

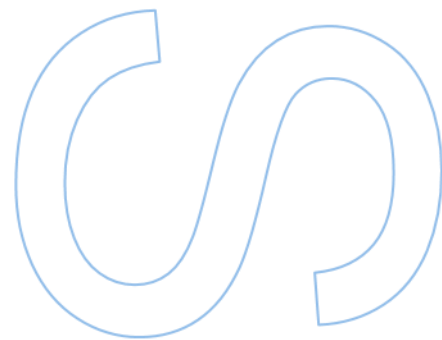
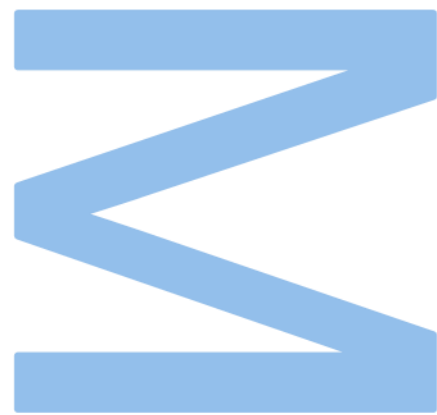
Lipolytic and browning activity during cancer cachexia: Are there molecular differences between male and female K14-HPV16 transgenic mice?

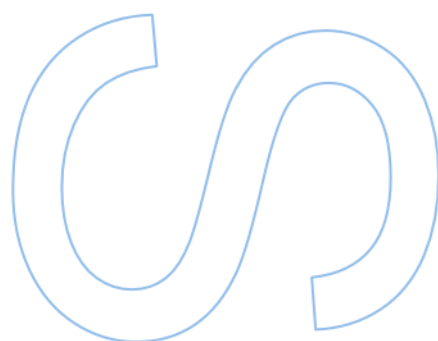
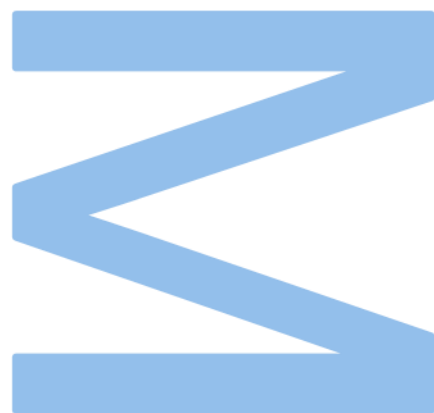
Inês Nunes Ribeiro da Mota

MSc Programme in Cell and Molecular Biology
Biology Department
2023

Supervisor

Professor Doctor Rui Manuel de Medeiros Melo Silva, PhD, head of the Molecular Oncology and Viral Pathology research group of the Research Centre of IPO Porto





Acknowledgements

The accomplishment of this thesis would not have been possible without the contribution of several people and institutions to whom I owe my gratitude.

Firstly, I would like to highlight the Faculty of Sciences of the University of Porto (FCUP), which enabled me to fulfil my childhood dream of becoming a biologist over the course of 5 years of Bachelor's and Master's studies. This institution will always hold a special place in my heart. Particularly, I would like to thank the scientific committee of the Master in Cell and Molecular Biology for their dedication and helpfulness, contributing to my passion for the field and consequently to my academic success.

Next, I would like to thank the Research Centre of IPO-Porto, and with great emphasis, my advisor, Professor Doctor Rui Medeiros, who welcomed me into the Molecular Oncology and Viral Pathology Group (GOMPV) and who granted me the opportunity and the resources to have my first contact with the field of oncological research and to carry out this exciting dissertation, instilling in me essential values for a researcher, namely critical spirit, proactivity, responsibility, and persistence. Furthermore, I extend my gratitude to the University of Trás-os-Montes and Alto Douro (UTAD), namely to Professor Doctor Paula Oliveira and to Master Beatriz Medeiros Fonseca, who were responsible for the management and sacrifice of the mice used in this study.

Additionally, my heartfelt acknowledgments to my workmate Setareh Satari for the companionship and cooperation, and to my lab supervisor, Joana Santos, who guided me step by step from the beginning of this project and taught me everything that was unknown to me in this field. She encouraged my autonomy and passed on her dedication, scientific rigor, and vast knowledge, which I greatly admire.

Last but certainly not least, my gratitude to my closest friends, which stood by me throughout this academic journey and with whom I grew, learnt and laughed, and the greatest appreciation goes to my family, who shaped me not in the academic sense, but in all other aspects that structure and dignify a human being and without whom everything would have been immensely more difficult to achieve.

Resumo

Introdução: A caquexia associada ao cancro (CAC) é uma síndrome complexa que afeta múltiplos órgãos, contribuindo para a redução da qualidade de vida e aumento da mortalidade de muitos doentes oncológicos. O seu cariz multifatorial dificulta o seu diagnóstico atempado e intervenções terapêuticas eficazes. O tecido adiposo é um dos órgãos altamente afetados pela caquexia, verificando-se tipicamente aumento de lipólise, “browning” e termogénese, particularmente nas fases iniciais da doença, o que conduz à perda de massa gorda e contribui para a disrupção de outros órgãos. As vias de sinalização adrenérgicas estão associadas com os processos referidos anteriormente – o recetor adrenérgico $\alpha 2A$ está associado com o armazenamento de lípidos, enquanto o recetor adrenérgico $\beta 3$ induz lipólise, termogénese e “browning” –, e evidência acumulada sugere que a sua desregulação possa estar na origem da remodelação do tecido adiposo durante a caquexia, no entanto a literatura ainda é controversa. Adicionalmente, pouco se sabe relativamente ao dimorfismo sexual do tecido adiposo no contexto de caquexia. Os modelos animais são uma ferramenta útil para estudar a patogénese da caquexia bem como o dimorfismo sexual, contudo a maioria dos que existem não se focam nas alterações do tecido adiposo, como é o caso do murganhos transgénicos K14-HPV16, validados como um modelo de perda muscular e inflamação na CAC. Nesse sentido, os objetivos deste trabalho são caracterizar ao nível molecular a remodelação do tecido adiposo, particularmente no que respeita à expressão dos recetores adrenérgicos, na caquexia associada ao cancro em murganhos K14-HPV16, bem como avaliar possíveis diferenças entre sexos.

Métodos: Os recetores adrenérgicos *Adra2a* e *Adrb3*, a proteína mitocondrial termogénica *Ucp1*, os reguladores de adipogénese *Cebpa* e *Pparg*, e as lipases *Pnpla2* e *Lipe* foram os marcadores moleculares do tecido adiposo selecionados. Os murganhos transgénicos K14-HPV16 (HPV+) e os murganhos controlo (HPV-) de ambos os sexos foram sacrificados em três momentos distintos (33, 42 e 50 semanas de idade), foram extraídas amostras de tecido adiposo de três regiões anatómicas diferentes (abdominal, inguinal e perirrenal) e o seu RNA total foi isolado e convertido em cDNA. Posteriormente, foi realizada a quantificação relativa da expressão génica dos marcadores moleculares em todas as amostras através da técnica qPCR. Os resultados obtidos foram depois analisados através do método Livak juntamente com o teste de Mann-Whitney, utilizando o programa IBM SPSS Statistics.

Resultados e Discussão: Os resultados observados foram de certa forma surpreendentes, uma vez que os murganhos caquéticos de ambos os sexos apresentaram expressão diminuída de *Adrb3* ($p < 0,05$) e *Ucp1* ($p < 0,01$) (indicando termogénese e “browning” diminuídos), das lipases *Pnpla2* ($p < 0,05$) e *Lipe* ($p < 0,05$) (sugerindo uma redução da lipólise) e dos marcadores de adipogénese *Cebpa* ($p < 0,05$) e *Pparg* ($p < 0,05$) (indicando diferenciação de adipócitos reduzida). Adicionalmente, a expressão de *Adra2a* e o rácio *Adra2a/Adrb3*, que estão associados com a acumulação lipídica e que expectavelmente estariam diminuídos na caquexia, estavam aumentados nos murganhos caquéticos em comparação com os controlo ($p < 0,05$). Estes resultados poderão refletir um mecanismo de retroregulação negativa ativado para compensar a perda de tecido adiposo que pode ter ocorrido previamente no curso da caquexia, enquanto a redução na diferenciação de adipócitos poderá contribuir mais para a perda de tecido adiposo nesta fase da doença. Além disso, ficou claro que o dimorfismo sexual em condições saudáveis é específico de cada tipo de tecido adiposo: nos murganhos controlo, o tecido adiposo abdominal revelou menor acumulação de lipídios e mais termogénese e lipólise em fêmeas do que em machos, consistente com a maioria da literatura; o tecido adiposo inguinal parece não apresentar dimorfismo sexual; o tecido adiposo perirrenal mostrou-se inesperadamente menos termogénico e lipolítico em fêmeas em comparação com machos. Porém, no que respeita aos murganhos caquéticos HPV+, o único tipo de tecido adiposo em que foi observado dimorfismo sexual foi o tecido adiposo perirrenal, que apresentou um padrão idêntico ao verificado nos controlo, assim como um maior rácio *Adra2a/Adrb3* ($p < 0,05$) em fêmeas, reforçando que as atividades termogénica e lipolítica no tecido adiposo perirrenal são maiores em machos HPV+ do que em fêmeas HPV+. Assim, é possível sugerir que o dimorfismo sexual relativamente ao tecido adiposo é menos pronunciado no contexto da caquexia do que em condições saudáveis.

Conclusão: Em suma, esta investigação produziu resultados relevantes para uma melhor compreensão dos mecanismos moleculares associados à disrupção do tecido adiposo na caquexia em cada um dos géneros. No entanto, futuramente será necessária investigação mais detalhada, nomeadamente ao nível da expressão proteica, para consolidar a interpretação destes resultados.

Palavras-chave: cancro; caquexia; tecido adiposo; *browning*; lipólise; adipogénese; sinalização adrenérgica; recetor adrenérgico alfa2A; recetor adrenérgico beta3; dimorfismo sexual; modelo tumoral; murganhos transgénicos K14-HPV16

Abstract

Introduction: Cancer-associated cachexia (CAC) is a complex multiple organ syndrome that largely contributes to reduced quality of life and increased mortality of many cancer patients. Its multifactorial nature makes its early diagnosis and effective therapeutic interventions challenging. Adipose tissue is one of the organs greatly affected by cachexia, typically through increased lipolysis, browning and thermogenesis, particularly on the onset of the disease, which leads to fat mass wasting and contributes to the disruption of other organs. Adrenergic signalling pathways are associated with the aforementioned processes – α 2A-adrenergic receptor is associated with lipid storage, while β 3-adrenergic receptor reportedly induces lipolysis, thermogenesis and browning –, and accumulating evidence suggests that their dysregulation may be at the origin of adipose tissue remodelling during cachexia, however, literature is still controversial. Additionally, little is known regarding sexual dimorphism of adipose tissue in the context of cachexia. Animal models are a valuable tool to study cachexia pathogenesis as well as sexual dimorphism, however, most models do not focus on adipose tissue alterations, as it is the case of K14-HPV16 transgenic mice, which are validated as a model of muscle wasting and inflammation in CAC. In this regard, this study's aims were to characterize at molecular level the adipose tissue remodelling, particularly regarding adrenergic receptors expression, in cancer-associated cachexia in K14-HPV16 mice, as well as to assess possible differences amongst sexes.

Methodologies: The adrenergic receptors *Adra2a* and *Adrb3*, the thermogenic mitochondrial protein *Ucp1*, the adipogenic key regulators *Cebpa* and *Pparg*, and the lipases *Pnpla2* and *Lipe*, were the selected adipose tissue molecular markers. K14-HPV16 transgenic mice (HPV+) and control mice (HPV-) from both sexes were sacrificed at three distinct timepoints (33, 42 and 50 weeks of age) and adipose tissue samples from three different anatomic regions (abdominal, inguinal and perirenal) were collected and its total RNA was isolated and converted to cDNA. Subsequently, relative quantification of the gene expression of the molecular markers in all samples was performed using qPCR. The results obtained were then analysed employing the Livak method along with Mann-Whitney using IBM SPSS Statistics.

Results and Discussion: The results found in this thesis were at some point surprising, as cachectic mice from both sexes exhibited decreased expression of *Adrb3* ($p < 0,05$) and *Ucp1* ($p < 0,01$) (pointing to decreased thermogenesis and browning), of the lipases *Pnpla2* ($p < 0,05$) and *Lipe* ($p < 0,05$) (suggesting reduced lipolysis) and of the markers

of adipogenesis *Cebpa* ($p < 0,05$) and *Pparg* ($p < 0,05$) (indicating reduced adipocyte differentiation). Moreover, *Adra2a* expression and *Adra2a/Adrb3* ratio, which are associated with lipid storage and were expected to be decreased in cachexia, were found increased in cachectic mice when compared to control ones ($p < 0,05$). This may constitute a mechanism of negative feedback activated in order to compensate for the loss of adipose tissue that may have occurred in earlier stages of cachexia, while low adipocyte differentiation may be contributing to adipose tissue wasting at this point of the disease. In a further matter, it was clear that sexual dimorphism in healthy conditions is adipose tissue depot-specific: in control mice, abdominal adipose tissue revealed lower lipid accumulation and higher thermogenesis and lipolysis in females than in males, consistent with most literature; inguinal adipose tissue seemed not to be sexual dimorphic; perirenal adipose tissue was unexpectedly less thermogenic and lipolytic in females compared to males. Concerning HPV+ cachectic mice, however, the only type of adipose tissue where sexual dimorphism was observed was perirenal adipose tissue, that displayed an identical pattern of that verified in controls as well as a higher *Adra2a/Adrb3* ratio ($p < 0,05$) in female mice, reinforcing that thermogenic and lipolytic activities in perirenal adipose tissue are higher in HPV+ males than in HPV+ females. Therefore, it is possible to hypothesize that sexual dimorphism regarding adipose tissue is less pronounced in the context of cachexia compared to the healthy state.

Conclusion: In summary, this research has produced relevant results for a better understanding of the molecular mechanisms associated with adipose tissue disruption in cachexia in each gender. However, further investigation, particularly at the protein expression level, will be needed in the future to solidify the interpretation of these findings.

Keywords: cancer; cachexia; adipose tissue; browning; lipolysis; adipogenesis; adrenergic signalling; alpha2A-adrenoceptor; beta3-adrenoceptor; sexual dimorphism; tumour model; K14-HPV16 transgenic mice

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List of Abbreviations

A

<i>Adra2a</i>	ALPHA 2A-ADRENERGIC RECEPTOR
α 2-AR	ALPHA 2-ADRENERGIC RECEPTORS
α 2A-AR	ALPHA 2A-ADRENERGIC RECEPTOR
α 2B-AR	ALPHA 2B-ADRENERGIC RECEPTOR
α 2C-AR	ALPHA 2C-ADRENERGIC RECEPTOR
AC	ADENYLYL CYCLASE
<i>Actb</i>	B-ACTIN
AT	ADIPOSE TISSUE
ATGL	ADIPOCYTE TRIGLYCERIDE LIPASE
ATP	ADENOSINE TRIPHOSPHATE

B

<i>Adrb3</i>	BETA 3-ADRENERGIC RECEPTOR
β -AR	BETA-ADRENERGIC RECEPTORS
β 1-AR	BETA 1-ADRENERGIC RECEPTOR
β 2-AR	BETA 2-ADRENERGIC RECEPTOR
β 3-AR	BETA 3-ADRENERGIC RECEPTOR
BAT	BROWN ADIPOSE TISSUE
BMI	BODY MASS INDEX

C

CAC	CANCER-ASSOCIATED CACHEXIA
cAMP	CYCLIC ADENOSINE MONOPHOSPHATE
cDNA	COMPLEMENTARY DEOXYRIBONUCLEIC ACID
cGMP	CYCLIC GUANOSINE 3',5'-MONOPHOSPHATE
<i>Cebpa</i>	CCAAT/ENHANCER BINDING PROTEIN ALPHA
C/EBP α	CCAAT/ENHANCER BINDING PROTEIN ALPHA

E

ER α	ESTROGEN RECEPTOR ALPHA
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F

FAS	FATTY ACID SYNTHASE
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FCUP FACULTY OF SCIENCES OF THE UNIVERSITY OF PORTO
FFA FREE FATTY ACID

G

G_i INHIBITORY G PROTEIN
G_s STIMULATORY G PROTEIN

H

H&E HEMATOXYLIN AND EOSIN
HPV HUMAN PAPILLOMAVIRUS
HSL HORMONE SENSITIVE LIPASE

I

IFN γ INTERFERON GAMMA
IGF1 INSULIN-LIKE GROWTH FACTOR 1
IL1 INTERLEUKIN 1
IL6 INTERLEUKIN 6
IL8 INTERLEUKIN 8

K

K14 KERATIN 14
K14-HPV16 KERATIN 14-HUMAN PAPILLOMAVIRUS 16

L

Lipe LIPASE E
LPL LIPOPROTEIN LIPASE

M

MAPK MITOGEN-ACTIVATED PROTEIN KINASE
MGL MONOACYLGLYCEROL LIPASE
mRNA MESSENGER RIBONUCLEIC ACID
Myf5 MYOGENIC FACTOR 5

N

NE NOREPINEPHRINE

NF-KB	NUCLEAR FACTOR-KB
NPY	NEUROPEPTIDE Y
P	
<i>Pgc1a</i>	PPARG COACTIVATOR 1 ALPHA
PGC1 α	PPARG COACTIVATOR 1 ALPHA
PKA	PROTEIN KINASE A
<i>Pnpla2</i>	PATATIN LIKE PHOSPHOLIPASE DOMAIN CONTAINING 2
<i>Pparg</i>	PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA
PPAR γ	PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA
PRDM16	PR DOMAIN CONTAINING 16
Q	
qPCR	REAL-TIME POLYMERASE CHAIN REACTION
R	
RNA	RIBONUCLEIC ACID
S	
SAT	SUBCUTANEOUS ADIPOSE TISSUE
SNS	SYMPATHETIC NERVOUS SYSTEM
SREBP-1C	STEROL REGULATORY ELEMENT BINDING PROTEIN-1C
T	
TAG	TRIGLYCERIDE
<i>Tbp</i>	TATA-BINDING PROTEIN
TNF α	TUMOUR NECROSIS FACTOR ALPHA
U	
UCP1	UNCOUPLING PROTEIN 1
UP	UNIVERSITY OF PORTO

V	
VAT	VISCERAL ADIPOSE TISSUE
W	
WAT	WHITE ADIPOSE TISSUE
Z	
ZAG	ZINC- α 2-GLYCOPROTEIN

Introduction

1. Cancer-associated Cachexia

Cachexia is a complex multifactorial syndrome that can occur due to a wide range of pathological conditions, such as cancer – cancer-associated cachexia (CAC) [1, 2]. According to the international consensus [3] it is characterized by wasting of cardiac and skeletal muscle accompanied or not by adipose tissue depletion, systemic inflammation, energy homeostasis imbalance (with superposition of the catabolic state over the anabolic one) and other metabolic abnormalities, that all together contribute to the evolution of this syndrome and consequently to a marked reduction in the quality of life of patients [1, 4, 5]. It is a condition that cannot be reverted by conventional or even parenteral nutritional support [6], and because there is a major interplay of several factors underlying it, it makes this a really hard-to-manage disease which currently has no available effective treatment options.

Cachexia can be understood as three distinct stages: pre-cachexia, cachexia and refractory cachexia (the most frequently diagnosed and the one that reflects the greatest damage to the patient and the shortest survival time) [3]. Currently, the most consensual criteria to obtain a diagnosis of cancer-associated cachexia is the observation of, at least, one of the three following indicators: 1) weight loss above 5% over the last six months (without experimenting starvation); 2) weight loss above 2% and a body mass index (BMI) < 20; 3) weight loss above 2% accompanied with appendicular skeletal muscle index accordant with sarcopenia [3].

Cancer-associated cachexia affects between 50% and 80% of oncologic patients depending on the cancer type, host factors [6] and tumour stage [4], but apparently not on the tumour size – the higher prevalence is found in pancreatic, prostate, gastric and lung cancers [6-8], but it is also observed in other cancer types as head-and-neck, breast, and so on [4]. CAC is responsible for increased susceptibility to infections, fatigue, loss of appetite, anti-cancer therapies lack of effect and side effects exacerbation [9], and it is the direct cause of nearly 20% of all cancer patients' deaths [2, 10]. However, CAC diagnosis is often overlooked or delayed [11], considering that metabolic abnormalities can start way earlier than physic manifestations [3, 12], and symptoms can be masked by other diseases, such as obesity [1]. For all this, CAC has been a target of research for decades, but there is still a lot to understand, considering its multifactorial

nature. Therefore, unravel as many molecular mechanisms as possible underlying CAC is of great interest and will help to develop more accurate and effective multidirectional therapies approaches to improve the prognosis of most cancer patients.

In the light of current knowledge, both tumour and host's immune system in response to the presence of the tumour release factors that are responsible for the origin and progression of CAC and are known as "pro-cachectic factors" [7, 9]. Pro-cachectic factors include inflammatory cytokines which induce the breakdown of muscle and adipose tissue, anorexigenic pathways and other metabolic dysfunctions [e. g. tumour necrosis factor alpha (TNF α), interferon gamma (IFN γ) and interleukin 1, 6 and 8 (IL1, IL6 and IL8)] [4, 7] and other catabolism-inducing factors (for instance, microRNAs and other non-coding RNAs) [11]. Furthermore, the overactivation of futile cycles, such as the Cori cycle that promotes the recycling of glucose in the liver to fuel the tumour, contributes to the increased resting energy expenditure associated with cancer and CAC [2, 6, 13]. It is also agreed that tumour competes with other organs and tissues for nourishment (i.e., biosynthetic substrates), thus the progression of cachexia benefits the tumour expansion through the release of biomolecules such as free fatty acids (FFAs), glucose and amino acids (e.g., glutamine) from the disruption of distinct tissues [6, 14].

Reduction of insulin and insulin-like growth factor 1 (IGF1) production, responsible for triggering anabolic signalling pathways that promote protein synthesis and suppress their degradation, is also associated with CAC by promoting the loss of muscle mass and adipose tissue [4]. Additionally, insulin and IGF1 resistance is frequently observed in cancer and cachectic patients [14]. The resistance to anabolic mediators experienced by several tissues during cachexia may be promoted by the tumour [4], which favours its own expansion, since tumours have a high number of receptors for anabolic factors, and also benefit from the metabolites (such as amino acids) released into the bloodstream resulting from the degradation of muscle and other tissues [14].

The discovery of accurate predictive biomarkers of cachexia is one of the main goals of the scientific community, as it would allow an early diagnosis of cachexia (bearing in mind that clear phenotypic manifestations often reflect an advanced stage of the disease) and would help to prevent its progression by directing the therapeutic approach [11]. At the moment, the most proposed biomarkers include cytokines, tumour-derived molecules, and markers of skeletal muscle breakdown [6]. On the one hand, the use of proteins (such as cytokines) represents an expensive and sluggish method due to their complex structures and the need to produce new, more specific antibodies, on the other hand, nucleic acids (e. g. microRNAs) have the advantage of their faster and easily

affordable methods of detection [11], however, none of them is yet reliable enough to be successfully applied in the clinics [6, 11].

In the research field, greatest attention has been given to the study of inflammation mediators and muscle loss, however, fat remodelling is also a hallmark of CAC [10]. Adipose tissue is not only the major body's energy storage, but also an important endocrine organ [15], thus alterations in its abundance and metabolism lead to pronounced consequences for the overall energy homeostasis of an individual [7]. Additionally, it is already established that adipose tissue disruption can precede and aggravate muscle depletion by the infiltration of circulating FFAs into the muscle tissue, resulting from the increased lipolysis in fat [16, 17].

There are two markedly distinct adipose tissue categories: white adipose tissue (WAT), the major triglycerides (TAGs) reservoir accounting for the body's main energy storage, and brown adipose tissue (BAT), rich in mitochondria and mainly responsible for thermogenesis through fatty acids oxidation [4, 13, 18]. Additionally, a third type of adipocytes can emerge, called beige or "brite" adipocytes, that originate from white adipocytes triggered to develop histologically and physiologically like the brown ones, in a process called "browning" [15]. Both the activation of the brown adipocytes and the recruitment of the beige ones (i.e. browning) contribute to the increment of thermogenesis, thus promoting increased body energy expenditure, which is strongly associated with CAC [2, 4, 19].

The adrenergic signalling pathways are major players in adipose tissue regulation, namely in the activation of lipolysis, thermogenesis and browning [2, 7]. Catecholamines are able to activate the three types of β -adrenergic receptors ($\beta_{1,2,3}$ -AR) promoting increased lipolysis in WAT and inducing thermogenesis in beige and brown adipocytes [7], but can also act contrary, stimulating lipid storage through α_2 -adrenergic receptors (α_2 -AR) [20]. Of all β -adrenoceptors, β_3 -AR is known to be the prevalent subtype in mice [21, 22], and its blockade has been proven to be effective in attenuating browning and CAC [23]. On the other hand, the homolog of the human α_2A -AR subtype seems to be the most expressed in mouse adipocytes [24, 25].

Sexual dimorphism in cachexia is already reported, with men being more susceptible to develop CAC than women, however most studies focus on muscle wasting [26, 27], with scarce information regarding adipose tissue metabolism. It is well-known that fat metabolism and distribution have great differences between genders [28, 29], which have already been linked with variations at adrenergic signalling level, once

adrenoceptors are a target of steroid hormones [20, 30]. Nevertheless, the available information regarding sex-specific fat remodelling during cachexia and how adrenergic receptors contribute to these distinct patterns is still insufficient and controversial [20, 28, 30]. Therefore, it is imperative to clarify the gender differences of adipose tissue molecular mechanisms in the context of CAC and understand how they modulate the disease's progression.

