



**HAL**  
open science

# The drawbacks of paternal obesity on sperm biology, preimplantation embryo morphokinetics, and its transgenerational impacts

Georges Raad

► **To cite this version:**

Georges Raad. The drawbacks of paternal obesity on sperm biology, preimplantation embryo morphokinetics, and its transgenerational impacts. Agricultural sciences. COMUE Université Côte d'Azur (2015 - 2019), 2016. English. NNT : 2016AZUR4143 . tel-01492786

**HAL Id: tel-01492786**

**<https://theses.hal.science/tel-01492786v1>**

Submitted on 20 Mar 2017

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Université de Nice-Sophia Antipolis

Ecole doctorale SVS ED85

Sciences-Vie-Santé

THESE DE DOCTORAT

Pour obtenir le grade de

Docteur en biologie de l'université de Nice-Sophia Antipolis

Spécialité: Recherche clinique et thérapeutique

Soutenue par: Georges RAAD

**The drawbacks of paternal obesity on the sperm biology, the preimplantation embryo morphokinetics, and its transgenerational impacts**

Directrice de thèse : Valérie GRANDJEAN

Co-directrice de thèse : Mira HAZZOURI

Soutenue le 14 Décembre 2016

Jury :

Rapporteurs : Sophie ROUSSEAU

Roger MIEUSSET



## Table of Contents

<b>THESIS ABSTRACT</b> .....	<b>6</b>
<b>RESUME</b> .....	<b>8</b>
<b>LIST OF FIGURES</b> .....	<b>10</b>
<b>LIST OF TABLES</b> .....	<b>13</b>
<b>CHAPTER1 THE DRAWBACKS OF OBESITY ON THE GLOMERULOPATHY, TESTICULAR HOMEOSTASIS AND SPERM EPIGENOME</b> .....	<b>14</b>
I- THE BODY WEIGHT HOMEOSTASIS .....	14
A- The human body homeostasis.....	14
B- The body weight management.....	18
B. 1 The metabolic energy network .....	18
B.1.1 The digestive system: intake, processing and distribution of foodstuffs.....	18
B.1.2 Cells and molecules implicated in glucose homeostasis and energy management	
19	
a) Energy storing cells .....	20
a. 1 The white adipose tissue .....	20
a. 2 The liver .....	22
b) Energy consuming cells .....	23
B. 2 The control of food intake .....	24
II- WEIGHT IMBALANCE: OBESITY COMPLICATIONS .....	25
A- The drawbacks of obesity on kidney homeostasis .....	28
A. 1 Kidney physiology .....	28
A. 1.1 Obesity-related glomerulopathy (ORG).....	31
A. 1.1.a The effect of obesity on the glomerular histology.....	31
A. 1.1.b Decline in renal function and obesity .....	31
a) Renal hemodynamics.....	31
b) Hyperglycemia and hyperlipidemia: .....	33
B- The drawbacks of obesity on testicular homeostasis .....	34
B. 1 Male reproductive system anatomy .....	34
B.1.1 The effects of obesity on testicular anatomy, sexual function and seminal plasma	
biochemistry .....	36
B. 2 Testicular histology and physiology.....	37
B.2.1 The interstitial compartment.....	38
a) Blood vessels .....	38
b) Leydig cells .....	38
b.1 Overview .....	38
b.2 Steroidogenesis .....	39
b.3 The effect of obesity on Leydig cell physiology and androgen homeostasis...40	

c)	Immune cells .....	41
c.1	Phagocytosis, proliferation and physiology of Leydig cells .....	41
c.2	Immunoregulation and inflammation .....	42
c.3	The effect of obesity on testicular immune cells .....	42
B.2.2	Tubular compartment .....	43
a)	Peritubular cells .....	44
b)	Sertoli cells .....	45
b. 1	Overview .....	45
b. 2	Cytological features .....	45
b. 3	Sertoli cell functions .....	45
b. 4	The effects of obesity on Sertoli cell functions .....	49
c)	Germ cells.....	52
c. 1	Spermatogonia.....	52
c. 2	The meiotic cells.....	54
c. 3	The spermiogenesis.....	55
c. 4	The impact of male obesity on sperm parameters and embryo development ..	58
III-	EPIGENETIC INHERITANCE OF PATERNAL ACQUIRED OBESITY .....	60
A-	Epigenetic control of gene expression during spermatogenesis and early embryogenesis	60
A. 1	DNA methylation.....	60
A. 1.1	DNA methylation reprogramming in mouse germ cells and pre-implantation embryos	62
A. 1.2	The drawbacks of abnormal DNA methylation profile in gametes .....	63
A. 2	DNA hydroxymethylation and DNA demethylation .....	65
A. 2.1	5-hmC dynamics during mouse spermatogenesis and embryo development ..	67
A. 3	Chromatin modifications.....	69
A. 3.1	Chromatin structure in somatic cells .....	69
A. 3.2	Chromatin remodelling during spermatogenesis .....	72
a)	Spermatogonia .....	72
b)	Meiotic cells.....	73
c)	Spermatids and spermiogenesis .....	74
c. 1	The incorporation of histones variants into the chromatin .....	74
c. 2	Histones hyperacetylation.....	74
c. 3	Replacement of histones by transition proteins .....	75
c. 4	Replacement of TP by protamine.....	75
d)	Sperm chromatin .....	76
d.1	Nucleoprotamine-bound sperm chromatin.....	77
d.2	Nucleosome-bound sperm chromatin .....	77
d.3	Matrix attachment regions (MARs).....	78
A. 4	Sperm RNAs.....	78

B-	Transgenerational inheritance of paternal acquired obesity .....	79
B. 1	Overview .....	79
B. 2	Possible mechanisms of intergenerational and transgenerational inheritance of paternal acquired obesity .....	81
IV-	RESEARCH AIMS .....	84
V-	BIBLIOGRAPHY.....	85
<b>CHAPTER 2 SELECTIVE PHYSIOLOGICAL AND MOLECULAR ALTERATIONS IN THE MOTILE SPERM FROM OBESE MEN .....</b>		<b>109</b>
I-	ABSTRACT .....	109
II-	INTRODUCTION.....	110
III-	MATERIALS AND METHODS .....	112
a)	Study population .....	112
b)	Anthropometric measurements .....	112
c)	Semen analysis.....	112
d)	Sperm isolation using swim-up technique.....	113
e)	Sperm morphology evaluation.....	113
f)	Sperm membrane integrity assessment .....	113
g)	Sperm chromatin structure assessment .....	113
h)	Histone retention assessment.....	114
i)	Sperm DNA extraction.....	114
j)	Sperm Global DNA 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) measurements .....	114
k)	Ovarian stimulation, oocytes retrieval, and ICSI.....	115
l)	Embryo culture and evaluation of time-lapse images .....	115
m)	Statistical analysis .....	115
IV-	RESULTS.....	117
a)	Characteristics of the cohort.....	117
b)	The effect of paternal obesity on the macroscopic semen parameters.....	117
c)	Description of the motile sperm-enriched fraction according to the paternal BMI and WC .....	117
d)	Higher histones retention in motile sperm-enriched fraction of obese men.....	121
e)	Altered sperm chromatin integrity in motile sperm-enriched fraction of obese men ...	122
f)	Global DNA hydroxymethylation in the motile sperm of obese men .....	123
g)	The effect of paternal obesity on pre-implantation embryo morphokinetics .....	124
h)	The effect of paternal obesity on embryo quality .....	124
V-	DISCUSSION .....	129
VI-	BIBLIOGRAPHY .....	134

<b>CHAPTER 3</b>	<b>INTERGENERATIONAL AND TRANSGENERATIONAL INHERITANCE OF PATERNAL ACQUIRED OBESITY AND ITS ASSOCIATED GLOMERULOPATHY AND SUBFERTILITY IN A MOUSE MODEL.....</b>	<b>141</b>
I-	ABSTRACT .....	141
II-	INTRODUCTION.....	142
III-	MATERIALS AND METHODS.....	144
	a) Animals and experimental design.....	144
	b) Body measurements .....	145
	c) Metabolic cage experiments .....	145
	d) Renal histology and special stains .....	145
	e) Quantification of renal pathology .....	145
	f) Assessment of sperm count and motility.....	146
	g) Evaluation of sperm morphology .....	146
	h) Sperm chromatin integrity.....	146
	i) Statistical analysis .....	146
IV-	RESULTS.....	147
	1- Exacerbation of the overweight phenotype and the adipose tissue volume after seven successive generations of HFD feeding .....	147
	2- Feeding five successive generations of male mice with a HFD negatively impacted the body weight and the adipose tissue volume of the CD6 and the CD7 males.....	148
	3- Progressive alterations in the body composition after seven successive generations of HFD feeding .....	148
	4- The drawbacks of HFD feeding during 5 generations on the body composition of the CD6 and CD7 males .....	149
	5- The amount of food intake in the different HFD and CD groups.....	149
	6- The effect of the exposure to the same obesogenic environment during seven successive generations on the water balance .....	151
	7- Physiological adaptation of the kidney to the continuous HFD stress during several generations.....	152
	8- The effect of exposure to HFD during successive generations on glomerular histology	152
	9- The impact of the continuous exposure to a HFD stress during several generations on the conventional semen parameters .....	155
	10- The changes in the sperm chromatin integrity across HFD and CD generations .....	157
V-	DISCUSSION .....	158
VI-	CONCLUSION AND PERSPECTIVES.....	161
VII-	BIBLIOGRAPHY .....	162
<b>CHAPTER 4</b>	<b>FINAL DISCUSSION AND PERSPECTIVES.....</b>	<b>167</b>
<b>APPENDIX.....</b>		<b>172</b>

## Thesis abstract

The body weight homeostasis is defined as the balance between the energy intake and the energy expenditure. This equilibrium is maintained due to the activity of different body systems such as the neuroendocrine system and the gastrointestinal tract. Obesity is a medical condition resulting from an excessive accumulation of adipose deposits. The pathological remodelling of the adipose tissue in obese subjects may lead to the development of several health problems such as hypertension, kidney disease, and type 2 diabetes. Unfortunately, the prevalence of obesity is increasing worldwide and of particular interest among young men of reproductive age. The impact of obesity on reproduction is well established in women; however it is not fully elucidated in men. Furthermore, accumulated evidence suggests that information from paternal environment such as acquired obesity remains in the sperm epigenome (non-coding RNAs, chemical modifications of paternally inherited histones, and DNA modifications) and can modulate the phenotype of the offspring. Therefore, a deeper comprehension of the drawbacks of male excessive fatness on the sperm molecular composition is needed.

The first aim of this thesis was to assess the impact of obesity on the molecular composition and on the physiology of the motile sperm. The semen samples were obtained from 96 men attending the A-clinic fertility center, Lebanon. Patients were categorized into three groups according to their body mass index (BMI) and waist circumference (WC): normal weight ( $18 < \text{BMI} < 24 \text{ kg/m}^2$  and  $\text{WC} < 90 \text{ cm}$ ), overweight ( $25 < \text{BMI} < 29.9 \text{ kg/m}^2$  and  $\text{WC} < 102 \text{ cm}$ ), and obese ( $\text{BMI} > 30 \text{ kg/m}^2$  and  $\text{WC} > 103 \text{ cm}$ ). We showed that the motile sperm of obese men had abnormal levels of paternally inherited histones and hypomethylated/hypohydroxymethylated DNA as compared to normal weight men. Subsequently, the embryos derived from the motile sperm of an obese father had an altered morphokinetic patterns when compared to those derived from normal weight one. Altogether, these results indicated that paternal obesity could affect the molecular composition of the motile sperm nucleus and negatively impact the pre-implantation embryo morphokinetics.

The second aim of this thesis was to evaluate the adaptive and evolutionary potential of non-genetic heritable mechanisms in experimentally controlled animal models. Using a high fat diet (HFD)-induced obesity mouse model, we have examined how feeding male mice with a high fat diet for multiple generations impacts the phenotype of the resulting mice. For that purpose, *C57BL/6* male mice were fed a high-fat diet (HFD) from weaning for up to 5 months during 5 successive generations. The resulting male offspring were raised either on a control diet (CD6 and CD7) or on a HFD (HFD6 and HFD7). Despite their genetic similarity, there was a gradual increase in the body weight, in the adipose tissue volume, in the severity of obesity-related glomerulopathy, and a significant decrease in the fertility status across HFD generations as compared to controls. Although the CD6 and CD7 male mice had never encountered an obesogenic environment, they had a heavier body weight and a larger adipose tissue, they

developed histological kidney lesions, and they had a reduced fertility potential when compared to the control group. These findings indicated that at each generation the offspring might retain residual effects of the paternal obesity within their cells that could be worsened when they were re-exposed to the same obesogenic environment.

In conclusion, our findings might add more insights into the current knowledge on the epigenetic signature transferred by the sperm of obese men to the oocyte during fertilization and into how biological systems might respond and adapt to continuous exposure to a HFD stress.

## Résumé

L'homéostasie du poids corporel est définie comme étant l'équilibre entre les apports et les dépenses énergétiques. Cet équilibre est maintenu grâce à l'activité des différents systèmes du corps tels que le système neuroendocrinien et le tractus gastro-intestinal. L'obésité est une condition médicale résultant d'une accumulation excessive de dépôts adipeux. Le remodelage pathologique du tissu adipeux chez les sujets obèses pourrait conduire à l'élaboration de plusieurs problèmes de santé tels que l'hypertension, les maladies rénales et le diabète de type 2. Malheureusement, la prévalence de l'obésité augmente dans le monde entier et en particulier chez les jeunes hommes en âge de procréation. L'effet de l'obésité sur la reproduction est bien établi chez les femmes; cependant, il n'a pas été complètement élucidé chez les hommes. En outre, plusieurs études ont suggéré que les informations de l'environnement paternel comme l'obésité acquise restent dans l'épigénome du spermatozoïde (ARNs non codants, des modifications chimiques des histones, et les modifications de l'ADN) et peuvent moduler le phénotype de la descendance. Pour toutes ces raisons, une compréhension plus approfondie des effets de l'obésité sur la composition moléculaire des spermatozoïdes est nécessaire.

Le premier objectif de cette thèse était d'évaluer l'effet de l'obésité sur la composition moléculaire et sur la physiologie des spermatozoïdes mobiles. Les échantillons de sperme ont été obtenus à partir de 96 hommes s'adressant au centre de fertilité 'A-clinic', Liban. Les patients ont été classés en trois groupes en fonction de leur indice de masse corporelle (IMC) et le tour de taille (WC): poids normal ( $18 < \text{IMC} < 24 \text{ kg/m}^2$  et  $\text{WC} < 90 \text{ cm}$ ), le surpoids ( $25 < \text{IMC} < 29,9 \text{ kg/m}^2$  et  $\text{WC} < 102 \text{ cm}$ ), et obèses ( $\text{IMC} > 30 \text{ kg/m}^2$  et  $\text{WC} > 103 \text{ cm}$ ). Nos résultats ont montré qu'il y a une rétention des histones plus élevée, et un ADN spermatique hypométhylé et hypohydroxyméthylé, dans les spermatozoïdes mobiles des hommes obèses par rapport à ceux des hommes non-obèses. Par conséquent, les embryons issus de spermatozoïdes mobiles d'un homme obèse avaient une cinétique de division embryonnaire altérée par rapport à ceux provenant des spermatozoïdes d'un homme de poids normal. En conclusion, ces résultats indiquent que l'obésité paternelle peut avoir des effets négatifs sur la composition moléculaire des spermatozoïdes et sur la morphocinétique embryonnaire précoce.

Le second objectif de cette thèse était d'étudier l'évolution et/ou la régression des maladies liées à l'obésité chez les souris mâles après avoir été exposé pendant plusieurs générations à un régime riche en graisses. Pour accomplir ce but, des souris mâles (C57BL/6) ont été nourries avec un régime riche en graisses (HFD) à partir du sevrage jusqu'à 5 mois et pendant 5 générations successives. La progéniture mâle de la sixième et la septième génération ont été soulevées, soit sur un régime contrôle (CD6 et CD7) ou sur un HFD (HFD6 et HFD7). Tout en partant du même fond génétique le phénotype des souris était différent à chaque génération. Il y a eu une augmentation progressive du poids corporel, du volume du tissu adipeux, de la sévérité de la glomérulopathie, et une diminution graduelle de l'état de fertilité entre les générations HFD par

rapport aux souris contrôles. Bien que les souris mâles CD6 et CD7 avait jamais rencontré un environnement obésogène, ils avaient un poids corporel plus élevé, un tissu adipeux plus volumineux, ils ont développé des lésions rénales histologiques, et ils avaient un potentiel de fertilité réduit par rapport au groupe de souris contrôles. Ces résultats indiquent que, à chaque génération la progéniture pourrait conserver les effets résiduels de l'obésité paternelle dans leurs cellules qui pourraient être aggravée quand ils ont été exposés de nouveau au même environnement obésogène.

En conclusion, nos résultats ont montrés qu'il pourrait y avoir une signature épigénétique spécifique transférée par les spermatozoïdes des hommes obèses à l'ovocyte lors de la fécondation. En plus, à l'aide d'un modèle murin on a pu démontrer comment les systèmes biologiques pourraient réagir et s'adapter à une exposition continue à un régime riche en graisses.

