_t in F1 (n=5), and CTRL (n=5) and HFD_t (n=4) in F2. Outliers were identified by ROUT (Q = 10%).

4.3.2. *HOXB13* expression tends to be increased in the prostate of the mice fed with HFD until adulthood and decreased in their F1

Heightened HOXB13 expression is present in the prostate of adult individuals and is implicated in PCa development due to its role in cell growth and proliferation. In addition, HOXB13 is associated with obesity on account of its role in lipogenesis. Given the significant roles that HOXB13 plays in the context of prostate physiology, particularly in relation to PCa and obesity, we postulated the potential impact of distinct dietary regimens on *HOXB13* expression. This hypothesis extended beyond the immediate progenitor generation, encompassing the offspring's prostate tissue as well, even when nourished with a standard chow diet (as presented in Figure 9).

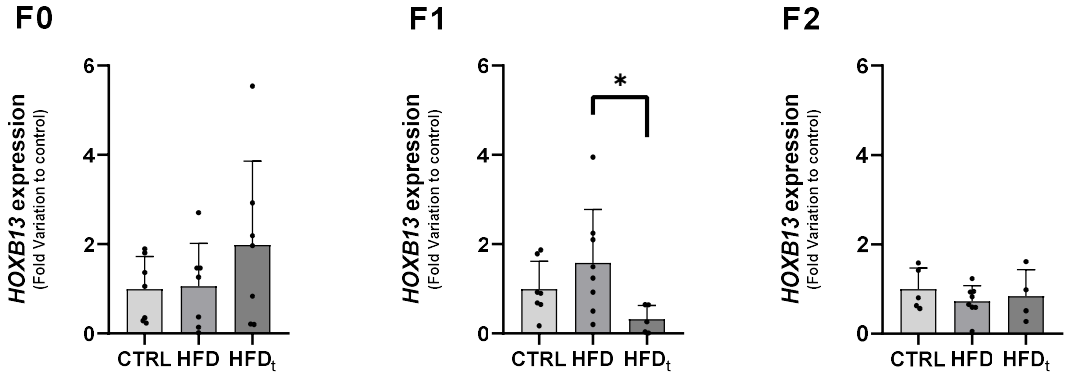

Figure 9: Expression of *HOXB13* in the prostate of mice fed with a standard chow (CTRL) or high-fat diet throughout life (HFD) or only until adulthood (HFD_t) of F0, F1, and F2. Data shows the expression of *HOXB13* in the prostate of mice of F0, F1, and F2. The expression was determined by qPCR. Results are expressed as mean ± SD (n=8 for each graph bar), with the exception of the groups in F0 (n=7), the CTRL (n=7) and HFD_t (n=5) in F1, and the CTRL (n=5) and HFD_t (n=4) in F2. Outliers were identified by ROUT (Q = 10%). Significantly different results ($p < 0.05$) are as indicated: * relative to CTRL).

In the HFD_t group of F0, *HOXB13* expression in the prostate shows a tendency towards an increase, relative to the CTRL group (from 1.98 ± 1.88 to 1.00 ± 0.72). However, for the offspring, represented in the F1, reversion of the diet presented a tendency towards a decrease of *HOXB13* expression in the prostate when compared to the levels detected in the prostate of mice fed only with standard chow (from 0.32 ± 0.31 to 1.00 ± 0.62). In addition, *HOXB13* expression in the prostate of those mice presented a significant decrease when compared with the expression detected in the prostate of mice from the HFD group (1.58 ± 1.19). In F2, no significant differences were found in *HOXB13* expression in the prostate between the CTRL group (1.00 ± 0.47) and HFD (0.73 ± 0.35) or HFD_t (0.84 ± 0.59) (Figure 9). Taking all into account and given the fact that HOXB13 is a crucial carcinogenesis hallmark, our findings suggest that an HFD or even a reversion of the diet may not be sufficient to revert the effects caused by the dietary changes, especially during early life, which appear to impact the expression of this PCa biomarker. This transcriptional change could suggest an increased risk for PCa development in the offspring when an HFD is present, even if transitory, during early life.

4.3.3. Relationship between *HOXB13* and *AR* expression in both F0 and F1 generation prostate tissues of mice

It is established that HOXB13 plays a pivotal role in regulating the AR gene through a complex interaction. HOXB13 can activate and repress distinct AR genes. This dynamic interplay between HOXB13 and AR is of paramount importance for orchestrating proper growth and finely tuning transcriptional activity within the prostate. With this significance in mind, our study sought to uncover a potential correlation between the expression

patterns of *HOXB13* and *AR* in the prostate of animals subjected to different dietary regimens. To achieve this, we conducted a thorough analysis using the Pearson Correlation test. This analytical approach allowed us to search for the potential link between the expression levels of *HOXB13* and *AR* transcripts in the prostate of animals subjected to different dietary regimens, shedding light on the potential relationship between these PCa biomarkers and prostate function and regulation. A positive correlation was found between the expression of *HOXB13* and *AR* in the prostate of the progenitor (F0) (Figure 10, F0, with a correlation coefficient of r=0.6068) and F1 (Figure 10, F1, with a correlation coefficient of r= 0.7341). However, no correlation was found in the prostate of F2 (data not shown). These results confirm a significant positive correlation between *HOXB13* and *AR* expression in the prostate of mice. The loss of this correlation in F2 suggests a switch in the interaction of *HOXB13* and *AR*, which could be related to the migration of the HFD effects in this generation of animals.

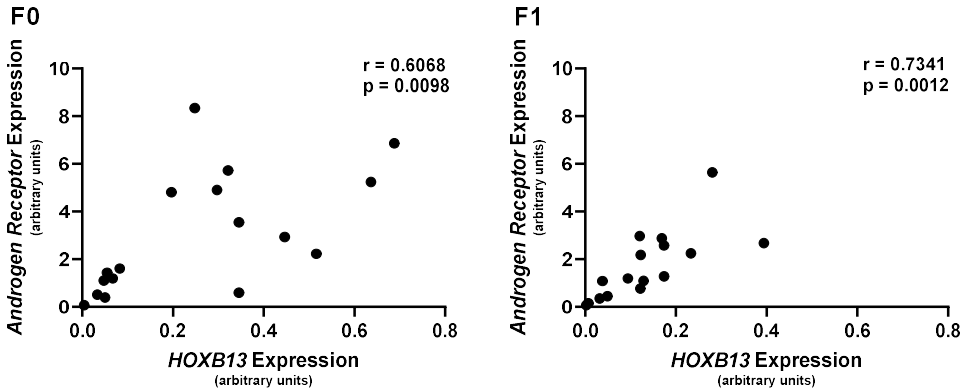

Figure 10: Pearson Correlation between *HOXB13* and *AR* expression in the prostate of mice fed with a standard chow (CTRL) or high-fat diet throughout life (HFD) or only until adulthood (HFD_t) of F0 and F1. Data shows the correlation between *HOXB13* and *AR* expression in the prostate of mice of F0 (n=17) and F1 (n=16) evaluated by Pearson Correlation. Outliers were identified by ROUT (Q = 10%). All P values < 0.05 were considered statistically significant.

4.4. *FTO* expression in the prostate is altered by the dietary regimens and is correlated with PCa biomarkers

FTO is a genetic factor linked to elevated BMI and adiposity. As a demethylase enzyme, FTO is implicated in the modulation of epigenetic m6A modifications, potentially influencing the expression of *HOXB13*. Such an association has been established in the context of endometrial and gastric cancers [250, 251]. Building upon this backdrop, we postulated that a similar interplay between *FTO* and *HOXB13* might manifest within the prostate tissue of mice subjected to different dietary regimens. *FTO* expression in the prostate presented the same tendencies as the expression of *HOXB13*, tending towards an increase in the HFD_t group in F0 (from 1.48 ± 1.07 to 1.00 ± 0.83 in the prostate of

mice fed with standard chow) and a decrease for the same group in F1 (from 0.28 ± 0.24 to 1.00 ± 1.08 in the prostate of CTRL mice). Additionally, *FTO* expression in the prostate of HFD_t mice presented a significant decrease in comparison to the expression detected in the prostate of mice from the HFD group (3.25 ± 2.76) (Figure 11).

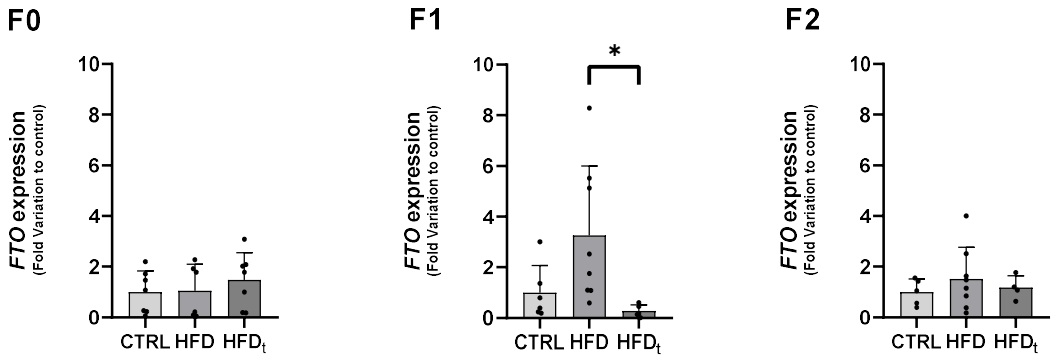

Figure 11: Expression of *FTO* in the prostate of mice fed with a standard chow (CTRL) or high-fat diet throughout life (HFD) or only until adulthood (HFD_t) of F0, F1, and F2. Data shows the expression of *FTO* in the prostate of mice of F0, F1, and F2. The expression was determined by qPCR. Results are expressed as mean ± SD (n=8 for each graph bar), with the exception of the groups CTRL (n=7), HFD (n=6), HFD_t (=7) in F0, the CTRL (n=6) and HFD_t (n=5) in F1, and CTRL (n=5) and HFD_t (n=4) in F2. Outliers were identified by ROUT (Q = 10%). Significantly different results ($p < 0.05$) are as indicated: * relative to CTRL).