Research in animal models proves to be an important tool to go forward in the understanding of CAC, as well as to test therapeutic options, since human biopsies are still invasive procedures, are harder to obtain, and might not reflect the “bigger picture” when it comes to assess all the changes that cachexia entails. K14-HPV16 transgenic mice carry the high-risk human papillomavirus 16 (HPV16) early region under the control of keratin 14 (K14) promotor and are a good model for CAC study as they progressively develop HPV-induced carcinogenesis and several cachexia features, coming closer to what is observed in humans [31]. Using this model, this thesis aims to clarify the involvement of key molecules in the cachexia-associated adipose tissue disruption, and to evaluate possible sexual dimorphisms in their expression, placing them as potential therapeutic targets to fight cachexia.

2. Characterization of Adipose Tissue

The adipose tissue (AT) in mammals is an organ diffused into several depots throughout the body that serves several purposes, being energy storage the most well-known [32]. Moreover, AT is now recognized as a very strong endocrine organ [33], and its functions include body temperature regulation, immunomodulation and angiogenesis, just to mention a few [34]. It accounts for about 20% of an individual's body mass, a number that can reach the 80% for obese people [34].

Adipose tissue can be classified as subcutaneous or visceral, depending on its anatomical position [34]. Besides that, according to its histological and physiological profile, there are two very distinct categories of adipose tissue – WAT and BAT –, and a third one that combines properties of them both called beige or “brite” (a fusion of brown and white) [34, 35]. Nonetheless, even considering adipocytes from the same histological category, their molecular and physiological profiles can differ (e. g. visceral but not subcutaneous white adipose depots are associated with increased risk of metabolic diseases) [36, 37].

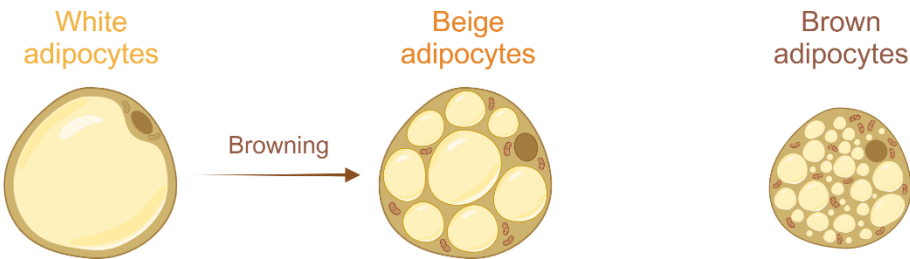
Adipogenesis is a complex process that can be understood as two main steps. The first one is the determination, in which the fate of pluripotent stem cells is established and thus they become pre-adipocytes. The second one is called the terminal differentiation, when pre-adipocytes develop all the properties of a mature adipocyte [37]. White and brown adipocytes are pointed to develop from different stem cell lineages: both BAT and skeletal muscle share the myogenic factor 5 positive (*Myf5*⁺) progenitor cells, whereas WAT putatively does not [34, 38, 39]. However, instigated by the fact that distinct adipose tissue depots arise at different postnatal development timings, a work conducted in mice surprisingly found out that there is a subgroup of white adipocytes that differentiates from *Myf5*⁺ progenitor cells, supporting the idea that adipocytes derived from different precursors coexist within a single fat depot [40].

Although adipogenesis results from the action of numerous molecules, peroxisome proliferator-activated receptor gamma (PPAR γ) and CCAAT/enhancer binding protein alpha (C/EBP α) are known as the two major players on this process [41], inducing the transcription of downstream adipogenic genes [42] and persisting at high concentrations during the life course of the mature adipocytes [43]. Based on several studies, it was concluded that the ability of C/EBP α to promote adipogenesis depends on the presence of PPAR γ , and that PPAR γ *per se* is sufficient to promote adipocyte differentiation [37, 41, 43]. Nevertheless, C/EBP α role should not be overlooked, as it is directly responsible for insulin sensitivity along with the induction of many essential genes for the development of adipose tissue [37], and it contributes to PPAR γ expression enhancement and maintenance [43]. Furthermore, C/EBP α was shown to be only required for the differentiation of white mature adipocytes, and unnecessary for brown adipogenesis [41, 44], although it is also expressed in brown adipocytes [45].

On the other hand, lipolysis contributes differently to adipose tissue dynamics. Lipolysis consists in the breakdown of TAGs that results in three free non-esterified fatty acids and glycerol that are subsequently released into the bloodstream to meet different requirements, that go from energy supply to insulin production regulation [46], or recycled within the adipocyte [46]. In WAT, this process is mainly prompted by fasting, while in BAT it occurs mostly due to cold exposure [47]. Besides lipoprotein lipase (LPL), that is secreted by adipose tissue and binds to the luminal surface of capillary endothelial cells to hydrolyse the plasma TAGs of circulating lipoproteins [48], three main enzymes catalyse intracellular lipolysis hierarchically: adipocyte triglyceride lipase (ATGL) [encoded by the gene patatin like phospholipase domain containing 2 (*PNPLA2*)] – the rate-limiting TAG lipase – removes the first FFA from the TAG, then hormone sensitive

lipase (HSL) [encoded by the gene lipase E (*LIPE*)] removes the second FFA, and finally monoacylglycerol lipase (MGL) catalysis the release of the last FFA and glycerol [8, 46, 49, 50]. Norepinephrine is considered the major lipolytic booster by activating the β -adrenergic signalling pathway, but other hormones are also described as relevant for lipolysis induction, for instance, thyroid stimulating hormone and growth hormone [51]. Inversely, insulin is reportedly the major antilipolytic factor, by downregulating intracellular cyclic adenosine monophosphate (cAMP) [51].

Below, the most significant differences between the three types of adipose tissue are exposed, which are then summed up in **Figure 1**.



Morphology	Single large lipid droplet; Low mitochondrial density	Phenotypic plasticity; Multiple small lipid droplets and medium mitochondrial density when active	Multiple small lipid droplets; High mitochondrial density
Vascularization	White depots present lower vascularization	-	Brown depots present higher vascularization
Anatomic location	Subcutaneous and visceral regions	Emerge within WAT depots (browning process)	Interscapular, supraclavicular, cervical, axillary, perirenal regions
Main functions and environmental triggers	Energy storage; Energy release (lipolysis) prompted by fasting; Endocrine	Lipolysis and thermogenesis (prompted by cold exposure or exercise); Endocrine	Lipolysis and thermogenesis (prompted by cold exposure or exercise); Endocrine
Hallmark proteins	Leptin	Uncoupling protein 1 (UCP1)	Uncoupling protein 1 (UCP1)
Effects of age	WAT depots increase with age	-	BAT depots decrease with age
Metabolic disease data	High ammounts associated with increased risk of obesity	-	High ammounts associated with decreased risk of obesity

Figure 1. Schematic representation and comparison of the main characteristics of white, beige and brown adipocytes. WAT: white adipose tissue; BAT: brown adipose tissue. (Created with BioRender.com)

2.1 White Adipose Tissue

WAT main function is energy store. It is composed by large adipocytes containing thin mitochondria and a single lipidic ‘droplet’ (unilocular) that occupies up to 90% of the cell space [39], in which TAG are accumulated and hydrolysed when energy is required [34].

Additionally, WAT has a major endocrine importance, producing a wide range of adipokines (peptides) and lipokines (lipids) with endogenous, paracrine and/or endocrine acting modes that are involved in inflammatory processes, the transport of other molecules, the fibrinolytic system, steroid metabolism and so on [34]. Leptin is probably the most studied adipokine and is preferentially expressed by WAT [39], mainly by subcutaneous depots [52]. Its secretion increases along with the accumulation of fat mass, and along with other physiologic functions, its primary role is promoting the feeling of satiety, thus considered an anorexigenic hormone [32, 53], and increasing energy expenditure [52, 54]. Contrary, in periods of fasting, the body’s energy demand increases and TAGs are hydrolysed to fuel other organs [32], thus leptin production is suspended, increasing the secretion of orexigenic factors such as the hormone ghrelin and the neuropeptide Y (NPY) [55]. Depending on its anatomical location, WAT depots can be divided into two categories: subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). SAT accounts for most of the fat and is located right under the skin condensed mainly in abdominal, gluteal and femoral regions in humans, and in inguinal and axillary regions in rats [15], whereas VAT is situated deeper around the organs, namely the kidney (perirenal AT), the gonads (perigonadal AT), the intestines (mesenteric and omental AT), the heart (epicardial and pericardial AT) and the vasculature (perivascular and periadventitial AT) [34, 56, 57].

WAT suffers gradual changes along with aging, more specifically, there is a decrease of subcutaneous WAT and visceral WAT trends to increase, leading to the development of metabolic pathologies [39, 58], and hyperplasia is tendentially replaced by hypertrophy over the years [34].

The main characteristics of white adipocytes are summarised in **Figure 1**.

2.2 Brown and Beige Adipose Tissue

BAT owns its colour to its high vascularization and specially to its adipocytes rich in mitochondria, which contain cytochromes [34]. In contrast with WAT, brown adipocytes

are multilocular, i. e. contain multiple small lipid vacuoles that are mainly used to fuel heat production by oxidative phosphorylation in mitochondria [59]. The main BAT depot in rodents is found in the interscapular region [60, 61], although it can also be found in the perirenal [62], pelvic and inguinal regions [63]. Similar to mice, human neonates have a pronounced interscapular BAT store, which greatly decreases along with age progression [60]. In addition, BAT in human adults can be found in some other locations both superficially (namely in supraclavicular, cervical, paravertebral and axillary areas) and deeply (especially in the perirenal region) [34, 62], however detection of BAT in human adults still faces some limitations due to the reduced size of its depots and to its plasticity that allows to both morphological and molecular shifts according to external cues [64].

BAT is mainly responsible for thermogenesis, that is, energy dissipation, thus playing a vital role in body's energy homeostasis [65]. Succinctly, FFAs resulting from lipolysis are directed to mitochondria, where ATP synthesis supposedly happens. However, uncoupling protein 1 (UCP1), a mitochondrial inner membrane protein which is a hallmark of brown adipocytes, dissipates the proton gradient of oxidative phosphorylation, disrupting ATP synthesis, and instead releasing energy in the form of heat [59, 66].

Thermogenesis is mostly driven by cold exposure through the β -adrenergic signalling pathway [23]. Cold stress leads to increased release of catecholamines – the endogenous ligands of adrenergic receptors, namely norepinephrine (NE) [67, 68] – by sympathetic nervous system (SNS), that highly innervates brown adipocytes [69]. When stimulated by norepinephrine binding, β 3-AR (the prevalent β -AR subtype in mice BAT [70]) induce adenylyl cyclase that increases cAMP intracellular levels which subsequently activates protein kinase A (PKA) [67]. PKA induces both HSL activation by phosphorylation (promoting lipolysis) and p38 mitogen-activated protein kinase (MAPK) pathway, that upregulates UCP1 gene expression [8, 22, 71]. Moreover, β -adrenergic stimulation also contributes for the expansion of BAT as it promotes the proliferation of brown fat precursor cells present in the stromal vascular fraction of BAT [69], namely β 1-AR [70].

Unlike WAT, BAT maximum levels are observed at birth, where thermogenesis plays a crucial role as it is harder for newborns to maintain the body temperature [39]. BAT was actually thought to be extinguished in adult life, but relatively recent studies showed that it remains active and vital in grown-ups. With aging, amount of brown adipocytes declines leading to metabolism and energy-related issues [58].

A few years ago, beige adipocytes have been discovered. Those adipocytes are diffused in WAT depots and can emerge both from the trans-differentiation of mature white adipocytes or from the differentiation of specific beige progenitor cells [72] when triggered by environmental stimuli such as exercise or cold exposure [34]. This process is called “browning”, as beige adipocytes present typical properties of brown cells: high mitochondrial content and expression of several brown specific genes, including the transcriptional regulator PR domain containing 16 (*Prdm16*) that induces the expression of thermogenic genes, such as *Ucp1* and PPAR γ coactivator 1 alpha (*Pgc1a*) [34, 66, 73, 74]. Recent findings suggest that beige cells arise primarily from *de novo* differentiation, at the time of the first cold exposure, and then are capable of interconverting between a white- and a brown-like phenotype, depending on external cues [75]. Furthermore, a study performed in mice concluded that cold stress induces beige adipocytes differentiation from perivascular progenitor cells via β 1-AR activation, whereas stimulation of β 3-AR leads to the emergence of beige adipocytes through the trans-differentiation of mature white adipocytes [76]. Consistently, β 3-AR agonists have been widely reported to induce UCP1 expression in WAT [70]. Subcutaneous WAT is reportedly more prone to undergo browning than visceral WAT [70]. Summing up, when active, beige adipocytes develop a multilocular morphology and contribute to body's energy expenditure and oxygen consumption through thermogenesis [34], otherwise their aspect is identical to white unilocular adipocytes. Although diverse WAT depots can display beige adipocytes, in rodents they are mainly found in inguinal WAT, whereas in humans they were detected in the supraclavicular region [62].

Both brown and beige adipocytes have generated great enthusiasm among scientific community because of their high capacity of wasting energy, that can be potentially useful to fight obesity [59, 64, 66]. **Figure 1** presents a summary of the main traits of brown and beige adipocytes.

3. Adipose Tissue in Cancer Cachexia

As previously mentioned, adipose tissue is markedly impaired in cancer cachexia even before the onset of muscle loss [17], a phenomenon that cannot be solely explained by decreased appetite, as it often begins before anorexia manifestation [7]. **Figure 2** summarily represents the processes underlying adipose tissue remodelling during CAC.

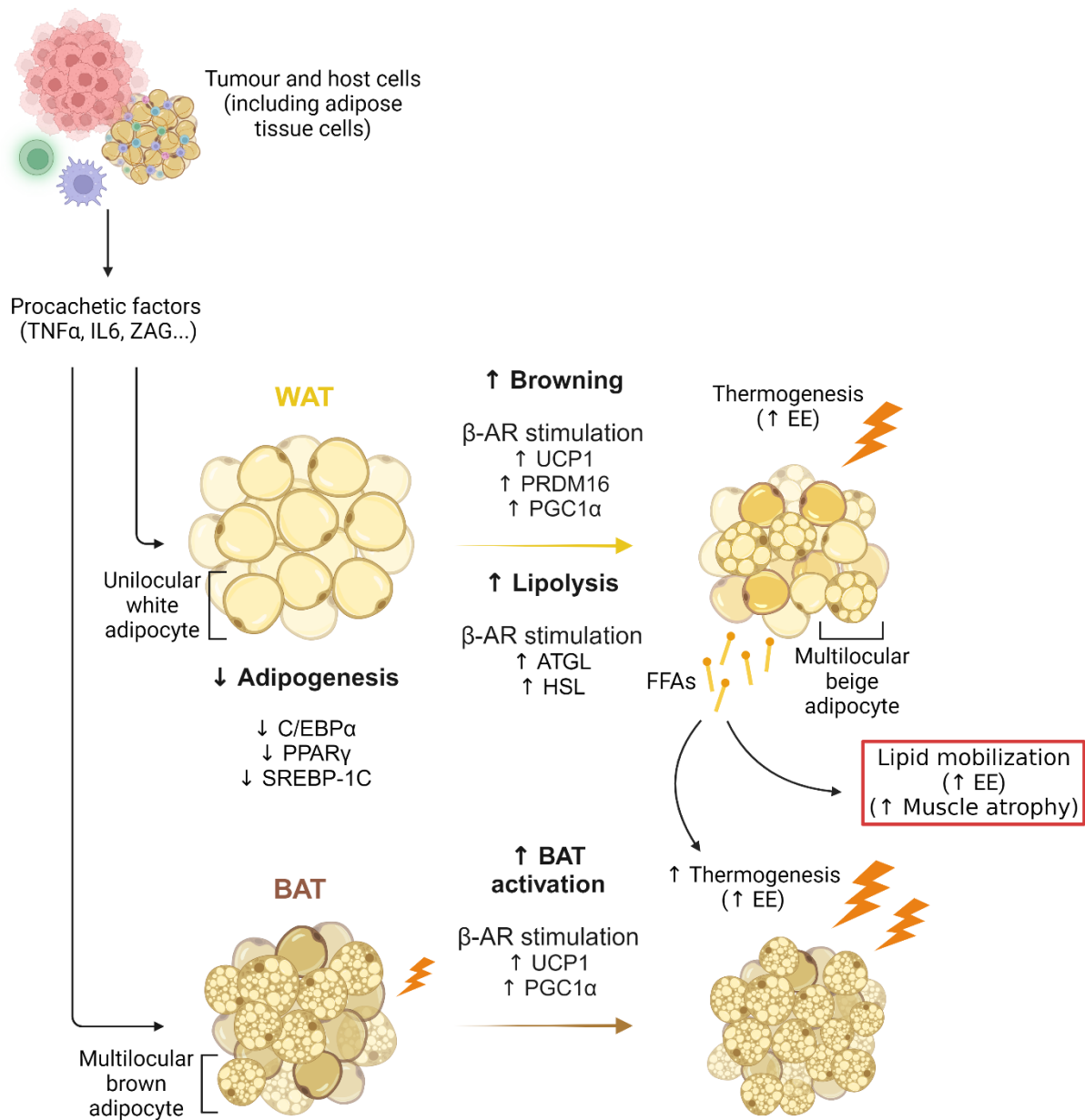


Figure 2. Schematic representation of some of the alterations occurring in adipose tissue during cancer-associated cachexia. ATGL: adipocyte triglyceride lipase; BAT: brown adipose tissue; β-AR: beta-adrenergic receptors; C/EBP α : CCAAT/enhancer binding protein alpha; EE: energy expenditure; FFAs: free fatty acids; HSL: hormone sensitive lipase; IL6: interleukin 6; PGC1 α : Pparg coactivator 1 alpha; PPAR γ : peroxisome proliferator-activated receptor gamma; PRDM16: PR domain containing 16; SREBP-1C: sterol regulatory element binding protein-1c; TNF α : tumour necrosis factor alpha; UCP1: uncoupling protein 1; WAT: white adipose tissue; ZAG: zinc- α 2-glycoprotein. (Created with BioRender.com)

As shown in **Figure 2**, pronounced lipolysis in cachectic patients has been particularly reported, associated with increased expression levels of ATGL and HSL when compared to healthy individuals [4, 7, 19, 77, 78]. Induction of lipolysis in CAC has been attributed

to inflammation driven by cytokines such as TNF α , IL6 and zinc- α 2-glycoprotein (ZAG), produced by host cells such as adipocytes and immune cells, and also by tumour cells [4, 79], as well as to natriuretic peptides and catecholamines [19]. Besides promoting fat loss, lipolysis contributes to an overall negative energy state, to skeletal muscle atrophy through the infiltration of circulating FFAs [17, 80], as well as to the recruitment of immune and stromal cells to the adipose tissue [13], all contributing to CAC progression.

In addition to lipolysis, increased thermogenesis caused by WAT browning and overactivation of BAT has also been associated with cachexia (**Figure 2**) [8, 18, 81]. Increased levels of UCP1, the hallmark of mitochondrial thermogenesis in adipose tissue, along with increased PRDM16 and PGC1 α expression, both markers of beige adipocytes development, were observed in several CAC models [8, 17]. WAT browning in cachexia has been pointed to be driven by chronic inflammation [13] and by stimulation of the β -adrenergic signalling [8, 18], and is responsible for amplifying energy expenditure and lipid mobilization, as FFAs serve as substrate for heat generation in mitochondria (as shown in **Figure 2**) [13]. However, the role of BAT and thermogenesis in cancer cachexia is not settled yet, since some experiments describe loss of brown fat mass, reduced *Adrb3* (β 3-AR encoding gene) expression and hypothermia in cachectic mice [80]. These conflicting results probably occur due to the employment of distinct models and cancer types, as well as due to the assessment of cachexia at different timepoints since BAT disruption likely occurs in the earlier stages of cachexia progression [80]. Some of the most important proinflammatory mediators underlying lipolysis, browning and thermogenesis during cachexia are explored next.

TNF α , also known as cachectin, can be produced by adipose tissue (adipokine), skeletal muscle [13], tumour and immune cells [19]. It has been shown to inhibit adipogenic factors such as *Cebpa* and *Pparg* [12], LPL activation and glucose transport (crucial for lipogenesis), to promote adipose tissue insulin resistance and to stimulate HSL, thus contributing to decreased adipogenesis and lipogenesis and enhanced lipolysis [12, 13]. Furthermore, it was demonstrated that TNF α acts in hypothalamus stimulating thermogenesis in BAT via sympathetic signalling [82], and it is responsible for the recruitment of monocytes to adipose tissue, which increases the levels of other circulating cytokines, such as IL6 and IL1 β [13]. IL6 is a stimulant of WAT browning and thermogenesis via induction of UCP1 expression, and it is also known to promote the catabolism and prevent the synthesis of lipids [4, 13]. However, depending on what type of cells it is targeting, IL6 can act as a pro- or as an anti-inflammatory cytokine [13]. Moreover, TNF α and IL6 can downregulate appetite by suppressing NPY production [4].

Moving on to ZAG, it is considered a lipid mobilizing factor that is highly expressed by several types of tumours, and apparently negatively correlated with fat mass [79, 83]. ZAG was found to drive lipolysis via the β 3-AR associated pathway, WAT browning and energy expenditure through upregulation of UCP1 [13, 83], and suppression of lipogenesis [79].

On the other hand, catecholamines and natriuretic peptides are considered the major hormonal mediators of human lipolysis, both leading to HSL stimulation [77, 78]. Natriuretic peptides, secreted by heart vasculature, were found to induce lipolysis in cachectic patients [13] by increasing cyclic guanosine 3',5'-monophosphate (cGMP), which in turn stimulates protein kinase G, thus promoting HSL activation [77]. In a different way, catecholamines are released by the sympathetic nervous system (SNS) and by binding to β -AR drive adenylyl cyclase rising, followed by increased production of cAMP that promotes both protein kinase A (PKA) phosphorylation, leading to HSL and ATGL stimulation [8], as well as UCP1 expression both in WAT (i. e. WAT browning) and BAT [18]. Summarily, these two differently activated signalling pathways result in lipolysis and thermogenesis/WAT browning enhancement [49].

Moreover, fat wasting can be linked to reduced adipogenesis and lipogenesis (**Figure 2**). Diminished expression of key regulators of the adipogenic programme, such as PPAR γ , C/EBP α and sterol regulatory element binding protein-1c (SREBP-1c) [10], and lipid storage mediators, for instance lipoprotein lipase (LPL) [4, 7], fatty acid synthase (FAS), among others [8], has been found in white fat of cachectic mice.

Following the previous reasoning, in cachectic subjects, fat depletion is expected to result in low leptin levels, which would induce orexigenic signalling. However, in the context of cachexia, the feedback mechanisms that regulate energy intake according to the adipose tissue amount seem to be disrupted [17], leading to decreased appetite in CAC patients and thus contributing to the progression of cachexia. Moreover, the role of leptin in CAC is still controversial, as some studies report a marked decrease in circulating leptin levels [54, 84] and in adipose tissue leptin mRNA levels [10], whereas others point to increased leptin levels in cachectic patients when compared to healthy individuals [52, 85]. Additionally, a study has demonstrated that the expected reduced circulating leptin concentration accompanied by increased production of the orexigenic factor NPY in hypothalamus are actually observed in the cachexia mouse model employed, however mice presented progressive hypophagia. Therefore, it is plausible that factors released by the tumour are able to bypass this orexigenic signalling pathway, or that some molecules downstream of NPY are dysregulated in cachexia [86].

3.1 The Role of Adrenergic Signalling

Adrenergic signalling pathways have been considered potential leading players contributing to adipose tissue rearrangement in cachexia [54, 87, 88].

β_1 and β_2 -AR are the classical beta adrenoceptors in humans, contrasting with the predominance of the β_3 -AR in mice, and it is still not clear whether the latter is able to induce lipolysis and thermogenesis in human adipose tissue due to its low expression [61]. Despite that, it has already been demonstrated that β_3 -AR could be relevant for the lipolytic and thermogenic activity in brown and beige human adipocytes [61].

The three β -AR types are G protein-coupled receptors [71], expressed in WAT and BAT [89], beyond several other tissues [90]. All of the β -AR are generally coupled to the stimulatory G protein subtype (Gs), that induce adenylyl cyclase (AC), cAMP and PKA activation, however some experiments have suggested that β_3 -AR in humans could also be linked to the inhibitory G protein subtype (Gi) that inhibits cAMP, pointing to a self-limiting system of cAMP stimulation [71]. Besides that, it has been consistently demonstrated that lipolysis is induced upon β_3 -AR stimulation, as so it is UCP1 expression through the activation of the p38 MAPK signalling cascade downstream of cAMP and PKA [67, 71, 89]. Moreover, thermogenesis relies on lipolysis activation, as without available FFAs, there is no substrate to fuel the oxidative phosphorylation that leads to heat production [71]. All these processes are summarised in **Figure 3**.

The evidence regarding the role of β -AR stimulation to the progression of cachexia are still not robust enough. It has already been shown that catecholamine levels were higher in a mouse model of CAC when compared with control mice [23]. Moreover, the use of β_3 -AR antagonists in cachectic mice were shown effective in repairing increased resting oxygen consumption [91], reducing WAT browning and attenuating cachexia [23], what was consistent with other findings that demonstrated the importance of adrenergic innervation to induce thermogenesis in BAT of cachectic mice, and that the blockade of β_3 -AR attenuated the cachexia wasting phenotype [2]. However, other papers reached different conclusions, pointing to a predominant role of inflammatory cytokines, such as IL6, to the CAC-associated adipose tissue phenotype [87, 92]. Again, this heterogeneity is likely attributable to differences in CAC pathophysiology among distinct organisms and tumour types.