## List of figures

### Chapter1 The drawbacks of obesity on the glomerulopathy, testicular homeostasis and sperm epigenome

Figure 1: Cellular communication and homeostasis .....	14
Figure 2: Organization of the integration systems. ....	17
Figure 3: The central role of pancreatic islets in the maintenance of the normal level of plasma glucose .....	20
Figure 4: The adipose tissue. ....	21
Figure 5: Relation between the white adipose tissue and metabolism.....	22
Figure 6: Regulation of glucose metabolism by the hepatocytes. ....	27
Figure 7: A diagram showing the possible regulators of food intake and energy balance.....	24
Figure 8: The prevalence (%) of obesity among adults in different countries. ....	26
Figure 9: The high prevalence (%) of overweight and obese among young population aged 5-17 years in both developed and developing countries .....	26
Figure 10: The central role of adipose tissue in the development of obesity-associated complications. ....	27
Figure 11: Anatomy of the nephron. ....	30
Figure 12: Regulation of blood Na <sup>+</sup> levels by the kidney . ....	30
Figure 13: Pathogenesis of obesity-related glomerulopathy. ....	32
Figure 14: Histological kidney sections .....	33
Figure 15: Sagittal view of testis and male pelvis .....	34
Figure 16: Histological section of the human testis .....	37
Figure 17: Illustration of the testicular interstitial tissue.....	38
Figure 18: Illustration of the cascade of events leading to male sexual hormone production by the Leydig cell. ....	39
Figure 19: The high-energy diet overconsumption affects the reproductive axis at the central and at the gonadal levels. ....	41
Figure 20: Interstitial immune cells secrete stimulatory and inhibitory factors that may regulate Leydig cell physiology. ....	42
Figure 21: Organization of the seminiferous tubule. ....	43
Figure 22: Scheme illustrating the function of sometheperitubular cells (PT) secreted factors....	44
Figure 23: Schematic presentation of the metabolic coupling between Sertoli cells and the germ cells. ....	47
Figure 24: Steroids homeostasis in the seminiferous tubule. ....	48
Figure 25: Illustration showing the compartmentalization of the seminiferous epithelium by the specialized junctions between the Sertoli cells.....	49
Figure 26: The drawbacks of excess high-energy diet consumption on testicular homeostasis ...	51
Figure 27: The cycle of mammalian germ cell development. ....	52

Figure 28: The role of retinoic acid in meiotic entry. ....	54
Figure 29: Illustration of the major events that occur during the meiotic division .....	56
Figure 30: The process of spermiogenesis .....	58
Figure 31: Flow diagram of methionine-folic acid metabolism. ....	60
Figure 32: The DNA methyltransferases functions. ....	61
Figure 33: The dynamics of DNA methylation during mouse gametogenesis and pre-implantation embryos. ....	63
Figure 34: Regulation of gene expression by DNA methylation and demethylation. ....	66
Figure 35: The cross-talk between the glucose metabolism and 5-hydroxymethylcytosine formation in normal and cancer cells .....	67
Figure 36: The dynamics of 5-hydroxymethylcytosine during mouse spermatogenesis and embryo development. ....	69
Figure 37: Chromatin structure.....	70
Figure 38: The major steps of spermatid chromatin remodelling during spermiogenesis. ....	75
Figure 39: The structure of the sperm chromatin. ....	76
Figure 40: Scheme showing different modes of non-genetic inheritance. ....	80
Figure 41: Pedigree pattern of acquired pathologies. ....	81
Figure 42: The impact of the lifestyle on the non-genetic paternal contribution to the embryogenesis. ....	84

**Chapter 2: Selective physiological and molecular alterations in motile sperm from obese men**

Figure 1: The study design. ....	116
Figure 2: Cytological assessment of sperm membrane integrity and morphology.....	120
Figure 3: Sperm histones retention as assessed by the aniline blue staining (AB).....	121
Figure 4: Sperm chromatin integrity as assessed by the toluidine blue staining (TB).....	122
Figure 5: Assessment of the global sperm DNA methylation status. ....	123
Figure 6: Assessment of the global sperm DNA hydroxymethylation status. ....	124
Figure 7: The different stages of pre-implantation embryo development.....	128
Figure 8: Figure showing two blastocysts. ....	129

**Chapter 3: Intergenerational and transgenerational inheritance of paternal acquired obesity and its associated glomerulopathy and subfertility in a mouse model**

Figure 1: Two pedigrees showing the high fat diet (HFD) .....	144
Figure 2: The body weight and the adipose tissue volume of the different HFD and CD groups. ....	147

Figure 3: Feeding male mice with a HFD during seven generations alters the glomerulus/capsule ratio and increases the matrix accumulation.....	153
Figure 4: Quantitative analysis of the glomeruli in CD6 and CD7 males compared to the controls at 16 weeks. ....	154
Figure 5: Pathological alterations in the kidney of mice on HFD (1-7) and control diet (CD6 and CD7) at 16 weeks.....	154
Figure 6: Graphs showing the sperm concentrations of the different HFD (a) and CD (b) generations.....	155
Figure 7: Graphs showing the percentage of spermatozoa with progressive motility, non-progressive motility, and the non-motile ones in the different HFD (a) and CD (b) generations. ....	156
Figure 8: The normal sperm morphology percentage in the different HFD and CD groups. ....	157
Figure 9: The mean percentage of TB positive cells in the different HFD (a) and CD groups (b). ....	158

## List of tables

### **Chapter 1 The drawbacks of obesity on the glomerulopathy, testicular homeostasis and sperm epigenome**

Table 1: The systems implicated in internal environment homeostasis in humans .....	15
Table 2: Table showing the classification of the overweight and obesity .....	25
Table 3: Table showing some of the sex gland secretions .....	35
Table 4: A summary of the phenotypes derived from DNMTs genes mutations. ....	64
Table 5: The biochemical properties of the histones . ....	71
Table 6: The different types and functions of the post-translational modifications of histones ...	71
Table 7: Table summarizing some of the epigenetic events that occur during spermatogonia differentiation from type A undifferentiated to type A differentiated in a mouse model .....	72
Table 8: A comparison between the epigenetic regulation in the embryonic stem cells and the spermatogonia .....	73

### **Chapter 2: Selective physiological and molecular alterations in motile sperm from obese men**

Table 1: Characteristics of the study population according to the paternal BMI and WC.....	118
Table 2: Comparison between the macroscopic semen parameters according to the paternal BMI and WC levels. ....	119
Table 3: Comparison between the sperm parameters in the raw semen and in the motile-enriched fraction post swim-up technique according to paternal BMI and WC levels .....	120
Table 4: The diagnosis of female infertility according to the paternal BMI and WC. ....	125
Table 5: The effect of obesity on the morphokinetic parameters of pre-implantation embryo development.....	126
Table 6: The effect of paternal obesity on the fertilization rate and the embryo development on day 4 and 5 of culture.....	127

### **Chapter 3: Intergenerational and transgenerational inheritance of paternal acquired obesity and its associated glomerulopathy and subfertility in a mouse model**

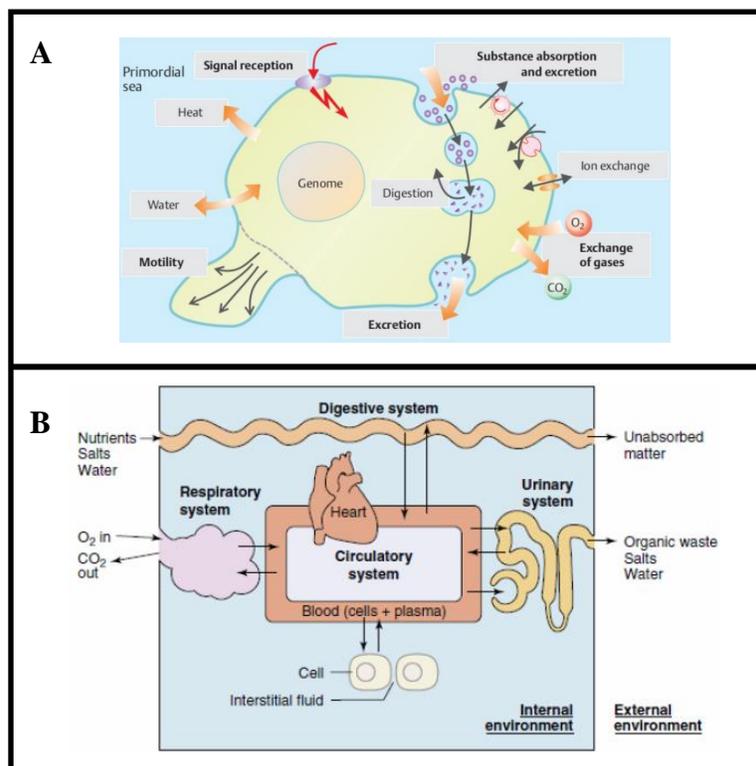
Table 1: Gross anatomy of the different HFD groups.....	148
Table 2: Gross anatomy of the different CD groups. ....	149
Table 3: Calories intake, water balance, and kidney physiology of different HFD groups. ....	150
Table 4: Calories intake, water balance, and kidney physiology of different CD groups. ....	151

# Chapter1 The drawbacks of obesity on the glomerulopathy, testicular homeostasis and sperm epigenome

## I- The body weight homeostasis

### A- The human body homeostasis

The evolution from aquatic unicellular organisms to land multicellular ones requires groupings of specialized cells into organs, which in turn are grouped into systems. To adapt and survive, the individual cells of the human body (multicellular organism) still need a milieu similar to that of the primordial sea. This medium is created by the extracellular body fluid. In this latter, specialized organs assure continuous absorption of nutrients and excretion of waste products via the urine and feces (**Figure 1**) (Despopoulos et al. 2003).



**Figure 1: Cellular communication and homeostasis**(A) Unicellular organisms can perform all the necessary functions to live (Despopoulos, et al. 2003). (B) Multicellular organisms have specialized organs to perform specific tasks to sustain their lives (extracted after permission from [www.santelvgar.blogspot.com](http://www.santelvgar.blogspot.com)).

In order to prevent depletion of essential elements and accumulation of toxic compounds, the body has developed a physiologic process called homeostasis (from the Greek *homeo-* meaning “same” and the Latin *stasis-* meaning “standing”). It is made up of physiologic auto-regulatory

mechanisms that maintain steady states of the body. The systems implicated in maintaining this equilibrium are summarized in **Table1** (Despopoulos et al. 2003).

**Table 1: The systems implicated in internal environment homeostasis in humans** (Despopoulos et al. 2003)

Body systems	Major organs	Functions
<b>Cardiovascular system</b>	<ul style="list-style-type: none"> <li>- heart</li> <li>- blood vessels</li> </ul>	<ul style="list-style-type: none"> <li>- Connects all the systems together</li> <li>- Exchanges compounds between blood and intercellular spaces</li> <li>- Regulates the body temperature</li> </ul>
<b>Digestive system</b>	<ul style="list-style-type: none"> <li>- Major organs: mouth, oesophagus, stomach, small and large intestine</li> <li>- Secondary organs: liver, pancreas, salivary glands and the gall bladder</li> </ul>	<ul style="list-style-type: none"> <li>- absorbs</li> <li>- Processes</li> <li>- Distributes</li> <li>- Excretes waste and toxins</li> </ul> <p style="text-align: right;">} water, salts...</p>
<b>Respiratory system</b>	<ul style="list-style-type: none"> <li>- Upper respiratory tract: nose, nasal cavity, pharynx and larynx</li> <li>- Lower respiratory cavity: trachea, bronchi, alveoli and the lungs</li> </ul>	<ul style="list-style-type: none"> <li>- Oxygen ( O<sub>2</sub>) intake</li> <li>- Carbon dioxide ( CO<sub>2</sub>) elimination</li> <li>- Regulates the internal environment (Osmolality, pH, ion concentrations...)</li> </ul>
<b>Excretory system ( urinary)</b>	<ul style="list-style-type: none"> <li>- Kidneys</li> <li>- Ureters</li> <li>- Bladder</li> <li>- Urethra</li> </ul>	<ul style="list-style-type: none"> <li>- Excretes the excess of water, salts, acids</li> <li>- Excretes the waste, toxins and foreign bodies</li> <li>- Regulates the internal environment (Osmolality, pH, ion concentrations...)</li> </ul>

Apart from the local regulation in each organ, these body systems can communicate together and with the external environment due to the coordinated control of regulatory systems. There are

three families of regulators: the nervous, endocrine and immune systems. The operation of these systems and the integration of information among them are responsible for the integrity of the organism or the homeostasis. This is accomplished by an intercellular communication that implements:

- A detector that responds to a chemical or physical stimulus
- Components that integrate the signals coming from the detector
- Molecular mechanisms responsible for the transmission of information among cells of the same system and / or different systems

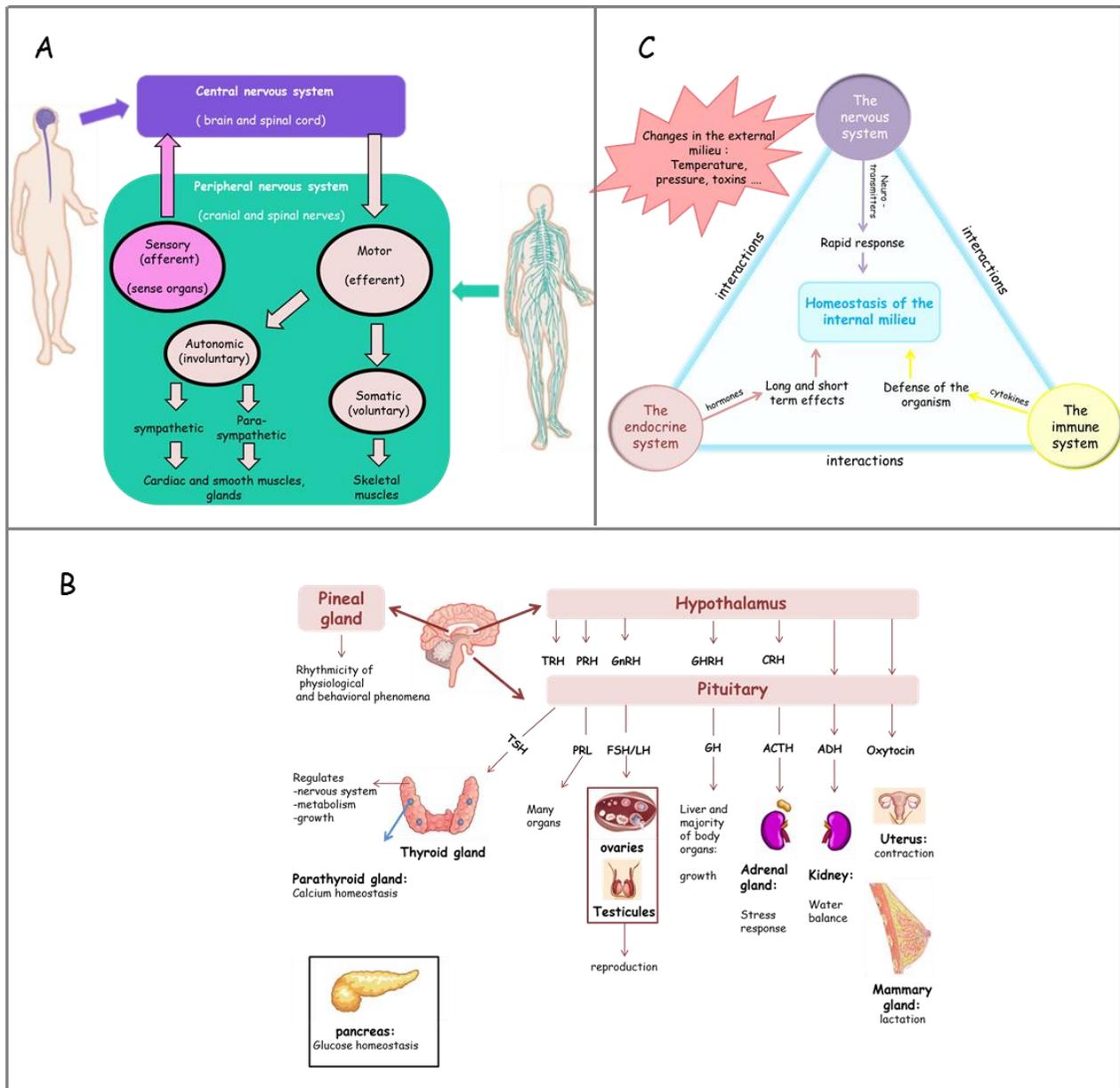
The intercellular communication signals are mainly of physical or chemical origin. At the fundamental level, the chemical signals are classified according to their site of secretion. The signals secreted in the interstitial fluid are the local paracrine and autocrine factors (growth factors as well as factors of the inflammatory reaction and cellular immunity), in addition to the chemicals diffusing in the synaptic space, which are termed neurotransmitters. However, the signals secreted in blood and using blood vessels to reach their cellular targets are the hormones and the antibodies. So, the concept of hormones is based on a physiological definition concerning the fluid compartment in which these signals are transported (Idelman et al. 2000).

According to the needs of the organism at any change in the internal or external environment, the mechanisms of adaptation and regulations are triggered:

1-The nervous system monitors the changes in the internal environment by the sensory receptors. It can regulate the altered parameters by controlling the effectors cells: cardiac muscle, gland cells, and smooth muscles of the internal organs (stomach, intestine...) and of the blood vessels. Moreover, the nervous system monitors the external milieu by controlling the skeletal muscles (body movements) and by modulating the behavior (eating, learning, looking for sex...)(**Figure 2A**) (Despopoulos et al. 2003).

2-The endocrine system is involved in lipids, proteins, and carbohydrates metabolism. In addition, it plays a crucial role in growth, fat stores, reproduction, thermoregulation, glucose homeostasis and adaptation to the fluxes in the external conditions (Despopoulos et al. 2003). The endocrine cell can function as a mechanoreceptor that is sensitive to the change in blood volume for instance or as a chemoreceptor that is sensitive to the ionic concentration ( $\text{Na}^+$ ,  $\text{K}^+$ ...) or to the hormonal substance. Endocrine cells secrete hormones into the blood that can act on distal target cells (**Figure 2B**) (Idelman et al. 2000).

3-The immune system is essentially involved in the defense of the body against external aggressions or internal changes (viral or malignant transformations). Immune cells can secrete molecules that regulate the activities of the neurons. In turn, these secreted molecules could be regulated by the hormones and the neurotransmitters (Figure 2C) (Idelman et al. 2000).



**Figure 2: Organization of the integration systems.**

(A) Anatomy of the nervous system. (B) Scheme showing the main hormones of the endocrine system, their site of secretion and their major target cells. (C) Interaction between the three integration systems to establish the homeostasis of the internal milieu. TRH= thyroid releasing hormone, TSH= thyroid stimulating hormone, PRH= prolactin releasing hormone, PRL= prolactin, GnRH= gonadotropin releasing hormone, FSH= follicle stimulating hormone, LH= luteinizing hormone, GHRH= growth hormone releasing hormone, GH= release hormone, CRH= corticotropin releasing hormone, ACTH= adrenocorticotropin hormone, ADH= antidiuretic hormone.

Nonetheless, a living being so complex and so organized, will eventually wear out and die. Therefore, humans like the majority of the species depend on the reproductive system to maintain their continuity. The copulation and the reproduction are assured by a group of female genital organs (ovaries, fallopian tubes, uterus, and vagina) and male genital organs (testicles, spermatic ducts, sex glands, and penis). Also, the physiology of the reproductive system is under the control of the regulatory systems (Brooker et al. et al. 1998) .