To further study a possible correlation between *FTO* and PCa biomarkers expression in the prostate of the animals subjected to the different dietary regimens, a Pearson Correlation test analysis was performed. A positive correlation was found between the expression of *HOXB13* and *FTO* in the prostate of F0 (Figure 12, F0, with a correlation coefficient of r=0.7714) and F1 (Figure 12, F1, with a correlation coefficient of r=0.7654). These findings validate a robust and noteworthy positive correlation between *HOXB13* and *FTO* expression within the mouse prostate. Elevated *FTO* expression corresponds to heightened *HOXB13* expression, underscoring the interconnected nature of these factors in the prostate. Notably, a positive correlation is evident between *AR* and *FTO* in both the prostate of F0 generation (Figure 13, F0, with a correlation coefficient of r=0.9113) and the F1 generation (Figure 13, F1, with a correlation coefficient of r=0.5905). Once again, no correlation was found between the expression of *HOXB13* and *FTO* nor *AR* and *FTO* in the prostate of animals of the F2 generation (data not shown), further supporting a switch in the interaction of these prostate mediators.

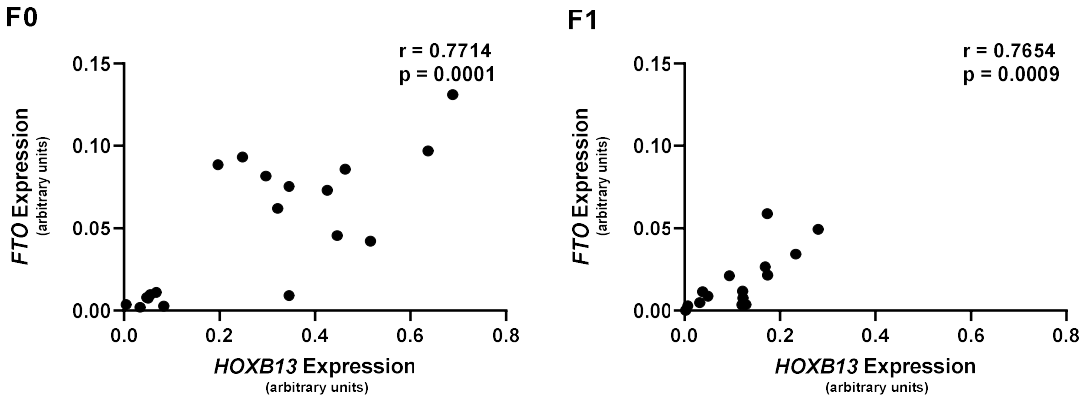

Figure 12: Pearson Correlation between *HOXB13* and *FTO* expression in the prostate of mice fed with a standard chow (CTRL) or high-fat diet throughout life (HFD) or only until adulthood (HFD_t) of F0 and F1. Data shows the correlation between *HOXB13* and *FTO* expression in the prostate of mice of F0 (n=19) and F1 (n=15) evaluated by Pearson Correlation. Outliers were identified by ROUT (Q = 10%). All P values < 0.05 were considered statistically significant.

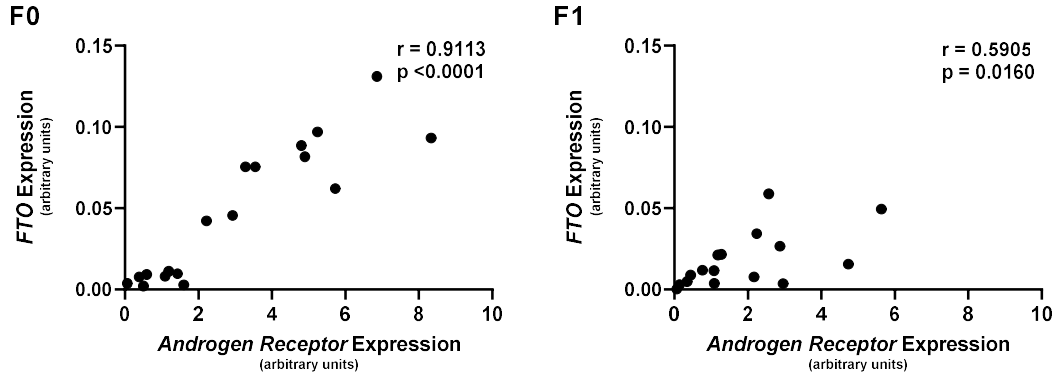

Figure 13: Pearson Correlation between *AR* and *FTO* expression in the prostate of mice fed with a standard chow (CTRL) or high-fat diet throughout life (HFD) or only until adulthood (HFD_t) of F0 and F1. Data shows the correlation between *AR* and *FTO* expression in the prostate of mice of F0 (n=18) and F1 (n=16) evaluated by Pearson Correlation. Outliers were identified by ROUT (Q = 10%). All P values < 0.05 were considered statistically significant.

4.5. Stability of *HOXB13* promoter methylation levels in the prostate is unaffected by dietary changes

It is established that hypermethylation of the HOXB13 promoter triggers a reduction in gene expression by inducing transcriptional silencing. This mechanism is occasionally linked to cancer development, serving as a hallmark of carcinogenesis. A pertinent study has highlighted that heightened *FTO* expression might modulate *HOXB13* expression by diminishing its methylation levels, particularly in gastric cancer [250]. Our work has shown that *HOXB13* expression confirmed the consistent presence of *HOXB13* transcripts in the prostates of mice across multiple generations (Figure 9). Thus, we then investigated the methylation patterns within the HOXB13 promoter to determine if it was possible to establish their connection with *HOXB13* expression, subsequently extending

to the relationship with *FTO* expression. This process encompassed DNA extraction followed by a comprehensive bisulfite treatment, effectively converting unmethylated cytosines into uracil. Subsequently, Methylation-Specific PCR (MSP) was executed to validate the existence of *HOXB13* promoter transcripts in mice prostate tissue (Figure 14) and to ensure the accurate conversion of all samples. Then, Quantitative MSP (qMSP) was conducted to gauge the levels of methylation within the HOXB13 promoter. This was achieved by employing a pair of specific primer sets designed via an-online software using the gene's promoter sequence – one set targeting methylated sequences and the other targeting unmethylated sequences. The outcomes of methylation analysis were depicted as a ratio between methylated and unmethylated levels. Intriguingly, alterations in the dietary regimen spanning several generations had an inconsequential effect on the methylation levels of the *HOXB13* promoter of the prostate. A faint inclination towards reduced methylation was observed in the HFD group (0.81 ± 0.27) in the F0 generation when in comparison with mice fed with standard chow (1.00 ± 0.20) and reversion diet (1.07 ± 0.23) (Figure 15). The overall implication suggests that fluctuations in prostate *HOXB13* expression may not be intimately linked to variations in promoter methylation levels.

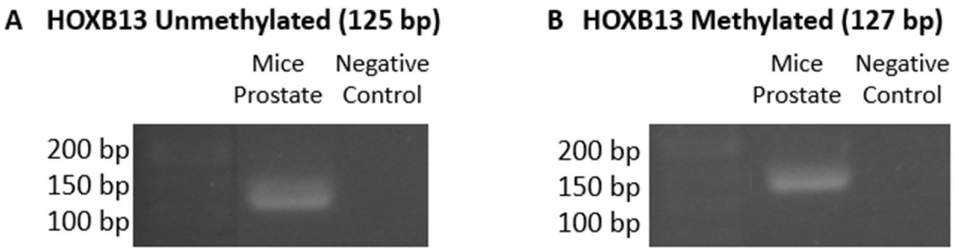

Figure 14: Identification of methylation-specific primers, HOXB13 unmethylated and HOXB13 methylated, in mice prostate tissue by MSP. MSP was used to identify the presence of unmethylated HOXB13 (A) and methylated HOXB13 (B) transcripts in mice prostate tissue. A cDNA-free sample was used as negative control.

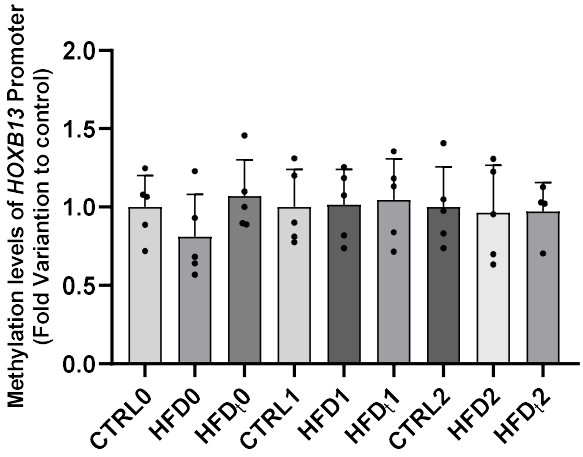

Figure 15: Methylation levels of *HOXB13* promoter in the prostate of mice fed with a standard chow (CTRL) or high-fat diet throughout life (HFD) or only until adulthood (HFD_t) of F0, F1, and F2. Data shows the methylation levels of *HOXB13* promoter in the prostate of mice. The expression was determined by qMSP. Results are expressed as mean ± SD (n=5 for each graph bar), with the exception of the group HFD_t in F2 (n=4).