Furthermore, beyond the notable role of β -AR associated pathways in promoting fat depletion and heat generation, as emphasized before [7], there is a different type of

adrenergic receptor, named $\alpha 2$ -adrenergic receptor, that includes the subtypes $\alpha 2A$, $\alpha 2B$ and $\alpha 2C$ ($\alpha 2A,B,C$ -AR) [93], that produce a distinct effect when stimulated by catecholamines. $\alpha 2$ -AR are negatively associated to adenylyl cyclase as they are coupled to the G_i subtype (**Figure 3**) [71] and upon catecholamine stimulation promote the reduction of intracellular cAMP concentration, suspending UCP1 expression [94] and lipolysis, thus contributing to lipid storage [20]. $\alpha 2A$ -AR was thought to be the only α -AR subtype expressed in humans, sharing a 92% similarity with its homolog receptor expressed in mice [24]. However, the expression of other α -AR in humans and rodents is now recognised [70]. Furthermore, $\alpha 2A$ -AR seems to be expressed in higher levels in humans than in murines [20], and its expression in human adults was observed to be much higher in WAT rather than in BAT, particularly in subcutaneous depots [70, 95, 96].

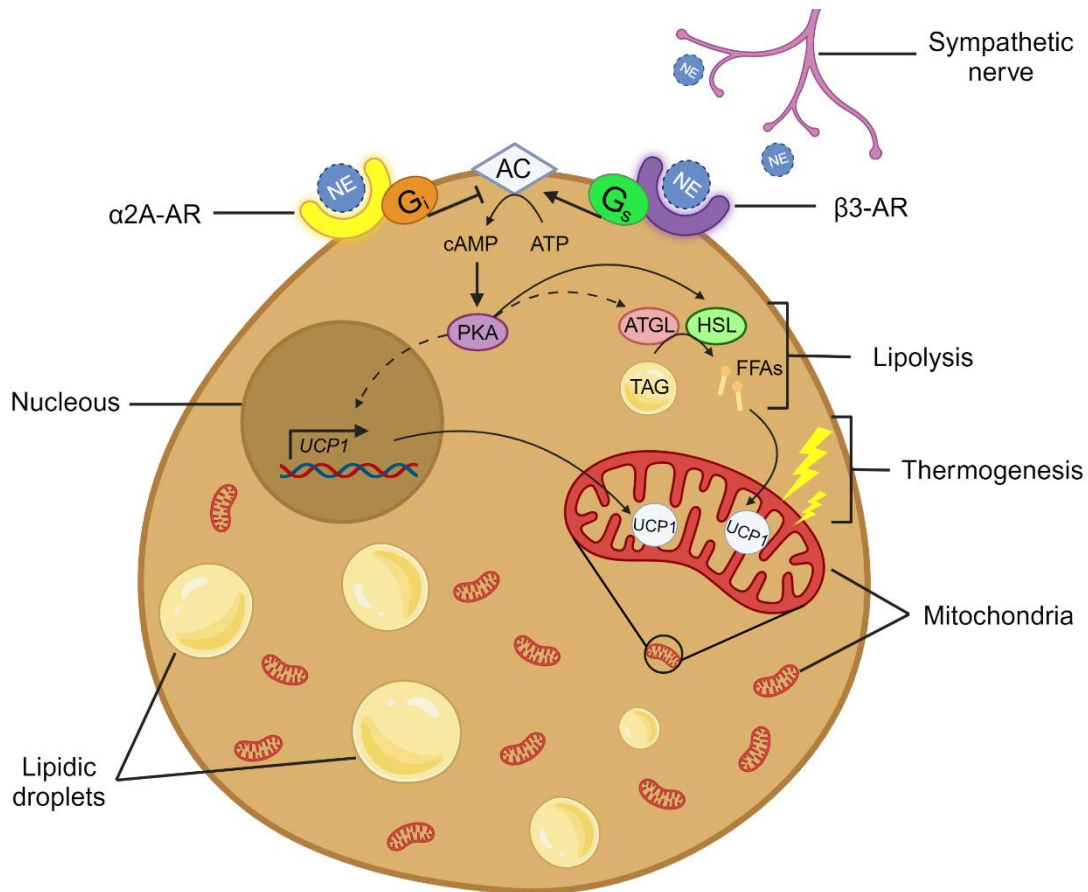


Figure 3. Schematic representation of some of the $\alpha 2A$ - and $\beta 3$ -adrenergic signalling pathways related with lipolysis and thermogenesis within an adipocyte. UCP1 and thermogenesis activation only occur in beige and brown adipocytes. Arrows denote activation; Dashed arrows denote indirect activation; Line with a dash at the end denotes inhibition. AC: adenylyl cyclase; ATGL: adipocyte triglyceride lipase; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; FFAs: free fatty acids; G_i : inhibitory G protein; G_s : stimulatory G protein; HSL: hormone sensitive lipase; NE: norepinephrine; PKA: protein kinase A; TAG: triglyceride; UCP1: uncoupling protein 1. (Created with BioRender.com)

Concluding, it is well established that the adrenergic signalling plays a leading role in the modulation of BAT physiology [71] and the distribution and relative amount of α 2- and β -AR in adipose tissue may determine which of them is activated by catecholamines, leading to the up or downregulation of intracellular levels of cAMP and the respective downstream effects [20, 71, 77]. In fact, a higher α 2A/ β 3-AR ratio has already been associated with obesity [20, 97], while a lower α 2A/ β 3-AR ratio is related with increased thermogenesis and lipolysis [20, 21, 98]. Particularly considering that the contribution of adrenergic receptors for CAC development is not well established yet, assessing the potential differences between cachectic and healthy subjects regarding the α 2A-AR/ β 3-AR ratio may be interesting and unveil promising results.

4. The Sexual Dimorphism in Adipose Tissue

Fat endocrine function, metabolism and distribution are undoubtedly sexually dimorphic and sex hormones have consistently been proposed to contribute to that phenomenon [28, 36, 99]. In fact, in addition to the gonads, oestrogens are also produced in adipose tissue of both males and females, through the action of aromatase and 17/ β -hydroxysteroid dehydrogenase, that convert androgens into oestrogens [82]. In spite of that, even before puberty, differences amongst sexes are observed, and other factors may contribute to that, such as the number of X chromosomes [28].

Women overall adipose tissue content is typically higher than men's [36, 100]. Moreover, throughout their reproductive life, women amass more white subcutaneous fat, whereby men present higher accumulation of visceral fat [101]. Nonetheless, after menopause this difference is attenuated, reinforcing the role of sex hormones in adipose tissue modulation [28, 36]. With respect to the anatomical location of the main BAT depots, however, no major differences have been observed between genders [101]. Leptin circulating levels, in turn, present sexual dimorphism in a species-specific manner, with women displaying higher levels than men [102], whereas male mice present higher levels than female [28, 30]. Accordingly, leptin expression is inhibited in men adipocytes upon testosterone treatment, while administration of 17 β -oestradiol produced the opposite effect in women fat cells [99].

It is important to have in mind that both males and females express progesterone, oestrogen and androgen receptors in their adipocytes [99], although the expression patterns vary depending on the sex, the fat depot, and the species [36]. Therefore, the action of sex hormones in adipose tissue is not only dependent on the hormone releasing

amount, but also on its receptors' quantity and distribution. For instance, in women, diabetes can be promoted by hyperandrogenism, while in men it can occur due to androgen deficiency [36].

As a further matter, differences in adipose tissue sympathetic innervation [28, 103], catecholamine-mediated lipolysis and $\alpha 2$ - and β -adrenoceptors distribution have been purposed to partly contribute to gender-specific adipose tissue profiles [28, 104], since it is established that adrenergic signalling is influenced by sex hormones [30, 94]. For instance, BAT of female rats seems to be more prone to β -adrenergic stimulation than that of males [101]. Furthermore, a study showed that cafeteria-diet feeding induced sex-dependent variations in lipolysis and in the ratio $\alpha 2A/\beta 3$ -AR both at mRNA and protein levels in retroperitoneal WAT of rats [20]. $\alpha 2A$ -AR expression was more upregulated in male, while downregulation of $\beta 3$ -AR was greater in female, therefore, the ratio $\alpha 2A/\beta 3$ -AR increased in both sexes, but in a higher extent in males, which points to a greater contribution of the $\alpha 2A$ -AR to the gender-associated differences in the context of obesity [20]. However, these mechanisms should be interpreted as fat depot-specific, since physiologic processes of adipocytes are known to vary depending on their anatomical location [20]. As an illustration, while in the inguinal adipose tissue the stimulation of $\beta 3$ -AR induced the brown adipocyte phenotype in both sexes, in gonadal WAT the same effect was only verified in female mice [103].

Oestrogens, the main female sexual hormone, have been associated with insulin sensitivity, reduction of inflammation [28] and protection against androgen-induced fat amassing [99], particularly via oestrogen receptor alpha ($ER\alpha$), one of the two nuclear oestrogen receptors [28, 105], that are present both in human and rodent adipose tissue [105]. Females express higher levels of $ER\alpha$ both in subcutaneous and visceral adipose tissue [105] and have higher density of neurons expressing $ER\alpha$ in subcutaneous adipose tissue than males [28]. Deletion of $ER\alpha$ is responsible for adiposity expanding in human and mice of either genders [28], and is also associated with higher propensity for obesity, namely via hypertrophy, reduced energy expenditure [106], impaired glucose tolerance and decreased insulin sensitivity in mice of both sexes [105]. Lack of the enzyme aromatase, responsible for the conversion of androgens into oestrogens, is associated with obesity, among other metabolic disorders, both in mice and human [99]. Moreover, oestradiol was also pointed to stimulate adrenergic signalling, promoting lipolysis in intraabdominal fat of ovariectomized rats [104], and different authors showed that the administration of an oestrogen receptor ligand in mice led to the upregulation of UCP1 in inguinal and gonadal WAT only in females [29].

Progesterone, another important female steroid hormone, was found to promote the differentiation of rodent brown adipocytes, along with the stimulation of adrenergic-induced *Ucp1* expression, although its action is probably oestrogen-dependent [94]. However, this effect faded with the employment of increasing hormone concentrations, suggesting that progesterone action on adipose tissue is dose-dependent. Indeed, during pregnancy, a period of high progesterone production, women display reduced activation of UCP1 [94]. Additionally, progesterone was responsible for decreasing α 2A-AR and increasing β -AR protein levels, mainly β 3-AR, thus promoting adrenergic-induced lipolysis in mice brown fat cells [21]. This steroid hormone is also pointed to have a contradictory role in BAT and mitochondria function. While it enhances the mRNA expression of key genes involved in BAT differentiation and mitochondrial biogenesis, simultaneously it negatively contributed to the mitochondrial programme [107].

Although most of the studies point to a greater susceptibility of females to undergo WAT browning [29, 101], they reportedly have higher amounts of BAT [98, 100], present larger mitochondria with bigger cristae [99, 101], higher thermogenic activity [101], higher metabolic rate per kilogram and higher UCP1 expression compared to males [28], it has already been reported that sexual dimorphism regarding browning and BAT activation exhibit different profiles depending on the causative stimuli [29].

On the other hand, androgens, the prevailing hormones in male, were found to promote adipogenesis and insulin sensitivity in men, whereas promoting insulin resistance in women adipose tissue [28]. Furthermore, male BAT express higher levels of lipogenic proteins accompanied with lower levels of thermogenic proteins [99], and testosterone was shown to inhibit *Ucp1* expression in rodent brown adipocytes stimulated by NE, in a dose-dependent manner. This could possibly be related to an inhibitory effect of testosterone to brown adipocyte differentiation, and to testosterone-induced upregulation of α 2A-AR, suppressing *Ucp1* levels and thermogenesis [94]. Additionally, testosterone was found to lower brown adipocytes *Ucp1* expression, by downregulating the transcription of genes related to the mitochondria biogenesis and function [107]. Though in a lesser extent, other sources of information, however, associate androgens with lipolysis and inhibition of adipocyte differentiation in men [108]. With regards to adrenergic pathways, testosterone also plays a controversial role. While it was found to promote the expression of the antilipolytic receptor α 2A-AR in rodents' intraabdominal and subcutaneous adipocytes [104], mice cultured brown adipocytes [21], and human WAT [109], it was also detected to stimulate the β -AR-associated lipolytic cascade [104].

Concluding, due to the contribution of multiple factors to the physiologic processes of adipose tissue, and due to its high heterogeneity, available data regarding adipose tissue sexual dimorphism is still controversial and has not yet been able to establish a solid profile of the molecular players involved in this matter.

4.1 The Impact of Biological Sex in Cancer Cachexia

Sex-associated differences in cancer cachexia are still poorly depicted, while greater efforts have been made towards muscle depletion comprehension [110, 111]. Concerning that, cachectic men have reportedly lower handgrip strength [110] and increased muscle fatigue [111] when compared to cachectic women.

Nonetheless, sexual dimorphism has also been found regarding leptin secretion, a well-known adipocytokine, during cancer and CAC progression. Leptin plasma levels showed a positive correlation with cancer-induced weight loss in men, by opposition to a negative correlation in women. Likewise, cachectic men displayed higher leptin levels comparatively with noncachectic men, whereas cachectic women presented lower leptin levels than noncachectic ones [85].

Finally, sex chromosomes seem to directly contribute towards sexual dimorphism in the pathophysiology of cancer cachexia. Several cachexia-related microRNAs genes were found to be located in the X chromosome, whereas only four have been detected in the human Y chromosome, however this matter has not been deeply explored yet [27].

5. K14-HPV16 Transgenic Mice

The use of mouse models to study human biological processes is a common practice nowadays, since the two species share 99% of their encoding genome sequences and great genomic evolutionary conservation [112]. Moreover, the recently developed ability of genetic engineering in mammals, including the possibility of inserting human genes in mice, has provided to the scientific research community great progressions towards the understanding of human diseases' pathophysiology and therapies testing [112].

In the cancer field, particularly, experimental mouse tumour models – which include transplantable tumours via syngeneic tumour cell lines, genetically engineered tumour models, and humanized mice through human tumour xenografts – have been crucial to the comprehension of both tumour's and host's antitumoral mechanisms, helping to

achieve new promising therapies [113]. Nevertheless, it should not be forgotten that cancer pathophysiology is a complex heterogenous process, and the molecular pathways taking place in rodent models are often not completely translatable to the human beings [113], that is why it takes a long way until it is possible to safely make it to the clinical practice.

Human papillomaviruses (HPVs) are one of the common causes of sexually transmitted infections [31] and can trigger a large set of injuries, from benign easily curable conditions to some specific cancers [114]. High-risk HPVs (such as HPV16 and HPV18) are associated with the development of the majority of cervical cancers, along with some other anogenital, head-and-neck [31] and skin cancers [115]. High-risk HPV oncoproteins can also promote cancer cachexia, through the activation of the transcription factor nuclear factor- κ B (NF- κ B) that leads to the activation of the ubiquitin ligases involved in the ubiquitin-mediated proteasome degradation pathway, thus stimulating skeletal muscle degradation [31].

Benefits of genetically engineered mouse tumour models include close reproduction of human tumour progression and immune response [113]. K14-HPV16 transgenic mice are an example of those models, which are generated by embryo microinjection [116] carrying the HPV16 early region genes under the control of the keratin 14 (K14) promoter (**Figure 4**), which leads to the HPV16 expression in all basal cells of the keratinized epithelia (where HPV infections naturally occur), and progressive development of HPV-induced carcinogenesis, not only in cervix, but also in cutaneous and head-and-neck characteristic locations [31]. Furthermore, both female [115, 117] and male [31] K14-HPV16 transgenic mice have previously shown a wasting syndrome with CAC typical phenotypic and molecular features, mainly regarding muscle wasting and inflammation (**Figure 4**). In more detail, in a previous work [31], male K14-HPV16 mice displayed a range of macroscopic cutaneous injuries, that went from hyperkeratosis to alopecia. Moreover, several histological damaging was observed, which included hyperplasia and dysplasia of the squamous epithelium in tongue and several skin regions, and some findings of well differentiated invasive squamous cell carcinomas (**Figure 4**). Additionally, total body weight of the transgenic mice started to gradually decrease compared to their control counterparts, from 19 to 21 weeks of age, culminating in a significant reduction when analysing the data from the whole experiment. Finally, gastrocnemius weights at the time of sacrifice (when mice were 31 to 33 weeks old) were also significantly reduced in transgenic mice [31], in accordance with the reduced grip strength observed in another study [117]. Furthermore, female K14-HPV16

transgenic mice displayed local and systemic inflammation, based on enhanced inflammatory cell infiltration in skin samples, increased levels of phosphorylated NF- κ B in muscle, leucocytosis, hepatitis and splenitis [115, 118].

For all this, K14-HPV16 transgenic mice was considered a good model for the study of HPV-induced cancer-associated cachexia [31, 115]. However, adipose tissue was not a parameter assessed by the previously mentioned studies. In that manner, the validation of the K14-HPV16 transgenic mice as a model of adipose tissue impairment in the context of CAC still lacks evidence.

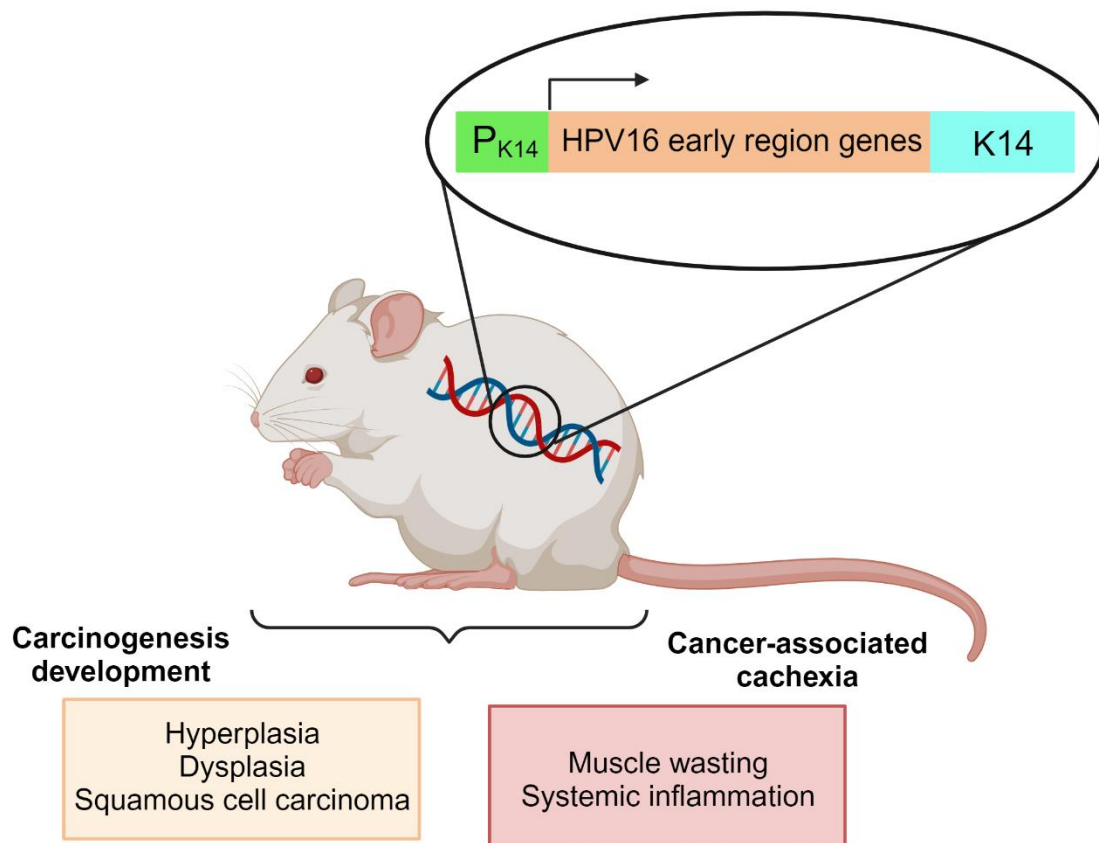


Figure 4. Schematic representation of the K14-HPV16 transgenic mouse and its main features. K14: keratin 14 gene; P_{K14} : Promoter of keratin 14 gene. (Created with BioRender.com)

Aims

The main aims of this work were the following:

1. To characterize at molecular level adipose tissue depletion in K14-HPV16 cachectic mice and to evaluate differences among male and female mice.
2. To verify if adrenergic signalling is differently activated among K14-HPV16 cachectic and wild-type mice and among male and female mice.

To achieve the main aims the following tasks were established:

1. To evaluate at mRNA level the expression of markers of lipolysis (*Pnpla2* and *Lipe*), browning and thermogenesis (*Ucp1*) and adipocyte differentiation (*Pparg* and *Cebpa*) in different types of adipose tissue of K14-HPV16 and wild-type mice (males and females).
2. To evaluate at mRNA level the expression of the adrenergic receptors *Adra2a* (related to lipid storage) and *Adrb3* (related to browning and thermogenesis) in different types of adipose tissue of K14-HPV16 and wild-type mice (males and females).
3. To evaluate at mRNA level the *Adra2a/Adrb3* ratio in different types of adipose tissue of K14-HPV16 and wild-type mice (males and females).

Methodologies

1. Mice

The present study was authorized both by the Portuguese Veterinary Directorate (approval number 014139-2022) and by the Ethics Committee of the University of Trás-os-Montes and Alto Douro (approval number 852-e-CITAB-2020), and was performed in accordance with domestic legislation (Decree-Law 113/2013, August 7) and European Directive 2010/63/EU on the protection of animals. The mice employed in this project were donated by Doctors Jeffrey Arbeit and Douglas Hanahan via the USA National Cancer Institute Mouse Repository, University of California, and were kept at the animal facilities of the University of Trás-os-Montes and Alto Douro. Mice were accommodated in polycarbonate cages with corncob beds (Ultragene, Santa Comba Dão, Portugal) and environmental enrichment, which were changed and cleaned once a week. All animals were housed under controlled conditions: temperature (23 ± 2 °C), relative humidity ($50 \pm 10\%$), photoperiod cycle of 12 hours of light (8 a.m.–8 p.m.) and 12 hours of darkness. All mice were provided *ad libitum* water and food, composed by a standard diet (Mucedola S.R.L., 4RF21 GLP Certificate, Milan, Italy).

2. Experimental design

The established model for the study of cancer cachexia K14-HPV16 transgenic mice (HPV+) [31], generated on an FVB/n background, and matched-control mice (HPV-) were employed. A total of 75 mice – 38 HPV+ and 37 control (HPV-) – were divided in 3 groups, which are presented in **Table 1**: Group 1 was sacrificed at 33 weeks of age (9 females HPV-, 10 males HPV+, 10 females HPV+), Group 2 was sacrificed at 42 weeks of age (5 males HPV-, 3 females HPV-, 2 males HPV+, 9 females HPV+) and Group 3 was sacrificed at 50 weeks of age (10 males HPV-, 10 females HPV-, 1 male HPV+, 6 females HPV+).

Group	Age of sacrifice	Control		HPV+	
		M	F	M	F
1	33 weeks	0	9	10	10
2	42 weeks	5	3	2	9
3	50 weeks	10	10	1	6

Table 1. Distribution of the control and HPV+ mice for the three experimental groups, according to the age of sacrifice. M: Male; F: Female.

3. Markers selection

The lipases adipose triglyceride lipase (*Pnpla2*) and hormone sensitive lipase (*Lipe*) were the chosen markers of lipolysis, as they are responsible for the first steps of triglyceride hydrolysis [8, 13, 119].

CCAAT/enhancer binding protein alpha (*Cebpa*) and peroxisome proliferator-activated receptor gamma (*Pparg*) were chosen as markers of adipocyte differentiation, particularly white adipocytes differentiation as for *Cebpa*, as they are crucial players in this process [43, 120].

Uncoupling protein 1 (*Ucp1*) was selected as one of the markers of thermogenesis and browning, as it is a hallmark of brown and beige adipose tissue [73, 74, 121]. Additionally, β 3-adrenergic receptor (*Adrb3*), the most prevalent of the three β -AR types in mice, was also designated to evaluate the thermogenesis and lipolysis in BAT as well as the browning process, as stimulation of β 3-AR associated signalling cascade is recognised as one of the most important ways to induce thermogenic and lipolytic activity in brown and beige depots, namely in the context of cancer associated cachexia [2, 23, 67].

α 2A-adrenergic receptor (*Adra2a*) was selected as it is responsible for promoting lipid accumulation [20]. Moreover, the ratio α 2A-AR/ β 3-AR, responsible for regulating adiposity and adrenergic response in adipose tissue, was selected as a target of study as it may contribute to adipose tissue impairment in CAC and because differences between sexes in this ratio have also been reported [20, 21].

Based on the available literature [122, 123], β -actin (*Actb*) and TATA-binding protein (*Tbp*), whose expression is reportedly stable in adipose tissue, were chosen as endogenous controls to evaluate the expression of the above-mentioned markers at RNA level.

4. Sample collection and maceration

After mice sacrifice, samples from adipose tissue (abdominal, inguinal, and perineal) were collected (as shown in **Figure 5**) into 250 μL of TripleXtractor reagent (Grisp®) and were subsequently macerated with surgical blades and pestles for sample homogenization.

