

# **The epididymis as a target of endocrine disruption: a metabolic perspective**

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# Resumo

A fertilidade masculina depende da eficiência do processo espermatogénico, assim como do conjunto de eventos que ocorrem durante a passagem dos espermatozoides pelo epidídimo e que são responsáveis pela sua maturação. Este órgão tem também um papel importante no armazenamento dos espermatozoides, aumentando a sua concentração, e conferindo-lhes proteção imunológica e contra o stress oxidativo. Para desempenhar as suas funções, o epidídimo depende do metabolismo da glicose como fonte de energia, e resultado da sua atividade secretora contribui para estabelecer a composição do fluido epididimal. Recentemente, foi demonstrado que existem várias vias energéticas ativas no epidídimo, como a glicólise, e vários metabolitos como o piruvato foram detetados no fluido epididimal. Além disso, o meio epididimal demonstrou ser rico em lípidos, uma classe de macromoléculas que desempenha um papel crucial na estrutura da membrana espermática influenciando a capacidade de fertilização. A atividade do epidídimo é regulada pela ação de hormonas, como os androgénios, os quais podem também influenciar o metabolismo deste órgão. Os disruptores endócrinos (EDCs) são compostos exógenos capazes de perturbar a normal sinalização hormonal. A vinclozolina (VNZ) é um EDC que atua como anti-androgénio e cuja ação demonstrou afetar a reprodução masculina. No entanto, o efeito da VNZ no epidídimo, nomeadamente na desregulação do metabolismo, é largamente desconhecido. A presente dissertação investiga o impacto da exposição à VNZ no metabolismo glicolítico e lipídico do epidídimo de ratos. Para este fim, seções da região *caput* do epidídimo foram mantidas em cultura *ex vivo* na presença ou ausência de VNZ 500  $\mu\text{M}$ . O consumo de glicose, a produção de lactato e a expressão e atividade de reguladores do metabolismo glicolítico e lipídico foram avaliados por ensaios espectrofotométricos e análise de Western blot. Os resultados obtidos mostraram que a VNZ aumentou o consumo de glicose pelas células da região *caput* do epidídimo, enquanto diminuiu a produção de lactato. Além disso, a VNZ ativou a biossíntese e a degradação de ácidos gordos. Estes achados demonstraram pela primeira vez que a VNZ desregula o metabolismo glicolítico e lipídico no epidídimo e alertam para o impacto que este e outros EDCs podem ter na qualidade dos espermatozoides e fertilidade masculina.

## **Palavras-chave**

Desreguladores endócrinos; epidídimo; (in)fertilidade masculina; metabolismo; vinclozolina

## Resumo alargado

A fertilidade masculina depende da eficiência do processo espermatogénico, assim como do conjunto de eventos que ocorrem durante a passagem dos espermatozoides pelo epidídimo e que são responsáveis pela sua maturação. O epidídimo tem também um papel importante no armazenamento dos espermatozoides, aumentando a sua concentração, e conferindo-lhes proteção imunológica e contra o stress oxidativo. Anatomicamente o epidídimo tem 3 regiões anatómicas distintas, o *caput*, *corpus* e *cauda*, as quais têm diferentes funções fisiológicas e contribuem diferencialmente para a maturação dos espermatozoides durante o trânsito neste órgão. A região do *caput* é a mais ativa em termos de síntese e secreção de proteínas, sendo responsável pela maioria das proteínas secretadas para o lúmen do epidídimo. Em termos de características histofuncionais, o epidídimo é constituído por seis tipos diferentes de células: *clear cells*, *principal cells*, *basal cells*, *narrow cells*, *apical cells* e *halo cells*. A atividade destas células no seu todo determina a regulação da concentração, maturação, armazenamento e viabilidade de espermatozoides. Para desempenhar as suas funções, o epidídimo depende do metabolismo da glicose como fonte de energia, e resultado da sua atividade secretora contribui para estabelecer a composição do fluido epididimal. Recentemente, foi demonstrado que existem várias vias energéticas ativas no epidídimo, como a glicólise e vários metabolitos como o piruvato foram detetados no fluido epididimal. e ainda que a atividade de enzimas-chave da via glicolítica varia ao longo do epidídimo, com maior atividade detetada no *caput*. Além disso, o meio epididimal demonstrou ser rico em lípidos, uma classe de macromoléculas que desempenha um papel crucial na estrutura da membrana espermática influenciando a capacidade de fertilização. A atividade do epidídimo é regulada pela ação de hormonas, como os androgénios, os quais podem também influenciar o metabolismo deste órgão. Na verdade, foi demonstrado que na ausência de androgénios, ocorre uma troca na fonte de energia metabólica usada pelas células do epidídimo que passam a utilizar lípidos por troca com os hidratos de carbono. Os disruptores endócrinos (EDCs) são compostos exógenos capazes de perturbar a normal sinalização hormonal. A vinclozolina (VNZ) é um EDC que atua como anti-androgénio e cuja ação demonstrou afetar a reprodução masculina. No entanto, o efeito da VNZ no epidídimo, nomeadamente na desregulação do metabolismo, é largamente desconhecido. A presente dissertação investiga o impacto da exposição à VNZ no metabolismo glicolítico e lipídico do epidídimo de ratos. Para este fim, seções da região *caput* do epidídimo foram mantidas em cultura *ex vivo* na presença ou ausência de VNZ 500  $\mu\text{M}$ . O

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# Abstract

Male fertility depends on the output of spermatogenic processes, as well as on the maturation events occurring during sperm passage throughout the epididymis. Epididymis plays an important role in storage of sperm, and also increasing their concentration and providing immunological and oxidative stress protection. In order to perform its functions, the epididymis depends on glucose metabolism as energy source, and also contributes by its secretory activity to establish the composition of the epididymal fluid. Recently, it was demonstrated that several energy pathways are active in this organ, namely glycolysis, and several metabolites like pyruvate are detected in the epididymal fluid. Also, the epididymal milieu has been shown to be rich in lipids, a class of macromolecules that plays a crucial role in the structure of sperm membrane influencing fertilization capacity. The activity of the epididymis can be regulated by hormones, like androgens, that can also have an influence on its metabolism. Endocrine disruptors (EDCs) are exogenous compounds capable of perturbing the normal hormone signalling. The EDC vinclozolin (VNZ) acts as anti-androgen and has been shown to affect male reproduction. However, the effect of VNZ in the epididymis, namely in the deregulation of metabolism is largely unknown. The present dissertation investigates the impact of VNZ exposure in the rat epididymis glycolytic and lipidic metabolism. For this purpose, *caput* epididymis sections were cultured *ex vivo* in the presence or absence of VNZ 500  $\mu$ M. Glucose consumption, lactate production, and the expression and activity of glycolytic and lipid metabolism regulators were evaluated by spectrophotometric assays and Western blot analysis. The results obtained showed that VNZ increased glucose consumption in the *caput* epididymis, whereas decreasing lactate production. Also, VNZ activated both fatty acid biosynthesis and degradation. These findings first demonstrated that VNZ disrupts glycolytic and lipid metabolism in the epididymis and highlight for the impact that this and others EDCs may have affecting sperm quality and male fertility.

# Keywords

Endocrine disruptors, epididymis, male (in)fertility, metabolism, vinclozolin.



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## Poster

Daniel Tavares, Mariana Feijó, Lara Fonseca, Ana Catarina Sousa, Sara Correia#, Sílvia Socorro#. Efeito Do Desregulador Endócrino Vinclozolina No Metabolismo Glicolítico Do Epidídimo. XVII Congresso Nacional da SPA / XVI Reunião Ibérica de Andrologia Medicina Sexual e Reprodução. 3-5 junho de 2022, Évora, Portugal.

## Scientific merit award in the team with work in this area

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# List of Abbreviations

ABP	Androgen-binding protein
ACC	Acetyl-CoA carboxylase
AhR	Arylhydrocarbon receptor
AR	Androgen receptor
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid protein
BEB	Blood-epididymis barrier
CAR	Constitutive androstane receptor
CO <sub>2</sub>	Carbon dioxide
CPT1A	Carnitine palmitoyl transferase I
DHA	Docosahexaenoic acid
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ED	Endocrine disruptor
EDC	Endocrine disrupting chemicals
EDTA	Ethylenediamine tetraacetic acid
ER	Estrogen receptor
FA	Fatty acid
FADH <sub>2</sub>	Flavin adenine dinucleotide
FASN	Fatty acid synthase
FSH	Follicle-stimulating hormone
GLUT	Glucose transporter
GnRH	Gonadotropin-releasing hormone
GPER1	G protein-coupled estrogen receptor
GPI	Glycosyl phosphatidyl inositol
HCO <sub>3</sub> <sup>-</sup>	Bicarbonate
LDH	Lactate dehydrogenase
LH	Luteinizing hormone
MCT	Monocarboxylate transporter
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NR	Nuclear receptor
PBS	Phosphate-buffered saline
PFK1	Phosphofructokinase 1
PPAR	Peroxisome proliferator-activated receptor
PVDF	Polyvinylidene difluoride
PXR	Pregnane X receptor
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TCA	Tricarboxylic acid cycle
TCDD	Tetrachlorodibenzo-p-dioxin
VNZ	Vinclozolin
WB	Western blot
WR	Working reagent



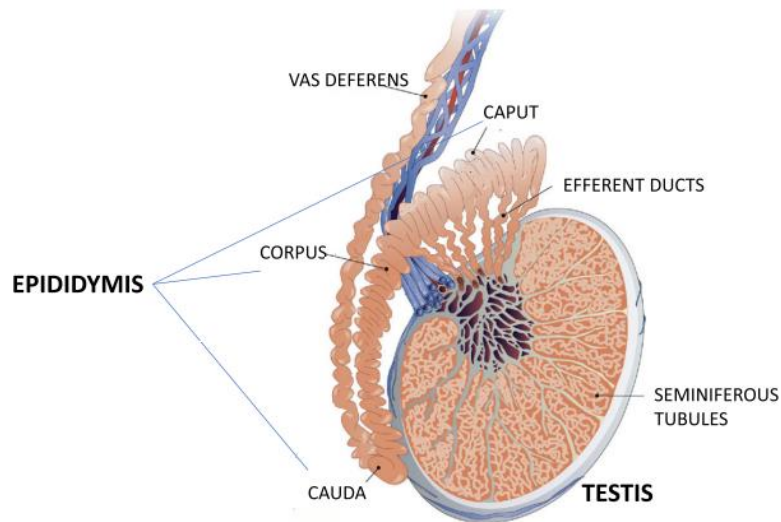
# ***I – Introduction***

## **1. Brief overview of epididymal anatomic features**

The anatomy of the male reproductive system shows great variability among vertebrate species with some of this diversity occurring in epididymis<sup>1</sup>. Nevertheless, overall in mammals the epididymis is divided into 3 distinct segments, namely the *caput*, *corpus* and *cauda* (figure 1)<sup>1-5</sup>. The epididymis is adherent to the testis by the epididymis-testicular connective tissue and attached to the testis cranial pole by the connective tissue tunica of the *caput*.