4.5.1. Methylation levels are correlated with *FTO* and *HOXB13* expression in the prostate of mice

Considering prior research and the uncharted interplay between *FTO* expression and *HOXB13* promoter methylation in the prostate, we embarked on an exploration of potential correlations. This encompassed investigating the plausible relationship between *FTO* expression and *HOXB13* promoter methylation, alongside probing the potential correlation between *HOXB13* expression and its promoter methylation levels in the prostate. To accomplish this, we conducted a comprehensive Pearson Correlation test analysis. The outcomes unveiled a noteworthy positive correlation between *FTO* expression and *HOXB13* promoter methylation levels in the prostate of both the F0 generation (Figure 16, F0, with a correlation coefficient of r=0.6039) and the F1 generation (Figure 16, F1, with a correlation coefficient of r=0.5995). Interestingly, this correlation was not observed in the F2 generation (data not shown). Furthermore, an additional positive correlation emerged between *HOXB13* expression and the methylation levels in the prostate of its promoter in the F0 generation (Figure 17, with a correlation coefficient of r=0.6153). Consequently, these findings substantiate a significant positive correlation between the expression of *FTO* and *HOXB13* genes and the methylation levels of *HOXB13* promoter within the murine prostate. Once again, this

correlation is lost on the F2 generation, which further suggests a change in the interaction of these factors.

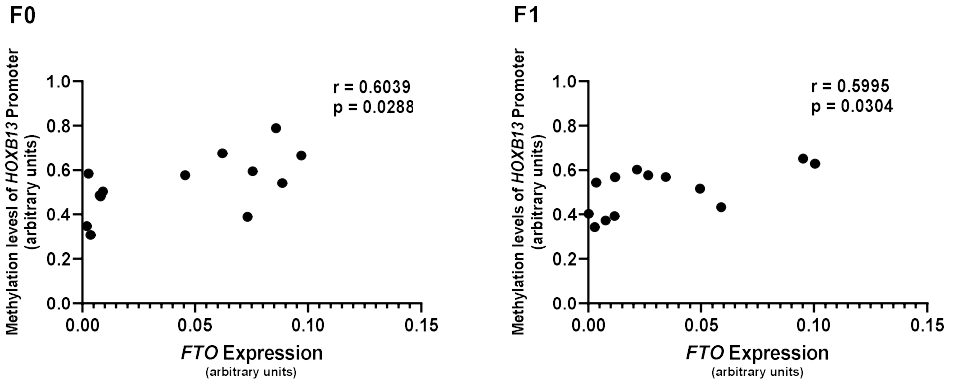

Figure 16: Pearson Correlation between *FTO* expression and *HOXB13* promoter methylation levels in the prostate of mice fed with a standard chow (CTRL) or high-fat diet throughout life (HFD) or only until adulthood (HFD_t) of F0 and F1. Data shows the correlation between *FTO* expression and *HOXB13* promoter methylation levels in the prostate of mice of F0 (n=13) and F1 (n=14) evaluated by Pearson Correlation. Outliers were identified by ROUT (Q = 10%). All P values < 0.05 were considered statistically significant.

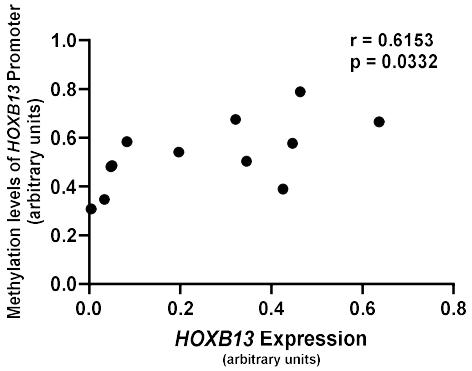

Figure 17: Pearson Correlation between *HOXB13* expression and *HOXB13* promoter methylation levels in the prostate of mice fed with a standard chow (CTRL) or high-fat diet throughout life (HFD) or only until adulthood (HFD_t) of F0. Data shows the correlation between *HOXB13* expression and *HOXB13* promoter methylation levels in the prostate of mice of F0 (n=13) evaluated by Pearson Correlation. Outliers were identified by ROUT (Q = 10%). All P values < 0.05 were considered statistically significant.

4.6. *TNF-α* expression is altered in the prostate of F1 and is correlated with *FTO* and PCa biomarkers expression

Obesity triggers a persistent low-grade state of inflammation through systemic changes induced. As a consequence, inflammatory biomarkers, such as TNF-α, manifest deviations in individuals with metabolic disorders. Specifically, TNF-α, a participant in inflammatory processes, exhibits escalated expression within adipose tissue. Given its pivotal role in tumorigenesis, TNF-α assumes potential relevance in the context of PCa development. Our hypothesis centred on the anticipation of altered *TNF-α* expression in

the prostate due to dietary modifications. Furthermore, we postulated the potential presence of correlations linking *TNF-α* expression with PCa biomarkers and *FTO* expression. The tool used to gauge *TNF-α* expression levels in the prostate was qPCR. Although we could not find any alteration regarding *TNF-α* expression in the animals exposed to different diets in F0, expression of *TNF-α* in the prostate showcased a significant alteration in the F1 generation. This alteration shows a decrease in expression of *TNF-α* in the prostate of mice subjected to HFD_t (0.22 ± 0.16) when compared with HFD (2.60 ± 2.19). This notable discrepancy that emerged between the prostate of mice fed with HFD throughout life or until adulthood, mirrors a trend akin to the fluctuations observed in *HOXB13* and *FTO* expression (Figure 18). This outcome strongly suggests that the changes in dietary regimen indeed prompted substantial shifts in the long-term inflammatory state of the prostate. In essence, these findings underscore the marked impact of dietary alterations on the inflammatory milieu, substantiating an interplay that holds potential implications for PCa dynamics.

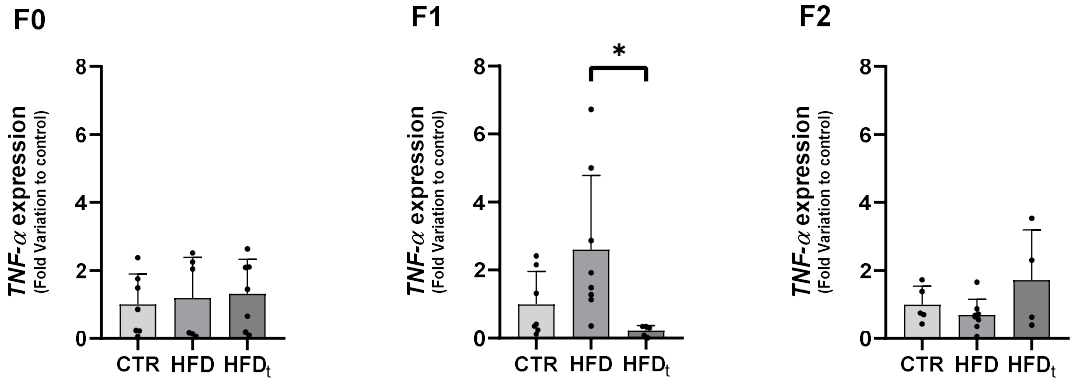

Figure 18: Expression of *TNF-α* in the prostate of mice fed with a standard chow (CTRL) or high-fat diet throughout life (HFD) or only until adulthood (HFD_t) of F0, F1, and F2. Data shows the expression of *TNF-α* in the prostate of mice of F0, F1, and F2. The expression was determined by qPCR. Results are expressed as mean ± SD (n=8 for each graph bar), with the exception of the groups CTRL (n=7), HFD (n=6), and HFD_t (n=7) in F0, CTRL (n=7) and HFD_t (n=5) in F1, and CTRL (n=5) and HFD_t (n=4) in F2. Outliers were identified by ROUT (Q = 10%). Significantly different results ($p < 0.05$) are as indicated: * relative to CTRL).

Given the intrinsic link between obesity and inflammation, our study sought to elucidate potential correlations in the prostate between *TNF-α* and the obesity-related gene, *FTO*, using the Pearson Correlation test. Additionally, we endeavoured to explore correlations between *TNF-α* and PCa biomarkers. Strikingly, a positive correlation emerged between *TNF-α* and *FTO* expression in the prostate within the F0 generation (Figure 19, F0, with a correlation coefficient of r=0.9049) as well as the F1 generation (Figure 19, F1, with a correlation coefficient of r=0.7027). Turning our attention to PCa biomarkers, we uncovered a positive correlation between *HOXB13* and *TNF-α* expression in the prostate

within the F0 generation (Figure 20, F0, with a correlation coefficient of r=0.6403) and the F1 generation (Figure 20, F1, with a correlation coefficient of r=0.7580). However, it is noteworthy that, in the prostate, in both *HOXB13* and *FTO*, the correlation with *TNF-α* appears to diminish in the F2 (data not shown). Interestingly, concerning the correlation between *AR* and *TNF-α* expression, a positive correlation is discernible solely within the F0 generation (Figure 21, with a correlation coefficient of r=0.8569). Thus, our results establish a positive correlation between obesity and inflammation in the prostate of animals fed with distinct dietary regimens, as well as between PCa biomarkers and *TNF-α*. This substantiates the linkage between PCa and inflammation, bolstered by the discernible impact of dietary changes on this interplay.