## **B- The body weight management**

The body weight is defined by the grand total of bones, organs, body fluids and adipose tissue. The percentage of fat mass and free fat mass compartments is subject to continuous modifications as a result of changes in reproductive status, growth, aging, fever and excessive sport. Asserting body weight homeostasis is crucial to defend against extravagant weight fluctuations(Gee et al. 2008).This equilibrium is managed by the metabolic energy network and the control of food intake.

### **B. 1 The metabolic energy network**

The energy is the ability of a system to perform work such as heart beating and respiration. The animals pick up energy mainly from protein, carbohydrate and fat metabolisms. After processing, the ingested foodstuffs could be transformed to energy-rich substances (adenosine triphosphate = ATP) and heat. Once ATP is hydrolyzed energy is produced and may be used as a fuel for cellular processes, muscle activity, ion transport, etc... An ideal diet should also provide water, minerals, vitamins and roughage (indigestible plant fibers: cellulose...), which facilitate the transit of food in the digestive tube.

The metabolic energy network is made up of a group of organs and cells involved in energy consumption, energy storage and regulation of energy usage. The cardinal goal of this network is to establish the metabolic homeostasis. On one hand, the supplied energy is used for different cellular processes; whereas an excess in energy supply will activate a cascade of events to store the energy in the white fat cells as triacylglycerol or in the liver cells as glycogen. On the other hand, the metabolic network will mobilize the energy from its stores when the supply is reduced. The main components of this network are (Despopoulos et al. 2003; Gee et al. 2008):

#### **B.1.1 The digestive system: intake, processing and distribution of foodstuffs**

Foodstuffs enter the body via the mouth where they are mixed with the saliva. Mammalian saliva is a heterogeneous biological fluid, composed of **water** (H<sub>2</sub>O), **solutes** (low concentrations of sodium (Na<sup>+</sup>) and chlorine (Cl<sup>-</sup>) / high concentrations of potassium (K<sup>+</sup>) and bicarbonate (HCO<sub>3</sub><sup>-</sup>

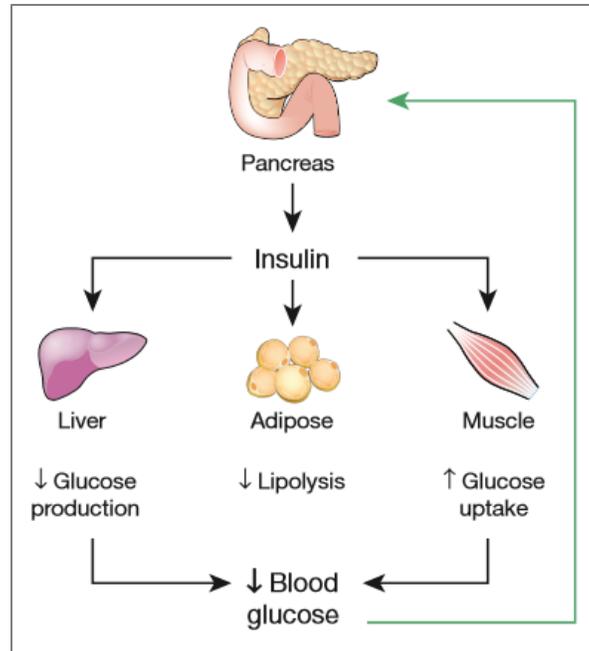
) and **proteins** (mucins,  $\alpha$ -amylase, lingual lipase, lysozyme, immunoglobulin A, growth factors and KalliKrein). Saliva constituents reflect its crucial functions. At first, water and mucins lubricate the food while eating thus facilitating its decomposition and swallowing. Besides that, digestion of starch and triglycerides start in the mouth due to presence of the  $\alpha$ -amylase and the lingual lipase respectively. Saliva is a hypotonic solution with low NaCl concentration that serves in maintaining the oral hygiene and rinsing the teeth and the taste receptors. Moreover, the high  $\text{HCO}_3^-$  content regulates the pH and helps in protecting the tooth, the upper gastrointestinal tract by buffering the oesophagus and neutralizing the acidity of gastric fluid during vomiting. In addition, the growth factors can repair the damaged oral tissues. Saliva also defends against different types of aggressors (Melvin et al. 2005; HETIZMAN et al. 2008; Ueda et al. 2013).

Next, the swallowed nutrients are transported via the oesophagus to the stomach where they are transformed into a liquid form termed chyme. The latter is periodically ejected to the small intestine, facilitating its decomposition by the pancreatic, hepatic and intestinal secretions. The resulting products (amino acids, free fatty acids, and monosaccharides) as well as water and vitamins are absorbed in the mucosa of the small intestine. Then the waste products and the excess of ions and water move down the large intestine; this organ serves as a last stop for fluid and minerals absorption, and as a storage area for feces (Despopoulos et al. 2003).

### B.1.2 Cells and molecules implicated in glucose homeostasis and energy management

Glucose is the essential carbon and energy source for the body. The ability of the organism to eliminate the excessive amount of glucose from the bloodstream following a meal (glucose tolerance) is of paramount importance. The failure in the establishment of glucose homeostasis in the blood may lead to severe complications such as hypertension and kidney failure.

After a meal, the high level of glucose in blood stimulates insulin secretion from the  $\beta$ -cells of the pancreas. The secreted insulin acts on the energy storage cells (adipocytes, skeletal muscle cells, and hepatocytes) to stimulate the uptake of the glucose. However, in the fasted state, the low level of glucose stimulates the secretion of glucagon from the pancreatic  $\alpha$ -cells in order to increase the concentration of plasma glucose (**Figure3**) (Schwartz et al. 2013).



**Figure 3: The central role of pancreatic islets in the maintenance of the normal level of plasma glucose (Schwartz et al. 2013).**

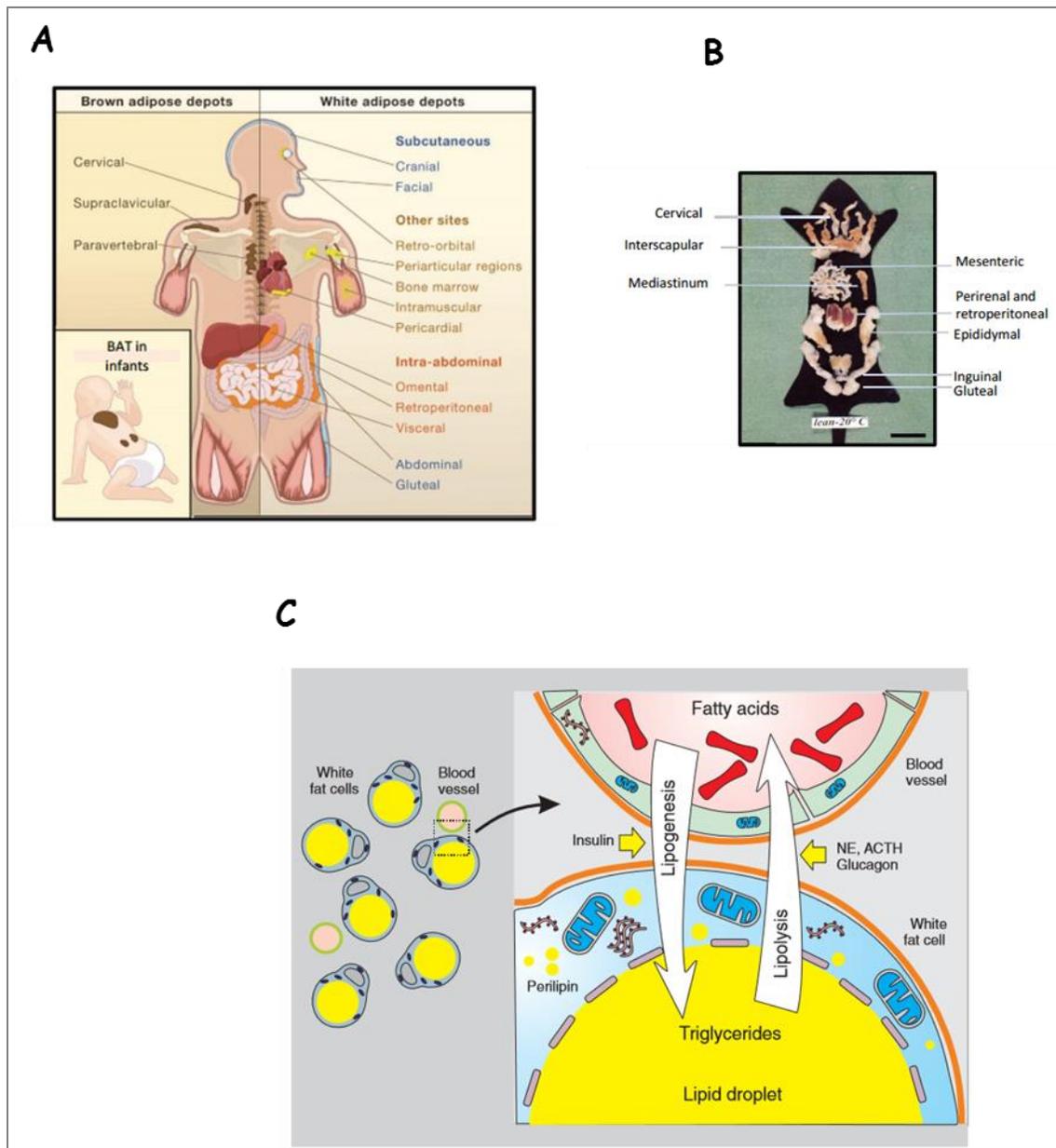
a) Energy storing cells

a. 1 The white adipose tissue

The white adipose tissue is one of the essential elements of the metabolic energy network. Grossly speaking, it is distributed throughout the body (**Figure 4A, 4B**). The white adipose tissue contains adipocytes, pre-adipocytes, white blood cells, endothelial cells, and fibroblasts (Wozniak et al. 2008). At the cytological level, the white adipocytes are spherical cells, characterized by a single lipid droplet made up of triacylglycerols generated from the metabolism of fat, carbohydrate, amino acids, and from the glucose uptake. Thus, the nutritional status of the body could modify the amount of lipid in this droplet (**Figure 4C**) (Berridge et al. 2012).

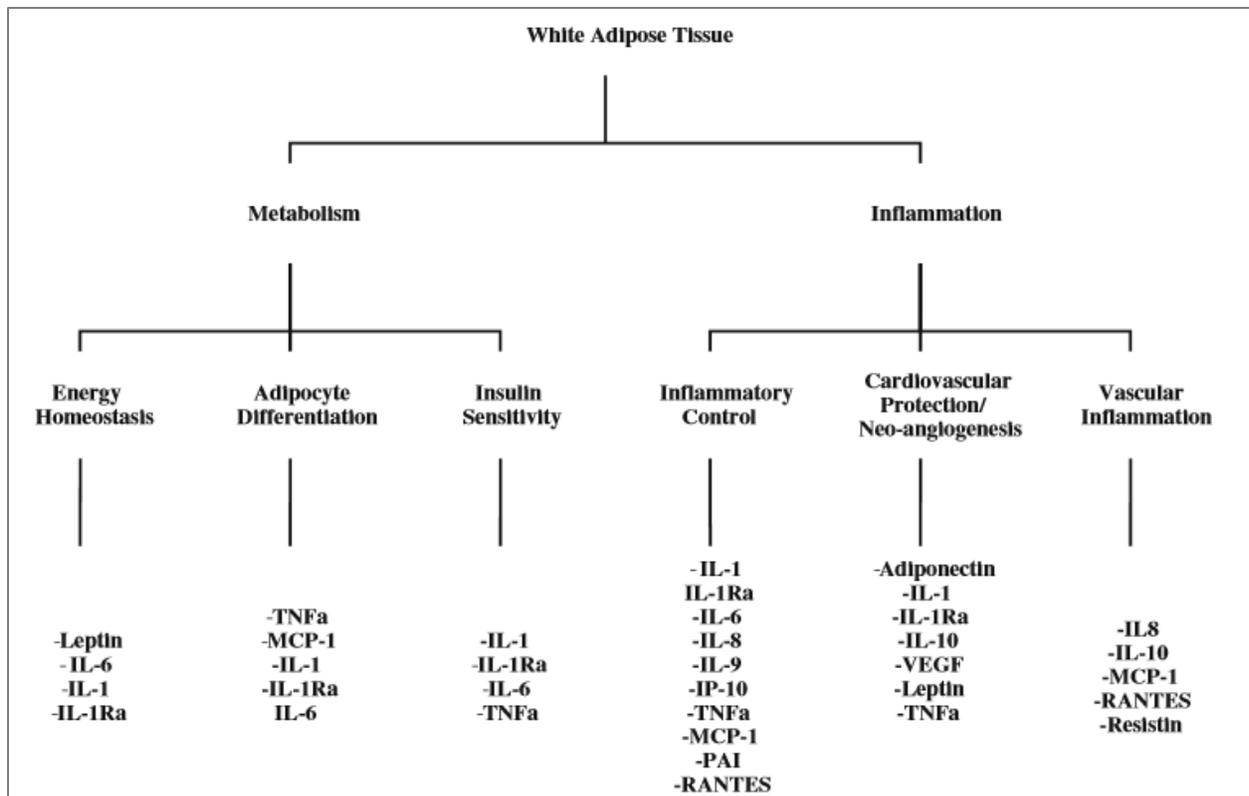
Following a meal, the secreted insulin binds to its receptor on the adipocyte, facilitates the glucose uptake from the blood, and drives lipogenesis in the white fat cells to store the energy. In contrast, when the energy is needed to accomplish a physiological process, several hormones (e.g. glucagon, adrenocorticotrophic hormone (ACTH), and norepinephrine (NE)) drive lipolysis and release fatty acids and glycerol into the blood (**Figure 4C**) (Berridge et al. 2012).

Moreover, the adipose tissue is also considered as an endocrine organ secreting hormones, proteins, and cytokines. These molecules are important mediators of metabolism and inflammation (**Figure 5**) (Wozniak et al. 2008).



**Figure 4: The adipose tissue.**

(A) In humans, the white adipose tissue is widely distributed in the body but mainly in subcutaneous and intra-abdominal areas. However, the brown adipose tissue is abundant in new born babies but in the adults it is present in cervical, supraclavicular, and paravertebral regions (Bagchi et al. 2012). (B) Scheme showing the distribution of the two types of adipose tissue in a mouse at 20 degree Celsius (Scale bar represents 2 cm) (Bagchi et al. 2012). (C) Illustration showing the role of adipocytes in establishing the metabolic homeostasis (Berridge et al. 2012).

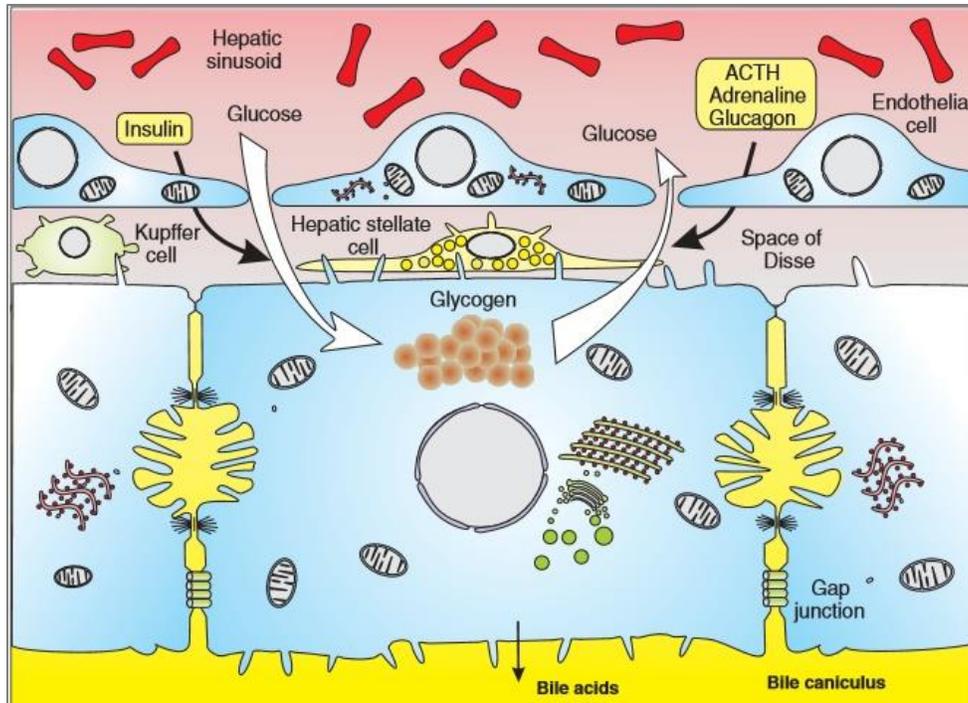


**Figure 5: Relation between the white adipose tissue and metabolism**

The white adipose tissue may regulate the metabolism by modulating the energy homeostasis, the adipocyte differentiation, and insulin sensitivity. Moreover, the white adipose tissue plays an important role in the inflammatory control, in protecting the cardiovascular system, and in vascular inflammation(Wozniak et al. 2008).

#### a. 2 The liver

The hepatocytes or liver cells have various crucial functions in the body; of particular interest is the maintenance of steady level of blood glucose (euglycaemia). After digestion, the nutrients are absorbed in the intestine and then transported to the liver via the portal vein. Subsequently, this hyperglycaemia induces hyperinsulinaemia that will stimulate the glucose uptake by the liver cells. In the hepatocytes, the glucose is stored as glycogen. In contrast, when the energy is required after fasting or due to stress, several hormones (e.g., ACTH, adrenaline, and glucagon) drive the glycogenolysis and gluconeogenesis; thus increase the hepatic glucose production (**Figure 6**)(Schwartz et al. 2013).