The mammalian epididymis is an exceptionally long, convoluted ductal system that establishes the communication between the testis and the *ductus deferens* (figure 1)<sup>2,6</sup>. Reaching lengths that can go from 1 to 80 meters (comparing mice to horses), the epididymis is the combination of 4 to 20 tubules, depending on the specie in question. These tubules are connected to the efferent ducts, which receive the secretions of seminiferous tubules from the rete testis, establishing the testicular-epididymis communication (figure 1)<sup>3</sup>. In all mammalian species examined to date, each region of the epididymis is further organized into lobules separated by connective tissue septa<sup>7</sup>. This tissue not only serves as an internal support for the organ itself, but also allows a selective expression of genes and proteins within the lobules<sup>7</sup>.

The distinct anatomic regions of the epididymis, *caput*, *corpus* and *cauda*, have different underlying physiological functions concerning sperm maturation during transit in the epididymis, which will be detailed in the next sections. Briefly, the *caput* is the region responsible for the absorption of the majority of testicular fluid and the most active in terms of protein synthesis and secretion<sup>6</sup>. Besides regional differences, and some specificities, these functional characteristics continue present in the *corpus* whereas the *cauda* is essentially the sperm storage reservoir<sup>6</sup>.



**Figure 1. Anatomy of the epididymis and its relationship with the testis<sup>4</sup>.**

## **2. Epididymal physiology**

### **2.1. General overview**

As said before, the epididymis is divided into three segments. The *caput* epididymis is most active region in terms of protein synthesis and secretion. It presents a higher concentration of secreted proteins, with around 70-80% of the total secreted proteins in the epididymis lumen resulting from the function of the *caput* epididymal cells. This region is also where a small portion of the sperm begins to demonstrate the ability to swim in a progressive manner and to recognize an oocyte<sup>6,8</sup>. Before reaching an optical level in the distal *cauda* segment, these functional characteristics are developed even further in the *corpus* epididymis<sup>6</sup>. Attributes like a large lumen and surrounding epithelial cells exhibiting strong absorptive activity, make the dominant function of the *cauda* epididymis a sperm storage reservoir<sup>6</sup>. Noteworthy, these two segments (*corpus* and *cauda*) have a small amount of expressed proteins when compared to *caput*<sup>6,9,10</sup>.

Considering the histofunctional features, the epididymis is made of six different types of cells: clear cells, principal cells, basal cells, narrow cells, apical cells and halo cells (figure 2), which contribute to the establishment and regulation for the concentration, maturation, storage and viability of spermatozoa<sup>8,11-13</sup>.

Principal cells comprise 65% to 80% of the total epithelial cell population and are responsible for synthesizing the majority of the proteins secreted into the epididymal lumen. This cell type also plays an important role in the transport and control of

protein concentration in the lumen<sup>12</sup>. Their morphology changes from a tall columnar structure in the initial segment to low cuboidal cells in the *cauda*. They have a prominent, branched, microvillus, absorptive border<sup>12,14</sup>.

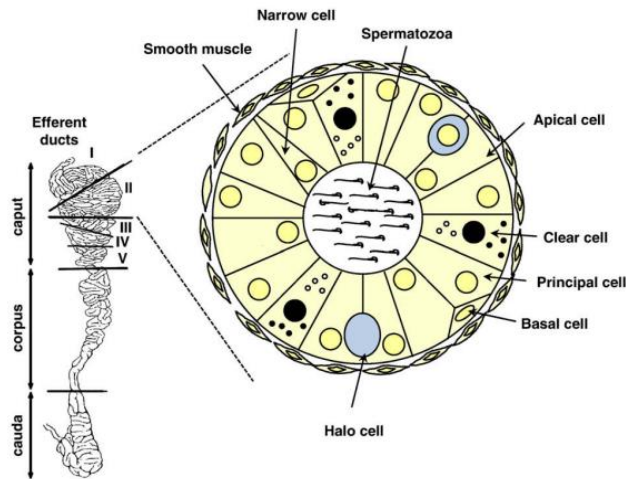
Apical cells are located in the epithelium of the *caput* and *corpus*. They have a lot of mitochondria in the apical cytoplasm (that may bulge slightly into the lumen) and their nuclei is oval to spheric<sup>12</sup>. Apical cells differ from principal cells and narrow cells in terms of protein expression, though little is known about their functions<sup>15</sup>. Nevertheless, it has been suggested that these cells may have an important role in the regulation of pH, as well as a possible role in the maintenance of spermatozoa in the quiescent state, through the production of enzymes of carbonic anhydrase family<sup>12,15,16</sup>.

Narrow cells are present within the epithelium of the *caput* and *corpus*. They have numerous apically located cup-shaped vesicles, performing endocytosis, and regulate the secretion of H<sup>+</sup> ions into lumen<sup>12,15</sup>. Narrow cells play an important role in degradation of specific protein and carbohydrates within their lysosomes and protect spermatozoa against electrolytic imbalance<sup>12,15,17,18</sup>.

Clear cells are large endocytic cells characterized by an apical region with numerous coated pits, vesicles, endosomes, multivesicular bodies, lysosomes, and lipid droplets<sup>12</sup>. They are interspersed between principal cells within the *caput*, *corpus* and *cauda* regions. The endocytic activity of clear cells is greater than that of any other cell type in the epididymis and is particularly active in the *cauda*<sup>8,12,19</sup>.

Basal cells are triangular and flat cells located against the basement membrane. Their plasma membrane is constituted by coated pits, which may be associated with the receptor-mediated endocytosis of factors from the blood or principal cells. Basal cells also have the ability to release paracrine factors, which regulate principal cell electrolyte transporter<sup>8,12,20</sup>.

Halo cells are present in all epididymal segments localized in the base of the epithelium and have a variable number of dense core granules. These cells are related to the immune system because they can be monocytes, helper T lymphocytes and cytotoxic T lymphocytes<sup>14</sup>.



**Figure 2. Different cell types of the caput epididymis<sup>13</sup>.**

The integrated action of the different cells types aforementioned, generates the specific microenvironment in the epididymis required to the sperm maturation<sup>21</sup>. This luminal environment is established by the epithelial cells by executing vectorial transepithelial transport of ions, nutrients, solutes and water<sup>11</sup>. The composition of the epididymal fluid is conditioned by proteins secreted by the epithelium, but also by the selective exchange of molecules between the lumen and blood circulation, determined by the blood-epididymis barrier (BEB)<sup>21,22</sup>, formed by the apical tight junctions between adjacent principal cells<sup>21</sup>. From all these actions, the epididymal fluid results in a complex mixture of inorganic and inorganic molecules. Moreover, it has been showed that the constituents of the epididymal fluid change as the fluid progresses along the epididymal tubule<sup>23</sup>. The inorganic ions, such as Na<sup>+</sup>, Cl<sup>-</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup> and HCO<sub>3</sub><sup>-</sup> decrease their concentration from *caput* to *cauda*, unlike of several unusual organic constituents namely, glycerylphosphorylcholine, carnitine, sialic acid, amino acids, glycosidases, and phosphatases, that increase their concentration levels along this organ<sup>23-26</sup>. Recently, the metabolite composition of the human epididymal fluid was characterized resulting from the activity of several energy pathways active in the epididymal cells of the *caput* region<sup>27</sup>. Ascorbate, dehydroascorbic acid,<sup>27</sup> glutathione and pyruvic acid are among the metabolite identified.

The pH of the epididymal lumen ranges from 6.5 to 6.8<sup>28</sup>.

## 2.2. The path for sperm maturation

The spermatozoa released by the testis are immature, acquiring potential of motility and ability to fertilize after 1 or 2 weeks of transit through the epididymis<sup>8,24,29</sup>. This organ is also responsible for the concentration of sperm and their transport towards *ductus deferens*<sup>5</sup>. Moreover, the epididymis is a sperm reservoir<sup>5</sup> and has a determinant action ensuring the quality of the ejaculated sperm. The epididymis plays a role nourishing sperm, and in the immunoprotection and protection against oxidative stress<sup>5</sup>.

During transit through the epididymis, sperm are in contact with the changes that occur in the epididymal lumen environment, including the release and absorption of fluids, ions and antioxidants<sup>8,29</sup>. The alterations on the composition of the epididymal fluid are reflected on sperm, namely on plasma membrane and protein payload<sup>8,24,29</sup>.

The acidic environment of the epididymis facilitates the transferring of proteins from epithelial cells to sperm, throughout extracellular vesicles, the exosomes, also known as “epididymosomes”<sup>11,29</sup>. It also helps in the maintenance of sperm quiescence by inhibiting some specific sperm channels and transporters<sup>11,29</sup>. Moreover, depending on the activity of transporters present at the membrane of epididymal cells, water removal from the luminal fluid increases sperm concentration in the epididymis, which is a very relevant step for fertility. It has been demonstrated that alterations in these transporters, such as the aquaporins water channels, is associated with impaired infertility<sup>30–32</sup>.

The alterations on the composition of the epididymal fluid are reflected on sperm that also undergoes some changes while passing in the different functional regions. This includes a set of structural and biochemical changes in the nucleus, mitochondria, acrosome, and plasma membrane, are reflected on sperm<sup>33</sup>. In the *caput* epididymis the cytoplasmic droplet is removed from the sperm cell and the resulting cell has virtually completely lost the cytosol that was present in an early spermatid<sup>34</sup>. Moreover, due to a series of sperm surface processing events, the sperm cell switches on some metabolic pathways and thus acquires motility characteristics<sup>34</sup>. However, the contribution of specific components of the epididymis energy metabolism to the sperm function still has some knowledge gaps.

Finally, in the *cauda* epididymis, sperm cells have attracted a number of proteins at their surface that are known to be involved in fertilization, for example, integral membrane proteins (the glycosyl phosphatidyl inositol (GPI)-anchored proteins)<sup>34</sup>. In

general, the maturation of sperm starts from a lower ionic environment from the *caput* to *cauda*, and in a progressively higher organic solute and protein environment<sup>3</sup>.

Because of this variety in the fluid composition in the 3 regions of the epididymis, and the consequent alterations on sperm, different rates of correlation between penetration of oocytes and the number of spermatozoa bound to them has been found. More spermatozoa from the *cauda* epididymis are able to bound oocyte compared with the spermatozoa from the *caput* or *corpus*<sup>35</sup>.