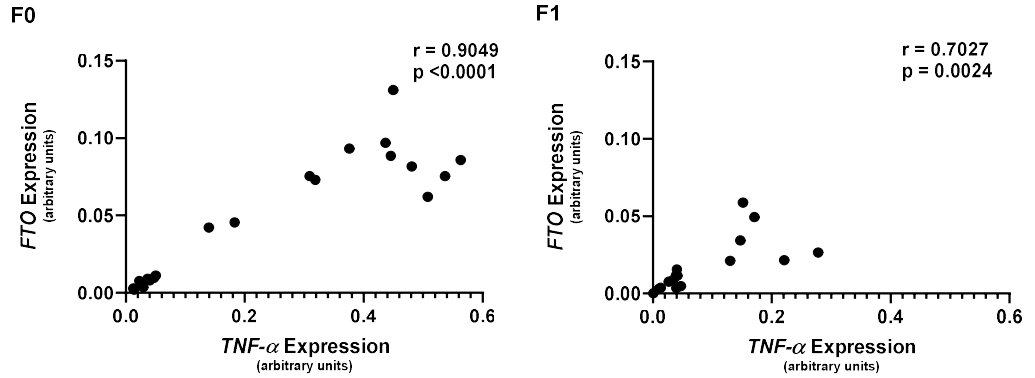

Figure 19: Pearson Correlation between *TNF-α* and *FTO* expression in the prostate of mice fed with a standard chow (CTRL) or high-fat diet throughout life (HFD) or only until adulthood (HFD_t) of F0 and F1. Data shows the correlation between *TNF-α* and *FTO* expression in the prostate of mice of F0 (n=20) and F1 (n=16) evaluated by Pearson Correlation. Outliers were identified by ROUT (Q = 10%). All P values < 0.05 were considered statistically significant.

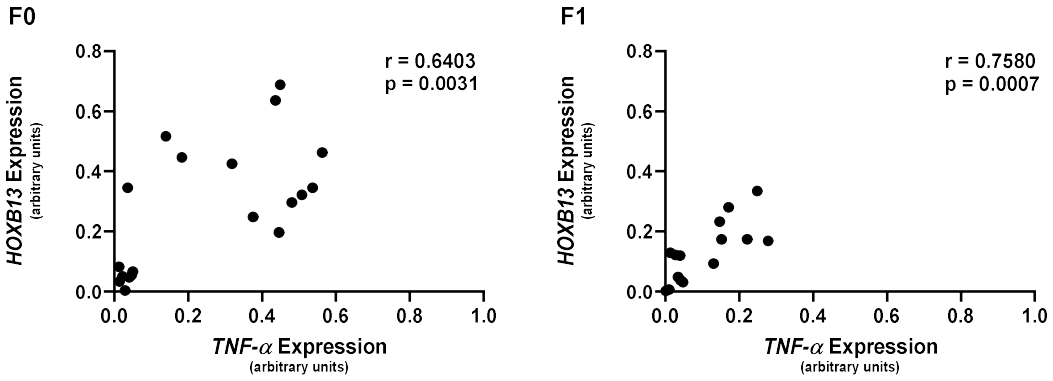

Figure 20*:* Pearson Correlation between *TNF-α* and *HOXB13* expression in the prostate of mice fed with a standard chow (CTRL) or high-fat diet throughout life (HFD) or only until adulthood (HFD_t) of F0 and F1. Data shows the correlation between *TNF-α* and *HOXB13* expression in the prostate of mice of F0 (n=19) and F1 (n=16) evaluated by Pearson Correlation. Outliers were identified by ROUT (Q = 10%). All P values < 0.05 were considered statistically significant.

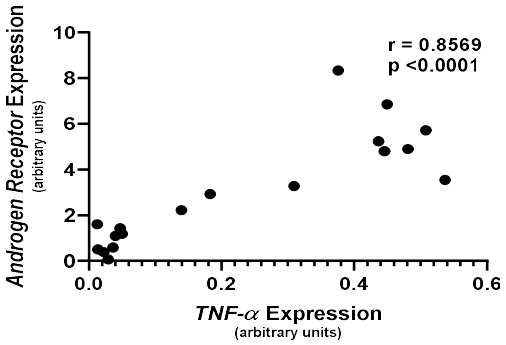

Figure 21: Pearson Correlation between *TNF-α* and *AR* expression in the prostate of mice fed with a standard chow (CTRL) or high-fat diet throughout life (HFD) or only until adulthood (HFD_t) of F0. Data shows the correlation between *TNF-α* and *AR* expression in the prostate of mice of F0 (n=18) evaluated by Pearson Correlation. Outliers were identified by ROUT (Q = 10%). All P values < 0.05 were considered statistically significant.

4.7. Lipid peroxidation levels are increased in the prostate of mice fed with HFD during their life and in their offspring

ROS are recognized culprits in inducing heightened OS when intertwined with obesity. In parallel, escalated OS is notably discernible in adipose tissue. Remarkably, OS has also been identified as a catalyst for PCa development. The PCa biomarker, HOXB13, is known to exhibit overexpression in tandem with an increase in OS through elevated ROS levels [295]. Likewise, overexpression of FTO has been linked to elevated OS and lipid accumulation [296]. Thus, we proposed to study OS related biomarkers in the prostate of mice subjected to the different dietary regimens and in the prostate of their offspring. Identification of OS-related biomarkers was accomplished via Slot-Blot for protein nitration and lipid peroxidation. The determination of protein nitration results in the prostate displayed no significant influence from dietary alterations in the F0 and their offspring. However, a propensity towards reduction of protein nitration levels was observed in the prostate of the mice from the HFD_t group of the F0 generation (0.15 ± 0.14) while a corresponding inclination towards elevation was evident in the same group in the F1 (1.92 ± 1.94) (Figure 22). In contrast, lipid peroxidation in the prostate displayed susceptibility to different diets across all generations. In the F0 generation, the HFD group exhibited a significant increase in prostate lipid peroxidation when compared to the CTRL group (7.76 ± 4.63 to 1.00 ± 1.36, respectively). Moreover, the HFD group in the F0 showed a significant increase in lipid peroxidation in the prostate when juxtaposed with the HFD_t group of the same generation, which experienced a noteworthy decrease (7.76 ± 4.63 to 1.63 ± 1.76, respectively). Furthermore, these significant differences persisted across the HFD groups of F1 and F2 generations and with the HFD_t groups of F1 and F2 generations (Figure 23). Collectively, the findings shed light on the potential

of an HFD to escalate OS in the prostate, particularly through heightened lipid peroxidation levels. Interestingly, indications suggest that the effects can be reversed in subsequent generations, revealing a noteworthy aspect of inheritance dynamics stemming from paternal exposure. Even though *AR*, *HOXB13*, *FTO*, *TNF-α* expression has been related with OS, no correlation was found between protein nitration nor lipid peroxidation and the PCa biomarkers nor *FTO* and *TNF-α* expression, across all generations and groups (data not shown).

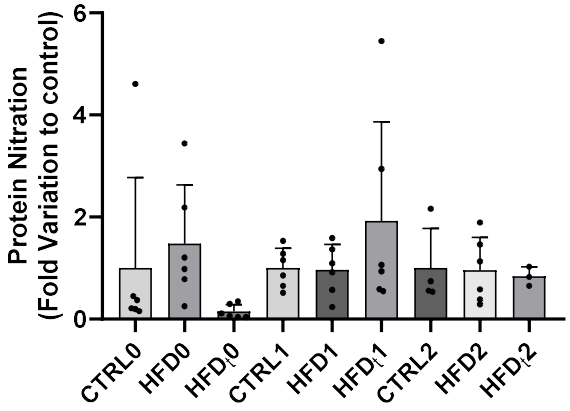

Figure 22: Evaluation of protein nitration in the prostate of mice fed with a standard chow (CTRL) or high-fat diet throughout life (HFD) or only until adulthood (HFD_t) of F0, F1, and F2. Data shows the analysis of protein nitration in the prostate of mice of F0, F1, and F2. The expression was determined by Slot-Blot. Results are expressed as mean ± SD (n=6 for each graph bar), with the exception of the groups CTRL (n=4) and HFD_t (n=3) in F2.