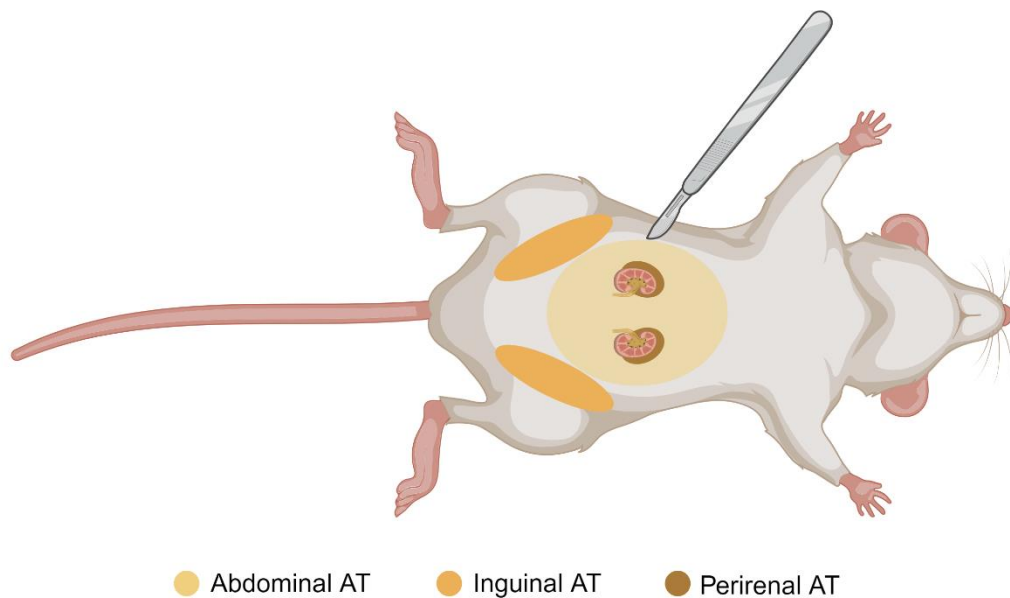


Figure 5. Schematic representation of the anatomic locations from where abdominal, inguinal and perirenal adipose tissue samples were collected. AT: adipose tissue. (Created with BioRender.com)

5. RNA isolation and quantification

Total RNA isolation was performed using GRS Total RNA Kit – Blood and Cultured Cells (Grisp®), adapted for RNA isolation from TripleXtractor. The protocol included the digestion of each sample with a mixture of 45 μL of DNase I reaction buffer with 5 μL of DNase I solution for 20 minutes at room temperature.

Subsequently, the RNA purity and concentration were analysed using NanoDrop Lite spectrophotometer (Thermo Scientific®). Purity is a parameter given by the A260/A280 ratio and concentration is expressed in $\mu\text{g}/\mu\text{L}$.

6. Reverse transcription of total RNA to single-stranded cDNA

Reverse transcription of 250ng of RNA from each sample to single-stranded cDNA was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems®), according to the manufacturer's protocol. Each reaction was performed in a final volume of 20,0 µL, containing 2,0 µL of 10X RT Buffer, 0,8 µL of 25X dNTP Mix (100 mM), 2,0 µL of 10X RT Random Primers, 1,0 µL of MultiScribe™ Reverse Transcriptase, 4,2 µL of Nuclease-free H₂O and 10,0 µL of RNA sample. Thermocycler conditions were the following: an initial 10 min step at 25°C, followed by 120 min at 37°C, and a final step at 85°C and were performed in a Biometra® Personal Cycler (Biocompare,USA). Negative controls lacking RNA were also included in all reactions.

7. Real-Time Polymerase Chain Reaction (qPCR)

Relative quantification of *Pnpla2*, *Lipe*, *Cebpa*, *Pparg*, *Ucp1*, *Adrb3* and *Adra2a* genes expression among the different adipose tissue samples of all mice were performed using a StepOne™One qPCR system. Each PCR reaction were carried out in a final volume of 10,0 µL, containing 3,5 µL of deionized water, 5,0 µL of TaqMan® Gene Expression Master mix (Applied Biosystems®), 0,5 µL of 20× TaqMan® Gene Expression Assays (Mm00503040_m1 for *Pnpla2*; Mm00495359_m1 for *Lipe*; Mm00514283_s1 for *Cebpa*; Mm00440940_m1 for *Pparg*; Mm01244861_m1 for *Ucp1*; Mm02601819_g1 for *Adrb3*; Mm00845383_s1 for *Adra2a*; Mm01205647_g1 for *Actb*; Mm01277042_m1 for *Tbp*) and 1,0 µL of cDNA sample. All reactions were performed in duplicate and had duplicated matching negative controls (containing the same reagents except cDNA), all done in the same plate. All targets and endogenous controls for each sample were also amplified in the same plate. Thermocycler conditions were the following: an initial denaturing step at 95°C for 10 min, followed by 45 cycles of 15 sec at 95°C, and a final step of 60 sec at 60°C. Duplicates with a Ct deviation value superior to 0.5 were excluded. In order to generate cycle threshold (Ct) values for all samples in each gene, it was used the analysis software for qPCR from the Thermo Fisher Connect platform (Thermo Fisher Scientific, Waltham, MA, USA), setting the same baseline and threshold consistently applied to each plate.

8. Statistical analysis

The expression of the genes was analysed using IBM SPSS Statistics for Windows (Version 29.0.0.0), employing the Livak method along with Mann-Whitney, once the data obtained from qPCR did not follow normality, and some of the groups of samples had a very reduced n.

Among *Tbp* and *Actb*, *Tbp* was the selected endogenous control for the normalization of our results, because its expression was the most stable, since it had the lowest standard deviation (SD = 1,18), and therefore, the lowest variability across samples.

Results

The results obtained regarding *Pnpla2*, *Lipe*, *Cebpa*, *Pparg*, *Ucp1*, *Adra2a* and *Adrb3* mRNA expression (as well as the ratio *Adra2a/Adrb3*) in all mice adipose tissue samples are presented below. Only the significantly different results between control and HPV+ mice, or between sexes are presented here.

The statistical analyses were performed comparing the expression of each gene between samples of the same type of adipose tissue (overlooking the different ages), or between samples of the same age (overlooking the different types of adipose tissue), because if both the type of adipose tissue and the age were taken into account in the same analysis, the amount of samples from each group would be too low to produce sufficiently solid results.

As there were no samples from control mice at the age of 33 weeks, differences between control and HPV+ male mice, and between sexes in the control group were not performed for that age.

1. Differences between wild-type (control) and HPV+ mice regarding molecular markers of adipose tissue

The significant differences found between samples from control and HPV+ mice followed an identical trend in male as well as in female mice. However, if in male mice differences were only found when analysing samples from inguinal adipose tissue and from the ages of 42 and 50 weeks (**Figures 6-8**), in female mice, significant differences were observed in the expression of almost all genes in all types of adipose tissue (abdominal, inguinal and perirenal) (**Figures 9-11**) and at all ages (33, 42 and 50 weeks) (**Figures 12-15**).

Table 2 gathers all the significant differences found between control and HPV+ mice.

1.1 Differences in male mice

Concerning the analysis performed in the three different types of adipose tissue, only regarding inguinal adipose tissue significant differences were found in the expression of the selected genes between control and transgenic male mice.

On the one hand, a significantly decreased expression of both *Adrb3* (3,19-fold, P-value < 0,001), encoding for β 3-adrenoceptor, responsible for inducing signalling pathways that lead to lipolysis and thermogenesis, and *Ucp1* (12-fold, P-value = 0,002), which encodes for a transmembrane mitochondrial protein essential for thermogenesis, was verified in HPV+ male mice, comparing to the control counterparts (**Figures 6a-b**, respectively).

On the other hand, the expression of *Adra2a*, the gene encoding for α 2A-adrenoceptor, which acts inhibiting cAMP production and thus limiting lipolysis and promoting lipid storage, was 1,77-fold increased in HPV+ male mice (P-value = 0,026) (**Figure 6c**). Accordingly, the *Adra2a/Adrb3* ratio, crucial for determining the adrenergic response of the adipocytes, displayed a significant increase in HPV+ male mice (P-value = 0,005) (**Figure 6d**).

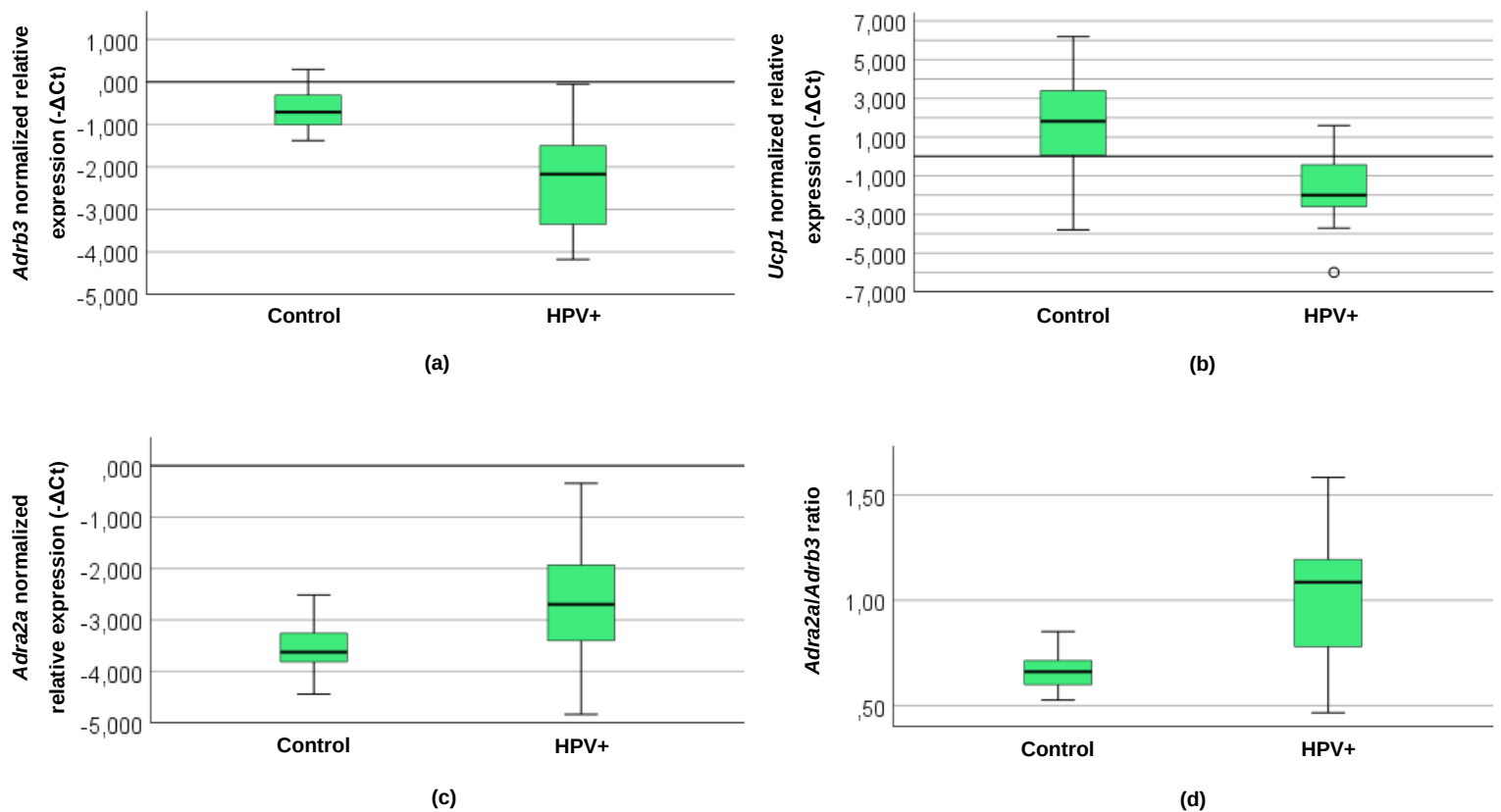


Figure 6. Normalized relative expression of **(a)** *Adrb3* (Control: n = 15, HPV+: n = 11, p < 0,001), **(b)** *Ucp1* (Control: n = 15, HPV+: n = 11, p = 0,002), **(c)** *Adra2a* (Control: n = 14, HPV+: n = 13, p = 0,026) and **(d)** levels of *Adra2a/Adrb3* ratio (Control: n = 14, HPV+: n = 11, p = 0,005) in inguinal adipose tissue samples from control versus HPV+ male mice.

As there were no samples from 33-week-old control mice, only samples with 42 weeks and 50 weeks of age were analysed. Analyses in both groups produced significant results.

Regarding the 42 weeks of age group, the expression of the adipocyte differentiation key regulator *Pparg* was 4,77-fold decreased (P-value = 0,025) in 42-week HPV+ males compared to control males (**Figure 7a**). Additionally, *Adrb3* expression was 6,06-fold decreased in HPV+ male mice when compared to the control ones (P-value = 0,025), and, consistently, *Adra2a/Adrb3* ratio was also increased in HPV+ males compared to control males (P-value = 0,037), as shown in **Figures 7b-c**, respectively.

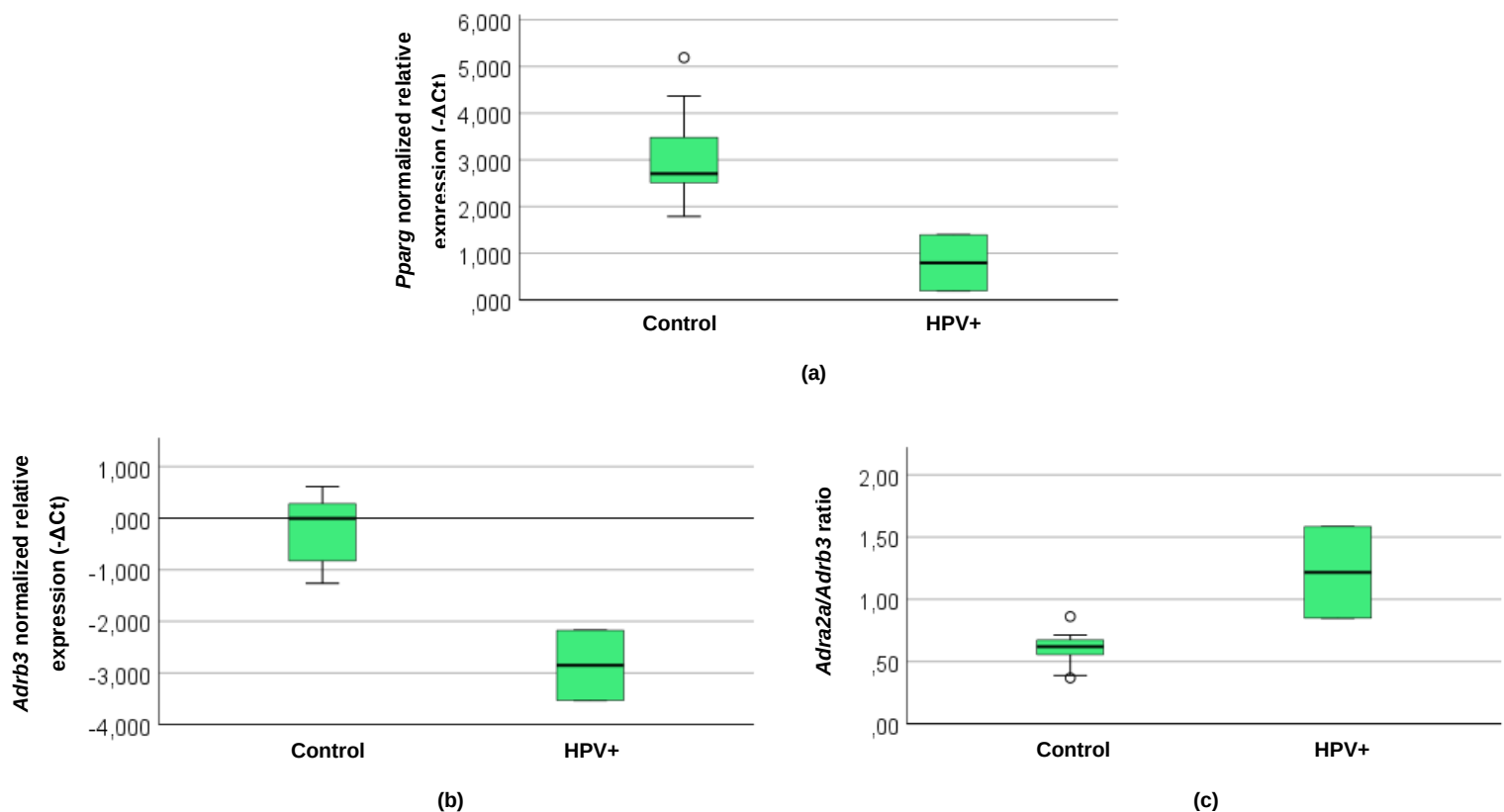


Figure 7. Normalized relative expression of **(a)** *Pparg* (Control: n = 14, HPV+: n = 2, p = 0,025), **(b)** *Adrb3* (Control: n = 14, HPV+: n = 2, p = 0,025) and **(c)** levels of *Adra2a/Adrb3* ratio (Control: n = 14, HPV+: n = 2, p = 0,037) in 42-week samples from control *versus* HPV+ male mice.

Furthermore, significantly reduced expression in HPV+ male mice from 42- and 50-week-old groups was verified in the other molecular markers of adipocyte differentiation *Cebpa* (6,85-fold in 42 weeks: P-value = 0,025, 18,04-fold in 50 weeks: P-value = 0,029), as well as in the lipases *Pnpla2* (2,58-fold in 42 weeks:

P-value = 0,025; 6,23-fold in 50 weeks: P-value = 0,037) and *Lipe* (6,35-fold in 42 weeks: P-value = 0,025; 9,25-fold in 50 weeks: P-value = 0,03), as presented in **Figures 8a-c**, respectively. It should be noted that the 42- and 50-week-old male groups only included two HPV+ samples, contrasting with several control samples. Therefore, these results should be approached with caution due to the limited sample size.

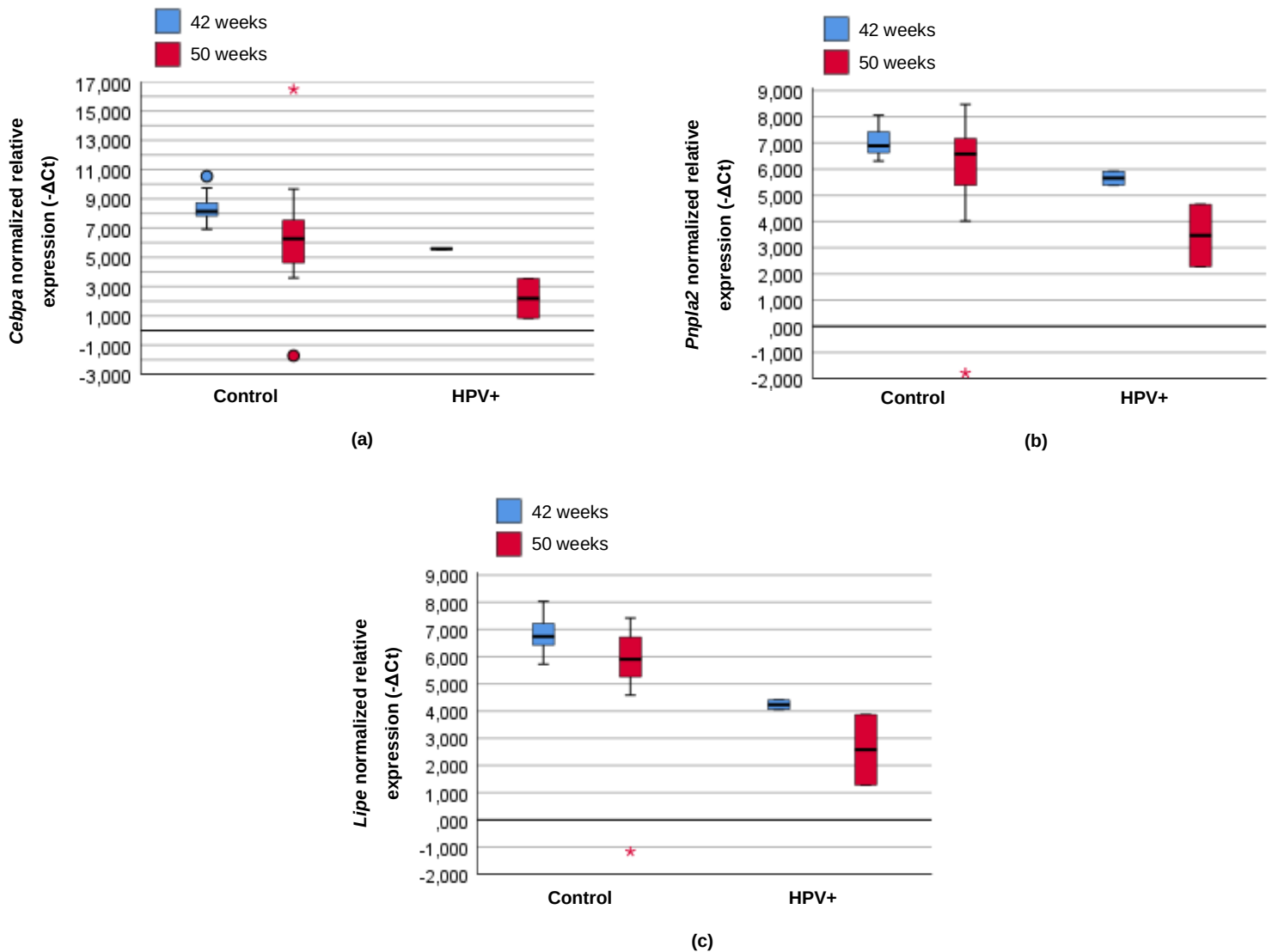


Figure 8. Normalized relative expression of (a) *Cebpa* (42 weeks: Control: n = 14, HPV+: n = 2, p = 0,025; 50 weeks: Control: n = 30, HPV+: n = 2, p = 0,029), (b) *Pnpla2* (42 weeks: Control: n = 14, HPV+: n = 2, p = 0,025; 50 weeks: Control: n = 29, HPV+: n = 2, p = 0,037) and (c) *Lipe* (42 weeks: Control: n = 14, HPV+: n = 2, p = 0,025; 50 weeks: Control: n = 29, HPV+: n = 2, p = 0,03) in 42- and 50-week samples from control versus HPV+ male mice.

1.2 Differences in female mice

In all types of adipose tissue, an increase of *Adra2a* expression (1,77-fold in abdominal adipose tissue: P-value = 0,026; 1,59-fold in inguinal adipose tissue: P-value = 0,007; 2,55-fold in perirenal adipose tissue: P-value = 0,006), as well as of the *Adra2a/Adrb3* ratio (abdominal adipose tissue: P-value = 0,001, inguinal adipose tissue: P-value < 0,001, perirenal adipose tissue: P-value < 0,001) was observed in HPV+ female mice, when compared to the control ones (**Figures 9a-b**).

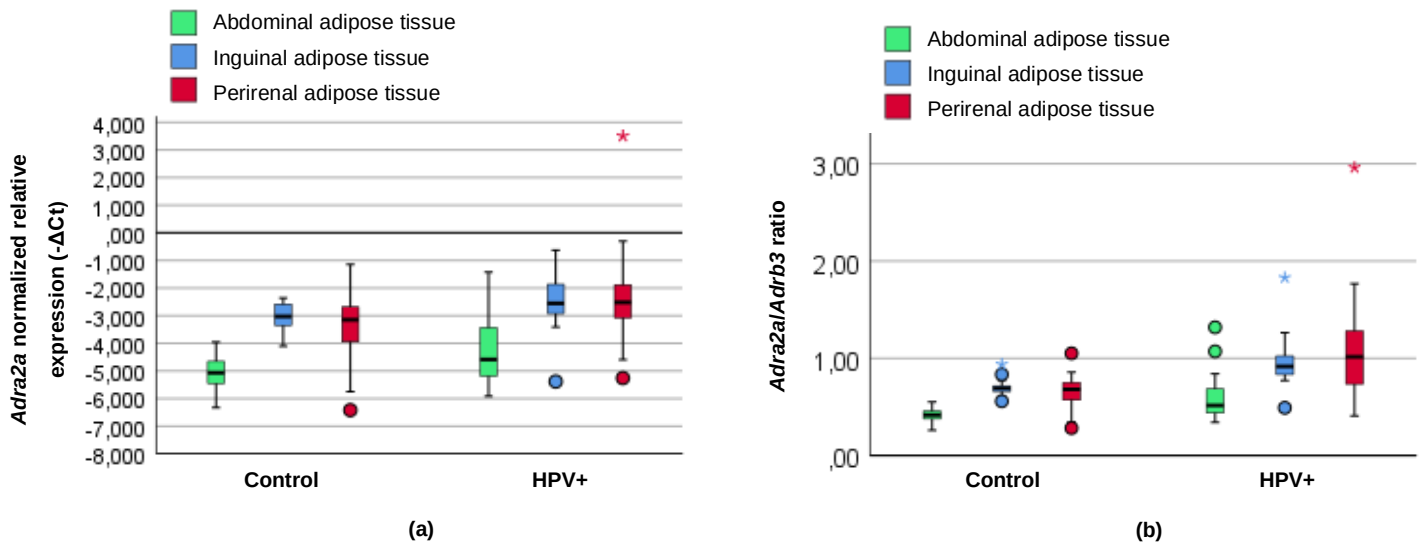


Figure 9. (a) Normalized relative expression of *Adra2a* (Abdominal AT: Control: n = 21, HPV+: n = 23, p = 0,026; Inguinal AT: Control: n = 20, HPV+: n = 25, p = 0,007; Perirenal AT: Control: n = 20, HPV+: n = 25, p = 0,006) and (b) levels of *Adra2a/Adrb3* ratio (Abdominal AT: Control: n = 21, HPV+: n = 23, p = 0,001; Inguinal AT: Control: n = 20, HPV+: n = 24, p < 0,001; Perirenal AT: Control: n = 20, HPV+: n = 25, p < 0,001) in abdominal, inguinal and perirenal adipose tissue samples from control *versus* HPV+ female mice.