### **2.3. Hormonal regulation of the epididymis**

The absorptive and secretory activity of the epididymis has been shown to be regulated by androgens, namely testosterone and dihydrotestosterone (DHT)<sup>36,37</sup>. The synthesis of some secretory proteins that play an important role in preparing the sperm for events of fertilization by modifying the surface characteristics, are also androgen-dependent. These steroid hormones (androgens) also have an important influence on the metabolism of the epididymis. As a matter of fact, it has been demonstrated that in the absence of androgens, the metabolic energy (ATP) is switch from carbohydrate to lipids<sup>37,38</sup>. Nevertheless, energy metabolic routes in the epididymis and how they contribute to sperm are not fully understood.

Testosterone is considered to be a male hormone, mostly because of its higher concentration in men, when compared to women. Like other steroids, testosterone is synthesized from cholesterol mostly in the gonads and adrenal cortex<sup>39</sup>. The concentration of testosterone in men depends on age and has been shown to decrease from 15.4 nmol/L at 19 years to 13.0 nmol/L in a 40 years old men<sup>40</sup>. This diminution is known to affect testicular output and is associated to diminished sperm density. However, the impact on the epididymis is much less studied.

Testosterone can enter the epididymis in 2 different ways. It can enter through the blood circulation, or can leave the *rete testis* and reach epididymis through the efferent ducts, bound to the androgen-binding protein (ABP). This protein has a higher concentration in the *caput* when compared to *cauda* of the epididymis, which can be explained because the *caput* also has the highest metabolic activity in the entire organ. Testosterone can also enter in the epididymis cells by passive diffusion, where it can be reduced to DHT. This metabolite is more potent than testosterone, and has been considered to have the main androgenic action in this organ. In the lumen of the epididymis the concentration of DHT is higher than that in the circulation, and as a

consequence of that, the ratio of testosterone to DHT declines as sperm passes through the epididymal duct<sup>36</sup>.

Besides the androgens, there are also other factors and hormones that play an important role in the regulation of the epididymis function. It is the case of prolactin, melatonin, vitamin D, progesterone, growth factors and estrogens<sup>36</sup>. This class of steroids are considered female hormones. In men, the serum concentration of estrogens is around 150 pmol/L compared to the 400 pmol/L found in the women<sup>41</sup>. However, nowadays there is much evidence that estrogens also play an important role in the male reproductive tract<sup>42</sup>. Germ cells and the epididymal sperm both express the P450 aromatase that synthesizes estrogens from androgens. namely, 17 $\beta$ -estradiol from testosterone<sup>41,42</sup>. High concentrations of 17 $\beta$ -estradiol have been found in the intratesticular fluid, and these hormones have shown an important role in spermatogenesis, erectile function and in modulating the libido<sup>43</sup>. However, the role of this steroid hormone in the male reproductive tract is still controversial, as there are also studies that demonstrate negative effects. For example, exposure to estradiol in early life affects spermatogenesis and impairs the potential reproductive system of the adult, causing adverse effects on male sexual differentiation<sup>44</sup>.

### **3. An insight on the Metabolism of epididymis**

To fertilize an oocyte sperm needs to mature in the epididymis and have the adequate supply of energy to ensure its survival and movement in the female reproductive tract after ejaculation<sup>45,46</sup>. As in other cell types, epididymis depends on glucose to obtain energy in form of ATP<sup>45,47</sup> for the normal epididymal function, and epididymal secretions into the epididymal lumen play a relevant nourishing sperm. In fact, it was recently demonstrated that several energy pathways are active in the *caput* region, which includes glycolysis, and pyruvate and, glycerophospholipid metabolism<sup>27</sup>. Moreover, it was shown that the activity of key enzymes of the glycolytic pathway varies along the epididymis, with the highest activity detected in the *caput*<sup>48</sup>. The presence of glycolytic enzymes in the epididymis has been shown to be essential for spermatozoa function, as these cells have a high glycolytic capacity and depend almost exclusively on hexoses (e.g. glucose, fructose) for energy<sup>45</sup>. For example, in the case of bovine, sperm in the epididymis appears to be more glycolytic than ejaculated sperm<sup>49</sup>.

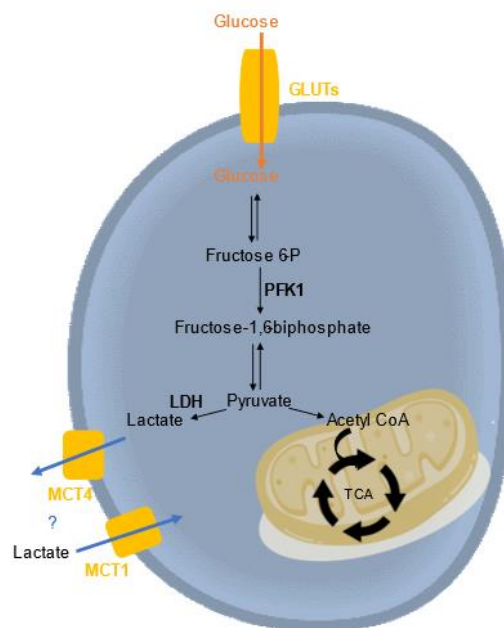
Glucose is uptake from the luminal milieu by the epididymal cells through the activity of GLUTs. GLUTs are proteins that transport monosaccharides and other small carbon compounds across the cell membrane. There are 14 GLUT proteins, divided into 3

classes based on the sequence similarity<sup>50</sup>. GLUTs 1-5 are the best studied with their activity and function being described in different cells and tissues. GLUT1 is expressed in erythrocytes, brain, and many other cell types, and is capable of transporting galactose, mannose and glucosamine. GLUT2 is the major transporter of hepatocytes and pancreas whereas GLUT3 is abundantly expressed in the brain and testis. GLUT4 is expressed in the muscle, heart and adipocytes. The GLUT5 isoform has a high specificity for fructose being found in the intestine<sup>50</sup>. It has been described the presence of GLUT1 and GLUT3 in the epididymis of human and rat<sup>45,51</sup>. The presence of GLUT2 was already described in the male reproductive tract, but only in the testis<sup>45,51</sup>.

In glycolysis (figure 3), one molecule of glucose is degraded into two molecules of pyruvate. This process is divided into 10 steps. In these steps there are 2 phases: the Preparatory Phase and the Pay-off Phase. The first phase begins with the phosphorylation of glucose and ends with its conversion into glyceraldehyde 3-phosphate. Furthermore, it is in this phase that phosphofructokinase 1 (PFK1) catalyses the irreversible conversion of fructose-6-phosphate into fructose-1,6-biphosphate, which is a rate-limiting step in glycolysis.

In the second phase, occurs the conversion of glyceraldehyde-3-phosphate into pyruvate. These reactions lead to the formation of ATP and NADH, and due to that, there is a recovery of the energy spent in the previous phase<sup>47</sup>. The pyruvate obtained can follow three different pathways: the tricarboxylic acid cycle (TCA) (in aerobic conditions, to be used in the electron transport chain), alcoholic fermentation (in conditions of hypoxia or anaerobioses it forms ethanol) and lactic fermentation. This last pathway converts pyruvate into lactate through the activity of lactate dehydrogenase (LDH) (figure 3). This reaction is supposed to occur mainly in anaerobic conditions<sup>52</sup> as in aerobic pyruvate is driven to oxidation in the mitochondria producing ATP. However, the somatic testicular cell, Sertoli cells, and similarly to cancer cells, follow the so-called Warburg effect, i.e. metabolize glucose to produce high amounts of lactate even in aerobic conditions<sup>53</sup>. Lactate has been shown to be the preferred energy substrate used by germ cells, and despite being present in the epididymis<sup>54</sup>, the production and availability of this substrate as a source of energy by epididymal sperm is not completely understood yet. Also, it is unknown if epididymal cells use the Warburg effect. Nevertheless, a report exists assuming that the different parts of the bovine epididymis may have sufficient LDH present to utilize lactate extensively as an energy source<sup>55</sup>.

Nevertheless, it is expected that the lactate produced is exported to the extracellular space through the activity of monocarboxylate transporters (MCTs)<sup>53</sup>. MCTs are responsible for the transport of several metabolites, such as pyruvate, lactate and ketone bodies, across cell membranes. Among the 14 isoforms of MCTs identified in different cells and tissues MCT1–4 are extensively studied<sup>56</sup>. MCT4 seems to be the one responsible for the export of lactate<sup>56</sup>. With this rationale, lactate is made available in the extracellular environment and could be used, instead of glucose, as an energy substrate by spermatozoa in the epididymis. However, MCTs are bidirectional, and, for example, MCT1 can uptake lactate from the extracellular space, which can be reused in the citric acid cycle<sup>53</sup> (figure 3). The presence of MCTs in the epididymis has not been reported yet, and the idea of lactate handling by epididymal cells needs confirmation.



**Figure 3. Schematic representation of the glycolytic pathway with identification of the main products and molecular targets.** Glucose enters the cell by the action of glucose transporters (GLUTs), being metabolized in the glycolytic pathway to fructose-1,6-bisphosphate by the action of phosphofruktokinase 1 (PFK1). The end product of glycolysis, pyruvate, is oxidized in the Tricarboxylic Acid Cycle (TCA). Alternatively, and especially under anaerobic conditions, pyruvate can be converted to lactate by the action of lactate dehydrogenase (LDH). Lactate is supposed to be exported to the extracellular environment by the action of monocarboxylate transporters (MCTs). The use of lactate as an energy substrate by epididymal cells needs confirmation as well as the expression of MCTs.

The lipid composition of sperm plasmatic membrane is modified during transit through the epididymis, with the remodelling events in lipid profile being crucial to maintain cellular integrity and ensure fertilization ability<sup>57</sup>. It has been suggested that these modifications are more likely to occur in the passage through the *caput* region, as sperm plasmatic membrane is flexible, facilitating lipid remodeling<sup>58</sup>. It is also established that the epididymis and the protein and lipid constituents of epididymal fluid determine the lipid remodeling of spermatozoa<sup>57,59,60</sup>. For this reason,

environmental factors that deregulate lipid metabolism in the epididymis can significantly hamper sperm function.

Lipids represent about 1,25% of the total weight of the epididymis, where 64% are formed by phospholipids, 12% by cholesterol and 24% by glycerides<sup>61</sup>. As comparison, mature sperm after epididymal transit are composed of 70% phospholipids, 25% neutral lipids (mainly cholesterol) and 5% glycolipids<sup>62</sup>.

Considering lipid metabolism there are two main pathways that are privileged depending on the presence/lack of glucose, i.e. the synthesis and the degradation of lipids.