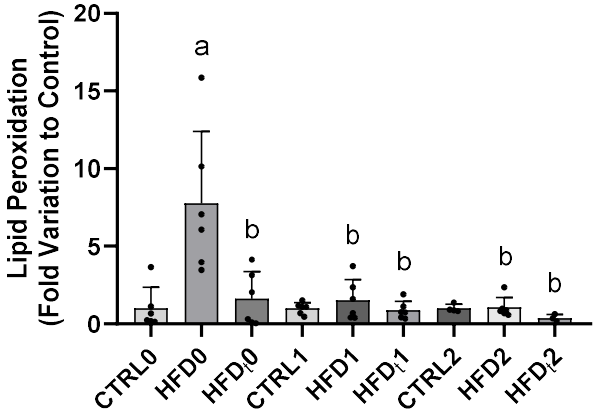

Figure 23: Evaluation of lipid peroxidation in the prostate of mice fed with a standard chow (CTRL) or high-fat diet throughout life (HFD) or only until adulthood (HFD_t) of F0, F1, and F2. Data shows the analysis of lipid peroxidation in the prostate of mice of F0, F1, and F2. The levels were determined by Slot-Blot. Results are expressed as mean ± SD (n=6 for each graph bar), with the exception of the groups CTRL (n=4) and HFD_t (n=3) in F2. Significantly different results ($p < 0.05$) are indicated as (a) relative to CTRL F0 and (b) relative to HFD F0.

5. Discussion

Nowadays, metabolic disorders have become a concern in the health of society due to their swiftly rising rates worldwide. Metabolic disorders are associated with homeostasis dysregulation and include diseases such as obesity, T2D, and cardiovascular disease [297]. Obesity is defined by WHO as an “abnormal or excessive fat accumulation that presents a risk to health” [35], with rates increasing worldwide, mainly due to society lifestyle changes, like the higher intake of high-fat and high-caloric diets and increased sedentary behaviours, which suit better the modern needs. WHO estimated that more than 1.9 billion adults aged 18 years and older are overweight, and of those, over 650 million have obesity. If this trend persists, by 2030, a majority of the global adult population will be grappling with excess weight [298]. Obesity precipitates a disruption in hormonal regulation, perpetuating a chronic low-grade inflammatory state coupled with OS [40, 43, 299]. The alterations triggered by obesity perturb the equilibrium in the release of adipokines and hormones, engendering the susceptibility to comorbidities such as cancer, diabetes mellitus, arterial hypertension, and cardiovascular diseases. Furthermore, obesity elicits metabolic shifts and disturbances in energy equilibrium [300]. Beyond these direct effects, genetic and epigenetic elements associated with obesity have the potential to be inherited across generations [171]. In fact, emerging research suggests that direct in-utero exposure or early postnatal contact with the maternal diet can induce metabolic changes that manifest later in life. Moreover, the nutritional status of both parents before conception exerts a substantial influence on the metabolic well-being of their offspring [301]. Notably, studies have demonstrated that an HFD can influence the phenotype of a third-generation female solely through the paternal lineage, thereby underscoring transgenerational inheritance rooted in the germline. The authors hypothesized if exposure to an HFD in mice could result in epigenetic modifications via the paternal germline in F3 offspring. Females of F3 presented a higher body size, which was transmitted via paternal lineage. Moreover, only this lineage transmitted the phenotype to F3 females. Curiously, this expression pattern was not detected in the maternal lineage [302]. Similarly, parental exposure to an HFD has been observed to impart metabolic phenotypic changes spanning up to the F2 generation. For this, rats were divided into two groups, the CTRL diet, and the HFD, to analyse possible changes in two following generations. F1 females presented a higher body weight via paternal lineage. Similar tendency was seen in their male offspring. Furthermore, reproductive changes were found in the grandsons, indicating that an HFD exposure can not only cause metabolic alterations in the progenitors but also in the offspring [303].

PCa is a heterogeneous disease, with its aggressive form being associated with progression and metastasis. Men with obesity present a higher risk of developing PCa, one of the most common and deadly cancers among men. Each year 1.6 million men are diagnosed with PCa, and 366,000 men die of the disease [28, 29]. The common treatment for PCa is based on surgery and radiotherapy, depending on each case [304]. PCa can exhibit hereditary traits, with estimated hereditary factors accounting for around 5-15% of cases. Hereditary PCa is often linked to gene mutations that follow an autosomal dominant inheritance pattern, leading to an early disease onset. Among these gene mutations, those in specific genes like HOXB13, BRCA2, CHEK2, ATM, MMR, BRCA1, PALB2, BRIP1, and NBS1 contribute to 0.6-6.25% of cases of hereditary PCa, while a portion of cases remains linked to genes that are yet unidentified [189]. Furthermore, mutations in DNA-repair genes have been proposed as potential genetic triggers for familial PCa [305]. However, up to now, no conclusive data has shown a correlation between the inheritance of factors related to obesity that could also contribute to the development of PCa. In men affected by obesity, PCa might be prone to misdiagnosis through conventional methods like PSA detection and rectal examinations, owing to the metabolic disruptions inherent to this disorder. Consequently, there arises a necessity for novel PCa biomarkers. The core objective of this work was twofold: firstly, to pinpoint distinct PCa biomarkers within the prostate tissue of mice and discern any correlation with obesity's onset; secondly, to ascertain whether alterations resulting from dietary modifications could be transmitted to subsequent generations, heightening the susceptibility to PCa development.

For this work, a transgenerational mice model was used. Animals were divided into three groups with different diets: standard chow (CTRL), HFD (carbohydrate: 35.7%, protein: 20.5%, and fat: 36.0%) and HFD_t (60 days HFD, plus 140 days standard diet), ad libitum for 200 days post-weaning. Mice were mated with lean females to generate F1 and F2, which were fed with a chow diet. Crisóstomo and colleagues studied the biometric data and metabolic function of this model, particularly the testicular metabolic profile [179, 306]. Body weight and fat mass were reduced by the dietary switch in the HFD_t group, reaching mean values of mice from the CTRL group. Hence, HFD mice were 50% heavier, when compared with CTRL and HFD_t. Higher glycaemia levels were shown by mice fed with HFD, whereas those of the HFD_t group presented fasting glycaemia levels in comparison with CTRL [179]. On the other hand, diet switch or weight loss did not restore the decreased sperm quality (viability and motility) caused by HFD exposure, even if metabolic syndrome development is avoided. Energy metabolism homeostasis was not restored by the diet switch in mice, having great differences between CTRL and

HFD [179, 306]. AMP, an important marker for metabolic homeostasis, presented a higher content in HFD and HFD_t mice, whereas adenosine, associated with lower testosterone levels and metabolic disorders, was decreased in HFD and HFD_t [179, 307]. Additionally, diet switch affected nucleic acid metabolism, induced by HFD in mice testicular. In fact, glutamate was increased in the testis of HFD_t mice, while glutamine, an indicative of metabolic dysfunction in Sertoli Cells, was higher in HFD and HFD_t. Contrastingly, testicular leucine was decreased in HFD_t, and even lower in HFD [179]. Furthermore, acetate, linked to the restoration of testicular function, was reduced in the testis of mice from HFD and HFD_t and succinate, associated with glucose intolerance, was elevated in HFD_t, when compared to HFD and CTRL [179, 308]. Consequently, the testis oxidative profile was not attenuated after diet correction. Glutathione, an important antioxidative component, was increased in testes from HFD and HFD_t. While, testicular betaine, crucial in antioxidant pathways and associated with both glucose and insulin resistance, was only increased in the HFD group. Concluding, both testicular pyruvate and glutamate metabolism, ethanol degradation and ammonia recycling were affected by HFD, with diet correction not being able to revert the detected effects [179]. Across all diets, while no significant differences were found in the levels of FSH, LH, E2, and testosterone, insulin was the only hormone affected in the serum of mice. Results showed that HFD effects until adulthood are not entirely reversed in β-cell function and insulin sensitivity. Nonetheless, insulin resistance was affected by the dietary regimens, mainly HFD displayed high plasma insulin. On the other hand, reversion of diet restored plasma insulin levels [306].

Firstly, the body weight of animals fed with three different diets, across three generations was analysed. The existing literature underpins the observation that exposure to an HFD precipitates amplified body weight, an augmented adipocyte count in adipose tissue, and more substantial fat accumulation [309-311]. Within the scope of this study, our findings in the initial generation align with these trends. The group exposed directly to the HFD throughout their life exhibited a heightened body weight in comparison to the CTRL group fed with standard chow. Intriguingly, the group subjected to HFD until adulthood and then reverted to a standard chow displayed a significant contrast with the HFD-exposed group yet exhibited a body weight akin to the CTRL group. As for the subsequent F1 and F2 generations, noteworthy reductions in body weight were evident in both the offspring of the HFD and HFD_t groups, showing that reversal of diet-induced effects can occur. Secondly, the presence of PCa biomarkers transcripts, *HOXB13* and *AR*, was confirmed in mice prostate tissue. Next, we analysed the expression of these biomarkers in the prostate tissue of animals exposed to different dietary regimens, across three