On the other hand, *Ucp1* expression was decreased in HPV+ female mice compared to control female mice in all types of adipose tissue, however the decrease was not significant for perirenal adipose tissue (40,54-fold decreased in abdominal adipose tissue: P-value < 0,001, 31,73-fold decreased in inguinal adipose tissue: P-value < 0,001) (**Figure 10**).

Moreover, in the three types of adipose tissue, HPV+ females exhibited a reduction in the expression of *Cebpa* (2,69-fold in abdominal adipose tissue: P-value = 0,004; 3,54-fold in inguinal adipose tissue: P-value < 0,001; 3,55-fold in

perirenal adipose tissue: P-value = 0,002), *Pparg* (2,26-fold in abdominal adipose tissue: P-value < 0,001; 1,79-fold in inguinal adipose tissue: P-value = 0,001; 2,71-fold in perirenal adipose tissue: P-value = 0,01), *Pnpla2* (2,25-fold in abdominal adipose tissue: P-value < 0,001; 2,53-fold in inguinal adipose tissue: P-value < 0,001; 4,13-fold in perirenal adipose tissue: P-value < 0,001), *Lipe* (4,71-fold in abdominal adipose tissue: P-value < 0,001; 1,14-fold in inguinal adipose tissue: P-value < 0,001; 3,7-fold in perirenal adipose tissue: P-value < 0,001) and *Adrb3* (2,29-fold in abdominal adipose tissue: P-value < 0,001; 2,89-fold in inguinal adipose tissue: P-value < 0,001; 4,17-fold in perirenal adipose tissue: P-value < 0,001) when compared to control females (**Figures 11a-e**).

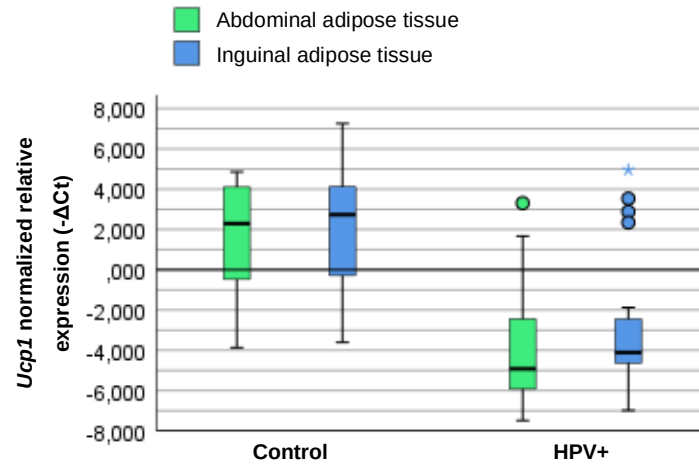
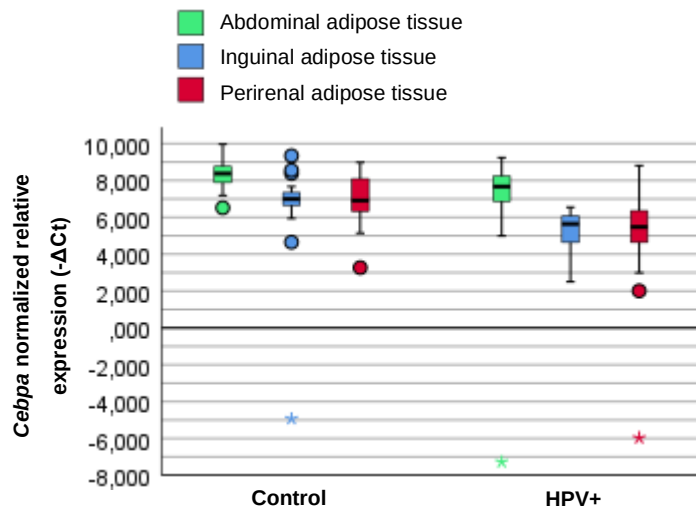
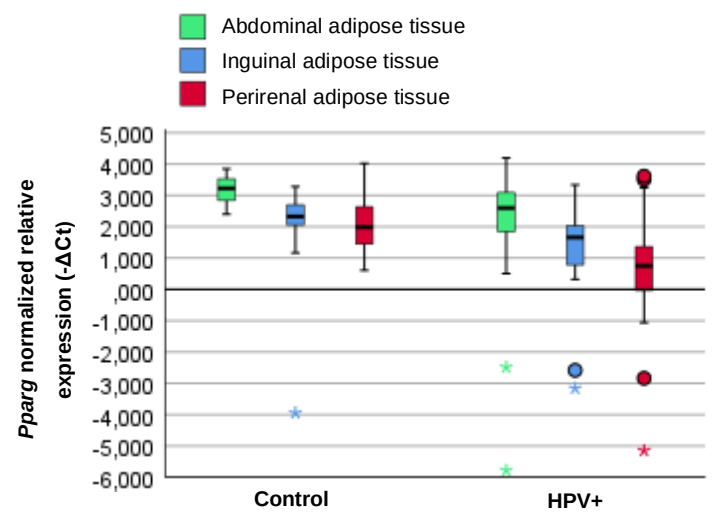


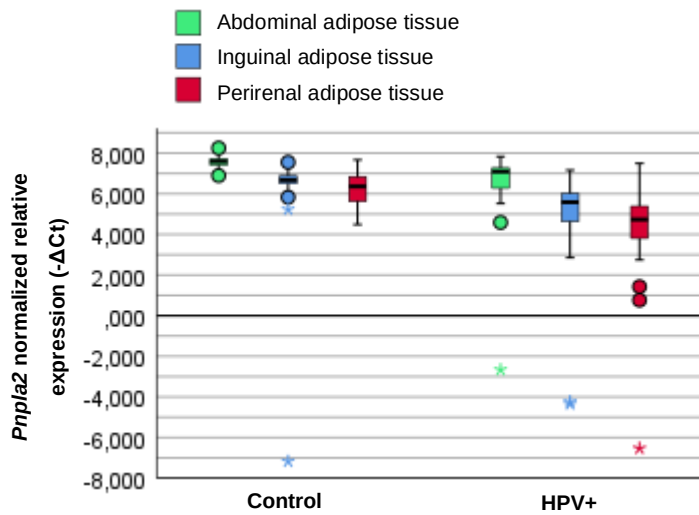
Figure 10. Normalized relative expression of *Ucp1* in abdominal and inguinal adipose tissue samples from control versus HPV+ female mice. (Abdominal AT: Control: n = 22, HPV+: n = 23, p < 0,001; Inguinal AT: Control: n = 21, HPV+: n = 22, p < 0,001)



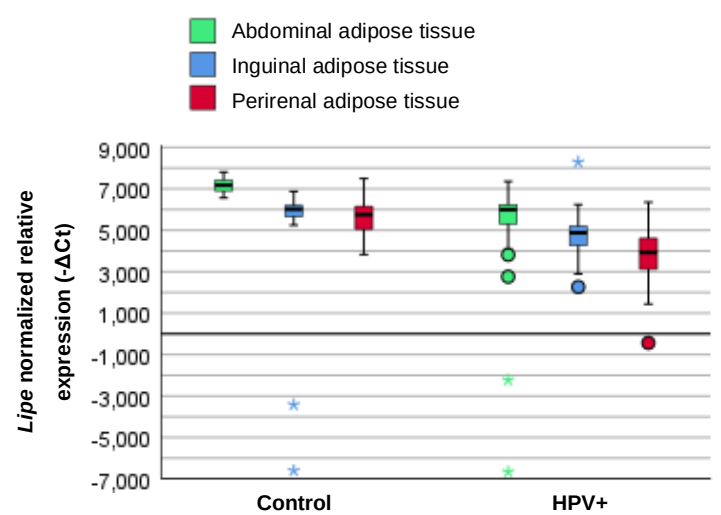
(a)



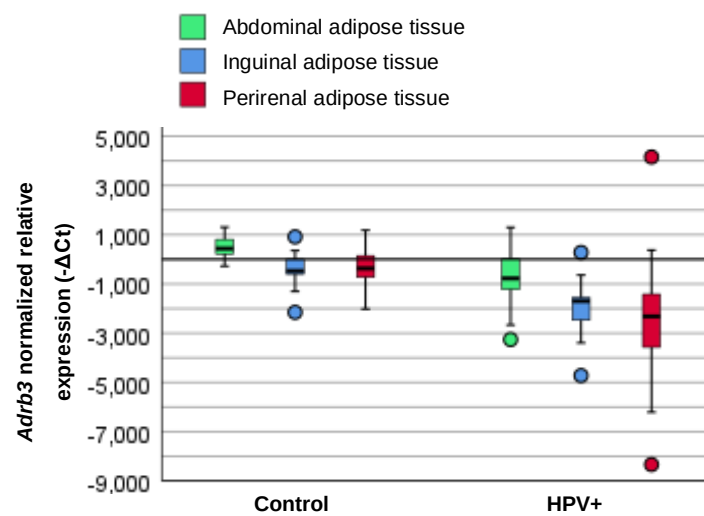
(b)



(c)



(d)



(e)

Figure 11. Normalized relative expression of **(a) *Cebpa*** (Abdominal AT: Control: n = 22, HPV+: n = 24, p = 0,004; Inguinal AT: Control: n = 21, HPV+: n = 24, p < 0,001; Perirenal AT: Control: n = 21, HPV+: n = 25, p = 0,002), **(b) *Pparg*** (Abdominal AT: Control: n = 22, HPV+: n = 24, p < 0,001; Inguinal AT: Control: n = 22, HPV+: n = 24, p = 0,001; Perirenal AT: Control: n = 22, HPV+: n = 25, p = 0,01), **(c) *Pnpla2*** (Abdominal AT: Control: n = 22, HPV+: n = 24, p < 0,001; Inguinal AT: Control: n = 22, HPV+: n = 25, p < 0,001; Perirenal AT: Control: n = 21, HPV+: n = 25, p < 0,001), **(d) *Lipe*** (Abdominal AT: Control: n = 22, HPV+: n = 24, p < 0,001; Inguinal AT: Control: n = 22, HPV+: n = 24, p < 0,001; Perirenal AT: Control: n = 22, HPV+: n = 25, p < 0,001) and **(e) *Adrb3*** (Abdominal AT: Control: n = 22, HPV+: n = 23, p < 0,001; Inguinal AT: Control: n = 21, HPV+: n = 24, p < 0,001; Perirenal AT: Control: n = 22, HPV+: n = 25, p < 0,001) in abdominal, inguinal and perirenal adipose tissue samples from control *versus* HPV+ female mice.

The results concerning the analysis of the samples grouped by age agreed with those found when analysing the samples by type of adipose tissue. *Ucp1* expression was significantly diminished in HPV+ females when compared to control ones at 42 and 50 weeks of age (24,95-fold in 42 weeks: P-value = 0,011; 32,61-fold in 50 weeks: P-value = 0,006), as shown in **Figure 12**.

Furthermore, when compared to control females, HPV+ female mice at all ages displayed a significant decrease in the expression of *Cebpa* (1,71-fold in 33 weeks: P-value = 0,009; 4,69-fold in 42 weeks: P-value = 0,001; 5,61-fold in 50 weeks: P-value = 0,005), *Pparg* (1,61-fold in 33 weeks: P-value = 0,029; 3,25-fold in 42 weeks: P-value < 0,001; 3,52-fold in 50 weeks: P-value = 0,03), *Pnpla2* (1,7-fold in 33 weeks: P-value = 0,007; 4,15-fold in 42 weeks: P-value < 0,001; 7,45-fold in 50 weeks: P-value = 0,001), *Lipe* (2-fold in 33 weeks: P-value = 0,003; 5,1-fold in 42 weeks: P-value < 0,001; 3,24-fold in 50 weeks: P-value = 0,001) and *Adrb3* (1,64-fold in 33 weeks: P-value = 0,015; 5,65-fold in 42 weeks: P-value < 0,001; 3,15-fold in 50 weeks: P-value < 0,001) (**Figures 13a-e**).

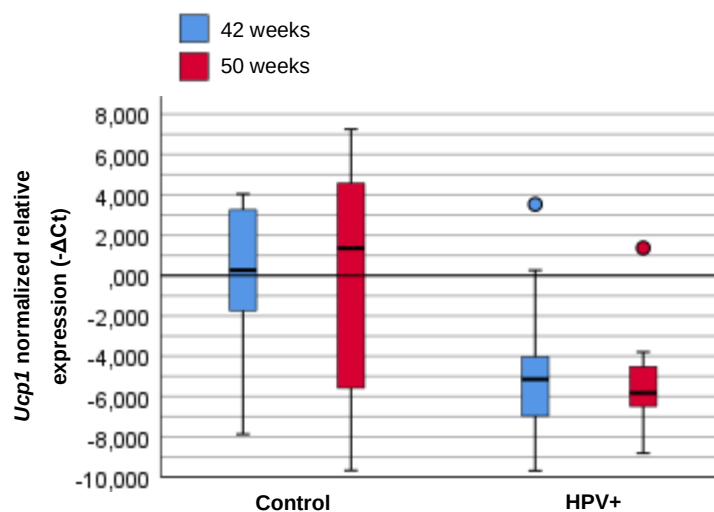


Figure 12. Normalized relative expression of *Ucp1* in 42 and 50-week samples from control *versus* HPV+ female mice. (42 weeks: Control: n = 9, HPV+: n = 25, p = 0,011; 50 weeks: Control: n = 28, HPV+: n = 15, p = 0,006)

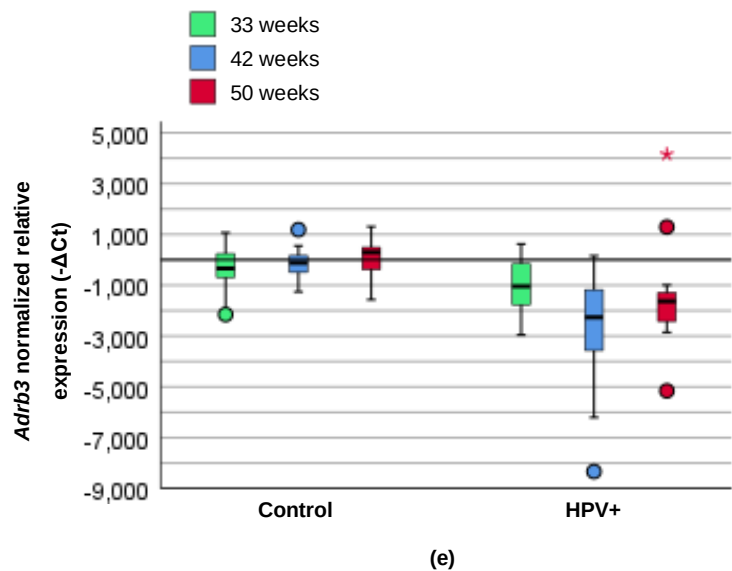
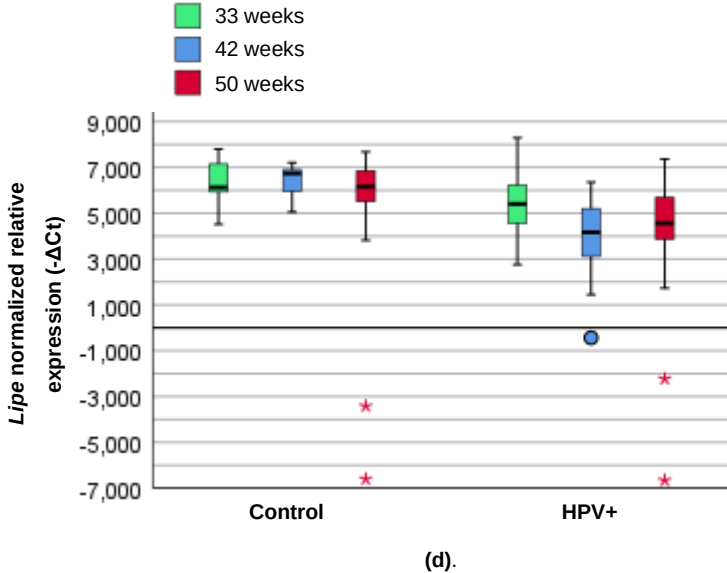
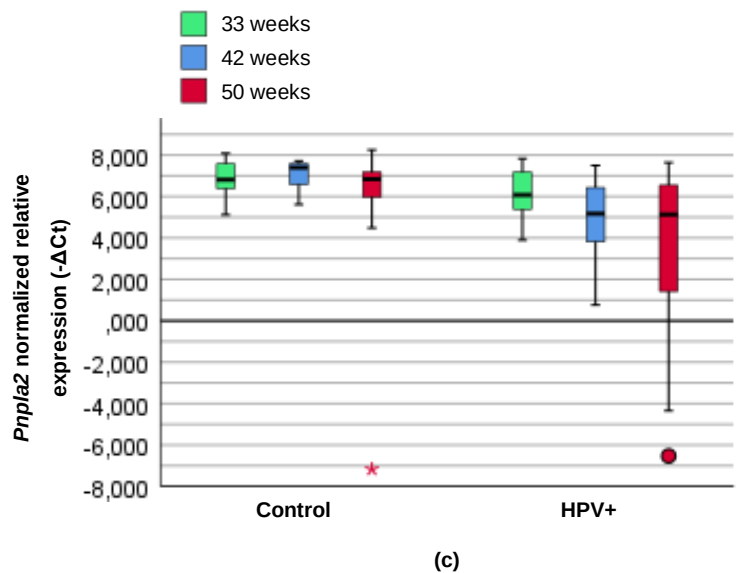
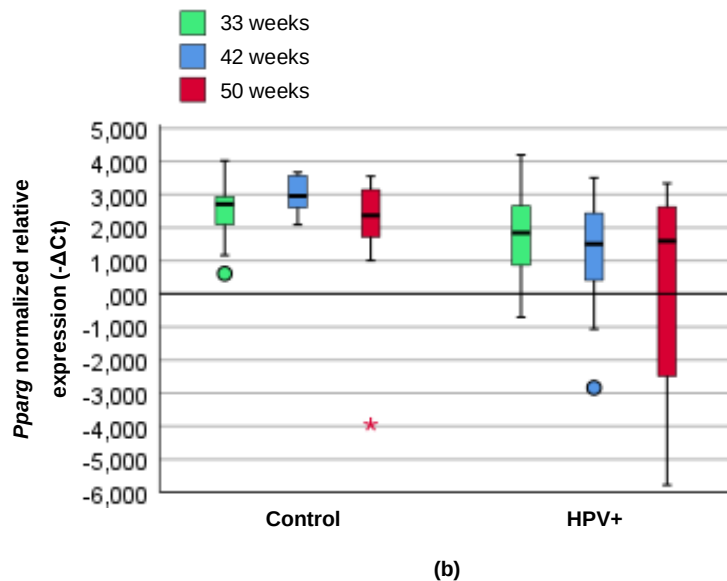
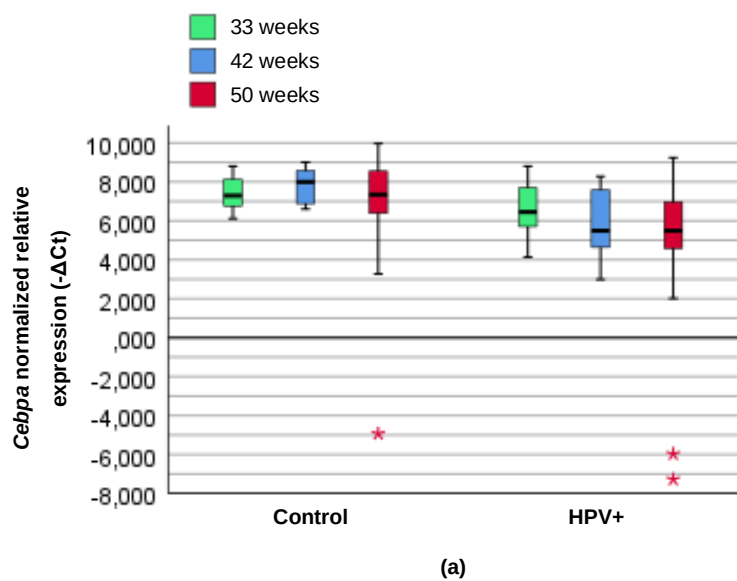


Figure 13. Normalized relative expression of **(a)** *Cebpa* (33 weeks: Control: n = 26, HPV+: n = 29, p = 0,009; 42 weeks: Control: n = 9, HPV+: n = 26, p = 0,001; 50 weeks: Control: n = 29, HPV+: n = 18, p = 0,005), **(b)** *Pparg* (33 weeks: Control: n = 27, HPV+: n = 29, p = 0,029; 42 weeks: Control: n = 9, HPV+: n = 26, p < 0,001; 50 weeks: Control: n = 30, HPV+: n = 18, p = 0,03), **(c)** *Pnpla2* (33 weeks: Control: n = 26, HPV+: n = 30, p = 0,007; 42 weeks: Control: n = 9, HPV+: n = 26, p < 0,001; 50 weeks: Control: n = 30, HPV+: n = 18, p = 0,001), **(d)** *Lipe* (33 weeks: Control: n = 27, HPV+: n = 29, p = 0,003; 42 weeks: Control: n = 9, HPV+: n = 27, p < 0,001; 50 weeks: Control: n = 30, HPV+: n = 19, p = 0,001) and **(e)** *Adrb3* (33 weeks: Control: n = 27, HPV+: n = 28, p = 0,015; 42 weeks: Control: n = 9, HPV+: n = 26, p < 0,001; 50 weeks: Control: n = 29, HPV+: n = 18, p < 0,001) in 33, 42 and 50-week samples from control versus HPV+ female mice.

On the other hand, as observed in males, *Adra2a* expression was found significantly higher in 33-week-old HPV+ females compared to control ones (1,98-fold, P-value = 0,002), as well as *Adra2a/Adrb3* ratio, which was increased in HPV+ female mice at all ages (33 weeks: P-value = 0,004, 42 weeks: P-value < 0,001, 50 weeks: P-value = 0,002) (**Figures 14 and 15**, respectively).

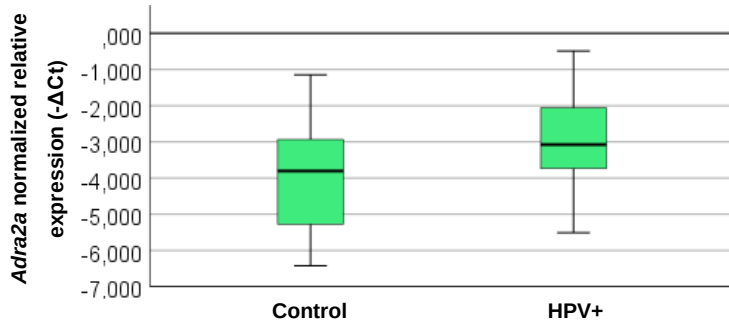


Figure 14. Normalized relative expression of *Adra2a* in 33-week samples from control versus HPV+ female mice. (Control: n = 26; HPV+: n = 29, p = 0,002)

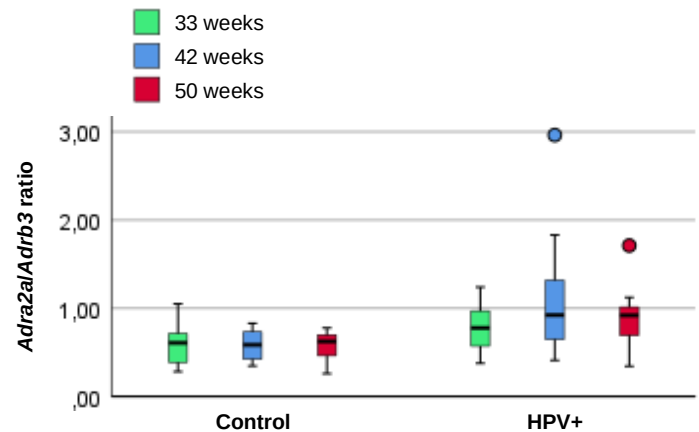


Figure 15. Levels of *Adra2a/Adrb3* ratio in 33, 42 and 50-week samples from control versus HPV+ female mice. (33 weeks: Control: n = 26, HPV+: n = 28, p = 0,004; 42 weeks: Control: n = 8, HPV+: n = 26, p < 0,001; 50 weeks: Control: n = 27, HPV+: n = 18, p = 0,002)

HPV+ mice vs. Control mice:

		Lipid storage	Thermogenesis		<i>Adra2a</i> / <i>Adrb3</i> ratio	Adipogenesis		Lipolysis	
		<i>Adra2a</i>	<i>Adrb3</i>	<i>Ucp1</i>		<i>Cebpa</i>	<i>Pparg</i>	<i>Pnpla2</i>	<i>Lipe</i>
Males	Abdominal AT								
	Inguinal AT	↑	↓	↓	↑				
	Perirenal AT								
	33 weeks								
	42 weeks		↓		↑	↓	↓	↓	↓
	50 weeks					↓		↓	↓
Females	Abdominal AT	↑	↓	↓	↑	↓	↓	↓	↓
	Inguinal AT	↑	↓	↓	↑	↓	↓	↓	↓
	Perirenal AT	↑	↓		↑	↓	↓	↓	↓
	33 weeks	↑	↓		↑	↓	↓	↓	↓
	42 weeks		↓	↓	↑	↓	↓	↓	↓
	50 weeks		↓	↓	↑	↓	↓	↓	↓

Table 2. Comparison of the expression of the adipose tissue molecular markers in HPV+ vs. control male mice (upper part of the table) and HPV+ vs. control female mice (lower part of the table) in each type of adipose tissue and age group. There were no 33-week-old control mice samples to perform the comparison. Green arrows denote increased expression in HPV+ mice compared to controls; Red arrows denote decreased expression in HPV+ mice compared to controls; AT: adipose tissue.