Acetyl-CoA derived from both carbohydrates and lipid metabolism (and also amino acids), is an intermediary fuelling the TCA in the mitochondrial matrix (figure 4). This intermediary can be completely oxidated in CO<sub>2</sub>, resulting in conservation of more energy, once the electrons pass to O<sub>2</sub> through the mitochondrial respiratory chain, providing the energy for ATP synthesis by oxidative phosphorylation<sup>47</sup>.

Acetyl-CoA is also a precursor for fatty acid synthesis (figure 4). This pathway starts with the formation of malonyl-CoA through an irreversible reaction catalysed by acetyl-CoA carboxylase (ACC). This enzyme has a prosthetic group, biotin, that acts as a temporary transporter of CO<sub>2</sub>, helping in the formation of malonyl-CoA. ACC activity is highly regulated allosterically by citrate and long-chain fatty acid, and the reaction catalysed is considered a limiting step in fatty acid synthesis. Also, ACC can be regulated by hormone action, namely by insulin, glucagon, cortisol, epinephrine and norepinephrine. In the epididymis, ACC is reported to be activated in fat tissue with a temperature around 30 °C. Moreover, under different physiological conditions (chronic diabetes or fasting), different forms of the catalytic activity of carboxylase exists, independent of polymerization<sup>63,64</sup>. However, very little is known considering the activity of this enzyme in the epididymis secretory cells.

Malonyl-CoA, with NADPH and by the activity of fatty acid synthase (FASN) can be converted into palmitate that is the main source for formation of long-chain fatty acids. Polyunsaturated fatty acid biosynthesis in epididymis cells and expression of FASN was described, for example in stallion<sup>65</sup>. In epididymal adipose tissue, lipogenic enzymes (ACC and FASN) reached maximum expression levels at 24 h after refeeding a carbohydrates/protein diet, compared to the 16 h in the liver<sup>66</sup>. Studies also suggested that the adipose tissue of epididymis is quite adaptive in response of changes in dietary status and hormonal manipulation: starvation results in a lowering of the activity of these two enzymes and of the rate of fatty acid synthesis; refeeding results in an

increase in these parameters<sup>67</sup>. For example, in rats, fatty acid deficiency was shown to increase the epididymal expression and activity of fatty acid desaturase, other enzyme of the fatty acid biosynthetic pathway<sup>68</sup>, which was linked to altered fatty acid profile in the spermatozoa during transport from the testis to *cauda* epididymis and significant increase in the number of abnormal spermatozoa. Furthermore, it is widely established that FASN and other enzymes of the fatty acid biosynthetic pathway are regulated by the action of androgens, which play a relevant role as metabolic regulators<sup>69</sup>.

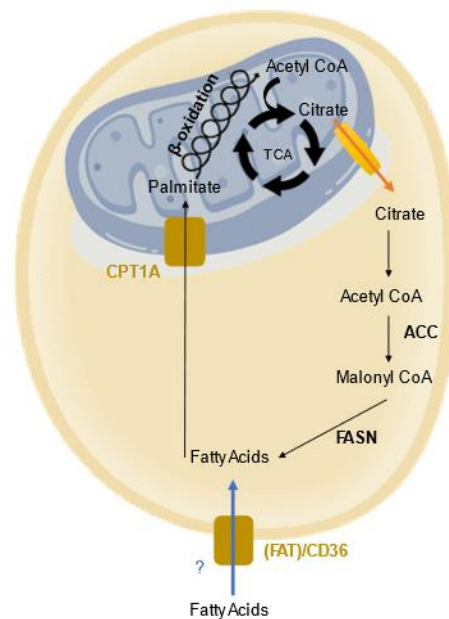
On the other hand, in bovine epididymis, acetate, a precursor of cholesterol, is more readily metabolized than glucose but less than other TCA intermediates<sup>70</sup>.

Malonyl-CoA also can regulate the carnitine palmitoyl transferase I (CPT1A), which allows the translocation of fatty acids into the mitochondria to  $\beta$ -oxidation (figure 4)<sup>47,71,72</sup>. Mitochondrial oxidation of fatty acids occurs in 3 steps:  $\beta$ -oxidation, the oxidation of acetyl groups to CO<sub>2</sub> in the citric acid cycle, and the oxidative phosphorylation<sup>47</sup>. In  $\beta$ -oxidation fatty acids undergo oxidative removal of successive units of two carbons in the form of acyl-CoA starting at the carboxylic end of the fatty acyl chain. The overall result is converting the 16-carbon chain of palmitate into eight two-carbon acetyl groups of acetyl-CoA molecules<sup>47</sup>. The energetic balance from palmitate can lead to 108 ATP, while energetic balance from glycolysis only lead to 32 ATP<sup>47</sup>.

The lipid composition and fatty acid content of the epididymal fluid has been characterized. For example, in bats the proportion of polyunsaturated fatty acids usually increases from the *caput* towards the *cauda* epididymis<sup>73</sup>. Similarly, a study in dogs identified higher contents of saturated fatty acids in the epididymal fluid from *cauda*, whereas the *caput*, *corpus* and *cauda* fluid was enriched in monounsaturated and polyunsaturated fatty acid, namely docosahexaenoic acid (DHA) and other fatty acids such as caprylic, capric, heptadecanoic and nervonic<sup>74</sup>.

The delivery of lipids by the epididymal cells has been suggested to occur via mechanisms of apocrine secretion through vesicles, the epididymosomes<sup>75</sup>. These vesicles undergo modifications along the transit in the epididymis, as it was observed that epididymosomes from the *cauda* are different from those in the *caput*<sup>75</sup>. Therefore, epididymosomes' lipid and protein content are continuously evolving. In several cell types, such as liver and adipocytes, as well as in lysosomes, there is evidence for the presence of membrane transporters involved in the efflux of fatty acids<sup>76</sup>. However, no reports exist on the activity of these transporters in the epididymis.

Considering the influx of fatty acids a variety of proteins have been associated with the transport of long-chain fatty acids, which is the case of the FA translocase (FAT)/CD36<sup>77</sup>. The CD36 is a multispecific membrane glycoprotein that has been postulated to have multiple functions<sup>78</sup>, including the membrane transport of long-chain fatty acids. It can bind to multiple ligands and can mediate different biological processes, acting as a signalling hub in angiogenesis and inflammatory response<sup>79</sup>. When expressed, this glycoprotein results in an increased rate of fatty acid uptake and increased rate of fatty acid metabolism<sup>80</sup>. Similarly to the efflux transporters, the presence and function of CD36 in epididymal secretory cells has not been described. However, in epididymal fat CD36 was shown to have an adaptive response to different fasting conditions<sup>81</sup>, like the other enzymes described. Also, previous findings of our research group has shown that the expression of CD36 is a target of regulation by androgens in prostate cells<sup>82</sup>.



**Figure 4. Schematic representation of the glycolytic pathway with identification of the main products and molecular targets.** Acetyl-CoA is converted into malonyl-CoA by the action of acetyl CoA carboxylase (ACC). Malonyl-CoA through the action of fatty acid synthase (FASN) can be converted into fatty acids. Malonyl-CoA can also regulate carnitine palmitoyl transferase I (CPT1A) that allows the translocation of fatty acids into the mitochondria. Here, through  $\beta$ -oxidation, palmitate is converted into acetyl-CoA. Citrate can enter the biosynthetic pathway by its conversion to acetyl-CoA, and cells can uptake fatty acids from the extracellular space by the activity of fatty acid transporters, such as CD36. The expression and activity of CD36 in the epididymis needs confirmation.

## **4. Endocrine Disrupting Chemicals (EDCs)**

### **4.1. Classification**

In 1996 the USA Environmental Protection Agency formally introduced the term endocrine disruptors (EDs). These are defined as exogenous compounds capable of altering the structure or function of the endocrine system, by compromising the synthesis, action, binding, transport, secretion or elimination of natural hormones, thereby affecting the hormone production, release and metabolism<sup>83-86</sup>. EDCs also can mimic the occurrence of natural hormones affecting cell, tissues, organisms and even populations or sub-populations of organisms<sup>83,84</sup>.

EDCs can be found in the most basic products, such as food, plastic bottles, cosmetics, toys and detergents<sup>86</sup>. The exposure can occur by different entry ways: by ingestion (that can bioaccumulate in fatty tissue due to lipophilicity), by inhalation (specially for some volatile chemicals and semi-volatile compounds) and by dermal uptake<sup>86</sup>.

EDs are usually found in fungicides (e.g. vinclozolin - which actions will be discussed in the next section), pesticides (e.g. methoxychlor) and in plasticizers (e.g. phthalates)<sup>87,88</sup>. EDCs can bind and are capable of interfere with the action of different hormones receptors, such as receptors for thyroid hormones, steroid receptors and also with receptors for protein hormones. For this reason, EDCs widely affect the endocrine system, and as expected, there is a much body of evidence that they also interfere with reproduction, in particular with the development and sex differentiation<sup>83,89</sup>. EDCs actions have been shown to have negative effects on the male reproductive tract by decreasing human adult sperm number and motility, as well as disrupting normal sperm morphology<sup>86,87</sup>. Interestingly, it was reported that in sperm treated with EDCs such as TCDD (tetrachlorodibenzo-p-dioxin), the ratio of Y/X live spermatozoa is significantly lower, due to a decrease in the number of Y spermatozoa in the presence of EDCs<sup>86,87</sup>. Several of these compounds also have been associated with birth defects like hypospadias and cryptorchidism, and prostate and testicular cancer.

### **4.2. Mechanisms of Action**

Endocrine disruptors exert their effects through five different mechanisms of action: nuclear receptors (NRs) binding, membrane receptors interaction, intracellular signalling pathways interaction, epigenetic modifications and altered expression of non-coding RNAs, and disruption of hormone synthesis and metabolism.

NRs recruit a set of coactivating proteins and the basal transcription machinery, by activating transcription and binding to hormonal response elements in the regulatory region of target genes<sup>90-92</sup>. The family of NRs includes several members, namely the sub-family of steroid hormone receptors, as is the case of estrogen receptors  $\alpha$  and  $\beta$  (ER $\alpha$  and ER $\beta$ ) and androgen receptors (AR). Other NR target of EDCs are hydrocarbon receptor (AhR), peroxisome proliferator-activated receptor (PPAR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR)<sup>90,91,93,94</sup>. AhR, CAR and PXR are important regulators of metabolism, by mediating the induction of cytochrome P450 enzymes and transporters<sup>90</sup>. The PPAR pathway modulates the expression of genes involved in adipogenesis, cellular differentiation and hormone and energy homeostasis<sup>95</sup>. The abnormal activation of each of these receptors can have deleterious consequences. If interfering with the ERs and AR, EDCs would hamper the normal action of estrogens and androgens, respectively. Thus, leading to consequences at reproductive and hormonal levels<sup>91</sup>.