generations (F0 to F2). AR is crucial for the development and correct function of the prostate, regulating different events such as proliferation, differentiation, and/or invasion. In PCa, AR expression is regulated by peptide and steroid hormones, which are altered due to hormonal dysregulation associated with the metabolic disorder. AR is over activated in PCa promoting cancer development and progression. Furthermore, AR expression is also regulated by cytokines, peptides involved in inflammatory processes, correlated with PCa development [312, 313]. In this work, *AR* expression was not affected in the prostate by the different diets in F0 nor in the prostate of their offspring, suggesting that the dietary changes studied do not have an impact on the expression of this PCa biomarker. Previous studies have reported that obesity and exposure to HFDs alter the levels of androgen and subsequently AR [314, 315]. The first study, conducted by Gromadzka-Ostrowska, divided rats into two groups, ones exposed to an HFD, and ones exposed to a high-lard diet, for 3 and 6 weeks. Additionally, the HFD was composed of 20% of grapeseed oil, rich in linoleic acid. The HFD group presented a higher AR density in the prostate when compared to the other group. It was then suggested that a shorter exposure to an HFD was able to promote AR density increase and possible carcinogenesis. Interestingly, it was also suggested that both HFD composition and time of exposure could influence androgens secretion [314]. In the second study, authors examined the effects of an HFD (20% fat) during 15 weeks in rats. Obese rats presented lower levels of plasma testosterone, as well as a 50% reduction in AR content in the prostate, in comparison to the CTRL group [315]. Since HFDs influence PCa and BPH development and progression, a study was conducted to evaluate the long-term effects of an HFD in rats. For this, rats were exposed to an HFD, composed of 20% fat, for 30 weeks, with the goal to induce obesity. After, the ventral prostate was examined for several parameters, including AR content. Results revealed body weight gain, as well as prostate increase, due to lower levels of plasma testosterone, in obese rats. Notably, it was concluded that prostate enlargement was established in the early obesity phases. Curiously, an increase of 125% in *AR* expression is registered [316]. In comparison to our work, the diet varied significantly in the percentage of fat, with 36.0–59.0%, and in exposure time of 200 and 60 days. Therefore, we hypothesize that both factors, composition of HFD and time of exposure, could influence the results here obtained.

Conversely, an elevated expression of *HOXB13* has been detected in the prostate, and intriguingly, this upregulation occurs independently of androgen influence. HOXB13 plays a pivotal role in maintaining the delicate equilibrium between cell growth and proliferation, influencing intricate mechanisms such as lipogenesis. Furthermore, this homeobox gene assumes a critical function in the proper developmental trajectory of the

prostate. The functional role of HOXB13 within prostate cells exhibits variation. For instance, in the PC3 cell line, HOXB13 seems to impede prostate cell growth by inhibiting androgen-mediated signalling. However, the story is different in the LNCaP cell line, where HOXB13 overexpression is closely associated with the progression of tumour growth [223, 317, 318]. Contrastingly to *AR*, *HOXB13* expression in the prostate was found to be altered by exposure to the different dietary regimens. In F0, *HOXB13* expression tends to increase in the prostate of animals fed with HFD until adulthood. Interestingly in F1, it tends to decrease in the prostate of animals from the same group, with a significant difference between the HFD and HFD_t groups. No changes were found in the *HOXB13* expression in the prostate of F2 generation from mice subjected to the different dietary regimens. HOXB13 is recognized for its direct involvement in repressing lipogenic transcriptional programs by enlisting the assistance of HDAC3, a histone deacetylase. This collaborative action effectively dampens *de novo* lipogenesis. Intriguingly, reduced *HOXB13* expression is linked to substantial lipid accumulation within PCa cells, with this occurrence standing as a hallmark of carcinogenesis [239, 240]. Hence, these findings underscore that the progeny of fathers exposed to an HFD, even with a subsequent transition to a standard diet during adulthood, remain susceptible to an elevated risk of PCa development. This vulnerability can be attributed to the alterations brought about in *HOXB13* expression, particularly during the early stages of development. These implications suggest that mere dietary correction might fall short of fully mitigating the impacts of an HFD in the prostate. Nonetheless, it is imperative to note that additional research is needed to solidify these conclusions and unravel the underlying mechanisms that orchestrate these effects.

HOXB13 is known to critically regulate AR, both positively and negatively by its interaction with chromatin in PCa cells [319]. While HOXB13 functions as a modulator of the AR curbing AR-mediated growth signalling within the domain of androgen control, it is worth noting that the interplay between HOXB13 and AR can manifest autonomously, even in the absence of androgens [225]. This observation underscores the pivotal role that HOXB13 assumes in propelling the progression of androgen-independent PCa. It was reported by Kim and colleagues that HOXB13 overexpression is present in the majority of cases of hormone-refractory PCa when compared to hormone-responsive PCa [225, 318]. Taking this into account, we analysed if a correlation between the expression of *HOXB13* and *AR* was present in mice prostate tissue, even after different dietary regimens, in the father and the offspring (F1 and F2). In F0 and F1, a positive correlation between *HOXB13* and *AR* in the prostate tissue was present, however, it was lost in F2. These findings imply that the impact of an HFD, as manifested through the

correlation between *HOXB13 and AR* expression in the prostate of the father, can be transmitted to their sons but not extended to their grandsons. A study by Zabalza and colleagues, analysed HOXB13 expression and its prognostic impact on tissue microarray of PCa. Authors found HOXB13 in more than half of interpretable tumours and a link to the AR expression. In fact, a positive correlation between these two biomarkers was seen in the PCa tissue samples, where overexpression of HOXB13 was followed by a higher expression of *AR* [229]. Therefore, our work reinforced the existence of a strong link between *HOXB13* and *AR* expression.

With the aim to correlate the development of obesity and PCa, we hypothesized that obesity-related genes expression can be altered. FTO is intricately linked with adiposity and metabolic disorders, encompassing conditions such as obesity and cancer. The function of FTO disrupts metabolic pathways, exerting influence over energy metabolism and the equilibrium within adipose tissue. Notably, escalated *FTO* expression within adipose tissue has been consistently associated with individuals grappling with obesity [320]. Through FTO demethylase function, this gene is associated with carcinogenesis and adipogenesis by different pathways. For example, via the FTO-mTOR axis, *in vivo* studies revealed that FTO participates in the mTORC1 signalling pathway. MEFs cells with loss of FTO presented reduced activation of mTORC1 pathway, lower translation rate, and higher autophagy, being this last factor a critical event in cancer progression [321, 322]. Consequently, an assessment of *FTO* expression within mice prostate tissue was conducted, followed by an exploration of potential correlations with PCa biomarkers. Intriguingly, the *FTO* expression profile unfolds with unexpected nuances. In the F0 generation, a propensity towards increased *FTO* expression is observed in the prostate of mice fed HFD until adulthood, which deviates from the anticipated pattern considering the typically elevated *FTO* expression linked to HFD groups. Moving to the F1, a noteworthy contrast emerges concerning *FTO* expression in the prostate of mice from HFD and HFD_t. Here, the prostate of mice fed with HFD until adulthood exhibits a tendency towards a decrease in *FTO* expression, whereas the prostate of HFD fed mice displays a tendency towards increased *FTO* expression. Once again, in the F2 generation, conspicuous shifts are notably absent, despite a tendency towards heightened *FTO* expression in the prostate of mice fed with HFD. Therefore, given FTO's classification as an obesity-related gene and the well-established association between obesity and PCa onset and development with individuals with obesity being at an elevated risk of the disease, we postulated the potential existence of a correlation between *FTO* expression and PCa biomarkers. Intriguingly, the trends observed in *FTO* expression align with those exhibited by *HOXB13*. Notably, FTO's activity is

interconnected with *HOXB13* expression through the removal of the m6A modification [250, 251], with heightened *FTO* expression being linked to an upsurge in *HOXB13* expression, as discussed previously. Through a similar pathway of the m6A modification, AR may also be related to FTO. Studies have shown that FTO has a crucial role in altering the proliferative ability of undifferentiated spermatogonia and maturation of Leydig cells in mice via AR regulation depending on m6A [323]. Likewise, it was reported that AR mRNA and protein expression can be affected by the m6A modification in PCa cells and altered levels in m6A modification may contribute to the development of PCa [324, 325]. Given this backdrop, we investigated to ascertain the presence of any correlation between *HOXB13*, *AR*, and *FTO* within mice prostate tissue. Remarkably, our analysis unveiled a positive correlation between *HOXB13* and *FTO* expression in the prostate of both the F0 and F1. A similar pattern was observed between *AR* and *FTO* expression in the prostate of those mice. Intriguingly, in F2, this correlation appeared to wane, hinting at the possibility that factors inherited by the sons could diminish across the grandsons, or that the effects instigated by the diet might undergo reversion. Therefore, it seems to indicate that a switch in the interaction of all these mediators may be happening in the prostate tissue in response to the dietary regimens or the transmission mechanisms. Notably, to our knowledge, this marks the pioneering instance where a positive correlation between *FTO* and either *HOXB13* or *AR* has been conclusively confirmed within mice prostate tissue. To unravel the underlying mechanisms responsible for the observed fluctuations in *HOXB13* expression within the prostate, we undertook experiments to determine the methylation levels within the *HOXB13* promoter. Promoter hypermethylation is known to lead to suppressed gene expression, a phenomenon linked to certain cancers, including PCa [260, 326]. As an example, in primary PCa, reduced methylation of the *HOXB13* promoter is associated with heightened *HOXB13* expression. Consequently, there exists a negative correlation between *HOXB13* expression and its methylation levels. [239]. Moreover, it has been suggested that *FTO* might decrease *HOXB13* promoter levels, increasing *HOXB13* expression [250]. Our findings reveal an absence of significant differences in the methylation levels of the *HOXB13* promoter in the prostate across the various diets and generations. This observation suggests that the shifts in *HOXB13* expression in the prostate might not be directly influenced by alterations in promoter methylation levels. Instead, it is plausible that the modification in the PCa biomarker expression could potentially be attributed to dietary changes or alternate pathways. Curiously, we ventured to explore the possibility of a correlation between the levels of methylation within the *HOXB13* promoter in the prostate and the expression of both *FTO* and *HOXB13*. In fact,