**Figure 6: Regulation of glucose metabolism by the hepatocytes.**

The hepatocytes manage their glycogen stores: on one hand, via the activity of the insulin (synthesis) and on the other hand, via glucagon, adrenaline, and ACTH (glycogenolysis). The ultimate goal is to maintain the euglycaemia (Berridge et al. 2012).

b) Energy consuming cells

The main three types of energy consuming cells are:

- The mammalian brain: It is the main glucose consumer organ of glucose (~5.6 mg glucose per 100 g human brain tissue per minute). The glucose is used by the neurones for cellular maintenance and for the production of neurotransmitters. Therefore, providing continuous and stable amount of glucose to the brain is essential for its normal physiology (Mergenthaler et al. 2013).
- The skeletal muscle cells: They transform the chemical energy into mechanical energy that is used during movements and exercises. In parallel, the skeletal muscle cells may also act as a glycogen storing cells under the control of insulin (Jensen et al. 2011).
- The brown adipose tissue: It exists in specific areas of the human body (**Figure 4A, 4B**), and it is mainly implicated in thermogenesis. The brown adipose tissue consumes the energy stored in the lipid droplets (triacylglycerols) to generate heat (Berridge et al. 2012).

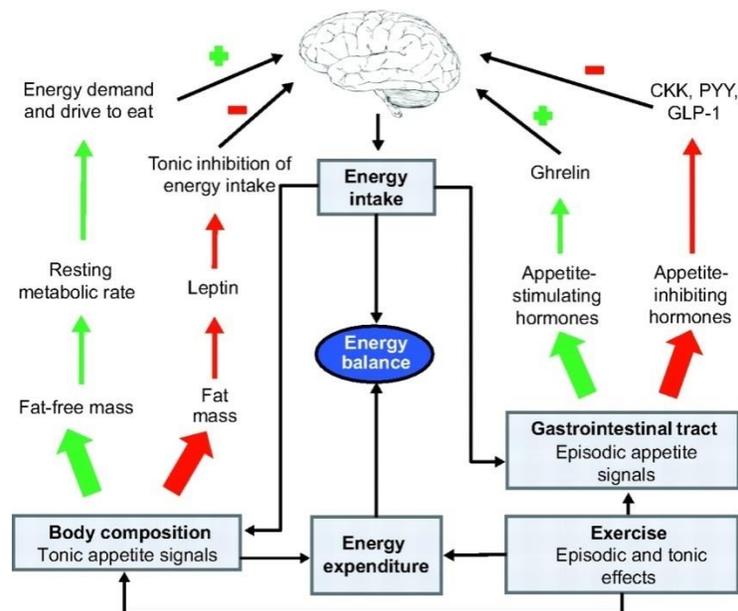
## B. 2 The control of food intake

Food intake brings to the body the energy needed to balance the amount of energy burned during various cellular processes. In order to maintain this energy balance, the feeding and the satiety are controlled by neuronal and endocrine signalling systems:

The feeding center in brain can respond to sensory stimulations (visual, taste, and smell) initiating the food uptake. Following the entry of the foodstuffs to the gastrointestinal tract, the stomach, the gut and the pancreas secrete hormones that may stimulate or inhibit the appetite; here are some examples (**Figure 7**):

- Ghrelin is a hormone secreted mainly by the stomach before the meal, and stimulates the feeding center in the brain
- Cholecystikin (CCK), glucagon like-peptide 1 (GLP-1), and peptide YY (PYY) are secreted in the gastrointestinal tract and transported *via* the blood to the brain, where they act as satiety signals.

In parallel, the body fat mass and fat-free mass can also participate in the regulation of energy intake. On one hand, the white adipocytes can ‘sense’ the content of their lipid droplets and secrete tonic hormones such as leptin (inhibits the feeding center in the brain) for long-term regulation of the energy stores. On the other hand, the daily physiological demand of energy (resting metabolic rate), body movements, and exercise can also regulate the food uptake to balance the energy expenditure (**Figure 7**) (Stensel et al. 2010; Parker et al. 2014; Blundell et al. 2015; Moehlecke et al. 2015).



**Figure 7: A diagram showing the possible regulators of food intake and energy balance**(Blundell et al. 2015).

## II- Weight imbalance: obesity complications

An imbalance between the energy intake and energy expenditure results in body weight fluctuations (e.g., gain or loss); the most common are overweight and obesity. Obesity is defined by an excessive accumulation of adipose tissue either generalized or localized within the body (Blundell et al. 2000; Proietto et al. 2004). Several ways could be used to assess the obesity such as: (De Lorenzo et al. 2016):

a- The body mass index (BMI):

It is a ratio of the weight to the squared height ( $BMI = \text{weight (Kg)} / \text{height (m}^2\text{)}$ ) of an individual, allowing to approximately determine the percentage of fat in the body. Based on this ratio, the people could be divided into five categories: underweight, normal weight, overweight, obese class I, obese class II, obese class III (Table 2).

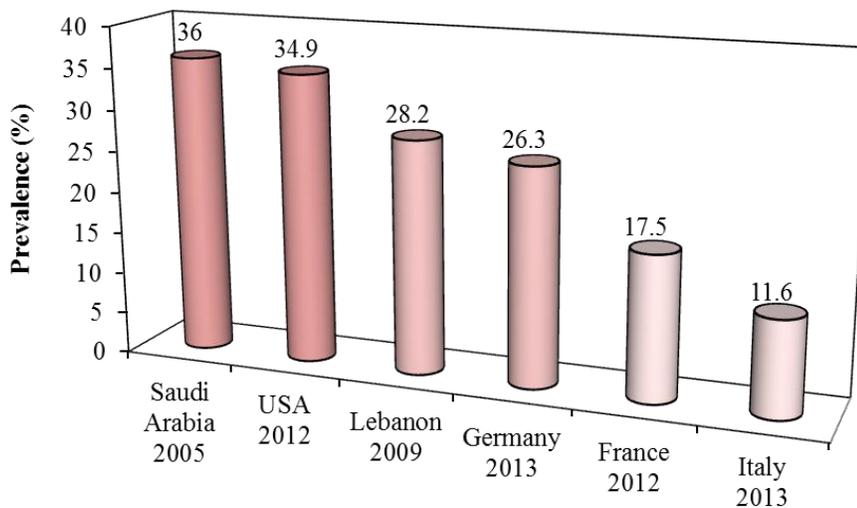
**Table 2: Table showing the classification of the overweight and obesity (De Lorenzo et al. 2016)**

Categories	Body mass index ( Kg/m <sup>2</sup> )
Underweight	< 18.5
Normal weight	18.5-24.9
Overweight	25.0-29.9
class I obesity	30.0-34.9
class II obesity	35.0-39.9
Class III, extreme obesity	≥ 40

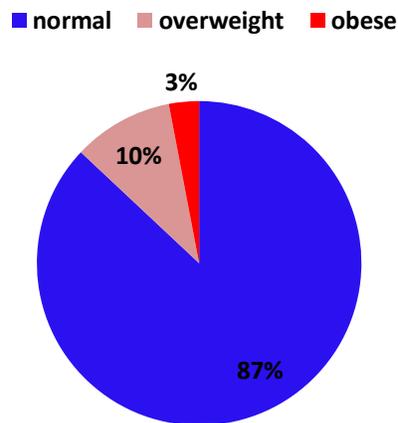
b- waist circumference (WC) :

The measurement of the waist circumference could be performed according to the National Health and Nutrition Examination Survey (NHANES) (measurement at the top of iliac crest) or according to the World Health Association (WHO) (midpoint between last palpable rib and top iliac crest) (Pettitt et al. 2012). The WC indicates the amount and the distribution of the fat within the body. Furthermore, a WC over 102 cm in men and over 88 cm in women indicates an excess in sub-cutaneous truncal-abdominal fat, termed android obesity. The “apple-shape” or android obesity is more common among men (WHO 2008).

Obesity has exceeded the stage of dietary measures and has reached alerting level worldwide. Recent reports revealed that the prevalence of obesity is high in different countries such as USA, Saudi Arabia and Lebanon (Figure 8 and 9) (Nasreddine and Naja et al. 2012; AL-Quwaidhi et al. 2014; Ogden et al. 2014; Vilet-Ostaptchouk et al. 2014). Moreover, the prevalence of obesity among young population aged 5-17 years and among young men of reproductive age is very high (Wu et al. 2006; Ng et al. 2014; McPherson et al. 2015).



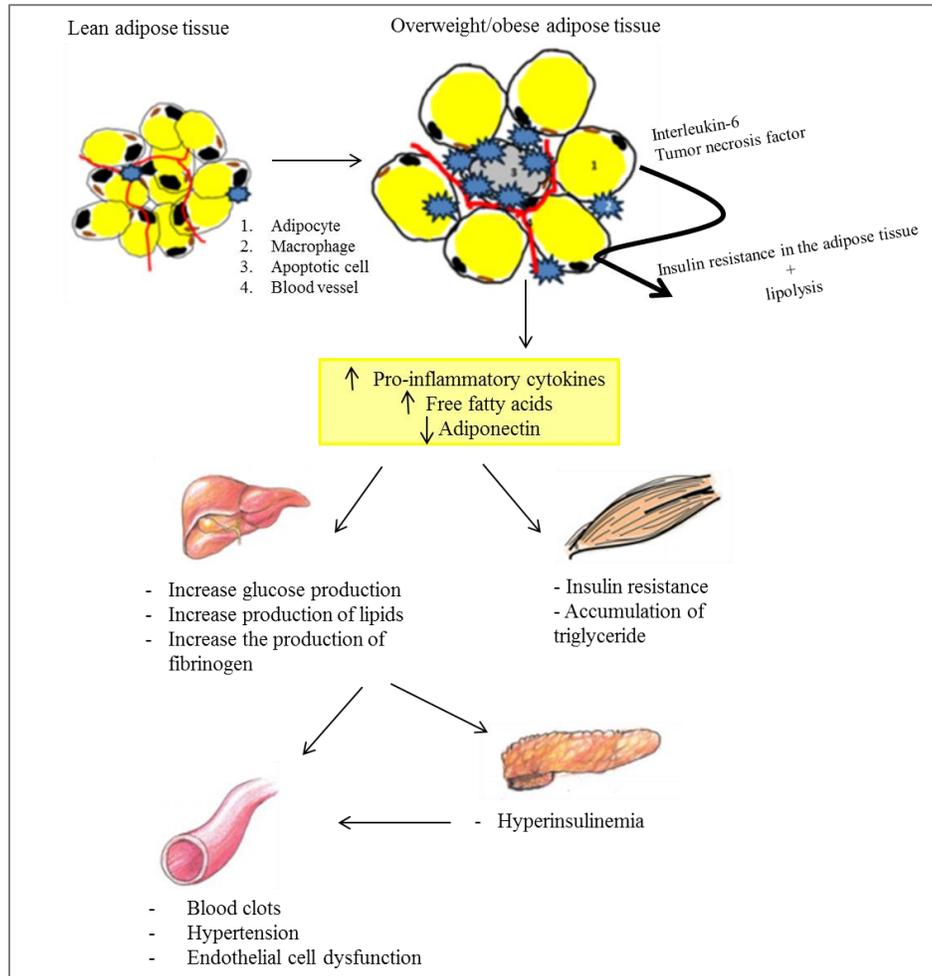
**Figure 8: The prevalence (%) of obesity among adults in different countries** (Nasreddine and Naja et al. 2012; AL-Quwaidhi et al. 2014; Ogden et al. 2014; Vilet-Ostaptchouk et al. 2014; Julia et al. 2016).



**Figure 9: The high prevalence (%) of overweight and obese among young population aged 5-17 years in both developed and developing countries**(Wu et al. 2006).

When the body weight increases, histological changes occur in the white adipose tissue such as an increase in the number and size of white adipocytes as well as in the number of infiltrated white blood cells and apoptotic adipocytes. These modifications are accompanied with an altered

secretion profile of the white adipose tissue (e.g., an increase in the levels of pro-inflammatory cytokines (tumor necrosis factor, and interleukin 6), an increase in the level of the free fatty acids, leptin, resistin, and decrease in the concentration of adiponectin) (Wozniak et al. 2008). Altogether, these alterations play a key role in the development of several pathologies such as type 2 diabetes mellitus (T2DM), cardiovascular diseases, respiratory diseases, and infertility (Golay et al. 2005; Fulleston et al. 2012). Of particular interest, obesity is one of the leading factors for the high prevalence (8.5%) of diabetes in Lebanon (Costanian et al. 2014) (**Figure 10**).



**Figure 10: The central role of adipose tissue in the development of obesity-associated complications.**

The expanded white adipose tissue (WAT) in obese subjects enhances the release of the interleukin-6 and the tumor necrosis factor resulting in insulin resistance in the WAT. Subsequently, the insulin resistance increases the lipolysis in the WAT. Moreover, the affected WAT releases high levels of pro-inflammatory cytokines, free fatty acids and low levels of adiponectin. These secretions promote insulin resistance and lipid accumulation in the muscle

cells. Also, they increase the production of glucose, lipid, and fibrinogen by the hepatocytes. Subsequently, the high level of plasmatic glucose will increase the insulin production by the pancreas leading to a hyperinsulinemia. This results in the loss of homeostasis in blood vessels (Eckel et al. 2005; Wozniak et al. 2008; Gauthier et al. 2010).

In the following sections we will focus on the effect of obesity on the urogenital apparatus, but mainly on the kidney and testicular homeostasis. This apparatus is characterized by a topographic and embryologic relationship between its organs (Hinrichsen et al. 1986).

### **A- The drawbacks of obesity on kidney homeostasis**

The increase in the prevalence of obesity parallels the increase in the chronic kidney disease worldwide (Sharma et al. 2014). As mentioned earlier, obesity can lead to the development of hypertension and type 2 diabetes which together account for approximately 70% of end-stage renal disease (Griffin et al. 2008).

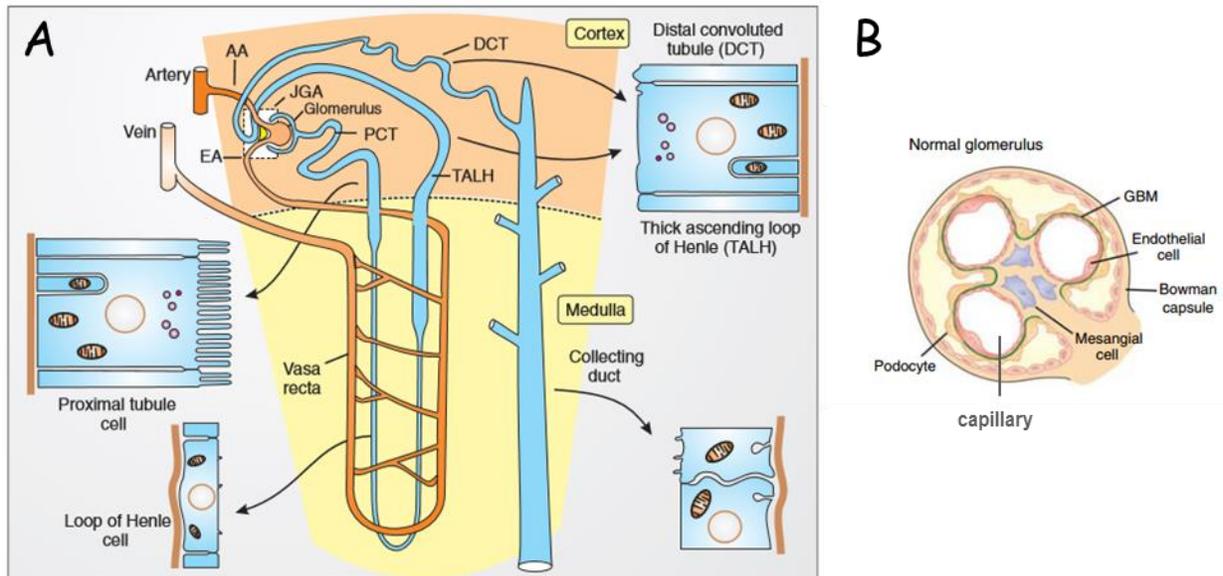
#### **A. 1 Kidney physiology**

The mammalian body contains 2 kidneys that are located in the retro-peritoneal region, on either side of the vertebral column. They play a pivotal role in the urine formation, in the excretion of metabolic waste products and toxic molecules, in the maintenance of Na<sup>+</sup> homeostasis, in osmoregulation, and also function as endocrine organs. At the histological level, each kidney is made up of approximately one million nephrons. Nephron is the functional autonomous unit of the kidney that has (**Figure 11A**) (Berridge et al. 2012):

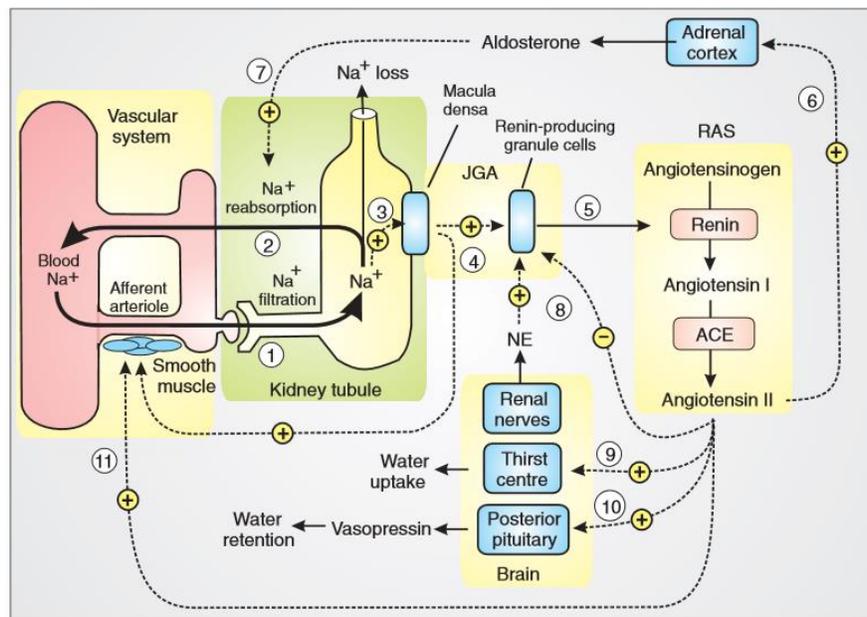
- An afferent arteriole which branches and narrows into capillaries to form the **glomerulus**. The endothelial cells of each capillary are surrounded by a basement membrane, and an outermost layer of podocytes. These three layers form the healthy glomerular filtration barrier. This filtration barrier helps to serially limit the escaping of serum large proteins (e.g., albumin) from the capillary loop; an abnormal protein leakage from the capillaries may lead to tubulointestinal injury and thus a decline in kidney function (**Figure 11 A, 11 B**) (Jefferson et al. 2008).
- Between the capillaries of the glomerulus there is a group of specialized cells termed **mesangial cells**. They are multifunctional cells; they can contract/relax and thus regulate the blood flow. They play a structural role in the kidney due to their contribution in the secretion and degradation of the extracellular matrix (such as: fibronectin, collagen IV, percalan, and laminin).
- The glomerulus is surrounded by a **Bowman's capsule**. The space created between the glomerulus and the Bowman's capsule is termed as **Bowman's space**. The blood plasma and all the small soluble molecules exit the capillaries and accumulate in the Bowman's space; thus creating the primary urine filtrate.