Several EDCs have been identified acting as agonists or antagonists of the ERs and AR, thus stimulating or inhibiting the activity of these steroid receptors. This can change dramatically the transcriptome and the protein network active in a cell in a given moment, which might have significant physiological repercussions. Also, EDCs have been shown to interfere with membrane receptors, affecting the process of signalling transduction through the 2<sup>nd</sup> messengers and the ion channels, for example. These actions have effects in a large variety of organs such as the gonads and the accessory sex organs (prostate and seminal vesicles in the men, uterus and mammary gland in women), the brain and the pituitary gland<sup>89</sup>.

EDCs can also interact with the steroid membrane receptors, mER $\alpha$ , mER $\beta$  and the G protein-coupled estrogen receptor (GPER1)<sup>96-98</sup>. The mER $\alpha$  and mER $\beta$  are classic nuclear estrogen receptors, ER $\alpha$  and ER $\beta$ , respectively, which act as ligand-activated transcription factors and have been shown to be translocated to the plasma membrane<sup>96-98</sup>. Estrogen-mediated signalling can be divided into genomic and non-genomic due to the direct or indirect binding to DNA of the estrogen-receptor complexes. In other hand, GPER1 has an important role in mediating fast responses to hormones like estrogens and through second messengers that can activate intracellular signalling cascade<sup>98-100</sup>.

In the cytosol, hormones can interact with other protein targets. Hormones, like estrogens, have mitogenic effects that can be linked to the activation of different signalling pathways, namely the Src/Ras/ERK pathway. This Src/Ras/ERK signaling

cascade is activated by the change of conformation of the kinase to an active state, that occurs due to the interaction of the cytosolic ERs with the c-Src kinase<sup>101</sup>.

EDCs also have been shown to influence the cell epigenetic panorama, at three main levels: microRNA expression, DNA methylation and post-translational histone modification<sup>87,102–107</sup>. Some studies were made to see the effect of VNZ during gestation and the results showed that it promotes a male germline epigenome reprogramming that can cause transgenerational diseases, due to the disrupting of the DNA methylation in sperm up to the next 3 generations<sup>87,106</sup>.

The last mechanism by which EDCs can exert their effects is the interference with the hormone synthesis and metabolism. EDCs interfere with the hormone bioavailability due to its activity in level of the transport and secretion or by disrupting the enzymatic pathways. EDCs interfere with aromatase by stimulating or inhibiting its activity, and also inhibits other p450 enzymes, being the most common the CYP1, CYP2, and CYP3, which are responsible for drug and steroid metabolism<sup>108–110</sup>. At last, EDCs also affect neuroendocrine homeostasis, mainly GnRH levels that control FSH and LH production<sup>111,112</sup>.

### **4.3. Antiandrogens in Endocrine Disruption: a focus on Vinclozolin**

Nowadays, humans are exposed to a panoply of substances that often cause health problems. One example of these harmful substances are pesticides that have several consequences, especially for the consumers of food, production workers and for formulators and other applicators. Pesticides residues cause a big public concern since they are found in food and water, and also because there are many evidence that fungicides, for example, have the ability to induce tumors<sup>113</sup>.

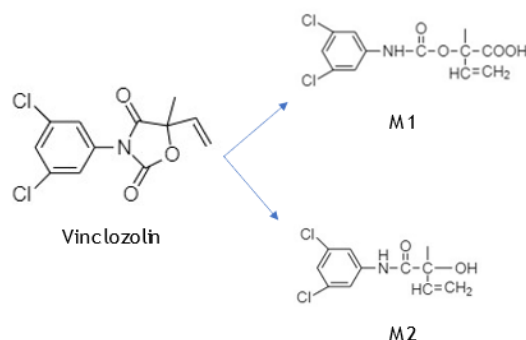
VNZ 3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-2, 4-oxazolidinedione) is a systemic dicarboximide fungicide used in several fruits and vegetables, ornamentals plants and also on turf grass to inhibit spore germination<sup>114–118</sup>. It can also be used to prevent grey mold and brown spot in fruit trees<sup>87,114–118</sup>.

VNZ has two metabolites, M1 ([2- [(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenic acid]) and M2 ([3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide]), mainly known as EDs. These products act as anti-androgens by its actions both as a 5 $\alpha$ -reductase inhibitor and as an AR antagonist<sup>87,116,119</sup>. There are studies showing that *in vitro*, these two metabolites competitively inhibit the AR and that *in vivo*, VNZ also inhibits gene expression dependent of AR and produce some anatomical defects<sup>116</sup>, like

the decline in human sperm counts and the increased incidence of urogenital malformations<sup>120</sup>.

M1 and M2 are strong AR antagonists with a  $k_i$  of 92 and 9,7  $\mu\text{M}$ , respectively, comparing to the weak ability of VNZ to compete for androgen binding to the AR which have a  $k_i > 700 \mu\text{M}$ . With this in mind, we can conclude that these two metabolites are primary causal agents for the *in vivo* actions of the VNZ<sup>87,121</sup>.

Several studies investigated the effects of VNZ administration in pregnant rats during the period of sexual differentiation. VNZ-treated female show malformations or functional alterations and the VNZ-treated males show female-like anogenital distance at birth, epididymal granulomas, suprainguinal ectopic testes, blind vaginal pouch, small to absent sex accessory glands, cleft phallus with hypospadias and birth retained nipples<sup>114,120</sup>. Our research group recently revised the effects of VNZ in male reproduction by a systematic review and meta-analysis<sup>114</sup>. Overall, VNZ is capable of decrease testosterone levels, increase the number of apoptotic germ cells, reduce testis and epididymis weight, decreased sperm number, daily sperm production and sperm motility<sup>114</sup>. However, the effects of VNZ in the *caput* epididymis metabolism are largely unknown.



**Figure 5. Schematic representation of the chemical structure of VNZ and its active metabolites.**

## ***II – Aim of Dissertation***

The testicular spermatozoa are immature and only acquire motility and the ability to fertilize oocyte after transit through the epididymis. Besides its role in concentrating and transport the sperm, the epididymis has crucial actions related to nourishing, immunoprotection and the protection of the male gamete from oxidative stress. Moreover, the epididymis is used as sperm reservoir. Overall, the epididymis function is achieved by the integrated actions of each anatomic region. It is the activity of *caput*, *corpus* and *cauda* that establishes the specific luminal environment due to the varied cell composition of the epithelium. Also, it has been demonstrated that hormone regulation, among other factors, influences the activity of epididymis. Indeed, the synthesis of some secretory proteins that play an important role in preparing the sperm for the events of fertilization by modifying the surface characteristics, are androgen-dependent.

Ultimately, to fertilize an oocyte, sperm needs to mature in the epididymis and have the adequate supply of energy to ensure its survival and movement in the female reproductive tract after ejaculation. This organ depends on glucose to obtain energy in form of ATP for the normal epididymal function, and epididymal secretions into the epididymal lumen play a relevant nourishing sperm. The presence of glycolytic enzymes in the epididymis has been shown to be essential for spermatozoa function, as these cells have a high glycolytic capacity and depend almost exclusively on hexoses for energy. Also, the epididymal milieu has been shown to be rich in lipids, and these macromolecules play a crucial role in the structure of sperm membrane influencing the fertilization capacity. For this reason, environmental factors that deregulate the metabolism of the epididymis can have deleterious effects on sperm function.

EDCs are exogenous compounds capable of altering the synthesis, action, binding, transport, secretion and elimination of natural hormones, affecting the production, release and metabolism. This class of compounds mimic the occurrence of natural hormones, disrupting the endocrine system affecting cells, organisms and populations. Substantial evidence exists linking EDCs with the decreasing of sperm number and motility, as well as abnormalities in sperm morphology in humans. The EDC VNZ, is a systemic dicarboximide fungicide, that acts as anti-androgen and has been shown to affect male reproduction. However, the effect of VNZ in the epididymis, namely in the deregulation of metabolism is largely unknown.

The present dissertation aims to study the impact of VNZ exposure in the rat epididymis glycolytic and lipidic metabolism. For this purpose, *caput* epididymis sections were cultured *ex vivo* in the presence or absence of VNZ 500  $\mu$ M and, glucose consumption and lactate production, as well as, the expression and activity of glycolytic and lipidic proteins were evaluated.

## ***III – Materials and Methods***

### **1. Animals and tissue collection**

Wistar rats (*Rattus norvegicus*) were obtained from Charles River (Barcelona, Spain) and handled in compliance with the European Union rules for the care and use of laboratory animals (Directive 2010/63/EU), and with the guidelines established by the “Guide for the Care and Use of Laboratory Animals”, published by the US National Institutes of Health (NIH Publication No. 85-23, revised on 1996). Rats were housed under a 12 h light:12 h darkness cycle, in a constant room temperature ( $20 \pm 2$  °C) with food and water available *ad libitum*. Three month-old male animals (n=7) were euthanized with carbon dioxide (CO<sub>2</sub>) and the epididymides removed, trimmed free of fat and washed in cold phosphate-buffered saline (PBS).

### **2. Culture of *caput* epididymis**

For each collected epididymis the three functional segments were identified, i.e. *caput*, *corpus* and *cauda*, and the *caput* region from the left epididymis (n=7) was placed in 6-well culture plates (VWR, Radnor, USA) containing 4 mL of pre-warmed RPMI (Sigma-Aldrich, UK) culture medium (control group). The contralateral *caput* epididymis was placed in RPMI culture medium containing 500 μM VNZ (n=7) (45705, Merck, UK). After 48 hours of culture in an atmosphere of 5% CO<sub>2</sub> at 37 °C, the control and VNZ-exposed *caput* epididymides were recovered from the medium, snap frozen in liquid nitrogen, and stored at -80 °C until protein extraction. In addition, epididymis culture medium was collected and stored at -80°C for the colorimetric quantification of glucose and lactate levels.

### **3. Quantification of Glucose and Lactate**

The glucose consumption and lactate production were determined through spectrophotometric assays using commercial kits (Spinreact, Girona, Spain) following the manufacturer’s protocols. For this purpose, the culture medium of the *caput ex vivo* cultures was collected at 0 h and 48 h from the control and VNZ-exposed groups. For glucose quantification, kit R1 buffer (92 mmol/L Tris, pH 7.4 and 0.3 mmol/L phenol) was used to dissolve R2 powdered enzyme mix (15000 u/L glucose oxidase, 1000 u/L peroxidase, 2.6 mmol/L 4-aminophenazone). In a 96 well-plate, 100 μL of this working solution were added to 1 μL of each sample from both control and VNZ-treated groups (n=7/group). Blank is composed of 100 μL working solution and the standard by 100 μL working solution with addition of 1 μL of the aqueous glucose calibration solution.