2. Differences between male and female mice regarding molecular markers of adipose tissue

Significant differences regarding adipose tissue molecular markers expression were found between sexes in both control and HPV+ groups. Nevertheless, whereas in control mice significant differences were found within all adipose tissue types (**Figures 16-20**) and within the 50-week-old group (**Figure 21**), in HPV+ mice genes were only differently expressed between sexes when comparing samples from perirenal adipose tissue (**Figures 22-23**).

Table 3 gathers all the significant differences found between male and female mice.

2.1 Differences in control mice

Significant differences between male and female mice were observed in each of the three different types of adipose tissue, although abdominal adipose tissue displayed the higher amount of differences, followed by perirenal adipose tissue, and then inguinal adipose tissue, that only revealed significant differences regarding the expression of one gene.

That being, concerning inguinal adipose tissue, only the expression of *Adra2a*, which accounts for lipid storage, was significantly different among sexes, being 1,39-fold increased in female compared to male mice (P-value = 0,019), as shown in the **Figure 16**.

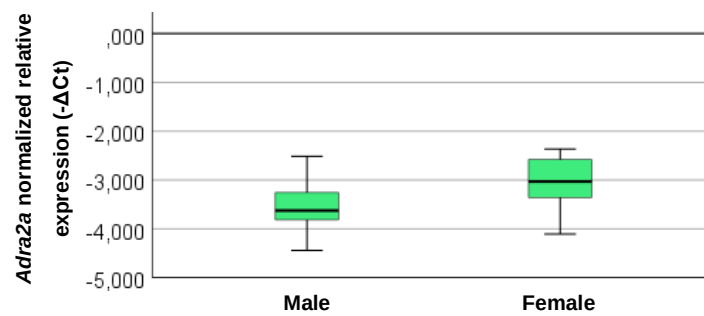


Figure 16. Normalized relative expression of *Adra2a* in inguinal adipose tissue samples from male versus female control mice. (Male: n = 14, Female: n = 20, p = 0,019)

Regarding abdominal adipose tissue, *Adra2a* expression was 2,61-fold decreased in females, compared to males (P-value = 0,001) (**Figure 17a**). Correspondingly, *Adra2a/Adrb3* ratio showed a significant decrease (P-value = 0,003) in females' abdominal adipose tissue (**Figure 17b**).

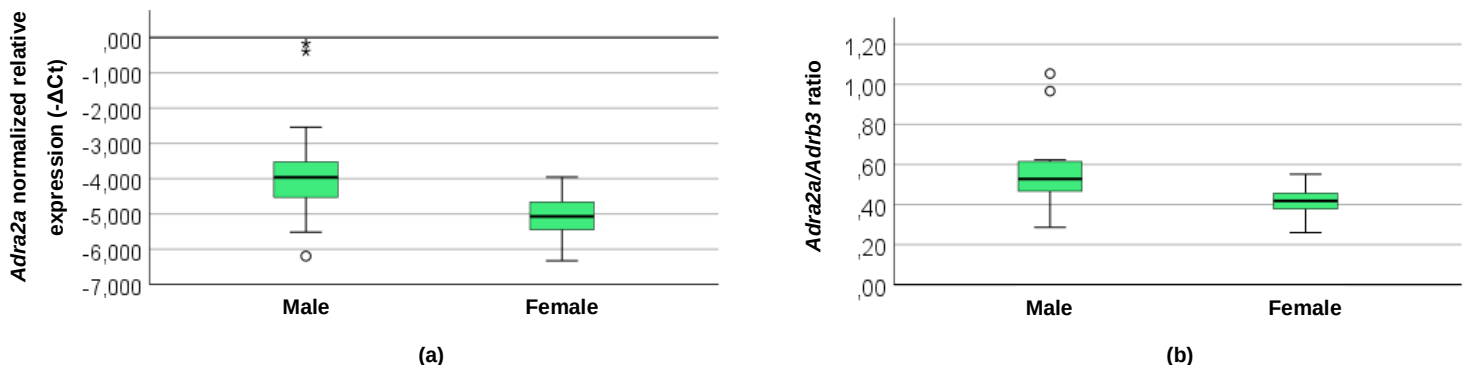


Figure 17. (a) Normalized relative expression of *Adra2a* (Male: n = 15, Female: n = 21, p = 0,001) and (b) levels of *Adra2a/Adrb3* ratio (Male: n = 15, Female: n = 21, p = 0,003) in abdominal adipose tissue samples from male *versus* female control mice.

Still about abdominal adipose tissue, females exhibited increased expression of the thermogenic marker *Ucp1* (46,28-fold increased, P-value < 0,001), of the adipocyte differentiation marker *Pparg* (1,24-fold increased, P-value = 0,014) as well as of the lipases *Lipe* (1,42-fold increased, P-value = 0,005) and *Pnpla2* (1,79-fold increased, P-value < 0,001). **Figure 18** shows the variation of the latter gene, while results concerning the former three genes will be presented together with those of perirenal adipose tissue in **Figures 20a-c**.

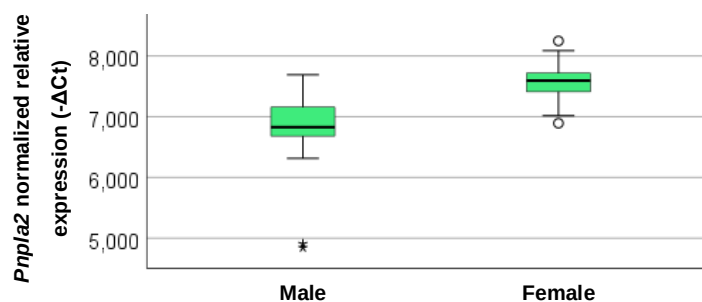


Figure 18. Normalized relative expression of *Pnpla2* in abdominal adipose tissue samples from male *versus* female control mice. (Male: n = 15, Female: n = 22, p < 0,001)

With reference to perirenal adipose tissue, an opposite pattern to that of abdominal adipose tissue was observed. The expression of the marker of thermogenesis and browning *Adrb3* was 2,88-fold decreased in control females compared to control males (P-value = 0,003) (**Figure 19**). Additionally, when

compared to control males, control female mice also displayed a decrease in *Ucp1* expression (25,19-fold decreased, P-value = 0,002) (**Figure 20a**) as well as in the expression of the adipocyte differentiation marker *Pparg* (2,49-fold decreased, P-value < 0,001) (**Figure 20b**) and of the lipase *Lipe* (1,4-fold decreased, P-value = 0,044) (**Figure 20c**). **Figures 20a-c** highlight the contrasting patterns between abdominal and perirenal adipose tissues, placing side by side the expression of the last three previously mentioned genes in both types of adipose tissue in each sex.

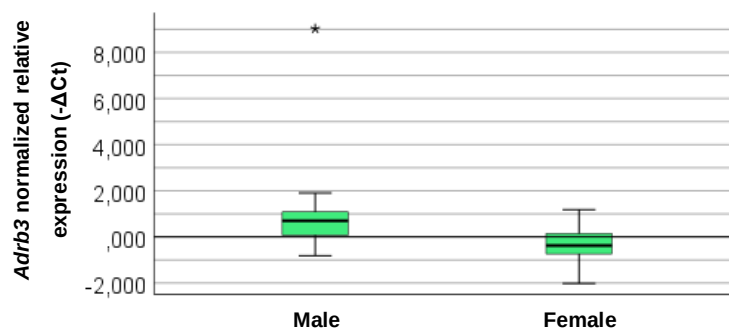


Figure 19. Normalized relative expression of *Adrb3* in perirenal adipose tissue samples from male versus female control mice. (Male: n = 14, Female: n = 22, p = 0,003)

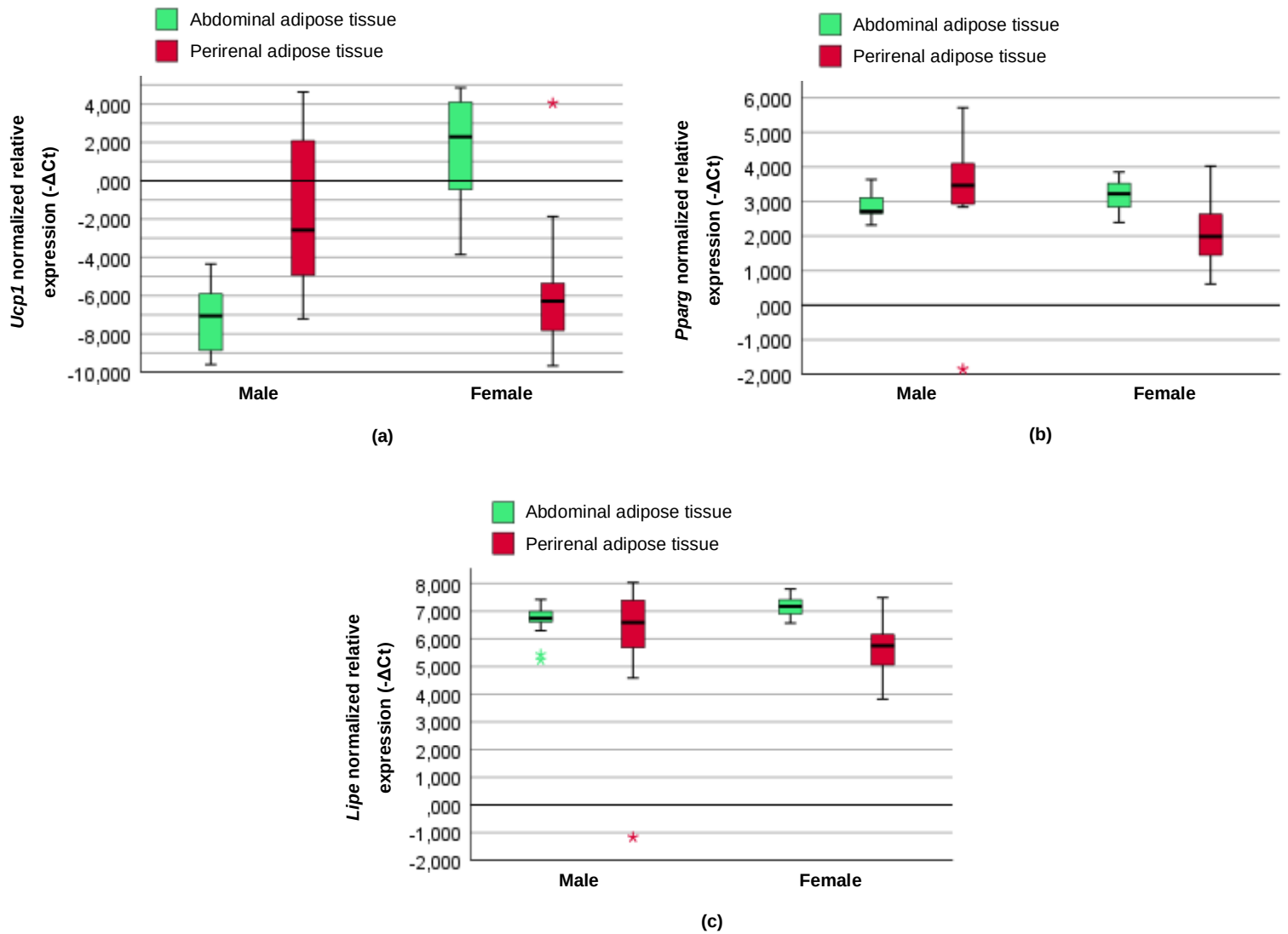


Figure 20. Normalized relative expression of (a) *Ucp1* (Abdominal AT: Male: n = 11, Female: n = 22, p < 0,001; Perirenal AT: Male: n = 11, Female: n = 19, p = 0,002), (b) *Pparg* (Abdominal AT: Male: n = 15, Female: n = 22, p = 0,014; Perirenal AT: Male: n = 15, Female: n = 22, p < 0,001) and (c) *Lipe* (Abdominal AT: Male: n = 15, Female: n = 22, p = 0,005; Perirenal AT: Male: n = 14, Female: n = 22, p = 0,044) in abdominal and perirenal adipose tissue samples from male versus female control mice.

Regarding control mice grouped by age, only at 50 weeks of age, the genes responsible for adipocyte differentiation *Cebpa* and *Pparg* displayed significant differences between sexes. If on the one hand, *Cebpa* expression was 1,48-fold increased in females (P-value = 0,038) (Figure 21a), on the other hand, *Pparg* displayed a 1,54-fold reduction in female compared to male mice (P-value = 0,046) (Figure 21b).

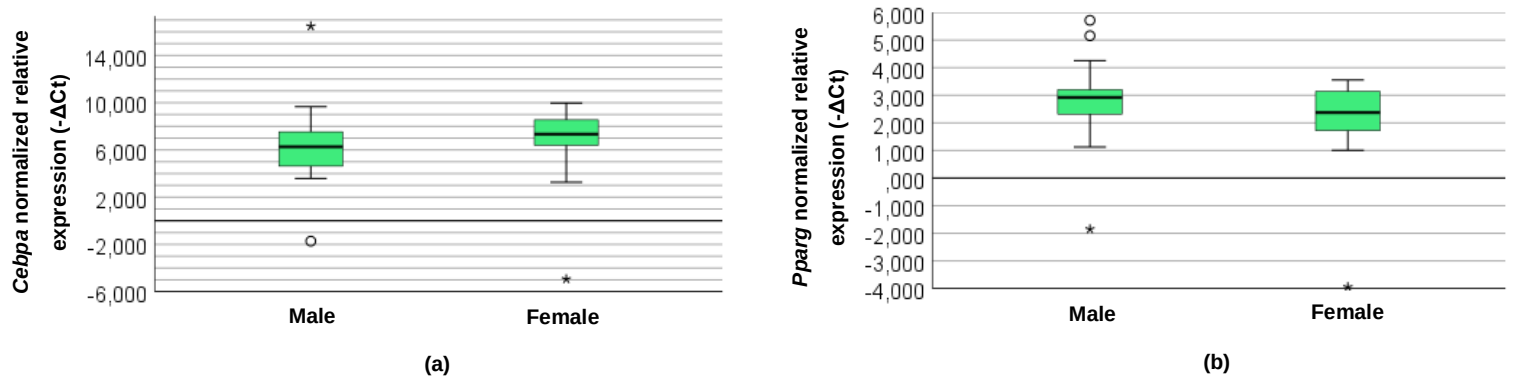


Figure 21. Normalized relative expression of (a) *Cebpa* (Male: n = 30, Female: n = 29, p = 0,038) and (b) *Pparg* (Male: n = 30, Female: n = 30, p = 0,046) in 50-week samples from male versus female control mice.

2.2 Differences in HPV+ mice

When the expression of molecular markers was analysed between male and female HPV+ mice, nearly all the significant differences that were observed in the control group disappeared. The exception were the HPV+ perirenal adipose tissue samples that displayed significant differences between sexes, in a pattern similar to that observed in the control mice. Concerning the three groups of ages, and the two other types of adipose tissue, no significant differences were found at all.

Therefore, regarding perirenal adipose tissue, similarly to what happened in the control group, *Adrb3* expression was 4,95-fold decreased in HPV+ females, when compared to HPV+ males (P-value = 0,016) (**Figure 22a**). The *Adra2a/Adrb3* ratio was also significantly increased (P-value = 0,019) in the perirenal adipose tissue of HPV+ females (**Figure 22b**), in agreement with the reduction of *Adrb3* expression.

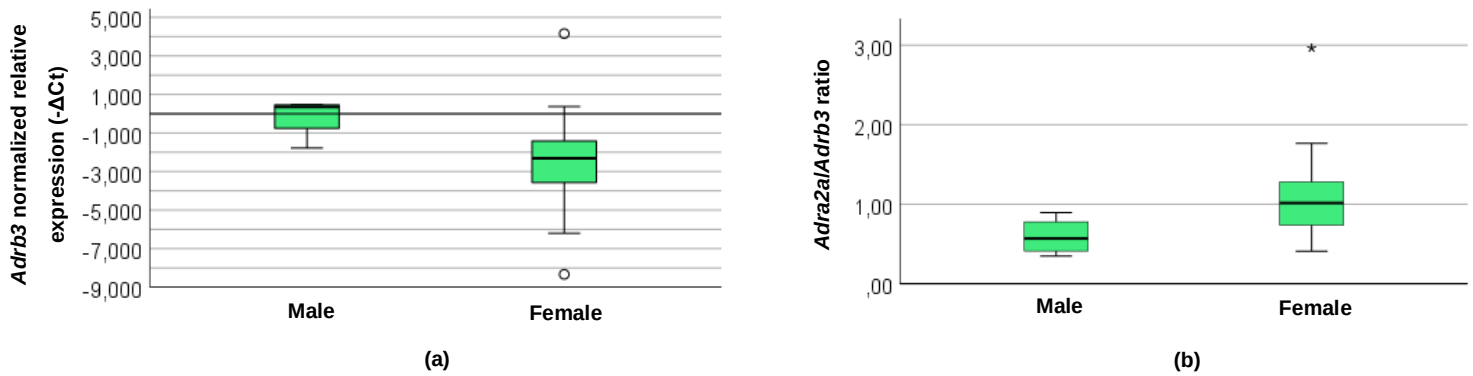


Figure 22. (a) Normalized relative expression of *Adrb3* (Male: n = 4, Female: n = 25, p = 0,016) and **(b)** levels of *Adra2a/Adrb3* ratio (Male: n = 4, Female: n = 25, p = 0,019) in perirenal adipose tissue samples from male *versus* female HPV+ mice.

Additionally, diminished expression of the differentiation markers *Cebpa* (6,97-fold decreased, P-value = 0,01) and *Pparg* (4,59-fold decreased, P-value = 0,027), and of the lipases *Pnpla2* (10,14-fold decreased, P-value = 0,004) and *Lipe* (7,34-fold decreased, P-value = 0,003), was observed in perirenal adipose tissue from HPV+ females when compared to HPV+ males, as shown in **Figures 23a-d**. Once more, it is important to take into consideration that, in this specific group, there were only four male samples, contrasting with twenty-five female samples. Therefore, these results should be approached with caution due to the limited sample size.

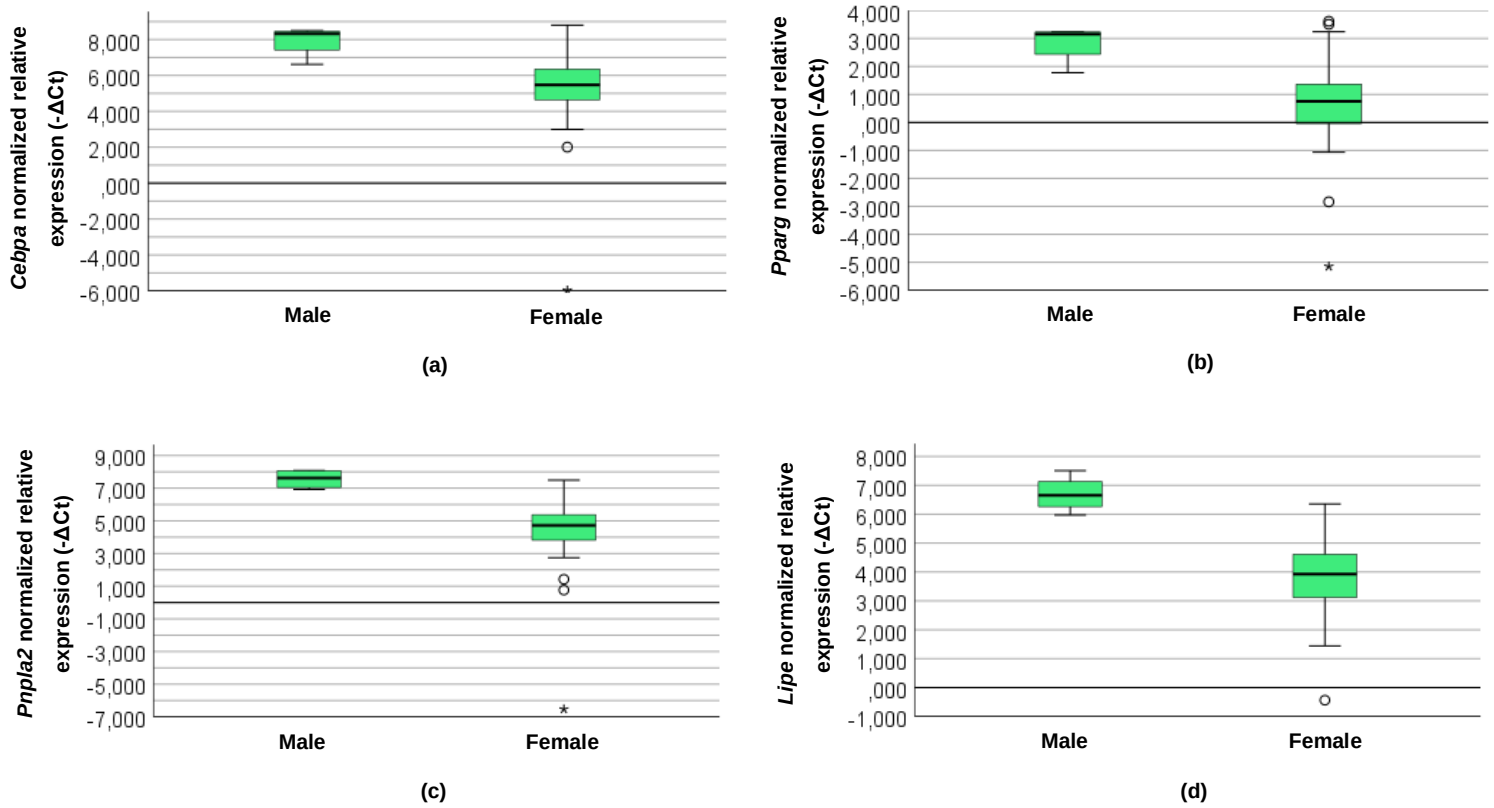


Figure 23. Normalized relative expression of **(a)** *Cebpa* (Male: n = 4, Female: n = 25, p = 0,01), **(b)** *Pparg* (Male: n = 4, Female: n = 25, p = 0,027), **(c)** *Pnpla2* (Male: n = 4, Female: n = 25, p = 0,004) and **(d)** *Lipa* (Male: n = 4, Female: n = 25, p = 0,003) in perirenal adipose tissue samples from male versus female HPV+ mice.

Female mice vs. Male mice:

		Lipid storage	Thermogenesis		Adra2a/ Adrb3 ratio	Adipogenesis		Lipolysis	
		<i>Adra2a</i>	<i>Adrb3</i>	<i>Ucp1</i>		<i>Cebpa</i>	<i>Pparg</i>	<i>Pnpla2</i>	<i>Lipe</i>
Control	Abdominal AT	↓		↑	↓		↑	↑	↑
	Inguinal AT	↑							
	Perirenal AT		↓	↓			↓		↓
	33 weeks								
	42 weeks								
	50 weeks					↑	↓		
HPV+	Abdominal AT								
	Inguinal AT								
	Perirenal AT		↓		↑	↓	↓	↓	↓
	33 weeks								
	42 weeks								
	50 weeks								

Table 3. Comparison of the expression of the adipose tissue molecular markers in female vs. male control mice (upper part of the table) and female vs. male HPV+ mice (lower part of the table) in each type of adipose tissue and age group. There were no 33-week-old control mice samples to perform the comparison. Green arrows denote increased expression in females compared to males; Red arrows denote decreased expression in females compared to males; AT: adipose tissue.

Discussion

Cancer-associated cachexia is a multiple organ wasting syndrome that affects up to 80% of cancer patients [4, 6] and contributes to decreased quality of life and increased mortality [1, 9]. Because CAC aetiology is multifactorial, it still lacks a well-defined molecular characterization and predictive biomarkers, thus its early diagnosis is infrequent in clinical practice, and efficient therapies are still an unmet need [6, 11].

Although research efforts have been made in order to better understand this pathology, adipose tissue impairment in the context of cachexia has not been a major target of study for a long time, favouring the study of muscle. However, that paradigm is gradually changing, as adipose tissue is now recognised as an important endocrine organ, which largely contributes to body energy balance [13, 19]. Several authors have already observed that adipose tissue damaging starts earlier than muscle wasting in cachexia [2, 8, 13], placing this organ as an important target of study to find cachexia predictive biomarkers and therapeutic targets. Lipolysis, non-shivering thermogenesis and browning are some of the processes that have been widely associated with adipose tissue disruption in CAC [2, 13]. Additionally, α - and β -adrenergic receptors, whose endogenous ligands are catecholamines, are known to be key players involved in the regulation of those mechanisms [2, 7]. β 3-AR, which induces lipolysis, browning and thermogenesis (specifically through the upregulation of *Ucp1* expression), is the prevalent subtype in mice [21, 22] and its activity has already been linked to cancer cachexia [2, 23, 68]. On the other hand, α 2A-AR is responsible for inhibiting lipolysis and *Ucp1* expression [20, 94]. The proportions of α 2- and β 3-AR in adipose tissue might regulate which of them is stimulated by catecholamines, leading to the respective outcomes [20]: a higher α 2A/ β 3-AR ratio is reportedly associated with obesity [20, 97]; whereas a lower ratio has been linked to increased thermogenesis and lipolysis [20, 21, 98]. Therefore, it is important to assess the ratio, besides the individual expression of each gene, to better understand the physiological processes taking place within the cells.