- Then the primary urine flows into the nephron tubules. These tubules are surrounded by a second capillary network. The latter is created from branches of the efferent arteriole that leaves the glomerulus. This capillary network around the tubules is essential for modifying the composition of the primary urine filtrate.
- The filtrated urine first enters the proximal convoluted tubule (PCT). It is responsible for water and  $\text{Na}^+$  reabsorption from the urine. That's why the cells of the PCT contain aquaporins, and a specialised plasma membrane suitable for isotonic fluid reabsorption.
- Next, the filtrate enters the thin descending part of loop of Henle. At this level, the water is also reabsorbed by the aquaporin of the descending portion cells. Consequently, the urine becomes highly concentrated.
- As the concentrated fluid moves up the thick ascending loop of Henle (TALH) the cells become tightly linked together; they can absorb sodium but they have less water permeability. Fascinatingly, the close association between the TALH and the arterioles of the glomerulus creates the juxtaglomerular apparatus (JGA). The cells of the TALH facing the JGA are specialised cells termed macula densa. They can detect the concentration of  $\text{Na}^+$  and  $\text{Cl}^-$  in the urine (**Figure 12**). When the concentration of  $\text{Na}^+$  in the TALH is too high it will draw water out of the body and decrease the blood pressure. In order to maintain a steady blood pressure and to prevent dehydration, the kidney activates auto-regulatory mechanisms:
  - A. The macula densa cells detect the high concentration of  $\text{Na}^+$  and release ATP. The latter will stimulate the renin-producing granular cells. Consequently, these cells secrete renin that will transform the angiotensinogen in the plasma into angiotensin I. Furthermore, the angiotensin-converting enzyme (ACE) converts angiotensin I into angiotensin II (**Figure 12**).
  - B. By itself, angiotensin II is a hormone that can perform various functions (**Figure 12**):
    - a) It stimulates the secretion of aldosterone by the adrenal gland. Aldosterone regulates the reabsorption of  $\text{Na}^+$  by the distal convoluted tubules (DCT).
    - b) In addition, the angiotensin II may act on the brain to drive water uptake, and to stimulate the secretion of the vasopressin which is responsible for water retention.
    - c) Plus, the angiotensin II and the ATP produced by the macula densa cells could act on the smooth muscle cells to increase blood vessel constriction.
    - d) Moreover, angiotensin II inhibits the secretion of renin by the renin-producing granular cells.

- The dilution of the urine continues in the DCT with  $\text{Na}^+$ ,  $\text{Cl}^-$ , and  $\text{Ca}^{2+}$  reabsorption. Finally, several DCT are connected to a collecting duct, where the cells of the upper region of the duct continue to reabsorb the  $\text{Na}^+$ . However, water reabsorption in collecting ducts is only possible via the influence of the vasopressin (Berridge et al. 2012).



**Figure 11: Anatomy of the nephron.**(A)The structural organisation of the nephron (Berridge et al. 2012).(B)The gross anatomy of normal healthy glomerulus (Jefferson et al. 2008).



**Figure 12: Regulation of blood  $\text{Na}^+$  levels by the kidney** (Berridge et al. 2012).

## A. 1.1 Obesity-related glomerulopathy (ORG)

The increase in the BMI was shown to be associated with pathological changes in the glomeruli histology and physiology (Griffin et al. 2008).

A. 1.1.a The effect of obesity on the glomerular histology (de Vires et al. 2014; Sharma et al. 2014; D'Agati et al. 2016):

- Glomerulomegaly (glomerular hypertrophy) is characterized by the increase in the glomerular diameter and/or volume measured on histological sections. The biopsy samples from obese individuals may show an increase in the diameter of the glomerular capillaries and the afferent arteriole. In response to the glomerulomegaly, the podocytes may show a mal-adaptive response to obesity by increasing their size (hypertrophic changes) and decreasing their density.
- Progressive glomerulosclerosis is defined as an increase in the extracellular matrix deposition and/ or sclerosis (fibrosis) between the capillaries of the glomerulus leading to their obliteration. The glomerulosclerosis occurs mainly at the glomerular vascular pole, but also it could develop at any site of the glomerulus. In a mice model of diet-induced obesity, histological sections of the kidney showed an increase in the thickness of the glomerular basement membrane, a mesangial matrix expansion, and an increase in the deposition of type IV collagen in the glomeruli. These findings indicate that the high fat diet may induce glomerular sclerotic injury in mice (Deji et al. 2008).
- Tubular damage and interstitial fibrosis are also common features in obese patients.

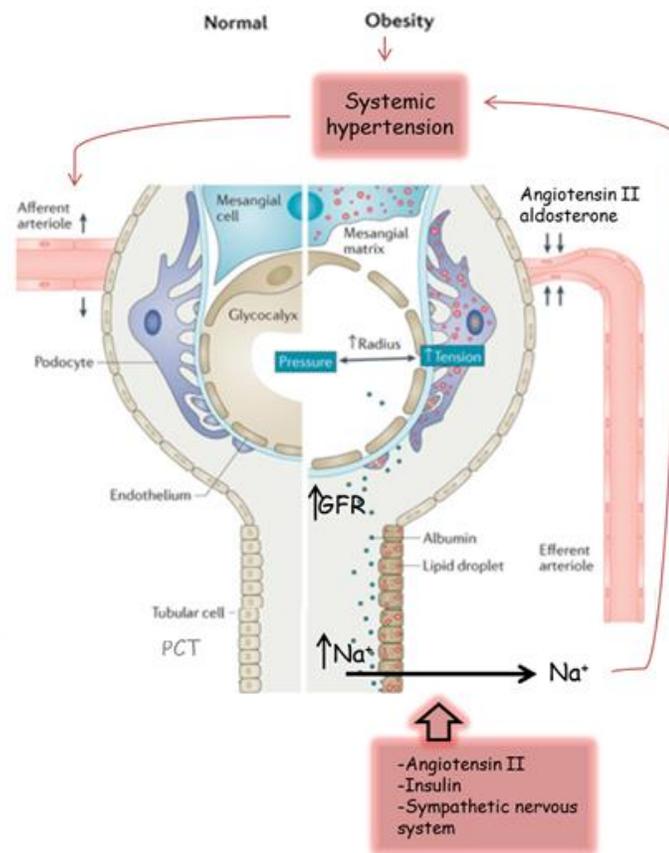
A. 1.1.b Decline in renal function and obesity

a) Renal hemodynamics (**Figure 13**):

The glomerular filtration rate (GFR) is defined as the total fluid volume filtered by the glomeruli of both kidneys per unit time (~ 180 L/day). Approximately, 99% of the GFR is reabsorbed by the nephron tubules and 1% is excreted (urine output ~ 1 to 2 L/day). Moreover, some indicators present in the plasma may help to measure the GFR. For instance, the creatinine could be used as an indicator since it does not alter the renal function and it is freely filtered, not reabsorbed by the tubules, and not metabolized by the kidney. However, glomerular hyperfiltration is an abnormal increase in the GFR of the kidneys (Helal et al. 2012). Of particular interest, several studies showed that obesity is generally associated with systemic hypertension, resulting in glomerular afferent arteriole vasodilation. The high blood flow may augment the pressure inside the capillaries which may lead to an increase in the GFR (Chagnac et al. 2000).

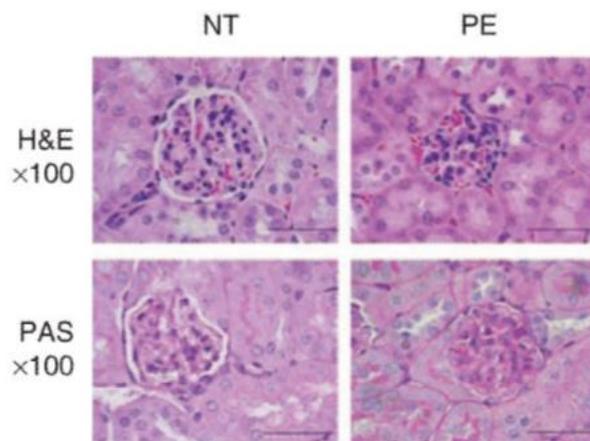
Moreover, this abnormal glomerular hyperfiltration in obese subjects is more likely correlated to an increase in sodium concentration in the tubules (Strazzullo et al. 2001). To counterbalance the loss of this high concentration of salt, the renin-angiotensin-aldosterone system is overactivated. The level of aldosterone and angiotensin II were shown to be higher in obese subjects (Engeli et al. 2004). On one hand, angiotensin II stimulates  $\text{Na}^+$  reabsorption in the PCT. On the other hand, aldosterone and angiotensin II may induce efferent arteriole vasoconstriction which may increase the pressure inside the capillary and thus promotes further glomerular hyperfiltration. BMI and WC are independent predictors of sodium reabsorption in the PCT (D'Agati et al. 2016).

Secondary to the excessive reabsorption of salt in the PCT, the low levels of  $\text{Na}^+$  in the urine could be detected by the macula densa of the TALH. Consequently, the tubuloglomerular feedback is deactivated resulting in further afferent arteriole vasodilation and thus glomerular hyperfiltration (D'Agati et al. 2016). Moreover, the glomerular hyperfiltration may affect the protein handling in the glomeruli. Epidemiological and observational studies demonstrated that the prevalence of proteinuria or macroalbuminuria is increased in obese patients (Valensi et al. 1996; Chen et al. 2004).



**Figure 13: Pathogenesis of obesity-related glomerulopathy.** The two major players in the development of ORG are the hypertension and the lipid accumulation in the kidney (D'Agati et al. 2016).

Altogether these hemodynamic changes in obese patients will lead to a glomerulomegaly with capillary growth, podocytes hypertrophy, detachment and apoptosis. Consequently, these histological lesions will develop the obesity-related glomerulosclerosis (D'Agati et al. 2016). Furthermore, chronic and serious hypertension (e.g., pre-eclampsia during pregnancy) may increase the risk of renal failure and present small and condensed glomeruli, narrowed Bowman's space, and occluded capillary lumen on histological sections of the kidney (Datta et al. 2004; Siddiqui et al. 2011) (**Figure 14**).



**Figure 14: Histological kidney sections.** Histological kidney sections stained with haematoxylin and eosin (upper panel) and with periodic acid Schiff (lower panel). These sections show the small glomerulus and the narrowed Bowman's space in the kidney derived from pregnant mice with pre-eclampsia (severe hypertension) (Siddiqui et al. 2011).

b) Hyperglycemia and hyperlipidemia:

In humans and mice, the adipose tissue increases the size and the number of its adipocytes as the body weight increases (Guo et al. 2004; Wozniak et al. 2008). It can secrete adipokines such as leptin, adiponectin, and resistin that could affect renal extracellular matrix, cellular hypertrophy and fibrosis in rodents (D'Agati et al. 2016). Moreover, obesity in humans and rodents may lead to the accumulation of lipids in the kidney “fatty kidney” (mainly in mesangial cells and tubular cells), resulting in inflammation, and fibrosis (**Figure 13**)(de Vires et al. 2014).

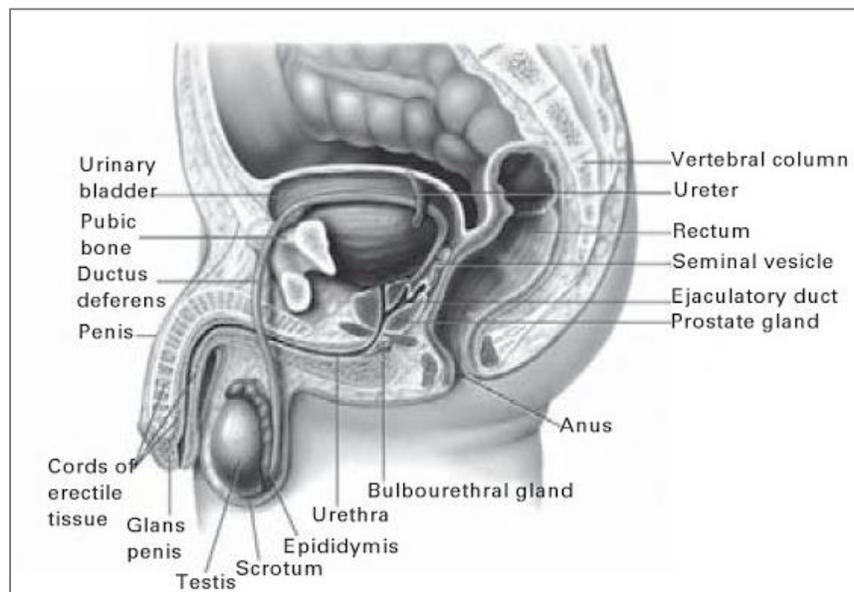
In addition, obesity may induce the development of type 2 diabetes leading to a hyperglycemia. High glucose levels could activate several pathways in the kidney (e.g., hexokinase pathway, polyol pathway) promoting an abnormal deposition of the extracellular matrix components such as fibronectin and collagen I and IV in the glomerulus(Sharma et al. 2013; Reidy et al. 2014). The diabetic-kidney disease (DKD) is a leading cause of the development of end-stage renal disease. The DKD is characterized by: arteriole hyalinosis, basement membrane thickening, mesangial cells hypertrophy, nodular glomerulosclerosis development, podocytes hypertrophy/loss, albuminuria followed by a decline in the GFR, tubulointestinal inflammation and fibrosis (Alsaad et al. 2006; Tervaert et al. 2010; Reidy et al. 2014).

## B- The drawbacks of obesity on testicular homeostasis

Andrology (from Greek *Andros*, man), is a multifaceted medical specialty dealing with normal and pathological aspects of male sexuality and reproductive health (Auger et al. 2009). Badly, there is an increase in the prevalence of male obesity among young men suffering from andrological disorders (Palmer et al. 2012) . The following section, will discuss in details the impact of obesity on the reproductive health of males.

### B. 1 Male reproductive system anatomy (Figure 15)

The testicles are twin oval-shaped gonads that produce male gametes (motile spermatozoa) and sexual hormones. Testicular functions are thermodependent ( $\sim 36^{\circ}\text{C}$ ) and sensitive to heat stress. That's why they are localised outside the body in the scrotum. The scrotum is a suspended thin skin sac, containing contracting muscles (dartos and cremaster) with minimal subcutaneous adipose tissue. These features plus the testis vasculature play a major role in the testicular thermoregulation, preventing hyperthermia and heat stress (Durairajanaygam et al. 2015). Furthermore, each testicle is attached to an epididymis characterized by sausage-shaped structure. The gametes mature as they pass through the epididymis and could be stored in it. The epididymis is connected to the vas deferens where the spermatozoa are mixed with the seminal vesicle secretions. Next, the spermatozoa are transported to the ureter due to the peristaltic contraction of vas deferens. In the ureter, the male gametes are exposed to the prostatic and Cooper gland secretions. The secretions of the different sex glands contribute to the complex content of the seminal plasma (**Table 3**). Finally, the semen (spermatozoa + seminal plasma) is ejaculated *via* the penile urethra (Caroppo et al. 2011).



**Figure 15: Sagittal view of testis and male pelvis** (Caroppo et al. 2011).

**Table 3: Table showing some of the sex gland secretions**

Sex gland	Molecules	Functions	References
Seminal vesicles	Fructose	Present at high concentrations (7.55-34.86 mM)	(Montagnon et al. 1982)
		Source of energy	(Owen et al. 2005)
	Insulin	Its concentration in the semen is higher than in serum ( $19 \pm 3\mu\text{U/ml}$ vs. $7.53\mu\text{U/ml}$ respectively)	(Hicks et al. 1973) (Paz et al. 1977)
		Largely unknown	(Lampiao et al. 2008)
		May enhance sperm motility	(Lampiao et al. 2008)
	Prostaglandins	Immune tolerance of male gametes in the female tract	(QUAYLE et al. 1989)
	Semenogelin	Gelatinous entrapment of ejaculated spermatozoa, thus playing a major role in semen coagulation	(Wang et al. 2015)
Fibronectin	Gelatinous entrapment of ejaculated spermatozoa, thus playing a major role in semen coagulation	(Wang et al. 2015)	
Prostate	Carnitine	Metabolism of fatty acids and antioxidant effect	(Abd-Allah et al. 2009)
	Calcium ions	Regulation of sperm physiology and modulation of oxidative stress	(Stru'inker et al. 2011)
	Zinc	Antioxidant, antibacterial, and protection against heavy metals (lead)	(Dissanayake et al. 2010)
	Prostate-specific antigen (PSA)	Digestion of the gel formed after ejaculation = liquefaction	(Wang et al. 2015)
Cooper gland	Lubricating fluid	Neutralize urine acidity and lubricate the urethra	(Choughtai et al. 2005)
By the sperm itself	Glucose	present in semen at a range of concentrations ( 0.22-16.37 mM)	(Bakos et al. 2010)
		Present endogenously within the sperm as glycogen/ source of energy	(BALLESTER et al. 2000)
	Insulin	Auto-regulation of glucose metabolism	(Aquila et al. 2005)