The plate was incubated for 10 minutes at 37 °C and the absorbance values were read at 505 nm (violet). The lactate production was evaluated following a similar methodology. R1 buffer (50 mmol/L PIPES, pH 7.5, 4 mmol/L 4-chlorophenol) was used to dissolve R2 powdered enzyme mix (800 u/L lactate oxidase, 2000 u/L peroxidase, 0.4 mmol/L 4-aminophenazone). In a 96 well-plate, 100 µL of the working solution were added to 1 µL of each sample from both control and VNZ-treated groups (n=7). Blank is composed of 100 µL working reagent and the standard by 100 µL working reagent with addition of 1 µL of the aqueous lactate calibration solution. The plate was incubated for 5 minutes at 37 °C and the absorbance values were read at 505 nm (violet). The glucose consumption and lactate production by epididymides cells in response to the VNZ were calculated in comparison with the glucose and lactate content at 0 h, and normalized per mg of tissue in each experimental group.

#### **4. Protein Extraction and Quantification**

All epididymis tissues (control and VNZ-treated) were homogenized in RIPA buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0 and 1 mM EDTA) supplemented with 1% protease cocktail inhibitors (Sigma-Aldrich) and 10% phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich). 3 µL of RIPA per mg of tissue was added to each sample, that was then macerated with cotton swabs to obtain small pieces of tissue. Samples were kept on ice for 40 min to 1 hour and mixed from time to time. Then, all samples were centrifuged at 14000 rpm, 4 °C, for 20 min, and the supernatant was collected to fresh tubes and kept at - 80 °C. Total protein concentration was determined through the Bicinchoninic Acid Protein (BCA) method, using Bovine Serum Albumin as standard.

#### **5. Western Blot**

Total proteins were denatured at 100 °C for 5 min using loading buffer (10% 2-Mercaptoethanol) and resolved on 12,5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). Membranes were incubated overnight at 4 °C with the rabbit anti-GLUT1 (1: 1000, CBL242, Sigma-Aldrich, Missouri, USA), rabbit anti GLUT3 (1: 1000, sc-30107, Santa Cruz Biotechnology, California, USA), rabbit anti-MCT1 (1: 1000, PA5-72957, Thermo Fisher, Massachusetts, USA), rabbit anti-LDH (1: 1000, Ab52488, Abcam, Cambridge, UK) and rabbit anti-PFK1 (1: 1000, sc-67028, Santa Cruz Biotechnology), rabbit anti-FasN (1: 1000, 3180S, Cell Signaling Technology, Massachusetts, USA), rabbit anti-ACC (1: 1000, 3662S, Cell Signaling

Technology), rabbit anti-CPT1A (1: 1000, Ab128568, Abcam), rabbit anti-CD36 (1: 1000, Ab64014, Abcam) primary antibodies. A mouse anti- $\beta$ -actin (1: 10000, 5441, Sigma-Aldrich) antibody was used for protein loading control in all WB analyses. Mouse-IgG $\kappa$  BP-HRP (1: 10000, SC-516102, Santa Cruz Biotechnology) and goat anti-rabbit IgG-HRP (1: 10000, 1706515, Bio-Rad) were used as secondary antibodies. Membranes were developed with enhanced chemiluminescence (ECL) substrate (Bio-Rad) for 5 minutes and immune-reactive proteins were detected with the ChemiDoc™ MP Imaging System (Bio-Rad). Bands density was assessed according to standard methods using the Image Lab 5.1 software (Bio-Rad) and normalized by division with the respective  $\beta$ -actin band density.

## **6. LDH activity assay**

The enzymatic activity of LDH was determined using a commercial kit (Spinreact) according to the manufacturer's instructions. The working reagent (WR) was prepared by mixing the assay kit's set buffer (imidazole, 65 mmol/L and pyruvate, 0,6 mmol/L) with an NADH (0,18 mmol/L) in a ratio of 4:1. In each well of a 96-well plate, 5  $\mu$ l of protein sample were added to 300  $\mu$ l of WR, and the plate was both mixed and incubated for 1 minute at 37 °C in a Bio Rad xMark™ Microplate Absorbance Spectrophotometer. An initial absorbance was read, followed by subsequent readings every minute for 5 minutes, still maintaining a constant temperature of 37 °C. The assay's principle consists in the fact that LDH catalyses the reduction of pyruvate by NADH (both present in the working reagent) into lactate. The rate of decrease in the concentration of NADH, measured photometrically at 340 nm, is proportional to the catalytic concentration of LDH present in the sample. The method was calibrated using an LDH positive control. The activity was calculated as U/L per mg of protein.

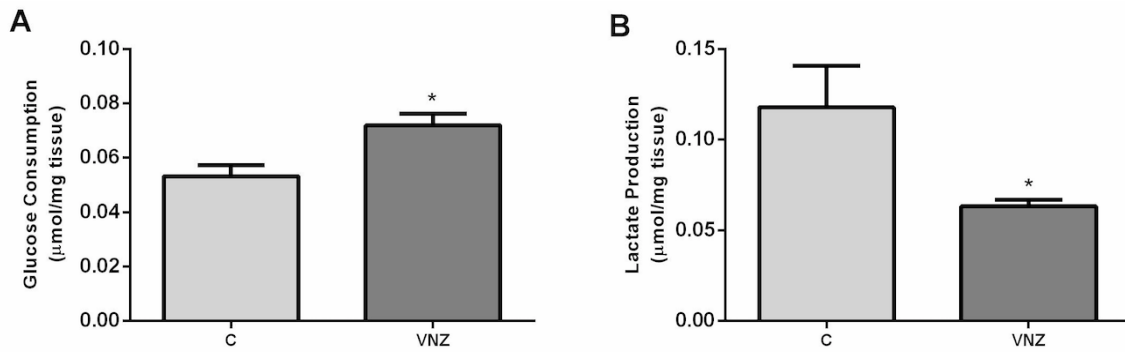
## **7. Statistical analysis**

The statistically significant differences between the control and VNZ-treated groups were assessed by the Unpaired Student's T-Test. The differences were considered significant when  $p < 0.05$ . Data analysis was performed using the GraphPad Prism v6.01 software (GraphPad Software, San Diego, California, USA). Experimental data are shown as mean  $\pm$  SEM.

## IV – Results

### 1. Glycolytic metabolism in the epididymis in the presence of VNZ

Glucose and lactate were quantified in the culture media to understand how glucose is metabolized by the epididymis, and how its metabolism is altered upon exposure to VNZ. Results of spectrophotometric analysis showed that glucose consumption (figure 6A) was increased in presence of VNZ when compared to the control group (Control:  $0.05325 \pm 0.004087$  vs. VNZ:  $0.07207 \pm 0.004194$   $\mu\text{mol}/\text{mg}$ ,  $p < 0.05$ ). The opposite happened with the lactate production (figure 6B), which has decreased with VNZ treatment (Control:  $0.1179 \pm 0.02322$  vs. VNZ:  $0.06326 \pm 0.003693$   $\mu\text{mol}/\text{mg}$ ,  $p < 0.05$ ).



**Figure 6. Glucose consumption (A) and lactate production (B) in the rat caput epididymis cultured for 48 h, in the presence and absence of 500  $\mu\text{M}$  VNZ.** Data are represented as mean  $\pm$  S.E.M. (n= 7 in each group). (\*) Statistically significant difference when compared with the control group in the absence of VNZ (\*  $p < 0.05$ ).

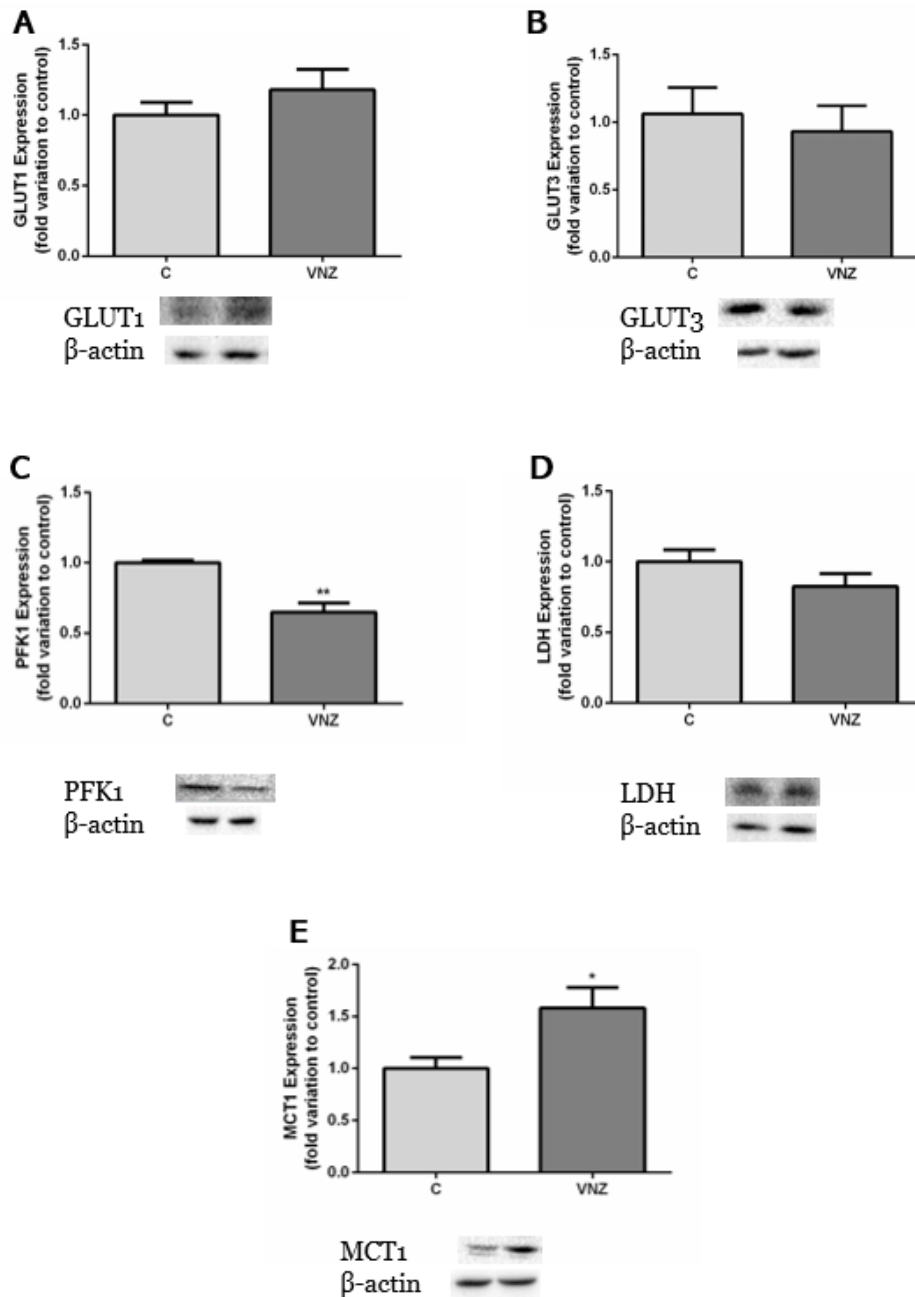
Glycolysis occurs inside the cytoplasm after glucose being uptake by plasma membrane glucose transporters (e.g. GLUT1) always in favour of the concentration gradient<sup>122</sup>. No significant differences were observed on the expression levels of GLUT1 (figure 7A) in consequence of VNZ exposure (control:  $1.00 \pm 0.08985$  vs. VNZ:  $1.179 \pm 0.1456$  fold variation). GLUT3 is another GLUT involved in the transport of glucose across plasma membranes in several cell types, including sperm<sup>123</sup>. GLUT3 expression levels (figure 7B) showed no significant differences between VNZ- treated and control epididymis (control:  $1.062 \pm 0.1947$  vs. VNZ:  $0.9319 \pm 0.1915$  fold variation).