Furthermore, although men have been associated with increased susceptibility for cachexia development, sexual dimorphism concerning CAC remains poorly explored and far from being understood, especially with respect to adipose tissue metabolism since greatest attention in that field has been given, once more, to muscle alterations [26, 27].

K14-HPV16 transgenic mice are a genetically engineered mice model that develops HPV-induced carcinogenesis in several anatomic regions accompanied by several characteristics of cachexia, mainly related to muscle wasting and inflammation [31, 115, 117]. Therefore, this work employed K14-HPV16 mice and matched-controls in an attempt to characterize at molecular level the changes occurring in adipose tissue during cancer-associated cachexia, and to evaluate the differences in the molecular profile of adipose tissue between sexes, both in wild-type and in HPV+ status. From now on, HPV+ mice are going to be referred to as cachectic mice as well, since K14-HPV16 model has already been proven to be a model of HPV-induced CAC by our group [31].

The results obtained when the expression of adipose tissue molecular markers was compared between control and cachectic mice show the same trend in both sexes: *Adra2a* expression and *Adra2a/Adrb3* ratio, associated with lipid storage through inhibition of lipolysis, were augmented in adipose tissue of cachectic mice, while genes positively related to thermogenesis and browning (*Ucp1* and *Adrb3*), lipolysis (*Pnpla2* and *Lipe*) and adipocyte differentiation (*Cebpa* and *Pparg*) were reduced. However, while in female mice these alterations were significant in all types of adipose tissue (abdominal, inguinal and perirenal), male mice only presented significant results in inguinal adipose tissue.

Although most studies point to increased lipases and *Ucp1* expression during cancer cachexia [4, 7, 19, 77], it is possible that the increase of lipolysis, browning and BAT activity, namely thermogenesis, occurs at the onset of cachexia pathogenesis – as proposed by several authors [2, 8, 81] – and that, at a later stage of the disease, feedback mechanisms, including increased *Adra2a* expression, might be activated to try to mitigate the loss of adipose tissue. Considering that even the samples from our youngest mice (33-week-old) already reflect an advanced state of the disease, according to a previous study from our group [31], it is acceptable to hypothesise that those feedback mechanisms may already be activated, resulting in decreased thermogenesis and lipolysis. Furthermore, it is likely that different types of tumours induce distinct cachexia-associated molecular patterns, and it should be bear in mind that, in other experiments, increased lipolysis and thermogenesis were identified in CAC triggered by tumour models different from the one employed here [2, 23, 124, 125]. Moreover, it should be contemplated that K14-HPV16 transgenic mice develop several distinct HPV-induced lesions [31], all of which can be differently contributing to cachexia.

Nevertheless, a previous work on a mouse model of pancreatic cancer cachexia found reduced *Ucp1* expression in cachectic mice, supporting the results achieved in this thesis

[126]. Moreover, since higher levels of catecholamines have been associated with cachexia [2, 8, 19, 83], the observed reduction in *Adrb3* expression in cachectic subjects may be supported by some studies suggesting that, instead of stimulating, norepinephrine downregulated *Adrb3* expression [21, 127].

Based on the results achieved here, it is also reasonable to hypothesize that the decreased expression of the *Adrb3* and *Ucp1* genes observed in cachectic mice may reflect a reduction in the quantity and/or size of active brown and beige adipocytes in the fat depots herein analysed. Thus, browning and energy expenditure through thermogenesis may not be associated with HPV16-induced cachexia, at least at this timepoint of the disease, as also purposed for the pancreatic cancer cachexia model previously mentioned [126]. Additionally, the reduction of the adipocyte differentiation process (based on decreased expression of *Cebpa* and *Pparg*) may be the more significant contribution to the disruption of adipose tissue at this stage of HPV16-induced cachexia. Ultimately, it is essential to highlight that the gene expression levels observed in this experiment might not necessarily reflect the levels of protein expression and activation, however it will be assessed in the near future.

Regarding the comparison between control and cachectic subjects, when male and female mice were grouped by age, both sexes exhibited significant differences in the expression of the molecular markers, which were in line with those observed when mice samples were clustered by adipose tissue type, and that have already been explored above.

The comparison of the expression patterns of the selected adipose tissue molecular markers between males and females was another aim of this study. Concerning that matter, differences between sexes were found both in control and in HPV+ transgenic mice.

Regarding abdominal adipose tissue, control females displayed increased *Ucp1* and lipases (*Pnpla2* and *Lipe*) expression than control males, pointing to increased thermogenic and lipolytic activity in females, in line with most literature [28, 29, 98-101]. Moreover, not only *Adra2a* expression, but also *Adra2a/Adrb3* ratio were significantly decreased in control female mice compared to control male mice, indicating that the proportion of β 3-AR to its antagonist α 2A-AR is higher in female, presumably leading to a higher sympathetic-induced β 3-AR activation in females, contrasting to a greatest contribution of α 2A-AR in males. These results are aligned with the higher accumulation of abdominal fat in males and the higher α 2A-AR levels reportedly associated with male

sex hormones in normal physiological conditions [21, 94, 101, 104, 109]. Additionally, *Pparg* expression was found increased in control females compared to control males. This gene is a key regulator of adipocyte differentiation, however, if it reflects enhanced white or brown adipocyte development in females' abdominal fat is questionable, since *Pparg* is reportedly involved in both brown and white adipogenesis, as well as in *Ucp1* production [19, 74].

On the other hand, when it comes to HPV+ mice, no significant differences were observed between male and female abdominal adipose tissue, therefore it is plausible to hypothesize that the mechanisms underlying cachexia make the molecular profiles of adipose tissue of both sexes more similar than they are in healthy conditions. It is likely that the changes resulting from cachexia occurring in females contribute more to the convergence of molecular profiles between both sexes in CAC. This is because when comparing abdominal adipose tissue between control and HPV+ (cachectic) males, no significant differences were observed at all. However, when it comes to abdominal adipose tissue of cachectic females, all molecular markers showed significant alterations, bringing their expression levels closer to those of control males: *Adra2a* expression and *Adra2a/Adrb3* ratio that were lower in control females compared to control males, significantly increases in HPV+ females compared to control females; *Ucp1*, *Pparg*, *Pnpla2* and *Lipe* expression which were higher in control females compared to males counterparts, present a significant reduction when comparing HPV+ to control female mice.

Regarding inguinal adipose tissue, differences between sexes were barely inexistent in both control and cachectic conditions, with the exception of *Adra2a* that is more expressed in control females than in males. This phenomenon may result from the fact that inguinal adipose tissue is not substantially influenced by the sex hormones, thus presenting a relatively identical gene expression pattern among genders.

With respect to perirenal adipose tissue, several differences were detected between control male and female mice however, contrarily from what was verified in abdominal tissue, almost all those differences persisted in cachectic mice. If in control mice the expression of *Adrb3*, *Ucp1*, *Pparg* and *Lipe* was lower in females than males, in cachexia the expression pattern was not very distinct: in HPV+ female mice *Ucp1* persisted reduced but lost its significance, the marker of adipocyte differentiation *Cebpa* and the lipase *Pnpla2* were also found significantly decreased as well as *Pparg* and *Lipe* and, in addition to diminished *Adrb3*, the *Adra2a/Adrb3* ratio was also significantly higher. This means that in perirenal adipose tissue, which is a typical anatomical region of BAT [34,

62], thermogenic activity, lipolysis, and adipogenesis are higher in male than in female, both in healthy and cachectic conditions. These are unexpected results, as most of the available literature points to higher BAT activity in females (especially higher $\beta 3$ -AR-induced thermogenesis) [101, 103], and lower thermogenesis (mainly through *Ucp1* downregulation by testosterone) and BAT differentiation in males, contrarily from what was found in this thesis [94, 107]. Nevertheless, the effect of androgens is quite questionable, since it has been suggested that they can also promote lipolysis and activation of β -adrenoceptors, instead of fat accumulation [104].

Concerning the assessment of sexual dimorphism at different groups of age, the results obtained did not produce relevant conclusions, since only 50-week-old control mice displayed significant differences in the expression of two genes, both related to adipogenesis: while *Cebpa* expression was found increased in females compared to males, *Pparg* expression, on the other hand, was decreased in female mice.

Regarding the adrenergic receptors it was possible to perceive, from the various analyses performed in this thesis, that the expression pattern of *Adrb3* was consistently aligned with *Ucp1* expression, whereas conversely, alterations of *Adra2a* expression and of *Adra2a/Adrb3* ratio were consistently inverse of those of *Ucp1*, suggesting that adrenergic signalling likely mediates *Ucp1* expression in this CAC model.

Regarding sexual dimorphism, each type of adipose tissue produced different results. Abdominal adipose tissue presented several sex-based differences in control subjects, pointing to greatest thermogenesis and lipolysis in females (commonly reported traits), which were not observed in cachectic mice. This may result from the fact that cachectic female mice exhibited differences in the expression of all molecular markers compared to controls, thus converging with the expression profile of males, who did not present any significant difference between healthy and cachectic individuals.

In inguinal adipose tissue, almost no differences were observed between male and female control mice. As both sexes presented similar gene expression changings in cachexia when compared to control circumstances, again there were found no sex-related differences in cachectic mice. These results are supported by some previous works corroborating the idea that inguinal adipose tissue does not present major sexual dimorphism regarding mitochondrial respiration nor sympathetic innervation (which is a contributing factor for thermogenesis) [29, 103].

Finally, perirenal adipose tissue, which reportedly represents one of the classical BAT depots both in mice and in humans [34, 62], showed remarkable sexual dimorphism

regarding the expression of several markers in wild-type subjects, suggesting lower levels of thermogenesis, lipolysis and adipogenesis in females, what is not in line with most previous literature. That profile was, in a general way, maintained when comparing cachectic males and females. Contributing to that are the facts that cachectic male mice did not display any significant difference from control male mice, while significant differences in the expression of nearly every gene were observed in cachectic female mice compared to their control counterparts, reinforcing the sex differences in the perirenal adipose tissue expression pattern that have already been verified in healthy conditions. These results can be supported by some previous studies [29, 101] reporting that under caloric deprivation females tend to reduce their energy expenditure, particularly thermogenesis, more than males, to compensate for insufficient energy intake. Although K14-HPV16 transgenic mice exhibit identical, or even higher food intake compared to wild-type mice [31], cachexia may induce malabsorption mechanisms [4], therefore it could be hypothesised that those mechanisms may lead to similar responses as those triggered by caloric restriction. Moreover, it may be that perirenal adipose tissue comprises a greater amount of brown adipocytes in males rather than in females.

Limitations of the Study

While this work provided interesting, surprising results, some limitations were verified in the course of the experiment. The assessment of mice's food intake, locomotor activity body temperature, and weights during mice's lifespan and at the time of sacrifice were not possible to collect for this study and these factors should be taken into consideration in future research, as they constitute essential information to strengthen the obtained results and the consequent assumptions. Furthermore, with respect to the experimental design, there was an initial goal of obtaining ten mice of each sex of each HPV status (HPV- and HPV+) for each age group, but it could not be achieved since the employed mice, especially HPV+ ones, displayed high levels of mortality from 42 weeks of age.

Additionally, by the time of the submission of this thesis, histological evaluation of the samples could not be performed due to logistic limitations and it is not possible to discern WAT from BAT only via relative quantification of gene expression (even *Ucp1*, which is a marker of BAT, can be expressed in white depots, and *Cebpa*, that is only required for WAT differentiation, can be also detected in brown adipocytes). Therefore, accurate distinction of the different types of adipose tissue as white or brown is still an unmet need that we aim to achieve soon.

Conclusion and Future Perspectives

This study reinforces the idea that cancer-associated cachexia molecular profiles are distinct according to the type of tumour in the cause of the disease. Based on this work, it is possible to conclude that the K14-HPV16 transgenic mouse model, which is already validated as a cancer cachexia model for muscle wasting and inflammation, also display significant adipose tissue molecular remodelling during CAC. In addition, the gene expression profiles in adipose tissue seem to be sex-specific in healthy conditions and to become more alike in cachexia. Adrenergic signalling may be related to the observed sexual dimorphism, as adrenergic receptors were differently expressed amongst genders, namely in the context of cachexia. Nonetheless, further research is needed to validate these results.

Near future perspectives include measurement of protein expression of the selected markers in the adipose tissue samples by immunohistochemistry, what will certainly help in the interpretation of the results obtained to date. Hematoxylin and eosin (H&E) staining is also going to be performed subsequently in the adipose tissue samples to help in their histologic distinguishment (brown *versus* white). Additionally, future research is expected to be performed with bigger cohorts to increase the reliability of the conclusions, and with younger subjects in order to evaluate the molecular changes taking place in adipose tissue at the onset of cachexia and compare them with those obtained in this thesis.

References

- [1] R. Dev, "Measuring cachexia-diagnostic criteria," (in eng), *Ann Palliat Med*, vol. 8, no. 1, pp. 24-32, Jan 2019, doi: 10.21037/apm.2018.08.07.
- [2] M. Petruzzelli *et al.*, "A switch from white to brown fat increases energy expenditure in cancer-associated cachexia," (in eng), *Cell Metab*, vol. 20, no. 3, pp. 433-47, Sep 2 2014, doi: 10.1016/j.cmet.2014.06.011.
- [3] K. Fearon *et al.*, "Definition and classification of cancer cachexia: an international consensus," (in eng), *Lancet Oncol*, vol. 12, no. 5, pp. 489-95, May 2011, doi: 10.1016/s1470-2045(10)70218-7.
- [4] S. Peixoto da Silva, J. M. O. Santos, E. S. M. P. Costa, R. M. Gil da Costa, and R. Medeiros, "Cancer cachexia and its pathophysiology: links with sarcopenia, anorexia and asthenia," (in eng), *J Cachexia Sarcopenia Muscle*, vol. 11, no. 3, pp. 619-635, Jun 2020, doi: 10.1002/jcsm.12528.
- [5] M. C. Seelaender and M. L. Batista, "Adipose tissue inflammation and cancer cachexia: the role of steroid hormones," (in eng), *Horm Mol Biol Clin Investig*, vol. 17, no. 1, pp. 5-12, Jan 2014, doi: 10.1515/hmbci-2013-0040.
- [6] J. M. Argilés, S. Busquets, B. Stemmler, and F. J. López-Soriano, "Cancer cachexia: understanding the molecular basis," (in eng), *Nat Rev Cancer*, vol. 14, no. 11, pp. 754-62, Nov 2014, doi: 10.1038/nrc3829.
- [7] C. Bing and P. Trayhurn, "Regulation of adipose tissue metabolism in cancer cachexia," (in eng), *Curr Opin Clin Nutr Metab Care*, vol. 11, no. 3, pp. 201-7, May 2008, doi: 10.1097/MCO.0b013e3282f948e2.
- [8] M. Tsoli, M. M. Swarbrick, and G. R. Robertson, "Lipolytic and thermogenic depletion of adipose tissue in cancer cachexia," (in eng), *Semin Cell Dev Biol*, vol. 54, pp. 68-81, Jun 2016, doi: 10.1016/j.semcdb.2015.10.039.
- [9] M. Petruzzelli and E. F. Wagner, "Mechanisms of metabolic dysfunction in cancer-associated cachexia," (in eng), *Genes Dev*, vol. 30, no. 5, pp. 489-501, Mar 1 2016, doi: 10.1101/gad.276733.115.
- [10] C. Bing *et al.*, "Adipose atrophy in cancer cachexia: morphologic and molecular analysis of adipose tissue in tumour-bearing mice," (in eng), *Br J Cancer*, vol. 95, no. 8, pp. 1028-37, Oct 23 2006, doi: 10.1038/sj.bjc.6603360.
- [11] J. M. O. Santos, S. Peixoto da Silva, R. M. Gil da Costa, and R. Medeiros, "The Emerging Role of MicroRNAs and Other Non-Coding RNAs in Cancer Cachexia,"

- (in eng), *Cancers (Basel)*, vol. 12, no. 4, Apr 19 2020, doi: 10.3390/cancers12041004.
- [12] M. L. Batista, Jr. *et al.*, "Adipose tissue inflammation and cancer cachexia: possible role of nuclear transcription factors," (in eng), *Cytokine*, vol. 57, no. 1, pp. 9-16, Jan 2012, doi: 10.1016/j.cyto.2011.10.008.
 - [13] S. I. Daas, B. R. Rizeq, and G. K. Nasrallah, "Adipose tissue dysfunction in cancer cachexia," (in eng), *J Cell Physiol*, vol. 234, no. 1, pp. 13-22, Jan 2018, doi: 10.1002/jcp.26811.
 - [14] P. E. Porporato, "Understanding cachexia as a cancer metabolism syndrome," (in eng), *Oncogenesis*, vol. 5, no. 2, p. e200, Feb 22 2016, doi: 10.1038/oncsis.2016.3.
 - [15] A. Sakers, M. K. De Siqueira, P. Seale, and C. J. Villanueva, "Adipose-tissue plasticity in health and disease," (in eng), *Cell*, vol. 185, no. 3, pp. 419-446, Feb 3 2022, doi: 10.1016/j.cell.2021.12.016.
 - [16] D. X. Cao *et al.*, "Role of beta1-adrenoceptor in increased lipolysis in cancer cachexia," (in eng), *Cancer Sci*, vol. 101, no. 7, pp. 1639-45, Jul 2010, doi: 10.1111/j.1349-7006.2010.01582.x.
 - [17] J. A. Vaitkus and F. S. Celi, "The role of adipose tissue in cancer-associated cachexia," (in eng), *Exp Biol Med (Maywood)*, vol. 242, no. 5, pp. 473-481, Mar 2017, doi: 10.1177/1535370216683282.
 - [18] M. Dong, J. Lin, W. Lim, W. Jin, and H. J. Lee, "Role of brown adipose tissue in metabolic syndrome, aging, and cancer cachexia," (in eng), *Front Med*, vol. 12, no. 2, pp. 130-138, Apr 2018, doi: 10.1007/s11684-017-0555-2.
 - [19] X. Sun, X. Feng, X. Wu, Y. Lu, K. Chen, and Y. Ye, "Fat Wasting Is Damaging: Role of Adipose Tissue in Cancer-Associated Cachexia," (in eng), *Front Cell Dev Biol*, vol. 8, p. 33, 2020, doi: 10.3389/fcell.2020.00033.
 - [20] I. Lladó *et al.*, "Gender effects on adrenergic receptor expression and lipolysis in white adipose tissue of rats," (in eng), *Obes Res*, vol. 10, no. 4, pp. 296-305, Apr 2002, doi: 10.1038/oby.2002.41.
 - [21] M. Monjo, A. M. Rodríguez, A. Palou, and P. Roca, "Direct effects of testosterone, 17 beta-estradiol, and progesterone on adrenergic regulation in cultured brown adipocytes: potential mechanism for gender-dependent thermogenesis," (in eng), *Endocrinology*, vol. 144, no. 11, pp. 4923-30, Nov 2003, doi: 10.1210/en.2003-0537.

- [22] J. M. Valentine *et al.*, " β 3-Adrenergic receptor downregulation leads to adipocyte catecholamine resistance in obesity," (in eng), *J Clin Invest*, vol. 132, no. 2, Jan 18 2022, doi: 10.1172/jci153357.
- [23] H. Xie *et al.*, "An immune-sympathetic neuron communication axis guides adipose tissue browning in cancer-associated cachexia," (in eng), *Proc Natl Acad Sci U S A*, vol. 119, no. 9, Mar 1 2022, doi: 10.1073/pnas.2112840119.
- [24] M. Lafontan and M. Berlan, "Fat cell alpha 2-adrenoceptors: the regulation of fat cell function and lipolysis," (in eng), *Endocr Rev*, vol. 16, no. 6, pp. 716-38, Dec 1995, doi: 10.1210/edrv-16-6-716.
- [25] J. Merlin *et al.*, "The PPAR γ agonist rosiglitazone promotes the induction of brite adipocytes, increasing β -adrenoceptor-mediated mitochondrial function and glucose uptake," *Cellular Signalling*, vol. 42, pp. 54-66, 2018/01/01/ 2018, doi: <https://doi.org/10.1016/j.cellsig.2017.09.023>.
- [26] X. Zhong *et al.*, "Sex specificity of pancreatic cancer cachexia phenotypes, mechanisms, and treatment in mice and humans: role of Activin," (in eng), *J Cachexia Sarcopenia Muscle*, vol. 13, no. 4, pp. 2146-2161, Aug 2022, doi: 10.1002/jcsm.12998.
- [27] X. Zhong and T. A. Zimmers, "Sex Differences in Cancer Cachexia," (in eng), *Curr Osteoporos Rep*, vol. 18, no. 6, pp. 646-654, Dec 2020, doi: 10.1007/s11914-020-00628-w.
- [28] E. Fuente-Martín, P. Argente-Arizón, P. Ros, J. Argente, and J. A. Chowen, "Sex differences in adipose tissue: It is not only a question of quantity and distribution," (in eng), *Adipocyte*, vol. 2, no. 3, pp. 128-34, Jul 1 2013, doi: 10.4161/adip.24075.
- [29] I. Gómez-García, J. Trepiana, A. Fernández-Quintela, M. Giralt, and M. P. Portillo, "Sexual Dimorphism in Brown Adipose Tissue Activation and White Adipose Tissue Browning," (in eng), *Int J Mol Sci*, vol. 23, no. 15, Jul 26 2022, doi: 10.3390/ijms23158250.
- [30] I. Lladó, M. E. Estrany, E. Rodríguez, B. Amengual, P. Roca, and A. Palou, "Effects of cafeteria diet feeding on beta3-adrenoceptor expression and lipolytic activity in white adipose tissue of male and female rats," (in eng), *Int J Obes Relat Metab Disord*, vol. 24, no. 11, pp. 1396-404, Nov 2000, doi: 10.1038/sj.ijo.0801390.
- [31] S. Peixoto da Silva *et al.*, "Human Papillomavirus 16-Transgenic Mice as a Model to Study Cancer-Associated Cachexia," (in eng), *Int J Mol Sci*, vol. 21, no. 14, Jul 16 2020, doi: 10.3390/ijms21145020.

- [32] S. Cinti, "Adipocyte differentiation and transdifferentiation: plasticity of the adipose organ," (in eng), *J Endocrinol Invest*, vol. 25, no. 10, pp. 823-35, Nov 2002, doi: 10.1007/bf03344046.
- [33] E. E. Kershaw and J. S. Flier, "Adipose tissue as an endocrine organ," (in eng), *J Clin Endocrinol Metab*, vol. 89, no. 6, pp. 2548-56, Jun 2004, doi: 10.1210/jc.2004-0395.
- [34] M. E. Frigolet and R. Gutiérrez-Aguilar, "The colors of adipose tissue," (in eng), *Gac Med Mex*, vol. 156, no. 2, pp. 142-149, 2020, doi: 10.24875/gmm.M20000356. Los colores del tejido adiposo.
- [35] R. K. Zwick, C. F. Guerrero-Juarez, V. Horsley, and M. V. Plikus, "Anatomical, Physiological, and Functional Diversity of Adipose Tissue," (in eng), *Cell Metab*, vol. 27, no. 1, pp. 68-83, Jan 9 2018, doi: 10.1016/j.cmet.2017.12.002.
- [36] B. Le Magueresse-Battistoni, "Adipose Tissue and Endocrine-Disrupting Chemicals: Does Sex Matter?," (in eng), *Int J Environ Res Public Health*, vol. 17, no. 24, Dec 15 2020, doi: 10.3390/ijerph17249403.
- [37] E. D. Rosen and O. A. MacDougald, "Adipocyte differentiation from the inside out," (in eng), *Nat Rev Mol Cell Biol*, vol. 7, no. 12, pp. 885-96, Dec 2006, doi: 10.1038/nrm2066.
- [38] C. Auger and S. Kajimura, "Adipose Tissue Remodeling in Pathophysiology," (in eng), *Annu Rev Pathol*, vol. 18, pp. 71-93, Jan 24 2023, doi: 10.1146/annurev-pathol-042220-023633.
- [39] C. H. Saely, K. Geiger, and H. Drexel, "Brown versus white adipose tissue: a mini-review," (in eng), *Gerontology*, vol. 58, no. 1, pp. 15-23, 2012, doi: 10.1159/000321319.
- [40] J. Sanchez-Gurmaches, C. M. Hung, C. A. Sparks, Y. Tang, H. Li, and D. A. Guertin, "PTEN loss in the Myf5 lineage redistributes body fat and reveals subsets of white adipocytes that arise from Myf5 precursors," (in eng), *Cell Metab*, vol. 16, no. 3, pp. 348-62, Sep 5 2012, doi: 10.1016/j.cmet.2012.08.003.
- [41] S. R. Farmer, "Transcriptional control of adipocyte formation," (in eng), *Cell Metab*, vol. 4, no. 4, pp. 263-73, Oct 2006, doi: 10.1016/j.cmet.2006.07.001.
- [42] J. K. Sethi and A. J. Vidal-Puig, "Thematic review series: adipocyte biology. Adipose tissue function and plasticity orchestrate nutritional adaptation," (in eng), *J Lipid Res*, vol. 48, no. 6, pp. 1253-62, Jun 2007, doi: 10.1194/jlr.R700005-JLR200.