### B.1.1 The effects of obesity on testicular anatomy, sexual function and seminal plasma biochemistry

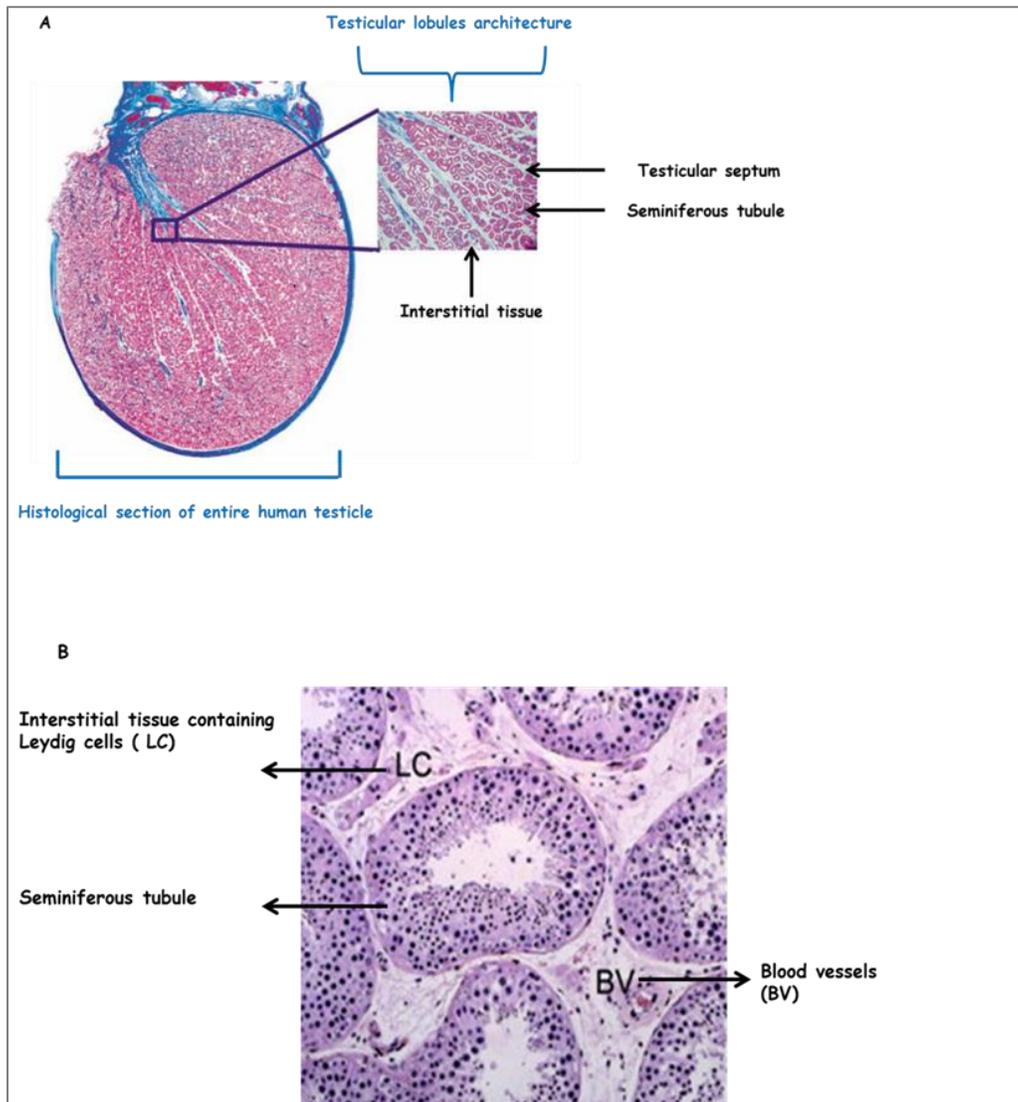
The sedentary life style and the increase in calories intake by obese men could impair their reproductive health. On one hand, male obesity may lead to the accumulation of scrotal fat; thus inducing testicular hyperthermia. The excess of subcutaneous adipose tissue in the scrotum may inhibit the sperm production by the testicles and increase the risk of infertility. However, surgical removal of the scrotal fat in obese patients improves the sperm count, motility, and morphology (Sahfik et al. 1981; Durairajanaygam et al. 2015). On the other hand, erectile dysfunction and reduced libido are common conditions among obese men (Bacon et al. 2006; Larsen et al. 2007; Traish et al. 2009). Interestingly, these functions could be restored after weight loss (Esposito et al. 2004).

Furthermore, the sex accessory glands and the seminal plasma components could also be affected by male obesity (Binder et al. 2015). The seminal plasma is an alkaline gelatinous fluid (pH ~ 7.2), made up of seminal vesicle secretions (~60% of the total semen), and prostate gland secretions (~20% of the total semen volume). Moreover, this biological fluid not only serves as a transport and survival medium for spermatozoa, but also can modulate the female reproductive tract; potentially influencing the embryo development and implantation. It was demonstrated in mice, pigs, and humans, that seminal plasma induces a post-mating inflammatory response in the female tract, facilitating the embryo implantation. In addition, several studies in mammals highlighted the role of seminal plasma in regulating fetuses growth trajectory (Robertson et al. 2005; Lane et al. 2014). In a mice model of diet induced obesity, Binder and colleagues showed that seminal vesicle proteins and metabolites are affected by obesity. Also, clinical data indicate that obese men are at higher risk to have a reduced semen volume (Eisenberg et al. 2014) and an altered seminal plasma biochemistry. The level of insulin, leptin, fructose, and interleukin 8 were found to be higher in the semen of obese men; whereas the adiponectin, progranulin, and alpha-glucosidase levels were lower (Martini et al. 2010; Lotti et al. 2011; Thomas et al. 2012; Leisegang et al. 2014). Plus, men with type 2 diabetes had a higher level of nitric oxide (NO) in the seminal plasma when compared to non-diabetic men. NO can lead to an oxidative stress at high concentration, and its presence in the seminal plasma of diabetic men was positively correlated to the presence of oxidized nucleoside of DNA (8-hydroxydeoxyguanosine (8-OHdG)). Further, malondialdehyde (MDA) which is one of the oxidative stress markers was detected at significantly higher level in the semen of men with type 2 diabetes when compared to non-diabetic ones. Altogether, these data open the question on the drawbacks of this altered seminal plasma composition in obese men on the spermatozoa, the uterine environment, and the embryo development.

Besides these alterations, several scientific papers hot spotted the correlation between obesity and prostate cancer. It was concluded that obese men are at greater risk to experience aggressive prostate cancer specific mortality (Discacciat et al. 2012; Moller et al. 2014).

## B. 2 Testicular histology and physiology

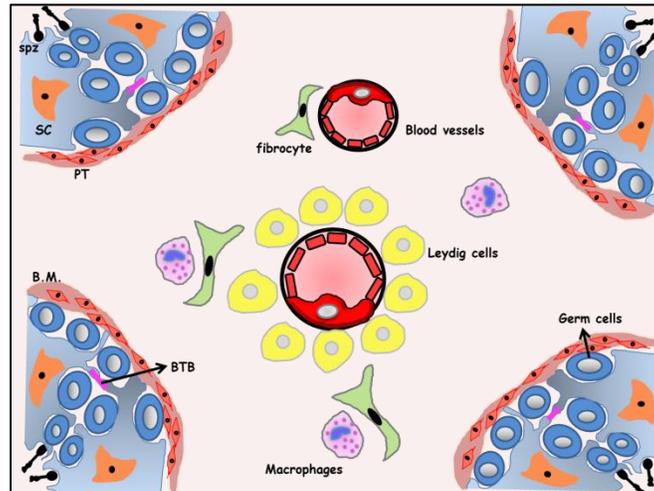
The human testis is divided by a group of septa into 250 to 300 lobules. Each lobule is composed of interstitial tissue and one to three seminiferous tubules (figure 16: A and B). The interstitial and the tubular compartments are histologically distinguishable from each other but are physiologically connected. All the processes involved in the production of male gametes take place in the tubular compartment, whereas the main function of the interstitial tissue is the production of male sex hormones. The integrity of both compartments is crucial for sperm cells (male gametes) differentiation (Weinbauer et al. 2010).



**Figure 16: Histological section of the human testis**(A)Histological section of entire human testicle (Weinbauer et al. 2010). (B)Histological section of the human testis (x10) showing the two testicular compartments (McLchlan et al. 2007).

### B.2.1 The interstitial compartment (Weinbauer et al. 2010)

The interstitial compartment occupies approximately 12-15 % of the testicular volume. It is composed of Leydig cells ( $200 \times 10^6$  Leydig cells per testis), immune cells, blood vessels, fibroblasts, and connective tissue (**Figure 17**).



**Figure 17: Illustration of the testicular interstitial tissue.** Spz= spermatozoa, PT = peritubular cell, BTB= blood-testis barrier.

#### a) Blood vessels

The testicular interstitial tissue contains small arteries, capillaries and small veins. The circulating nutrients, oxygen and hormones leave the blood vessels and diffuse into the interstitial and tubular compartment. In contrary, the metabolic and cellular wastes are carried away by the small veins (Jones et al. 2013). It was suggested that diabetes in men and rats may increase the wall thickness of blood vessels, and modify the vascular density, thus altering the testicular homeostasis (Villarroel-Espindola et al. 2015).

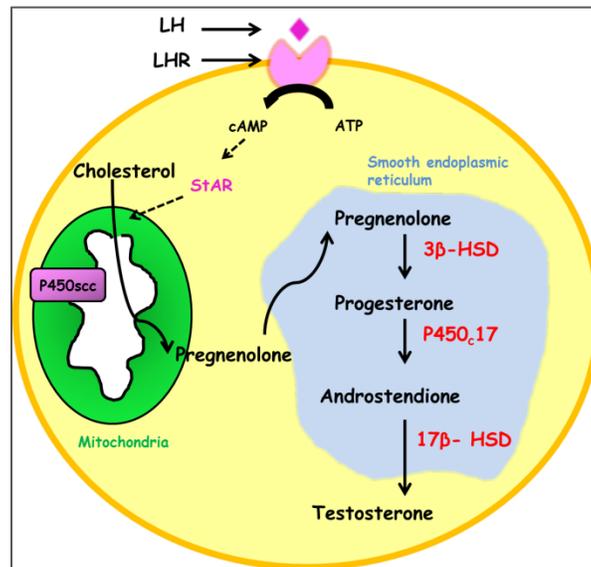
#### b) Leydig cells

##### b.1 Overview

Leydig cells were first described in 1850 by Franz Leydig. These cells secrete the most important male steroid hormone called testosterone via steroidogenesis. The latter, is a group of enzymatic reactions leading to the production of testosterone from cholesterol. Moreover, leydig cells secrete insulin-like factor 3 (INSL3) which is a local regulator of testicular functions. At the cytological level, Leydig cells are rich in smooth endoplasmic reticulum and mitochondria. These two organelles are mainly implicated in steroid production. Lipid droplets are also present in the cytoplasm and provide the starting materials for testosterone formation (Weinbauer et al. 2010).

## b.2 Steroidogenesis

Leydig cells contain the machinery for androgen (testosterone) production. They have a direct access to the blood vessels, permitting the uptake of the luteinizing hormone (LH) and cholesterol from the circulation to produce male sexual hormone. The cascade of events leading to the synthesis of testosterone is summarized in **(Figure 18)**. The produced testosterone can diffuse to the interstitial and tubular compartment to regulate the process of spermatogenesis. Also, the testosterone is secreted into the blood. Testicular androgen plays important roles in brain masculinization and sexual behavior, in modulating the larynx growth, in the stimulation of erythropoietin synthesis in the kidney, in maturation of male sexual organs, hair growth, in regulating the bone and muscle mass, and in protein synthesis in the liver.

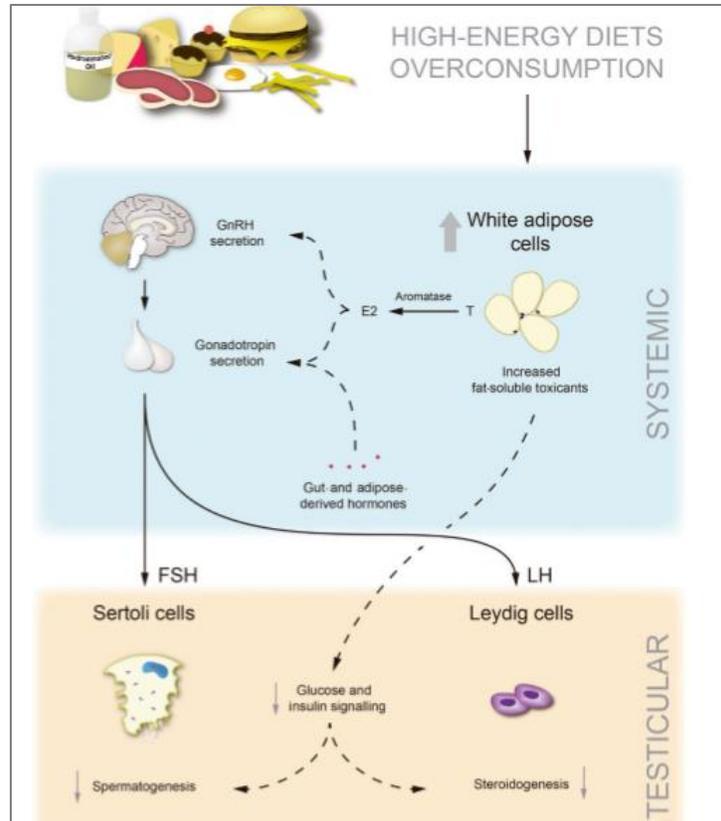


**Figure 18: Illustration of the cascade of events leading to male sexual hormone production by the Leydig cell.** The luteinizing hormone (LH) binds to its receptor (LHR) on the plasma membrane of the Leydig cell and activates it. This will lead to the transformation of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP can stimulate a signaling cascade that will activate a group of genes involved in the steroidogenesis process. Free cholesterol could be transported to the mitochondria after the synthesis of the steroidogenesis acute regulatory protein (StAR). Inside this organelle, the cholesterol will be transformed to pregnenolone *via* the activity of cytochrome P450 side chain cleavage (P450<sub>scc</sub>). Pregnenolone is subsequently, transported to the smooth endoplasmic reticulum and metabolized by several enzymes (3β-hydroxysteroid dehydrogenase (3β-HSD), steroid 17 alpha-hydroxylase/17,20 lyase (P450<sub>c17</sub>), and 17β-hydroxysteroid dehydrogenase (17β-HSD)) to form testosterone (Zirkin et al. 2000).

### b.3 The effect of obesity on Leydig cell physiology and androgen homeostasis

The physiology of the testis is under the control of the endocrine system. Furthermore, the hormone produced by the testis regulates the action of the hypothalamus-pituitary-testis axis. In men with normal weight, the hypothalamus releases the gonadotropin-releasing hormone (GnRH) that will bind to its receptor on the pituitary gonadotrophs stimulating the release of LH and follicle stimulating hormone (FSH). LH can stimulate the Leydig cells to produce testosterone. The circulating testosterone is mainly bound to the albumin or to the sex hormone binding globulin (SHBG) synthesized by the liver. The SHBG reduces the clearance rate of the androgen and regulates their access to target tissues. In addition, the free circulating testosterone can be transformed in the adipose tissue into oestrogen (E2) under the activity of an enzyme called aromatase. High oestrogen level can reduce the GnRH secretion from the hypothalamus (Michalakis et al. 2013).

In obese men, the excess fat mass and the hyperinsulinemia may lead to a decrease in the SHBG production by the liver. This will increase the amount of free testosterone available for conversion into E2 by the accumulated fat mass. In 2005, Pitteloud et al., demonstrated that insulin resistance can also inhibit the testosterone secretion by Leydig cells. These pathophysiological changes could explain the increase in E2 concentration and the decreased level of testosterone and gonadotropins in obese men (Pitteloud et al. 2005; Michalakis et al. 2013) . In parallel, glucose and lipid homeostasis are essential for normal Leydig cell functioning. Alteration in glucose levels and consumption of high amounts of unhealthy fats may inhibit the testosterone production and induce Leydig cell apoptosis (**Figure 19**) (Amrolia et al. 1988; Lu et al. 2003; Rato et al. 2014).



**Figure 19: The high-energy diet overconsumption affects the reproductive axis at the central and at the gonadal levels.** Solid lines represent stimulatory effects; dashed lines represent inhibitory effects (Rato et al. 2014).

c) Immune cells (Weinbauer et al. 2010):

Interstitial immune cells play an important role in the testicular homeostasis. There is approximately one macrophage for every 10-50 Leydig cells. The main functions of these immune cells are:

c.1 Phagocytosis, proliferation and physiology of Leydig cells

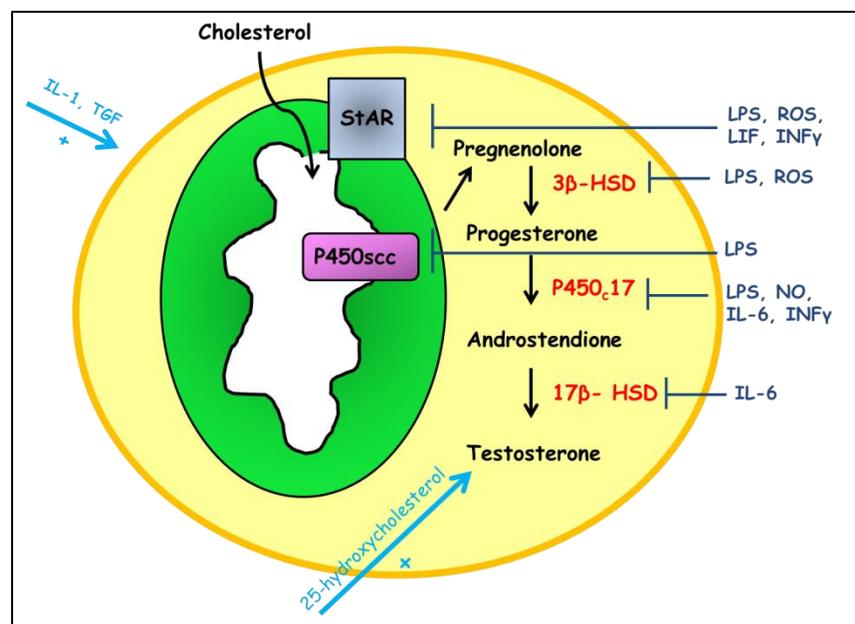
Macrophages and monocytes are potential sources for regulatory factors such as interleukin-1 (IL-1) and tumour growth factor (TGF) that may modulate Leydig cell phagocytosis, proliferation and differentiation. Interestingly, testicular macrophages can stimulate the steroid production by Leydig cells *via* the secretion of 25-hydroxycholesterol.

### c.2 Immunoregulation and inflammation

Inflammatory response against bacterial and viral infection can occur in the testicular interstitial tissue. Interstitial leukocytes can secrete pro-inflammatory cytokines such as interleukin 6 (IL-6), interferon gamma (IFN- $\gamma$ ), reactive oxygen species (ROS), and prostaglandins that could negatively affect the spermatogenesis and the steroidogenesis (**Figure 20**). Likewise, testicular mast cells could be activated and secrete a serine protease tryptase. This product can stimulate the collagen production by interstitial fibroblast, resulting in fibrosis.