a statistically significant positive correlation emerged for both genes. *FTO* expression was positively correlated with *HOXB13* promoter methylation levels in the prostate of mice from F0 and F1, being this correlation lost in F2. As previously discussed, *FTO* was proposed to decrease *HOXB13* methylation in gastric cancer [250]. However, our results suggest that a higher *FTO* expression is associated with higher *HOXB13* methylation levels in the prostate. On the other hand, an unexpected result was the positive correlation between the *HOXB13* promoter methylation levels and *HOXB13* expression in the prostate of F0, where an increase in the methylation levels was associated with increased gene expression. Even though this result contradicts what was discussed before concerning promoter hypermethylation and decreased gene expression, recent studies have found that hypermethylation is also associated with increased gene expression. A study by Spainhour and colleagues demonstrated that certain genes could have a complex methylation signalling, which affected gene expression. The work analysed the correlation between gene expression and DNA methylation on an individual CpG axis, through the Cancer Genome Atlas database. Authors found a positive correlation between methylation levels within the promoter and gene expression, contradicting the common association [327]. Others have also reported the same association. Indeed, different molecular mechanisms can be involved in gene activation or DNA methylation, as reviewed by Smith and colleagues [328]. For example, WT1 can act as either a tumour suppressor or oncogene in various types of cancers. This gene possesses two promoters, WT1 and alternative WT1 (AWT1). In acute myeloid leukemia derived cell lines, AWT1 promoter presented a switch towards hypermethylation, which was associated with increased *WT1* expression. In fact, the epigenetic modification was specific to myeloid-lineage malignancies [329]. Additionally, it has been suggested that a coregulatory model of WT1 and AWT1 promoters could exist where the expression might be modulated by GATA2-binding enhancer module [329]. Moreover, survivin (BIRC5) is involved in apoptosis and cell cycle regulation. It is expressed in various adult tissues and tumour types, being overexpressed in endometrial cancer. BIRC5 promoter region presents a CpG island, already shown to be epigenetically regulated. Through methylation-specific RT-qPCR and pyrosequencing, the methylation status of BIRC5 was analysed in endometrial tumour samples [330]. Results revealed that promoter hypermethylation was correlated with higher mRNA expression in endometrial tumour samples. Further, it was found that demethylation, induced by decitabine in wild-type HCT116 colon cancer cells, resulted in BIRC5 repression. In addition, DNA methylation reduced the binding of p53, a repressive factor, to the BIRC5 promoter. Concurrently,

this indicates that inhibition of DNA-repressor interactions can enhance gene transcription [330].

Recognizing that obesity engenders a persistent low-grade inflammatory condition, we found it pertinent to incorporate an inflammatory biomarker into our analysis. We aimed to assess the expression levels of this marker and subsequently establish correlations with the PCa biomarkers. In this context, we turned to TNF-α, a pro-inflammatory cytokine. Its association with obesity arises from the inflammatory cascades that result from the metabolic disruptions characteristic of this disorder. Moreover, TNF-α also emerges as a pertinent player in PCa, exerting influence over tumour development dynamics [331, 332]. Within the framework of our study, a notable shift in *TNF-α* expression was evident within the prostate of the F1 generation. Specifically, a discernible difference emerged between the mice of the HFD group when compared with those in the HFD_t group, with the prostate of F1 mice from the HFD group displaying an elevated expression. This aligns with findings from existing research, where augmented TNF-α expression is linked to an inflammatory state stemming from obesity or HFD exposure [333, 334]. Baek and colleagues studied if the sclerostin expression was related to bone loss induced by obesity. For this, male mice were exposed for 12 weeks to an HFD (60% kcal of fat). Mice fed with HFD presented increased body weight and body fat mass, as well as higher TNF-α levels. In addition, TNF-α is known to participate in bone loss through NF-kB activation. Concurrently, authors concluded that obesity linked to increased TNF-α production could upregulate sclerostin expression through the NF-kB signalling pathway, resulting in bone loss [333]. Similarly, in another work, obese rats were exposed for 6 months to an HFD or a CTRL diet. Then, based on their growth, rats were divided into obesity group and obesity resistant group. Both groups presented higher levels of TNF-α, when compared to CTRL. The authors indicated that no difference is detected between obese rats fed with HFD or the obesity resistant group [334]. These outcomes reinforce and support our results. Conversely, escalated levels of this inflammatory biomarker are closely correlated with an elevated risk of PCa development in the offspring. This observation is consistent with the findings of Al-Fartosy & Ati, where PCa patients exhibited markedly higher levels of TNF-α in comparison to both BPH patients and individuals without health issues [335]. Inflammation induced by obesity increases the levels of pro-inflammatory cytokines, such as TNF-α, which play a role in invasion, metastasis, disease progression, therefore promoting carcinogenesis and disease progression [336, 337]. Given that the notable discrepancy in *TNF-α* expression mirrors the patterns observed in both *HOXB13* and *FTO* expression in prostate, and taking into account the intricate interplay among obesity,

inflammation, and PCa, we extended our research to evaluate potential correlations between those markers. We explored the potential links between *TNF-α* and *FTO*, alongside the PCa biomarkers. Interestingly, we identified a positive correlation between *TNF-α* and *FTO* expression in the prostate of both the F0 and F1. However, this correlation dissipated in the F2. These findings provide added weight to the existing correlation between obesity and inflammation in the prostate. In a related study by Saucedo and colleagues, it was observed in peripheral blood samples of women with gestational diabetes mellitus, that the FTO risk allele A was associated with heightened TNF-α levels. Curiously, this group also presented lower adiponectin levels. Furthermore, the FTO risk allele C was linked to weight gain and higher TNF-α levels [338]. Another study by Samaras and colleagues also correlated the expression of *TNF-α* and *FTO* in subcutaneous adipose tissue to insulin resistance [339]. In our experimental setting, *TNF-α* and *HOXB13* also shows a positive correlation in the prostate of F0 and F1, with no correlation regarding to F2, suggesting an association between inflammation and a higher risk of PCa development in those animals. As of now, there is no established direct correlation between *TNF-α* and *HOXB13*. Furthermore, the underlying mechanism governing their association remains to be elucidated. It is well-established that TNF-α triggers the activation of NF-kB, a pivotal transcription factor renowned for its role in orchestrating inflammatory processes. NF-kB exerts its influence by instigating the expression of genes associated with diverse functions like cell proliferation, inflammation, and apoptosis. These very processes are recognized to be activated within numerous tumours. Consequently, the involvement of TNF-α in carcinogenesis is conceivable through the pathway of NF-kB activation, underscoring the potential impact of their correlation [51, 340]. On the other hand, HOXB13 has been associated with NF-kB in PCa. Results by Kim and colleagues showed that PCa invasion and metastasis were promoted by HOXB13, which led to the stimulation of NF-kB signals. The authors further showed that in the presence of TNF-α, NF-kB activity was stimulated by HOXB13 overexpression [341]. Another study conducted on transgenic mice skin revealed that an elevation in TNF-α levels was observed concomitantly with the overexpression of HOXB13. In fact, HOXB13 was proposed as a potential transcription factor for TNF-α, offering a compelling hint at their potential regulatory relationship [342]. In our experimental approach, *AR* presented a positive correlation with *TNF-α* only in the prostate of F0 mice. In PCa cells, TNF-α may interfere with the inhibition of AR sensitivity, thereby giving the start to an androgen-independent state in PCa [343]. A relevant study by Ko and colleagues elucidated a notable connection. In human prostate cells, they observed that NF-kB, activated by TNF-α, acted to inhibit the

expression of the *AR*. This inhibition spanned across both mRNA and protein levels, consequently diminishing androgen sensitivity in the androgen-dependent LNCaP cells [344]. In another study involving mice and LNCaP cells, it was observed that elevated glucose levels had a direct impact on the reduction of both AR mRNA and protein levels. This effect was attributed to the modulation of NF-kB activity. Further, the combined influence of glucose and TNF-α exhibited a synergistic effect, contributing to the significant decrease in AR levels. The implications of these findings suggest that TNF-α plays a pivotal role in PCa by orchestrating the reduction of AR levels through the NF-kB pathway [345]. Given the results from these prior studies, our findings come as a surprise due to the unexpected correlation observed between *AR* and *TNF-α* in the prostate of mice. However, our experimental design incorporated an animal model in which mice were subjected to three distinct diets, prominently including HFD during all life or only until adulthood. This dietary variability could potentially account for the nuanced outcomes we have uncovered.