- [43] E. D. Rosen *et al.*, "C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway," (in eng), *Genes Dev*, vol. 16, no. 1, pp. 22-6, Jan 1 2002, doi: 10.1101/gad.948702.
- [44] H. G. Linhart *et al.*, "C/EBPalpha is required for differentiation of white, but not brown, adipose tissue," (in eng), *Proc Natl Acad Sci U S A*, vol. 98, no. 22, pp. 12532-7, Oct 23 2001, doi: 10.1073/pnas.211416898.
- [45] S. Rehnmark, P. Antonson, K. G. Xanthopoulos, and A. Jacobsson, "Differential adrenergic regulation of C/EBP alpha and C/EBP beta in brown adipose tissue," (in eng), *FEBS Lett*, vol. 318, no. 3, pp. 235-41, Mar 8 1993, doi: 10.1016/0014-5793(93)80519-z.
- [46] P. Arner, "Human fat cell lipolysis: biochemistry, regulation and clinical role," (in eng), *Best Pract Res Clin Endocrinol Metab*, vol. 19, no. 4, pp. 471-82, Dec 2005, doi: 10.1016/j.beem.2005.07.004.
- [47] L. K. Markussen *et al.*, "Lipolysis regulates major transcriptional programs in brown adipocytes," *Nature Communications*, vol. 13, no. 1, p. 3956, 2022/07/08 2022, doi: 10.1038/s41467-022-31525-8.
- [48] "Balasubramanian S, Aggarwal P, Sharma S. Lipoprotein Lipase Deficiency. [Updated 2023 Jul 3]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK560795/>."
- [49] A. Yang and E. P. Mottillo, "Adipocyte lipolysis: from molecular mechanisms of regulation to disease and therapeutics," (in eng), *Biochem J*, vol. 477, no. 5, pp. 985-1008, Mar 13 2020, doi: 10.1042/bcj20190468.
- [50] J. Ogasawara *et al.*, "The Molecular Mechanism Underlying Continuous Exercise Training-Induced Adaptive Changes of Lipolysis in White Adipose Cells," (in eng), *J Obes*, vol. 2015, p. 473430, 2015, doi: 10.1155/2015/473430.
- [51] P. R. Kalra and S. Tigas, "Regulation of lipolysis: natriuretic peptides and the development of cachexia," (in eng), *Int J Cardiol*, vol. 85, no. 1, pp. 125-32, Sep 2002, doi: 10.1016/s0167-5273(02)00241-3.
- [52] M. Mannelli, T. Gamberi, F. Magherini, and T. Fiaschi, "The Adipokines in Cancer Cachexia," (in eng), *Int J Mol Sci*, vol. 21, no. 14, Jul 9 2020, doi: 10.3390/ijms21144860.
- [53] G. Fantuzzi, "Adipose tissue, adipokines, and inflammation," (in eng), *J Allergy Clin Immunol*, vol. 115, no. 5, pp. 911-9; quiz 920, May 2005, doi: 10.1016/j.jaci.2005.02.023.

- [54] M. J. Tisdale, "Mechanisms of cancer cachexia," (in eng), *Physiol Rev*, vol. 89, no. 2, pp. 381-410, Apr 2009, doi: 10.1152/physrev.00016.2008.
- [55] H. Suzuki, A. Asakawa, H. Amitani, N. Nakamura, and A. Inui, "Cancer cachexia-pathophysiology and management," (in eng), *J Gastroenterol*, vol. 48, no. 5, pp. 574-94, May 2013, doi: 10.1007/s00535-013-0787-0.
- [56] B. Ahmed, R. Sultana, and M. W. Greene, "Adipose tissue and insulin resistance in obese," (in eng), *Biomed Pharmacother*, vol. 137, p. 111315, May 2021, doi: 10.1016/j.biopha.2021.111315.
- [57] Q. Luong, J. Huang, and K. Y. Lee, "Deciphering White Adipose Tissue Heterogeneity," (in eng), *Biology (Basel)*, vol. 8, no. 2, Apr 11 2019, doi: 10.3390/biology8020023.
- [58] M. Y. Ou, H. Zhang, P. C. Tan, S. B. Zhou, and Q. F. Li, "Adipose tissue aging: mechanisms and therapeutic implications," (in eng), *Cell Death Dis*, vol. 13, no. 4, p. 300, Apr 4 2022, doi: 10.1038/s41419-022-04752-6.
- [59] A. Fenzl and F. W. Kiefer, "Brown adipose tissue and thermogenesis," (in eng), *Horm Mol Biol Clin Investig*, vol. 19, no. 1, pp. 25-37, Jul 2014, doi: 10.1515/hmbci-2014-0022.
- [60] C. Hepler and R. K. Gupta, "The expanding problem of adipose depot remodeling and postnatal adipocyte progenitor recruitment," (in eng), *Mol Cell Endocrinol*, vol. 445, pp. 95-108, Apr 15 2017, doi: 10.1016/j.mce.2016.10.011.
- [61] C. Cero, H. J. Lea, K. Y. Zhu, F. Shamsi, Y. H. Tseng, and A. M. Cypess, "β3-Adrenergic receptors regulate human brown/beige adipocyte lipolysis and thermogenesis," (in eng), *JCI Insight*, vol. 6, no. 11, Jun 8 2021, doi: 10.1172/jci.insight.139160.
- [62] L. Y. Cheong and A. Xu, "Intercellular and inter-organ crosstalk in browning of white adipose tissue: molecular mechanism and therapeutic complications," (in eng), *J Mol Cell Biol*, vol. 13, no. 7, pp. 466-479, Oct 21 2021, doi: 10.1093/jmcb/mjab038.
- [63] A. M. Mesa *et al.*, "Identification and characterization of novel abdominal and pelvic brown adipose depots in mice," (in eng), *Adipocyte*, vol. 11, no. 1, pp. 616-629, Dec 2022, doi: 10.1080/21623945.2022.2133415.
- [64] R. Singh, A. Barrios, G. Dirakvand, and S. Pervin, "Human Brown Adipose Tissue and Metabolic Health: Potential for Therapeutic Avenues," (in eng), *Cells*, vol. 10, no. 11, Nov 5 2021, doi: 10.3390/cells10113030.

- [65] K. Ikeda, P. Maretich, and S. Kajimura, "The Common and Distinct Features of Brown and Beige Adipocytes," (in eng), *Trends Endocrinol Metab*, vol. 29, no. 3, pp. 191-200, Mar 2018, doi: 10.1016/j.tem.2018.01.001.
- [66] U. D. Wankhade, M. Shen, H. Yadav, and K. M. Thakali, "Novel Browning Agents, Mechanisms, and Therapeutic Potentials of Brown Adipose Tissue," (in eng), *Biomed Res Int*, vol. 2016, p. 2365609, 2016, doi: 10.1155/2016/2365609.
- [67] C. Tabuchi and H. S. Sul, "Signaling Pathways Regulating Thermogenesis," (in eng), *Front Endocrinol (Lausanne)*, vol. 12, p. 595020, 2021, doi: 10.3389/fendo.2021.595020.
- [68] S. T. Russell, K. Hirai, and M. J. Tisdale, "Role of beta3-adrenergic receptors in the action of a tumour lipid mobilizing factor," (in eng), *Br J Cancer*, vol. 86, no. 3, pp. 424-8, Feb 1 2002, doi: 10.1038/sj.bjc.6600086.
- [69] W. Wang and P. Seale, "Control of brown and beige fat development," *Nature Reviews Molecular Cell Biology*, vol. 17, no. 11, pp. 691-702, 2016/11/01 2016, doi: 10.1038/nrm.2016.96.
- [70] B. A. Evans, J. Merlin, T. Bengtsson, and D. S. Hutchinson, "Adrenoceptors in white, brown, and brite adipocytes," (in eng), *Br J Pharmacol*, vol. 176, no. 14, pp. 2416-2432, Jul 2019, doi: 10.1111/bph.14631.
- [71] B. Cannon and J. Nedergaard, "Brown adipose tissue: function and physiological significance," (in eng), *Physiol Rev*, vol. 84, no. 1, pp. 277-359, Jan 2004, doi: 10.1152/physrev.00015.2003.
- [72] Q. Zhu, Y. A. An, and P. E. Scherer, "Mitochondrial regulation and white adipose tissue homeostasis," (in eng), *Trends Cell Biol*, vol. 32, no. 4, pp. 351-364, Apr 2022, doi: 10.1016/j.tcb.2021.10.008.
- [73] M. P. Marinovic *et al.*, "Crotamine induces browning of adipose tissue and increases energy expenditure in mice," *Scientific Reports*, vol. 8, no. 1, p. 5057, 2018/03/22 2018, doi: 10.1038/s41598-018-22988-1.
- [74] L. Rui, "Brown and Beige Adipose Tissues in Health and Disease," (in eng), *Compr Physiol*, vol. 7, no. 4, pp. 1281-1306, Sep 12 2017, doi: 10.1002/cphy.c170001.
- [75] M. Shao *et al.*, "Cellular Origins of Beige Fat Cells Revisited," (in eng), *Diabetes*, vol. 68, no. 10, pp. 1874-1885, Oct 2019, doi: 10.2337/db19-0308.
- [76] Y. Jiang, D. C. Berry, and J. M. Graff, "Distinct cellular and molecular mechanisms for β 3 adrenergic receptor-induced beige adipocyte formation," (in eng), *Elife*, vol. 6, Oct 11 2017, doi: 10.7554/eLife.30329.

- [77] T. Agustsson *et al.*, "Mechanism of increased lipolysis in cancer cachexia," (in eng), *Cancer Res*, vol. 67, no. 11, pp. 5531-7, Jun 1 2007, doi: 10.1158/0008-5472.Can-06-4585.
- [78] P. Arner and D. Langin, "Lipolysis in lipid turnover, cancer cachexia, and obesity-induced insulin resistance," (in eng), *Trends Endocrinol Metab*, vol. 25, no. 5, pp. 255-62, May 2014, doi: 10.1016/j.tem.2014.03.002.
- [79] S. Elattar, M. Dimri, and A. Satyanarayana, "The tumor secretory factor ZAG promotes white adipose tissue browning and energy wasting," (in eng), *Faseb j*, vol. 32, no. 9, pp. 4727-4743, Sep 2018, doi: 10.1096/fj.201701465RR.
- [80] M. Lemecha *et al.*, "Lcn2 mediates adipocyte-muscle-tumor communication and hypothermia in pancreatic cancer cachexia," (in eng), *Mol Metab*, vol. 66, p. 101612, Dec 2022, doi: 10.1016/j.molmet.2022.101612.
- [81] K. L. Klierer, J. Y. Ke, M. Tian, R. M. Cole, R. R. Andridge, and M. A. Belury, "Adipose tissue lipolysis and energy metabolism in early cancer cachexia in mice," (in eng), *Cancer Biol Ther*, vol. 16, no. 6, pp. 886-97, 2015, doi: 10.4161/15384047.2014.987075.
- [82] J. P. Deslypere, L. Verdonck, and A. Vermeulen, "Fat tissue: a steroid reservoir and site of steroid metabolism," (in eng), *J Clin Endocrinol Metab*, vol. 61, no. 3, pp. 564-70, Sep 1985, doi: 10.1210/jcem-61-3-564.
- [83] C. Bing and P. Trayhurn, "New insights into adipose tissue atrophy in cancer cachexia," (in eng), *Proc Nutr Soc*, vol. 68, no. 4, pp. 385-92, Nov 2009, doi: 10.1017/s0029665109990267.
- [84] M. Takahashi, M. Terashima, A. Takagane, K. Oyama, H. Fujiwara, and G. Wakabayashi, "Ghrelin and leptin levels in cachectic patients with cancer of the digestive organs," (in eng), *Int J Clin Oncol*, vol. 14, no. 4, pp. 315-20, Aug 2009, doi: 10.1007/s10147-008-0856-1.
- [85] I. Wolf *et al.*, "Adiponectin, ghrelin, and leptin in cancer cachexia in breast and colon cancer patients," (in eng), *Cancer*, vol. 106, no. 4, pp. 966-73, Feb 15 2006, doi: 10.1002/cncr.21690.
- [86] C. Bing, S. Taylor, M. J. Tisdale, and G. Williams, "Cachexia in MAC16 adenocarcinoma: suppression of hunger despite normal regulation of leptin, insulin and hypothalamic neuropeptide Y," (in eng), *J Neurochem*, vol. 79, no. 5, pp. 1004-12, Dec 2001, doi: 10.1046/j.1471-4159.2001.00639.x.
- [87] A. Y. Gandhi, J. Yu, A. Gupta, T. Guo, P. Iyengar, and R. E. Infante, "Cytokine-Mediated STAT3 Transcription Supports ATGL/CGI-58-Dependent Adipocyte

- Lipolysis in Cancer Cachexia," (in eng), *Front Oncol*, vol. 12, p. 841758, 2022, doi: 10.3389/fonc.2022.841758.
- [88] K. C. Fearon, D. J. Glass, and D. C. Guttridge, "Cancer cachexia: mediators, signaling, and metabolic pathways," (in eng), *Cell Metab*, vol. 16, no. 2, pp. 153-66, Aug 8 2012, doi: 10.1016/j.cmet.2012.06.011.
- [89] W. Cao, A. V. Medvedev, K. W. Daniel, and S. Collins, "beta-Adrenergic activation of p38 MAP kinase in adipocytes: cAMP induction of the uncoupling protein 1 (UCP1) gene requires p38 MAP kinase," (in eng), *J Biol Chem*, vol. 276, no. 29, pp. 27077-82, Jul 20 2001, doi: 10.1074/jbc.M101049200.
- [90] M. Grazia Perrone and A. Scilimati, " $\beta(3)$ -Adrenoceptor agonists and (antagonists as) inverse agonists history, perspective, constitutive activity, and stereospecific binding," (in eng), *Methods Enzymol*, vol. 484, pp. 197-230, 2010, doi: 10.1016/b978-0-12-381298-8.00011-3.
- [91] S. L. Brooks, A. M. Neville, N. J. Rothwell, M. J. Stock, and S. Wilson, "Sympathetic activation of brown-adipose-tissue thermogenesis in cachexia," (in eng), *Biosci Rep*, vol. 1, no. 6, pp. 509-17, Jun 1981, doi: 10.1007/bf01121584.
- [92] I. Pototschnig *et al.*, "Interleukin-6 initiates muscle- and adipose tissue wasting in a novel C57BL/6 model of cancer-associated cachexia," (in eng), *J Cachexia Sarcopenia Muscle*, vol. 14, no. 1, pp. 93-107, Feb 2023, doi: 10.1002/jcsm.13109.
- [93] A. Bouloumié, P. Valet, D. Daviaud, H. Prats, M. Lafontan, and J.-S. Saulnier-Blache, "Adipocyte $\alpha 2A$ -adrenoceptor is the only $\alpha 2$ -adrenoceptor regulated by testosterone," *European Journal of Pharmacology: Molecular Pharmacology*, vol. 269, no. 1, pp. 95-103, 1994/09/15/ 1994, doi: [https://doi.org/10.1016/0922-4106\(94\)90031-0](https://doi.org/10.1016/0922-4106(94)90031-0).
- [94] A. M. Rodriguez, M. Monjo, P. Roca, and A. Palou, "Opposite actions of testosterone and progesterone on UCP1 mRNA expression in cultured brown adipocytes," (in eng), *Cell Mol Life Sci*, vol. 59, no. 10, pp. 1714-23, Oct 2002, doi: 10.1007/pl00012499.
- [95] P. Mauriege, J. Galitzky, M. Berlan, and M. Lafontan, "Heterogeneous distribution of beta and alpha-2 adrenoceptor binding sites in human fat cells from various fat deposits: functional consequences," (in eng), *Eur J Clin Invest*, vol. 17, no. 2, pp. 156-65, Apr 1987, doi: 10.1111/j.1365-2362.1987.tb02395.x.
- [96] G. Tavernier *et al.*, "Molecular mechanisms underlying regional variations of catecholamine-induced lipolysis in rat adipocytes," (in eng), *Am J Physiol*, vol.

- 268, no. 6 Pt 1, pp. E1135-42, Jun 1995, doi: 10.1152/ajpendo.1995.268.6.E1135.
- [97] P. Valet *et al.*, "Expression of human alpha 2-adrenergic receptors in adipose tissue of beta 3-adrenergic receptor-deficient mice promotes diet-induced obesity," (in eng), *J Biol Chem*, vol. 275, no. 44, pp. 34797-802, Nov 3 2000, doi: 10.1074/jbc.M005210200.
- [98] S. Rodriguez-Cuenca *et al.*, "Sex-dependent thermogenesis, differences in mitochondrial morphology and function, and adrenergic response in brown adipose tissue," (in eng), *J Biol Chem*, vol. 277, no. 45, pp. 42958-63, Nov 8 2002, doi: 10.1074/jbc.M207229200.
- [99] C. Quarta, R. Mazza, R. Pasquali, and U. Pagotto, "Role of sex hormones in modulation of brown adipose tissue activity," (in eng), *J Mol Endocrinol*, vol. 49, no. 1, pp. R1-7, Aug 2012, doi: 10.1530/jme-12-0043.
- [100] F. Mauvais-Jarvis, "Sex differences in metabolic homeostasis, diabetes, and obesity," (in eng), *Biol Sex Differ*, vol. 6, p. 14, 2015, doi: 10.1186/s13293-015-0033-y.
- [101] M. Keuper and M. Jastroch, "The good and the BAT of metabolic sex differences in thermogenic human adipose tissue," (in eng), *Mol Cell Endocrinol*, vol. 533, p. 111337, Aug 1 2021, doi: 10.1016/j.mce.2021.111337.
- [102] N. Viguerie *et al.*, "Determinants of human adipose tissue gene expression: impact of diet, sex, metabolic status, and cis genetic regulation," (in eng), *PLoS Genet*, vol. 8, no. 9, p. e1002959, Sep 2012, doi: 10.1371/journal.pgen.1002959.
- [103] S. N. Kim, Y. S. Jung, H. J. Kwon, J. K. Seong, J. G. Granneman, and Y. H. Lee, "Sex differences in sympathetic innervation and browning of white adipose tissue of mice," (in eng), *Biol Sex Differ*, vol. 7, p. 67, 2016, doi: 10.1186/s13293-016-0121-7.
- [104] Y. Giudicelli, M. N. Dieudonne, D. Lacasa, Y. N. Pasquier, and R. Pecquery, "Modulation by sex hormones of the membranous transducing system regulating fatty acid mobilization in adipose tissue," (in eng), *Prostaglandins Leukot Essent Fatty Acids*, vol. 48, no. 1, pp. 91-100, Jan 1993, doi: 10.1016/0952-3278(93)90015-o.
- [105] J. R. Bracht, V. J. Vieira-Potter, R. De Souza Santos, O. K. Öz, B. F. Palmer, and D. J. Clegg, "The role of estrogens in the adipose tissue milieu," (in eng), *Ann N Y Acad Sci*, vol. 1461, no. 1, pp. 127-143, Feb 2020, doi: 10.1111/nyas.14281.
- [106] M. Blüher, "Importance of estrogen receptors in adipose tissue function," (in eng), *Mol Metab*, vol. 2, no. 3, pp. 130-2, 2013, doi: 10.1016/j.molmet.2013.07.001.

- [107] S. Rodríguez-Cuenca, M. Monjo, M. Gianotti, A. M. Proenza, and P. Roca, "Expression of mitochondrial biogenesis-signaling factors in brown adipocytes is influenced specifically by 17beta-estradiol, testosterone, and progesterone," (in eng), *Am J Physiol Endocrinol Metab*, vol. 292, no. 1, pp. E340-6, Jan 2007, doi: 10.1152/ajpendo.00175.2006.
- [108] M. W. O'Reilly, P. J. House, and J. W. Tomlinson, "Understanding androgen action in adipose tissue," (in eng), *J Steroid Biochem Mol Biol*, vol. 143, pp. 277-84, Sep 2014, doi: 10.1016/j.jsbmb.2014.04.008.
- [109] S. L. Schmidt, D. H. Bessesen, S. Stotz, F. F. Peelor, 3rd, B. F. Miller, and T. J. Horton, "Adrenergic control of lipolysis in women compared with men," (in eng), *J Appl Physiol (1985)*, vol. 117, no. 9, pp. 1008-19, Nov 1 2014, doi: 10.1152/japplphysiol.00003.2014.
- [110] K. Norman, N. Stobäus, J. Reiß, J. Schulzke, L. Valentini, and M. Pirlich, "Effect of sexual dimorphism on muscle strength in cachexia," (in eng), *J Cachexia Sarcopenia Muscle*, vol. 3, no. 2, pp. 111-6, Jun 2012, doi: 10.1007/s13539-012-0060-z.
- [111] R. N. Montalvo, B. R. Counts, and J. A. Carson, "Understanding sex differences in the regulation of cancer-induced muscle wasting," (in eng), *Curr Opin Support Palliat Care*, vol. 12, no. 4, pp. 394-403, Dec 2018, doi: 10.1097/spc.0000000000000380.
- [112] D. Nguyen and T. Xu, "The expanding role of mouse genetics for understanding human biology and disease," (in eng), *Dis Model Mech*, vol. 1, no. 1, pp. 56-66, Jul-Aug 2008, doi: 10.1242/dmm.000232.
- [113] G. Dranoff, "Experimental mouse tumour models: what can be learnt about human cancer immunology?," (in eng), *Nat Rev Immunol*, vol. 12, no. 1, pp. 61-6, Dec 2 2011, doi: 10.1038/nri3129.
- [114] J. Doorbar, "Model systems of human papillomavirus-associated disease," (in eng), *J Pathol*, vol. 238, no. 2, pp. 166-79, Jan 2016, doi: 10.1002/path.4656.
- [115] R. M. Gil da Costa *et al.*, "HPV16 induces a wasting syndrome in transgenic mice: Amelioration by dietary polyphenols via NF-κB inhibition," (in eng), *Life Sci*, vol. 169, pp. 11-19, Jan 15 2017, doi: 10.1016/j.lfs.2016.10.031.
- [116] J. M. Arbeit, K. Münger, P. M. Howley, and D. Hanahan, "Progressive squamous epithelial neoplasia in K14-human papillomavirus type 16 transgenic mice," (in eng), *J Virol*, vol. 68, no. 7, pp. 4358-68, Jul 1994, doi: 10.1128/jvi.68.7.4358-4368.1994.

- [117] J. M. O. Santos *et al.*, "Dimethylaminoparthenolide reduces the incidence of dysplasia and ameliorates a wasting syndrome in HPV16-transgenic mice," (in eng), *Drug Dev Res*, vol. 80, no. 6, pp. 824-830, Sep 2019, doi: 10.1002/ddr.21565.
- [118] I. Paiva *et al.*, "MicroRNA-21 expression and susceptibility to HPV-induced carcinogenesis - role of microenvironment in K14-HPV16 mice model," (in eng), *Life Sci*, vol. 128, pp. 8-14, May 1 2015, doi: 10.1016/j.lfs.2015.02.020.
- [119] L. Li, B. Li, M. Li, and J. R. Speakman, "Switching on the furnace: Regulation of heat production in brown adipose tissue," (in eng), *Mol Aspects Med*, vol. 68, pp. 60-73, Aug 2019, doi: 10.1016/j.mam.2019.07.005.
- [120] E. D. Rosen, "The transcriptional basis of adipocyte development," (in eng), *Prostaglandins Leukot Essent Fatty Acids*, vol. 73, no. 1, pp. 31-4, Jul 2005, doi: 10.1016/j.plefa.2005.04.004.
- [121] K. L. Marlatt and E. Ravussin, "Brown Adipose Tissue: an Update on Recent Findings," (in eng), *Curr Obes Rep*, vol. 6, no. 4, pp. 389-396, Dec 2017, doi: 10.1007/s13679-017-0283-6.
- [122] F. Almeida-Oliveira, J. G. B. Leandro, P. Ausina, M. Sola-Penna, and D. Majerowicz, "Reference genes for quantitative PCR in the adipose tissue of mice with metabolic disease," (in eng), *Biomed Pharmacother*, vol. 88, pp. 948-955, Apr 2017, doi: 10.1016/j.biopha.2017.01.091.
- [123] X. Fan *et al.*, "High-Fat Diet Alters the Expression of Reference Genes in Male Mice," (in eng), *Front Nutr*, vol. 7, p. 589771, 2020, doi: 10.3389/fnut.2020.589771.
- [124] S. Kir *et al.*, "Tumour-derived PTH-related protein triggers adipose tissue browning and cancer cachexia," (in eng), *Nature*, vol. 513, no. 7516, pp. 100-4, Sep 4 2014, doi: 10.1038/nature13528.
- [125] C. Bing, M. Brown, P. King, P. Collins, M. J. Tisdale, and G. Williams, "Increased gene expression of brown fat uncoupling protein (UCP)1 and skeletal muscle UCP2 and UCP3 in MAC16-induced cancer cachexia," (in eng), *Cancer Res*, vol. 60, no. 9, pp. 2405-10, May 1 2000.
- [126] K. A. Michaelis *et al.*, "Establishment and characterization of a novel murine model of pancreatic cancer cachexia," (in eng), *J Cachexia Sarcopenia Muscle*, vol. 8, no. 5, pp. 824-838, Oct 2017, doi: 10.1002/jcsm.12225.
- [127] T. Bengtsson, B. Cannon, and J. Nedergaard, "Differential adrenergic regulation of the gene expression of the beta-adrenoceptor subtypes beta1, beta2 and

beta3 in brown adipocytes," (in eng), *Biochem J*, vol. 347 Pt 3, no. Pt 3, pp. 643-51, May 1 2000.