### c.3 The effect of obesity on testicular immune cells

The mouse model of diet-induced obesity is a widely used model to mimic the acquired obesity in humans. A recent study showed that mice on high fat diet (HFD) had an alteration in the testis inflammatory profile. These obese mice had an increased level of pro-inflammatory cytokines mRNA expression (TNF- $\alpha$ , MCP-1, and F4/80) in the testis. This pathogenic inflammation may alter the testicular physiology (Zhang et al. 2015).

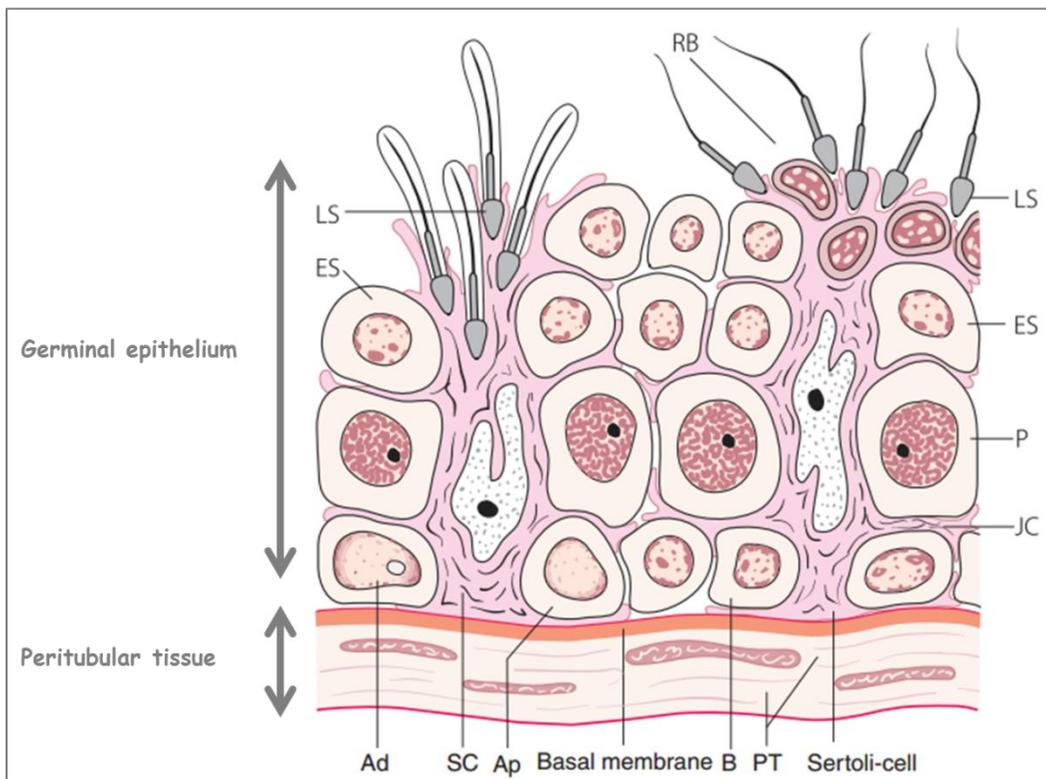


**Figure 20: Interstitial immune cells secrete stimulatory and inhibitory factors that may regulate Leydig cell physiology.**(1) Reactive oxygen species (ROS), lipopolysaccharide (LPS), interferon- $\gamma$  (IFN- $\gamma$ ), leukemia inhibitory factor (LIF) can inhibit the Steroidogenic acute regulatory protein (StAR) (2) 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) could be inhibited by: ROS, and LPS (3) P450<sub>scc</sub> may be inhibited by: LPS (4)LPS, IL-6, IFN- $\beta$ , and nitrite oxide (NO) may inhibit P450<sub>c17</sub> (5) 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD) may be inhibited by IL-6.(Hales et al. 2002).However, IL-1 and TGF can regulate the proliferation and

differentiation of leydig cells whereas 25-hydroxycholesterol may stimulate the testosterone production.

### B.2.2 Tubular compartment

The total number of seminiferous tubules is about 600 per testis and the length of each seminiferous tubule is approximately 60 cm. Thus, the tubular compartment occupies approximately 60 to 80 % of the testicular volume. The seminiferous tubule is made up of peritubular tissue (basal membrane and peritubular cells) and germinal epithelium (Sertoli cells and germinal cells) (**Figure 21**).

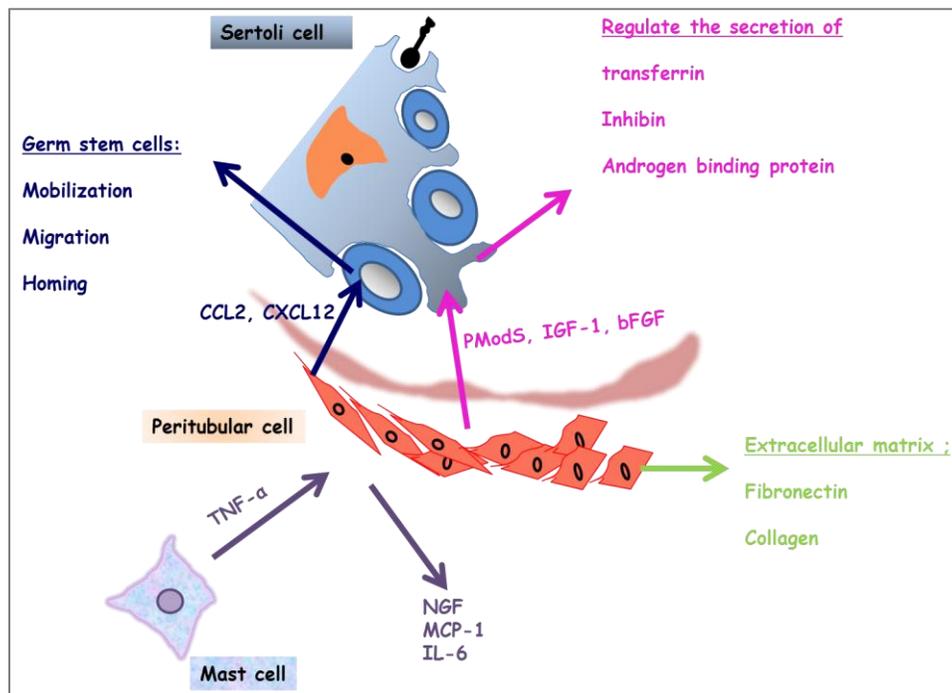


**Figure 21: Organization of the seminiferous tubule.**

RB: residual body, LS: late elongated spermatid, ES: elongating spermatid, P: pachytene spermatocyte, JC: junctional complexes of the BTB, PT: peritubular cells, B: type B spermatogonia, Ap: type A pale spermatogonia, SC: Sertoli cell, Ad: type A dark spermatogonia (Weinbauer et al. 2010).

a) Peritubular cells

The wall of seminiferous tubule is covered by several layers of elongated cells. These peritubular cells (PT) have the capacity to contract leading to the movement of fluid and sperm from the tubule. This could be explained by the expression of several receptors for the regulators of cell contractions by the PT such as endothelin, angiotensin, vasopressin, oxytocin, prostaglandins and androgenic steroids. Moreover, adrenomedullin relaxant peptide secreted by Sertoli cells can induce peritubular cell relaxation. Besides that, human and rodents PT cells secrete a wide range of factors that can regulate Sertoli cell secretome, extracellular matrix composition, germ stem cells regulation and modulate the inflammatory response (**Figure 22**) (Weinbauer et al. 2010). However, a decrease in spermatogenic activity in the seminiferous tubule is associated to loss of PT contractility and increase of collagen deposition in the peritubular wall. This fibrotic remodelling is a hallmark of testicular dysfunction(Albrecht et al. 2009; Flenkenthaler et al. 2014) and might be induced by diabetes (Villarroel-Espindola et al. 2015).



**Figure 22: Scheme illustrating the function of some the peritubular cells (PT) secreted factors.**

The tumour necrosis factor alpha (TNF- $\alpha$ ) secreted by interstitial mast cells can stimulate the secretion of immunomodulation factors such as nerve growth factor (NGF), monocyte chemoattractant protein-1 (MCP-1), and interleukin-6 (IL-6) by the PTC. Moreover, the human and rodent PT can secrete molecules implicated in extracellular matrix formation like fibronectin and collagen. Importantly, the PT may modulate the secretion of transferrin (which transports iron to the germ cells), inhibin, and androgen binding protein (which stimulates the seminiferous

epithelium and carries androgens to the epididymis) by the Sertoli cell. This modulation is possible due to specific factors such as PMOdS (peritubular modifying substance), insulin-like growth factor-1 (IGF-1), and bFGF secreted by the PT (Albrecht et al. 2009). Also, the analysis of the PTC secretome showed an important presence of two chemokines (C-C motif chemokine 2 (CCL2) and stromal cell derived factor 1 (CXCL12)) that may play a role in germ stem cells mobilization, migration, and homing. (Albrecht et al. 2009; Flenkenthaler et al. 2014)

## b) Sertoli cells

### b. 1 Overview

The Sertoli cells are the somatic cells of the seminiferous tubules, which are closely linked to germ cells and enable their protection and development. They were first described by the Italian scientist Enrico Sertoli in 1865. The normal testis contains 800 to 1200 x 10<sup>6</sup> Sertoli cells, which represents approximately 35 to 40 % of the germinal epithelium volume (Weinbauer et al. 2010).

### b. 2 Cytological features

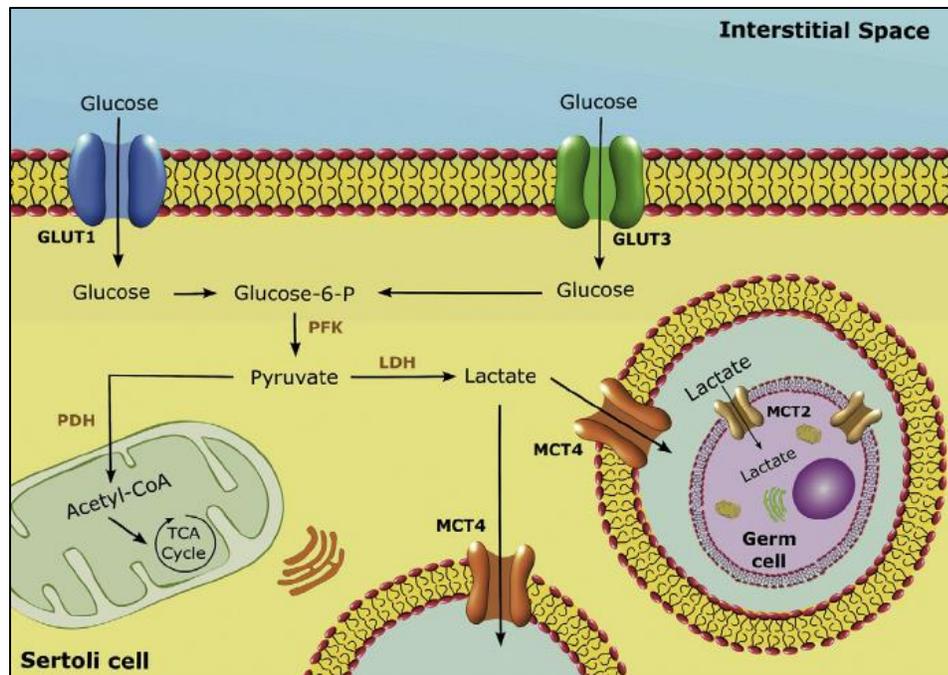
The Sertoli cell is a large pyramidal cell which extends over the entire height of the seminiferous epithelium from basement membrane until the lumen of the tubule. Its nucleus is large, triangular, and presenting visible nucleoli in testis biopsy slides. In the seminiferous epithelium, the germ cells are surrounded by the ramifications of these Sertoli cells. Furthermore, the cytoplasm is rich in smooth endoplasmic reticulum indicating the presence of steroid synthesis, as well as rough endoplasmic reticulum which is a mark of protein synthesis. In addition, the cytoplasm contains a developed Golgi apparatus implicated in the management of secretory products, an important number of lysosomal granules involved in the process of phagocytosis in addition to microtubules and intermediate filaments used by the Sertoli cells for shape adaptation (Weinbauer et al. 2010).

### b. 3 Sertoli cell functions

The Sertoli cells play a crucial role in the development of germ cells and in the regulation of spermatogenesis. They receive the hormonal messages (FSH and testosterone) and the local signals (autocrine and paracrine), allowing them to secrete other factors that modulate their own functions, and those of germ cells and Leydig cells (Weinbauer et al. 2010).

The Sertoli cells perform several functions:

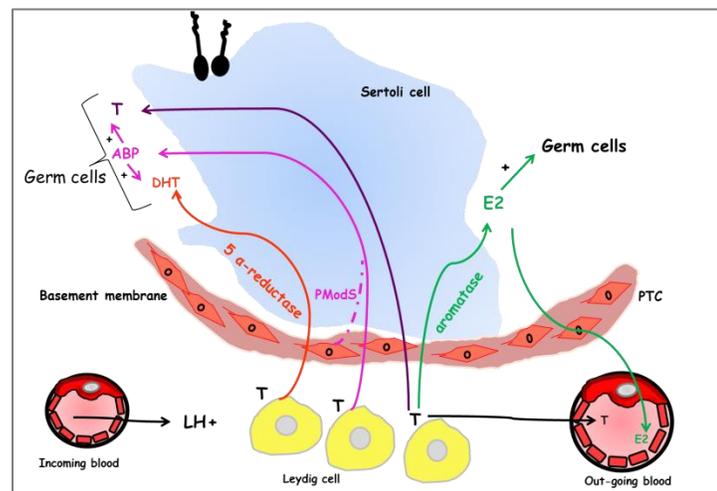
- **They are in charge of the final testicular volume.** In adults, Sertoli cells are mitotically inactive, so the number of these cells is created before puberty. They define the final testicular volume. In addition, Sertoli cells have the capacity to support a fixed number of germ cells. In men, each Sertoli cell is connected to approximately 30-50 germ cells (Neto et al. 2016). This binding is under the control of testosterone. Low testosterone levels in obese men may lead to retention and phagocytosis of spermatids and thus decrease mature sperm count (O Mc Pherson et al. 2015).
- **They secrete the transferrin and the ferritin, which transport the iron to the germ cells.** Testicular iron homeostasis is essential for germ cells DNA synthesis and mitochondriogenesis (Leichtmann-Bardoogo et al. 2012). It was shown that transferrin secretion by Sertoli cells is under the control of insulin, IL-1, IL-6, TNF- $\alpha$ , PMODS, bFGF, and IGF-1 (Huleihel et al. 2004, Dias et al. 2014).
- **They provide the energy substrates necessary for the germ cell survival.** Sertoli cells can uptake glucose *via* glucose transporters (GLUTs) under the control of androgens, FSH and insulin. After this internalization, the glucose could be converted to pyruvate *via* an enzyme called phosphofructokinase (PFK). On one hand, pyruvate could be converted by pyruvate dehydrogenase (PDH) to acetyl-coenzyme A, which enters the mitochondria to generate ATP. On the other hand, pyruvate could generate lactate *via* the activity of lactate dehydrogenase (LDH). Since the germ cells cannot metabolize glucose they will uptake the generated lactate by Sertoli cells. The monocarboxylate transporter (MCT) will facilitate the lactate internalization in the germ cells in order to produce ATP (**Figure 23**)(Dias et al. 2014).



**Figure 23: Schematic presentation of the metabolic coupling between Sertoli cells and the germ cells.** Abbreviations: GLUT1 and GLUT3, glucose transporter 1 and 3; MCT2 and MCT4, monocarboxylate transporter 2 and 4; TCA, tricarboxylic acid; PFK, phosphofructokinase; LDH, lactate dehydrogenase, PDH, pyruvate dehydrogenase (Dias et al. 2014).

- **They secrete the seminiferous tubular fluid and they regulate its composition.** This fluid creates a suitable environment for spermatogenesis and for the transports of the sperm cells in the testis. At the biochemical level, it is an acidic fluid, rich in water, sodium ( $\text{Na}^+$ ), chloride ( $\text{Cl}^-$ ), and potassium ( $\text{K}^+$ ) (Rato et al. 2010). The other constituents of the tubular fluid are bicarbonate, magnesium, inositol, carnitine, glycerophosphorylcholine, amino acids, and several proteins. All together, these compounds make the tubular fluid of unique composition in which the germ cells are immersed.
- **They have the ability to eliminate the defective germ cells and the sperm residual bodies by phagocytosis.** This biological process is important to maintain a constant ratio of germ cells to Sertoli cells, to eliminate the leakage of toxic compounds by degenerating cells and residual bodies, and to supply energy to the Sertoli cells (Hai et al. 2014).

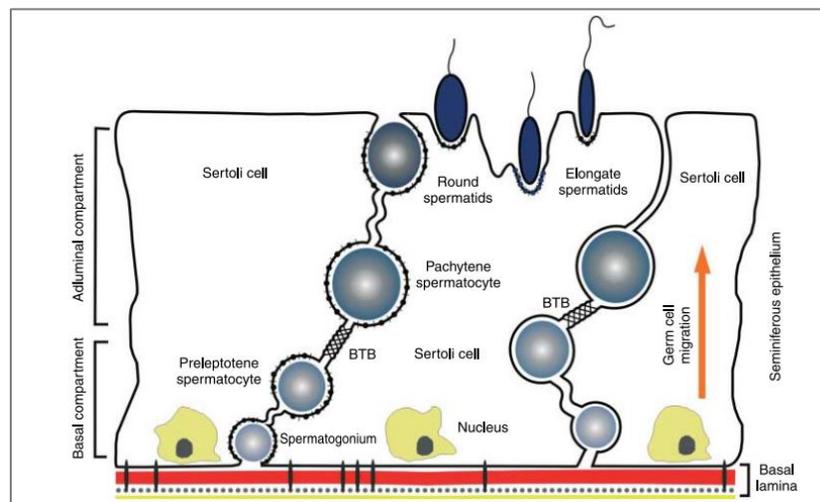
- **They play an important role in seminiferous tubule steroids homeostasis.** Under the influence of the circulating LH, the Leydig cells may produce testicular androgen (testosterone). At first, the testosterone can be metabolized in the Sertoli cell by the aromatase to produce estradiol (E2). The testosterone /E2 ratio may play an important role in the spermatogenesis. Secondly, the testosterone is a precursor for the production of a highly biologically active hormone dihydrotestosterone (DHT) by the 5  $\alpha$ -reductase in the Sertoli cell. In addition, the testosterone and the PModS (secreted by the PTC) can stimulate the secretion of the androgen-binding protein (ABP) by the Sertoli cells. It is a carrier protein that binds to testosterone and to DHT to regulate their bioavailability in the tubular fluid, and to control the process of spermatogenesis (**Figure 24**)(Jones et al. 2013).