Conversely, the link between obesity, PCa, and OS has drawn significant attention. Obesity has been intricately tied to the generation of ROS, potentially driving elevated OS levels. These escalated OS levels have been implicated in the dysregulation of adipokines and the initiation of pro-inflammatory processes. Notably, pro-inflammatory cytokines like TNF-α have been identified as catalysts for ROS production, creating a plausible connection to heightened OS levels and an accelerated rate of lipid peroxidation [44, 346]. Within the context of PCa, the role of OS becomes paramount in its developmental trajectory, with lipid peroxidation also being intertwined with the progression of cancer [347, 348]. Thus, we assessed OS within the prostate tissue of mice, employing measurements of protein nitration and lipid peroxidation. Intriguingly, the analysis of protein nitration did not yield any statistically significant differences. Nevertheless, notable trends emerged with the F0 of the HFD group displaying a tendency towards increased protein nitration in the prostate tissue, while the animals only fed with HFD until adulthood exhibiting a similar inclination in the F1. Considering the prevailing association between obesity, HFD exposure, and the modulation of protein nitration, our findings are somewhat unexpected, especially within the context of the F0 and F1 and the comparative analysis of the exposed groups. For instance, the examination of placental tissue in individuals with obesity revealed markedly elevated levels of protein nitration in comparison to their lean counterparts. This observation was attributed to the presence of inflammatory cytokines, which hinted at a potential interplay between these proteins and the inflammatory milieu [349]. Furthermore, the impact of HFDs on OS was evident in rodent brain tissue, where HFD-induced alterations in protein

nitration were paralleled by corresponding shifts in inflammatory signalling [350]. Thus, one might suggest that the HFD-exposed group would display elevated levels of protein nitration in the prostate tissue, in line with the expected effects of OS. Meanwhile, the modulation of lipid peroxidation in the prostate of mice proves to be a multi-generational outcome of dietary changes. Particularly noteworthy is the significant disparity observed between the notably high levels of lipid peroxidation in the prostate of the HFD group in the F0 when compared to the levels detected in both, the prostate of mice fed with HFD until adulthood and those fed with standard chow during all life. Equally remarkable are the pronounced contrasts between the levels detected in the prostate of mice from the HFD group in F0 and both, in the prostate of the HFD and HFD_t groups in the subsequent generations (F1 and F2). Lipid peroxidation intricate relationship with obesity has been firmly established, notably in its potential involvement in metabolic disorders. The connection is rooted in the understanding that obesity fosters OS, prompting modifications in the lipid membrane and the subsequent production of 4-hydroxynonenal (HNE). This compound, an outcome of membrane lipid peroxidation, demonstrates notably higher levels in individuals grappling with obesity [351]. In a notable study involving adult mice subjected to a seven-week exposure to an HFD, a consequential upsurge in lipid peroxidation was observed, attributed to OS impact on the hippocampus [352]. Moreover, the linkage between lipid peroxidation provoked by OS and its implication in PCa and its progression has been firmly established [353]. Our findings reinforce this hypothesis since we found compelling evidence indicating that an HFD is intricately associated with an augmented incidence of lipid peroxidation, predominantly manifesting in the F0. Intriguingly, the implications of our results extend even further, implying a heightened susceptibility to PCa among the offspring of fathers who were exposed to an HFD. This underscores the far-reaching consequences of dietary habits, potentially affecting subsequent generations in terms of PCa risk. In essence, our study amplifies the call for a comprehensive understanding of how dietary factors not only impact immediate health but might also exert lasting effects on the health trajectories of future generations.

Chapter IV

Conclusion

6. Conclusion

Obesity is closely intertwined with disruptions in hormonal balance, the persistence of chronic low-grade inflammation, and OS. This multifaceted interplay culminates in the emergence of various coexisting health conditions, including cancer. Thus, obesity is a risk factor for the initiation and progression of carcinogenesis, underscoring its potential to foster cancer development. Moreover, the epigenetic modifications associated with both obesity and cancer play a pivotal role in enabling cancer cells to adapt to new environmental conditions, facilitating their advancement and survival. Significantly, the cues inherited by offspring from parents grappling with obesity perpetuate a propensity for obesity development in the progeny. Consequently, a growing apprehension surfaces concerning the potential inheritance of influential factors to the offspring's health. This concern reverberates particularly in the context of the possibility that inherited factors could fuel the emergence of additional coexisting conditions, including PCa. Notably, understanding the intricate nexus of associated factors and the underlying mechanisms is an ongoing challenge, as the comprehensive dynamics of this relationship remain enigmatic.

In this study, we studied the repercussions of an HFD exposure, throughout life or until adulthood, and its subsequent reversal across three generations on two PCa biomarkers: AR and HOXB13. Our research encompassed their expression levels in mice prostate tissue, alongside the exploration of the obesity-associated gene, FTO, HOXB13 promoter methylation levels, the inflammatory status, and OS markers. To our knowledge, our study stands as the first to unveil a significant correlation between *FTO* and *HOXB13* expression in mice prostate tissue, as well as the correlation between these genes and *HOXB13* promoter methylation levels. Our findings reveal that while *AR* expression remained largely unaffected, the expression of *HOXB13* was indeed influenced by the dietary changes in the F0 and F1. Notably, a positive correlation was detected between these two PCa biomarkers during F0 and F1, likely attributed to HOXB13's regulatory impact on AR. These initial outcomes underscore the alteration in a PCa biomarker's expression due to HFD exposure. Intriguingly, the intervention in the diet (correction from HFD to standard chow in adulthood) seemed insufficient to entirely reverse the molecular and metabolic changes induced by prior dietary habits. Our observations further indicated that a transgenerational risk of PCa might be at play, particularly in fathers and their sons, as the effects seem to diminish or dissipate in the subsequent generation (F2). Thus, we explored the role of FTO, a gene associated with obesity and recently linked to HOXB13, in the prostate of mice subjected to those

different dietary regimens. It revealed significant perturbations in its expression in the prostate of mice during F0 and F1, mirroring the tendencies observed with *HOXB13*. Subsequently, a positive correlation was detected between *FTO* and *HOXB13* in the prostate of F0 and F1 mice, although this connection weakened in F2. These outcomes, for the first time, validate the association between an obesity-related gene and a PCa biomarker within mice prostate tissue. Notably, FTO's function as a demethylase, capable of boosting *HOXB13* expression, was supported by the finding that *FTO* expression aligned with decreased *HOXB13* methylation levels in the prostate. Intriguingly, *HOXB13* promoter methylation levels in the prostate of mice subjected to distinct dietary regimens yielded unexpected results. Contrary to conventional expectations, the correlation between methylation levels and *HOXB13* expression in the prostate was positively significant in the F0 but not in the subsequent generations, defying the common notion that hypermethylation represses gene expression. Furthermore, a positive correlation is present in mice prostate tissue between *FTO* expression and *HOXB13* promoter methylation levels in the F0 and F1, however not in F2. Considering obesity role in instigating a chronic low-grade inflammatory status, we studied the expression of *TNF-α*, a hallmark inflammatory biomarker, in the prostate tissue. Our analysis disclosed significant alterations in prostate *TNF-α* expression during F0 and F1, underscoring a shift towards heightened inflammation in mice prostate tissue, as anticipated in cases of HFD exposure and obesity. Moreover, our results underscored the connections between inflammation and PCa biomarkers. Particularly noteworthy was the positive correlation established between *TNF-α* and *FTO* in prostate tissue, as well as *TNF-α* and *HOXB13* in the F0 and F1, while such correlations weakened in the F2. Remarkably, *TNF-α* exhibited a positive correlation with *AR* expression in the prostate solely in F0, further reinforcing the intricate links between inflammation and PCa biomarkers. Finally, we studied OS in the prostate and found that while dietary changes did not significantly affect protein nitration, lipid peroxidation displayed pronounced alterations across all generations. This concurs with the understanding that obesity-driven inflammation fosters the generation of ROS, thereby elevating OS levels and ensuing lipid peroxidation. In summary, our study provides a comprehensive analysis of the intricate interplay between dietary habits, gene expression, epigenetics, inflammation, and OS within the context of PCa biomarkers. The nuances uncovered underline the complexity of these relationships and highlight the potential transgenerational impact of obesity-associated factors on PCa risk.

This study sheds light on the heightened risk of PCa in fathers exposed to an HFD during all life or until adulthood, extending to their sons and their grandsons. Moreover, our

findings underscore the enduring impact of early-life HFD exposure, manifesting as metabolic and molecular alterations that prove resistant to reversal through dietary corrections in both the F0 and the subsequent generations that were not exposed. Strikingly, however, in the F2, the detected effects appeared to either recede or disappear. This intricate pattern underscores the transgenerational dynamics of dietary influence. Furthermore, our results expose the critical intersection of obesity with PCa risk and development. Our findings establish an undeniable link between obesity and the multifaceted effects associated with the disease, encompassing hormonal dysregulation, inflammatory responses, and OS. With traditional PCa diagnosis and the physiological alterations prompted by obesity interacting in complex ways, there emerges a compelling case for the inclusion of additional PCa biomarkers or parameters when evaluating individuals with obesity. The implications of our study emphasize the pivotal role of paternal health in shaping the well-being of subsequent generations. This underscores the urgency for further exploration of this intergenerational relationship and the underlying mechanisms that drive it. To deepen our comprehension of the intricate interplay between obesity and PCa, a forward trajectory of research beckons. This could entail investigations involving diverse models, including human PCa cell lines, to expand the scope of our understanding. Furthermore, delving into other pertinent pathways such as the Wnt signalling pathway in the prostate of these animals would be an intriguing avenue, given that *HOXB13* expression is recognized to fuel this pathway and heighten tumour development. In conclusion, this study underscores the complex interplay between obesity and PCa risk. The transgenerational ramifications of dietary habits, the intricate molecular mechanisms, and the potential avenues for further research all highlight the urgent need for a more holistic approach to understanding and addressing the links between obesity and PCa, and the possible transgenerational impact.

Chapter V

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7. References

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