Inside the cell, glucose is converted into pyruvate through a cascade of enzymatic reactions. The conversion of fructose-6-phosphate into fructose-1,6-biphosphate, a reaction catalysed by PFK1, is one of the most important steps in glycolysis since it determines the flux through the glycolytic pathway<sup>47,124</sup>. For this reason we analysed

PFK1 expression levels (figure 7C), which were shown to be significantly diminished in the VNZ-treated group compared to control (control:  $1.000 \pm 0.01768$  vs. VNZ:  $0.6492 \pm 0.06538$  fold variation,  $p < 0.01$ ).

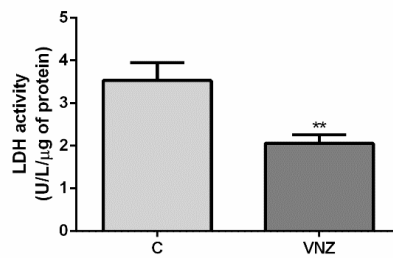
Another central player in the glycolytic metabolism is the LDH enzyme that is responsible for the reversible conversion of pyruvate into lactate. LDH expression levels (figure 7D) showed no significant alterations in response to VNZ treatment (control:  $1.00 \pm 0.0832$  vs. VNZ:  $0.9424 \pm 0.08851$  fold variation).

MCT1 mediates the uptake of lactate across cell membrane<sup>125</sup>, playing a relevant role in lactate handling. VNZ significantly increased MCT1 expression levels in the epididymis (figure 7E) compared with the control group (control:  $1.000 \pm 0.1047$  vs. VNZ:  $1.580 \pm 0.1947$  fold variation,  $p < 0.05$ ).



**Figure 7. Expression of GLUT1 (A), GLUT3 (B), PFK1 (C), LDH (D) and MCT1 (E) proteins in the rat caput epididymis cultured for 48 h, in the presence or absence of 500  $\mu$ M VNZ.** Data are represented as mean  $\pm$  S.E.M. (n= 7 in each group). Results are expressed as fold-variation to the control group in the absence of VNZ after normalization of protein expression with  $\beta$ -actin. (\*) Statistically significant difference when compared with the control group (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ). Representative blots are shown in bottom panels.

LDH activity (figure 8) was shown to be significantly diminished in VNZ-treated epididymis compared to control group (control:  $3.532 \pm 0.4214$  vs. VNZ:  $2.057 \pm 0.2033$  U/L/ $\mu$ g,  $p < 0.01$ ).

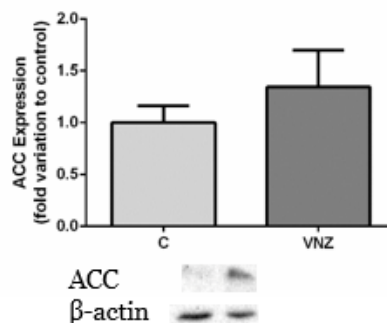


**Figure 8. LDH activity in the rat caput epididymis cultured for 48 h, in the presence and absence of 500 μM VNZ.** Data are represented as mean ± S.E.M. (n= 7 in each group). (\*) Statistically significant difference when compared with the control group in the absence of VNZ (\* p < 0.01).

## 2. Lipidic metabolism in the epididymis in the presence of VNZ

The epididymal milieu has been shown to be rich in lipid composition, and these macromolecules play a crucial role in the structure of sperm membrane influencing the fertilization capacity<sup>57</sup>. Therefore, we decided to investigate how lipid metabolism in the epididymis is affected by VNZ exposure.

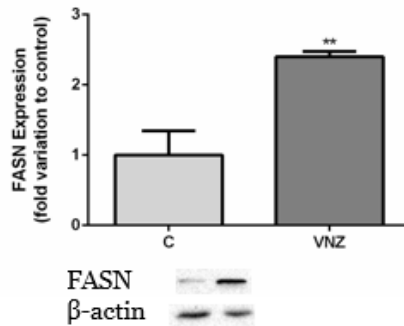
ACC is a target enzyme in fatty acid biosynthesis. It is a biotin-dependent enzyme and through its catalytic activity, as a carboxyltransferase and biotin carboxylase<sup>126</sup>, catalyses the carboxylation of acetyl-CoA to produce malonyl-CoA, a substrate for the formation of fatty acids. No significant alterations were observed on the expression levels of ACC in the *caput* epididymis (figure 9) in consequence of exposure to VNZ (control: 1.000 ± 0.1618 vs. VNZ: 1.344 ± 0.3559 fold variation).



**Figure 9. ACC expression in the rat caput epididymis cultured for 48 h, in the presence or absence of 500 μM VNZ.** Data are represented as mean ± S.E.M. (n= 7 in each group). Results are expressed as fold-variation to the control group in the absence of VNZ after normalization of protein expression with β-actin. A representative blot is shown in bottom panel.

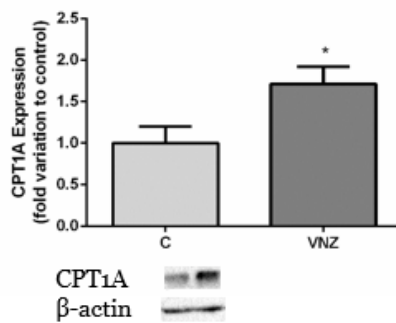
FASN is the central player in fatty acid biosynthesis, catalysing the synthesis of palmitate from acetyl-CoA and malonyl-CoA, into long-chain saturated fatty acids. The expression of this enzyme (figure 10) was shown to be highly increased in the presence

of VNZ compared with the control group (control:  $1.000 \pm 0,3449$  vs. VNZ:  $2,402 \pm 0,07106$  fold variation,  $p < 0.01$ ).



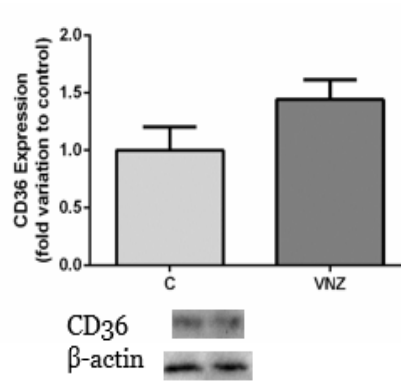
**Figure 10. FasN expression in the rat caput epididymis cultured for 48 h, in the presence and absence of 500  $\mu$ M VNZ.** Data are represented as mean  $\pm$  S.E.M. (n= 7 in each group). Results are expressed as fold-variation to the control group in the absence of VNZ after normalization of protein expression with  $\beta$ -actin. (\*\*) Statistically significant difference when compared with the control group in the absence of VNZ (\*\*  $p < 0.01$ ). A representative blot is shown in bottom panel.

CPT1A is the enzyme responsible for the formation of acyl carnitines by catalysing the transfer of the acyl group of a long-chain fatty acyl-CoA from coenzyme A to l-carnitine<sup>127,128</sup>, being a key player in fatty acid b-oxidation. WB analysis showed that VNZ significantly increased CPT1A expression levels (figure 11) in the caput epididymis compared with the control group (control:  $1.000 \pm 0.2002$  vs. VNZ:  $1.713 \pm 0.2093$  fold variation,  $p < 0.05$ ).



**Figure 11. CPT1A in the rat caput epididymis cultured for 48 h, in the presence and absence of 500  $\mu$ M VNZ.** Data are represented as mean  $\pm$  S.E.M. (n= 7 in each group). Results are expressed as fold-variation to the control group in the absence of VNZ after normalization of protein expression with  $\beta$ -actin. (\*) Statistically significant difference when compared with the control group in the absence of VNZ (\*  $p < 0.05$ ). A representative blot is shown in bottom panel.

We also investigated the effect of VNZ on the expression of fatty acid membrane transporters, namely CD36, an integral membrane glycoprotein that mediates the uptake of long-chain fatty acids through cell membrane and other membrane-bound organelles<sup>79</sup>. WB analysis showed no significant alterations on the expression of CD36 (figure 12) in response to VNZ treatment (control:  $1.000 \pm 0.2045$  vs. VNZ:  $1.443 \pm 0.1692$  fold variation).



**Figure 12. CD36 expression in the rat caput epididymis cultured for 48 h, in the presence and absence of 500  $\mu$ M VNZ.** Data are represented as mean  $\pm$  S.E.M. (n= 7 in each group). Results are expressed as fold-variation to the control group in the absence of VNZ after normalization of protein expression with  $\beta$ -actin. A representative blot is shown in bottom panel.

## V – Discussion

Epididymis has an important role in sperm maturation, as well as in sperm storage, and plays important actions increasing sperm concentration, nourishing sperm and providing immunological and oxidative stress protection<sup>5,8</sup>. To ensure its survival and movement, sperm needs a supply of energy that can be obtained in the epididymis by glucose metabolism<sup>44</sup>. Lipidic profile is also crucial to maintain cellular integrity and ensure fertilization ability<sup>57</sup>. Therefore, maintaining the integrity of the epididymis's metabolism and function is a crucial part to ensure sperm quality. Nevertheless, the metabolism and the metabolic routes active in the epididymis cells are still poorly investigated.

The epididymis is a target of hormone regulation that controls several of its functions including, the secretory/reabsorptive activities and metabolism. Exposure to hormone mimicking/antagonizing substances, the EDCs, can interfere with the male reproductive system, affecting sexual differentiation, triggering birth defects, and decreasing sperm number and mobility<sup>83,89</sup>. However, the deleterious effects of EDCs on the epididymis function is almost unknown.

This dissertation investigated the effect of VNZ, an anti-androgenic EDC that has been shown to have a significant impact on male fertility<sup>120</sup>, on the epididymis function. We focused on the VNZ actions affecting the glycolytic and lipid metabolism of the *caput* region, as this is the most active region in terms of hormonal regulation<sup>6</sup>. For this purpose, *ex vivo* cultures of *caput* epididymis were established based on a protocol previously developed by the research group<sup>129</sup>. This methodological approach with *ex vivo* culture of *caput* epididymis has been successfully used to study the organ morphology and function. For example, it was used for the analysis of Ca<sup>2+</sup> fluxes with the epididymis maintaining functional activity<sup>129</sup>.

The results obtained showed that exposure to 500  $\mu$ M VNZ increased glucose consumption (figure 6A), suggesting that under the influence of this anti-androgen epididymis is using glucose as a fuel to its metabolic activity. Only few studies have focused on the metabolic activity of epididymis, but it was reported that androgens exert a profound influence on the intermediary metabolism in the epididymis. In the presence of these steroid hormones, the epididymis rely substantially on lipid metabolism, whereas in the absence of androgens there is a switch to a complete dependence on carbohydrate as the metabolic fuel<sup>37</sup>, which is some in line with our findings using the anti-androgen VNZ.

Although glucose consumption was increased, no significant differences were observed on GLUT1 and GLUT3 expression (figure 7A and 7B). GLUT1 exerts its main role in the brain<sup>50</sup>, being capable of transporting glucose, galactose, mannose and glucosamine<sup>50</sup>, but its presence in the testis and epididymis has been reported<sup>130,131</sup>. GLUT3 is present in the sperm and has been shown to be highly expressed in the epididymis of human and rat<sup>45,50,51</sup>. This GLUT isoform controls glucose uptake and metabolism necessary for sperm maturation and motility<sup>132,133</sup>, but its functionality in the epididymis was not reported. Other GLUTs, such as GLUT2, have been detected in rat epididymis<sup>131</sup>. GLUT2 is the major glucose transporter in hepatocytes having a high capacity for glucose transport and is also very sensitive to glucose fluctuations<sup>50,131</sup>. Moreover, it was suggested that this GLUT isoform is important in male fertility and epididymis metabolism<sup>131</sup>. Gathering all these data, it can be considered that VNZ increases glucose consumption through the upregulation of the expression of other GLUTs, namely GLUT2.

PFK1, other key player in metabolizing glucose, determines the flux through the glycolytic pathway<sup>124</sup>. VNZ treatment decreased PFK1 expression in the *caput* epididymis (figure 7C), as indicated by the WB results. Considering the observed increase in glucose consumption upon exposure to VNZ, the diminished PFK1 expression seems contradictory. However, no definitive conclusion may be taken as we have not determined PFK1 activity, which may be augmented regardless of protein expression.

Glycolysis ends with the formation of pyruvate, which can follow different pathways<sup>52</sup>, namely the conversion to lactate by the activity of LDH<sup>52</sup>. The conversion of pyruvate to lactate occurs in different cells under anaerobiosis and is a typical feature of cancer cells even in aerobic conditions, being known as the Warburg effect<sup>134</sup>. This particularity of using glucose and pyruvate to produce lactate also has been found in the testicular cells Sertoli cells, being relevant to supply germ cells with this energy substrate<sup>53</sup>. Herein, we sought to investigate if VNZ could affect lactate production by epididymal cells. Although no difference was observed on LDH expression (figure 7D), treatment with VNZ decreased LDH activity (figure 8) and lactate production (figure 6B). The reduced lactate content is surely correlated with the decreased LDH activity in the *caput* epididymis. Lactate has been shown to be the preferred energy substrate used by germ cells, and identified as a constituent of the epididymal fluid<sup>54</sup>. However, the production and availability of this substrate as a source of energy for sperm provided by the epididymis has remained unknown. Our findings suggest that VNZ exposure may alter the epididymis metabolic profile compromising lactate accumulation in the epididymal

fluid, which can negatively affect sperm functionality, as this has been indicated as a survival factor and the preferred energy substrate for germ cells<sup>131</sup>. The importance of lactate production by epididymis cells is corroborated by a report showing that the bovine epididymis has sufficient LDH to utilize lactate extensively as an energy source<sup>55</sup>.

The increased glucose consumption, and reduced lactate production observed, together with the diminished PFK1 expression and decreased LDH activity, allow us to speculate that VNZ-treated epididymis is not using glucose into the glycolytic pathway. Storage of glucose (glycogen synthesis) in the epididymis has not been described but the secretion of glycogen by epididymis epithelial cells is known since 1954<sup>135</sup>.

MCT1 is a monocarboxylate transporter that mediates the uptake of lactate across cell membrane<sup>125</sup>. The presence of MCTs in the epididymis has not been reported yet. Therefore, we decided to analyse MCT1 expression. WB analysis demonstrated that VNZ increases MCT1 expression (figure 7E) in the *caput* region. This result led us to hypothesize that lactate is entering into epididymis cells and can be used as metabolic fuel. In fact, lactate can be converted back into pyruvate, which can follow the TCA and end up generating energy from oxidative phosphorylation<sup>47</sup>. Further research is necessary to completely ascertain this question.

Overall, our results indicated that VNZ increases glucose consumption whereas decreasing lactated production in the *caput* epididymis. However, further research is needed to be completely ascertain how VNZ disrupts the glycolytic pathway. Concerning lipid metabolism, the expression of four major targets involved in the biosynthetic (ACC and FASN) and catabolic (CPT1A) pathways, and fatty acid uptake (CD36) was analysed.

VNZ-treated *ex vivo caput* epididymis presented an increased expression of FASN (figure 10), and ACC expression also was slightly increased though not significant (figure 9). Therefore, we can assume that fatty acid synthesis is increased in epididymal cell in the presence of the anti-androgen VNZ, as ACC and FASN catalyse rate limiting steps in fatty acid synthesis<sup>66</sup>. These results were somehow surprising because FASN and other enzymes of the fatty acid biosynthetic pathway are known targets of the upregulation by androgens in other cell types, namely, human prostate cells<sup>69</sup>. This suggests that different mechanisms are activated in the epididymis and highlight for the relevance of EDCs affecting epididymis function.

Long-chain fatty acids translocate to the mitochondria through CPT1A, undergoing subsequently multiple  $\beta$ -oxidation cycles, which generate ATP, NADH and FADH<sub>2</sub>. The results obtained of WB analysis demonstrated that VNZ increased CPT1A expression, suggesting that the presence of this anti-androgen also stimulated the activity of lipid catabolic pathways in the epididymis. The (un)balance between fatty acid synthesis and  $\beta$ -oxidation in the epididymis is likely to have translation to sperm affecting membrane composition and the fertility potential. Sirtuins are a family of mammalian histone deacetylases (class III) known to be involved in metabolic regulation. Moreover, sirtuin 4 has been implicated in the maintenance of this equilibrium by repressing fatty acid oxidation while promoting lipid anabolism<sup>136</sup>, which raises the question about their action in the epididymis.

Fatty acids are a component of the epididymal fluid displaying a regional difference from the *caput* to *cauda* regions<sup>23</sup>, which are enriched in polyunsaturated and saturated fatty acids, respectively. The uptake of long-chain fatty acids from the extracellular space is mediated by the activity of CD36. Based on the results obtained, it is possible to hypothesize that  $\beta$ -oxidation is augmented with the lipids used coming from the intracellular reservoir, as CD36 expression is not significantly altered in the presence of VNZ. This suggested driven of lipids to the catabolic pathway is supported by a study showing that in the absence of androgens epididymal cells switch from carbohydrate to lipids as the ATP source<sup>37,38</sup>.

Altogether, our findings indicate that VNZ disrupts the metabolism of the *caput* epididymis, increasing glucose consumption whereas decreasing lactate production, and activating both fatty acid biosynthesis and degradation. Although preliminary, and somehow contradictory, this dissertation results highlight the impact of EDCs hampering the metabolic activity of the epididymis, and consequently sperm function. Further investigation is necessary to completely understand the deregulatory actions of VNZ and other EDCs in the epididymis metabolism, and how this is repercussed on sperm quality and male fertility.

## ***VI – Conclusions and Future Perspectives***

The present dissertation showed that exposure to VNZ culminates in the deregulation of glycolytic and lipid metabolism of epididymis. The obtained findings indicate that VNZ exposure disrupts the metabolism of the *caput* epididymis, by increasing the consumption of glucose and decreasing lactate production, as well as activating both fatty acid biosynthesis and degradation.

The research outcomes of this dissertation are fundamental to start elucidating the effect that VZN exposure may have in disrupting epididymal metabolism and impairing male fertility. Moreover, the obtained results highlight the impact of EDCs hampering the metabolic activity of the epididymis, and, consequently, sperm function, which is important for awareness related to human health.

Concerning VNZ actions, full comprehension of the deregulation induced on glycolytic metabolism will be extremely important. For example, we still do not know how VNZ affects the activity of certain regulatory enzymes, such as PFK1, which will be mandatory to confirm if glucose is being metabolized in glycolysis or driven to storage in the form of glycogen. With the same rationale, would be critical to investigate the expression and activity of glycogenin and glycogen synthase, major enzymes involved in glycogen synthesis. Also, would be interesting to study the production of pyruvate by analysing pyruvate kinase. Finally, to better investigate the glycolytic pathway, analysis with the Seahorse XF Analyzer would be envisaged because it measures oxygen consumption rate and extracellular acidification rate, as well as explores the component of oxidative phosphorylation.

In what concerns lipid metabolism, the finding that VNZ activates both fatty acid biosynthesis and degradation, is somehow intriguing and deserves to be deeply investigated. A component that could shed light on this is the analysis of sirtuins in the epididymis, as these enzymes have been implicated in the maintenance of the balance between biosynthetic and catabolic pathways. On the other hand, other enzymes and transporters involved in fatty acids synthesis and  $\beta$ -oxidation could be analysed. The metabolic processes in the epididymis are highly unknown, thus, investigation of other metabolic routes (e.g. amino acids, glycogen and phosphate pentoses), should also be considered.

Ultimately, this dissertation and the results obtained herein are the basis for establishing *in vivo* studies. This approach will definitely determine if the VNZ effects disrupting epididymis metabolism affect male fertility, by analysing sperm parameters and sperm-oocyte.

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