**Figure 24: Steroids homeostasis in the seminiferous tubule.** Luteinizing hormone (LH), Testosterone (T), Androgen-binding protein (ABP), dihydrotestosterone (DHT), Estradiol (E2), peritubular cells (PTC), peritubular modifying substance (PModS).

- **Follicle stimulating hormone (FSH) regulates Sertoli cell physiology.** The GnRH hormone secreted by the hypothalamus can stimulate FSH and LH secretion by the pituitary gland. The circulating FSH binds to its receptor on Sertoli cell and control virtually all the functions related to spermatogenesis. Interestingly, FSH controls the expression of crucial molecules for sperm production such as inhibin B (negative regulator of FSH secretion), anti-mullerian hormone (AMH= function remains unclear in adult testis), aromatase, androgen-binding protein (ABP), steroidogenesis-stimulating protein (STP = stimulate steroid production in Leydig cells)....(Jones et al. 2013).

- **They secrete growth factors, cytokines and antioxidants.** Glial cell-derived neurotrophic factor (GDNF), TGF- $\alpha$ , TGF- $\beta$ , EGF, jagged-1/2, Interleukin1 (IL-1), and Interleukin 6 (IL-6) are Sertoli cells-derived growth factors and cytokines that regulate germ cells differentiation (Hai et al. 2014; Neto et al. 2016). Moreover, Sertoli cells may protect the germ cells from the oxidative stress *via* its antioxidants such as superoxide dismutase, glutathione reductase, transferase, and peroxidase (Bauche et al. 1994).
- **They create the blood testis barrier.** The blood testis barrier (BTB) is a structural boundary between interstitial capillaries and the interior of the seminiferous tubules. It is made up of specialized junctions between adjacent Sertoli cells dividing the seminiferous epithelium into two compartments. The basal compartment where the early germ cells are located and the adluminal compartment containing the later stages of maturing germ cells. The functions of the BTB are to control the exchange of large molecules between the metabolically separated compartments, to isolate and to prepare the special milieu for germ cell differentiation, and to protect the germ cells from the immune system (**Figure 25**)(Wagner et al. 2008).



**Figure 25: Illustration showing the compartmentalization of the seminiferous epithelium by the specialized junctions between the Sertoli cells**(Wagner et al. 2008).

b. 4The effects of obesity on Sertoli cell functions (**Figure 26**):

As mentioned earlier, the increase in the peripheral testosterone aromatization may modify the testosterone/E2 ratio and thus inhibit LH and FSH secretion. Consequently, it affects inhibin B and AMH secretion. It was shown in obese men, with a high BMI, that the hormones involved in the regulation of Sertoli cells functions (FSH, inhibin B, AMH) were lower when compared to non-obese individuals (Michalakis et al. 2013; O Mc Pherson et al. 2015; Neto et al. 2016).

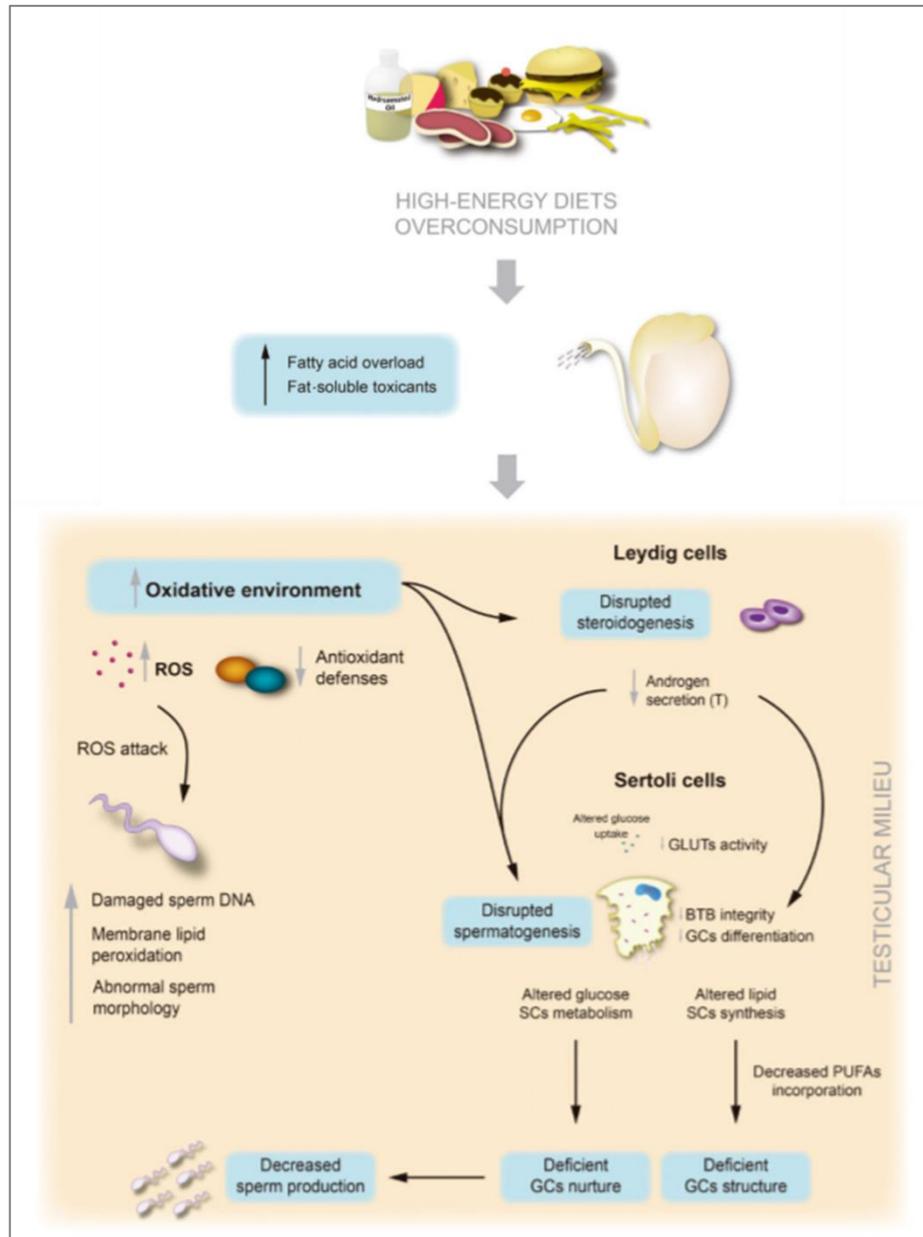
Moreover, obesity may alter Sertoli cell metabolism and consequently will affect the germ cells. On one hand, insulin stimulates several activities in the Sertoli cell, mainly carbohydrate metabolism. Lactate is produced by Sertoli cell under the control of insulin; it has an anti-apoptotic effect and it is the main energy source for germ cells. In a diabetic-like environment with insulin resistance, insulin-deprived Sertoli cells may decrease lactate production thus affecting spermatogenesis (Olivera et al. 2012). Recently, Rato and collaborators showed that feeding rats with a high-energy diet may lead to an increase in the expression of glucose transporters and glycolytic enzymes in Sertoli cell. Altogether, these changes increased the level of lactate and subsequently the oxidative stress in these cells (Rato et al. 2014). In addition, testicular biopsies from diabetic men showed vacuolization of Sertoli cells that may alter testicular glucose homeostasis (Alves et al. 2012)

On the other hand, lipid metabolism plays an important role in the testicular tissue. Sertoli cells can uptake fats by passive diffusion through the plasma membrane, by protein facilitated transport, and from the phagocytosed cells. Sertoli cells may utilize the internalized lipids to produce energy (ATP) or to generate polyunsaturated fatty acids (PUFAs). The PUFAs are essential for germ cells plasma membrane fluidity and flexibility and thus for fertilization. The excessive consumption of saturated fats present in the unhealthy diets may lead to their accumulation in the testicular cells. This abnormal accumulation of saturated fats affects the process of spermatogenesis and the physiology of the mature sperm cells (Rato et al. 2014).

Another complication derived from obesity is the testicular oxidative stress. By definition, oxidative stress is induced by the imbalance between the reactive oxygen species (ROS) and the ROS scavengers. Interestingly, the process of spermatogenesis is associated with a high rate of oxygen consumption and consequently important ROS production by the mitochondria. The hyperglycaemia and hyperlipidemia may exacerbate this outcome. The intracellular accumulation of lipids increases the rate of  $\beta$ -oxidation of the fatty acids. However, when testicular mitochondria are overloaded, they will be stressed. Under this kind of stress, the mitochondria reduce the ATP production and induce ROS overproduction. The high level of free ROS may attack and affect the PUFAs on the sperm plasma membrane, testicular proteins, mitochondrial and genomic DNA...In parallel, the high-energy diets may reduce the testicular antioxidant system by reducing the expression of ROS-detoxifying enzymes in the testis such as proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$  (PGC-1 $\alpha$ ) and sirtuin 3 (SIRT3). Altogether, these findings reveal that overconsumption of food may induce an oxidative stress in the testis and thus impairs sperm quality (Rato et al. 2014).

It's important to note that, the BTB can modulate to a certain extent the glucose and lipid uptake regarding their availability in the blood (Alves et al. 2012). However, histological analysis of the testis, from mice on high fat diet, showed that obesity may alter the BTB integrity (Fan et al. 2015). So, obesity may bypass the BTB adaptive mechanisms to the

changing environment and affect its main functions in selective transport and permeability (Alves et al. 2012).

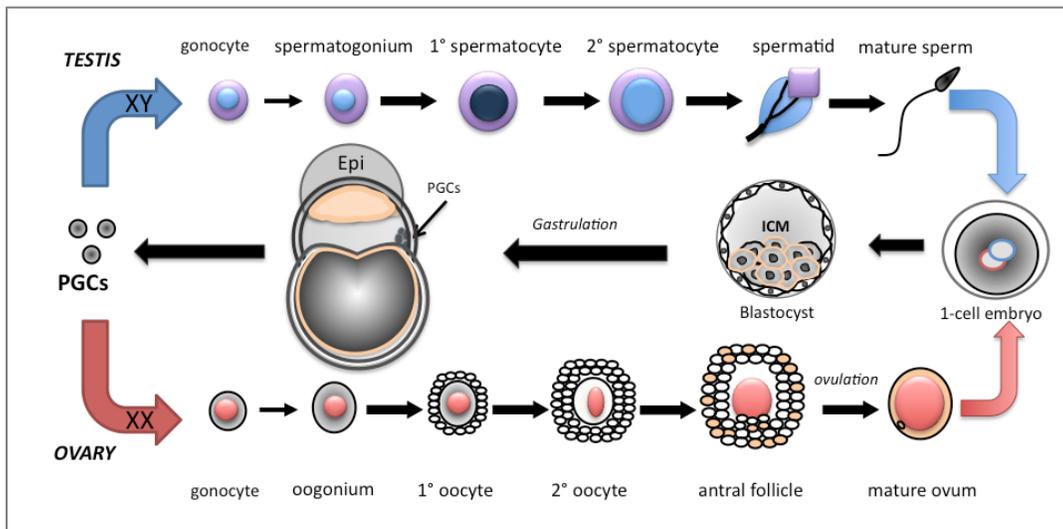


**Figure 26: The drawbacks of excess high-energy diet consumption on testicular homeostasis (Rato et al. 2014).**

c) Germ cells

Sexual reproduction requires the fusion of two haploid cells (containing 23 chromosomes each, in humans) from two parents to generate a diploid single cell called zygote (containing 46 chromosomes). The zygote (from the Greek “*zygotos*”- meaning “pairing”) is a totipotent single cell that has the potential to develop into a multicellular blastocyst. This latter is made up of 2 layers: the trophoblast and the inner cell mass containing the embryonic stem cells (ESC). Furthermore, the blastocyst may undergo gastrulation where a group of cells specializes into primordial germ cells (PGC). Once the PGCs are specified, they migrate to the gonad and differentiate into adult male germ cells (AGSC). The mature haploid sperm cells originate from the multiplication, the cytoplasmic differentiation, and the nuclear remodelling of AGSC in a process called spermatogenesis (**Figure 27**) (Ramathal et al. 2011). The spermatogenesis could be divided into four phases:

- a- The **spermatogoniogenesis**: this phase involves the proliferation (mitotic divisions) and the differentiation of the germ stem cells (spermatogonia).
- b- The resulting spermatogonia undergo two rounds of **meiotic divisions** resulting in haploid germ cells (spermatids).
- c- The **spermiogenesis** is a crucial phase where the round non-motile spermatid is transformed to a flagellated sperm.
- d- Finally, the sperm is released from the germinal epithelium into the tubular lumen by a process called **spermiation**.



**Figure 27: The cycle of mammalian germ cell development** (Ramathal et al. 2011).

c. 1Spermatogonia

Spermatogonia lie directly on the basement membrane of the seminiferous tubule. More precisely, they belong to the basal compartment of the germinal epithelium created by the BTB. The spermatogonia are the final germline stem cells rising from primordial germ cells (PGC).

They are characterized by a self-renewal capacity, and their commitment to spermatogenesis. The spermatogonia are classified as:

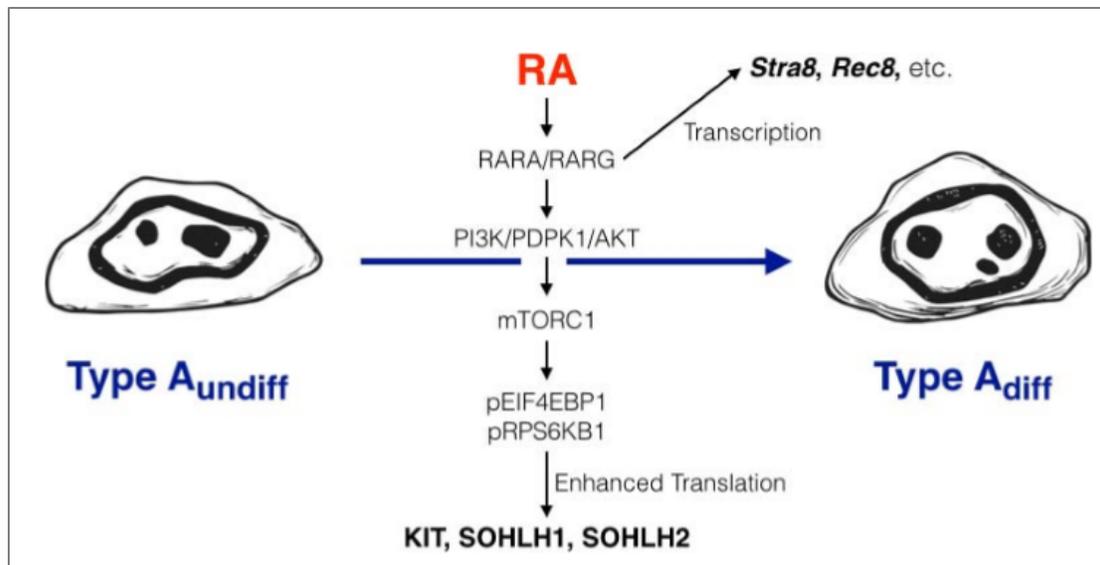
- **Dark type A ( $A_d$ ):** They just proliferate when there is a drastic decrease in the stock of spermatogonia (Ehmechke et al. 2006).
- **Pale type A ( $A_p$ ):** They renew themselves by mitosis to maintain the stock of spermatogonia and they can differentiate into Type B spermatogonia.  $A_p$  spermatogonia are analogous to the undifferentiated type A spermatogonia ( $A_{undiff}$ ) in rodents.
- **Type B spermatogonia:** These are differentiated cells that divide by mitosis to produce primary spermatocytes. The primary spermatocytes divide meiotically to give rise to secondary spermatocytes and spermatids.

❖ Regulation of spermatogonial self-renewal capacity and differentiation :

The germinal epithelium homeostasis is defined by the balance between germ cell proliferation and differentiation. The transcription profile of the self-renewing spermatogonia (spermatogonia stem cells (SSCs)) and those committed to spermatogenesis is distinct. The SSCs are characterized by the high expression of receptors for the cytokines secreted by the interstitial immune cells such as colony-stimulating 1 receptor (*Csf1r*), Interleukin 10 receptor (*Il10r*), and the tumour necrosis factor receptor (*Tnfr*). Moreover, they selectively express proto-oncogenes and tumor suppressors giving them a higher self-renewal capacity when compared to the spermatogonia committed to spermatogenesis (**Table7 and 8**) (Hammoud et al. 2014).

In contrast, spermatogonial differentiation and meiosis initiation requires vitamin A/retinol. Only, the spermatogonia devoted to gametogenesis can respond to the retinoic acid (RA) (vitamin A metabolite). The circulating vitamin A is metabolized by two successive reactions: retinol is converted to retinal by retinol dehydrogenases then the retinal is converted to RA by retinaldehyde dehydrogenases (e.g., *Aldh2*). It was shown that the aldehyde dehydrogenases and the retinoic acid receptor G (RARG) are overexpressed in differentiating spermatogonia, indicating a putative role of RA in the male gametogenesis (**Table7 and 8**).

When the RA binds its receptors RARA/RARG, it can stimulate the transcription of Stra8 (stimulated by retinoic acid, overexpressed in differentiated spermatogonia), Rec8 (meiotic recombination protein, provides the structural maintenance of the chromosomes), and other genes implicated in meiosis. In parallel, RARs can activate the PI3K/PDK1/AKT/mTORC1 signalling pathway, leading to the translation of the repressed mRNA such as KIT, SOHLH1, SOHLH2 (spermatogenesis and oogenesis specific basic helix-loop-helix 1 and 2). Once activated SOHLH 1 and 2 induce the genes important for spermatogonial differentiation. (**Table7, Figure 28**) (Suzuki et al. 2012; Hammoud et al. 2014; Busada et al. 2015).



**Figure 28: The role of retinoic acid in meiotic entry.** Retinoic acid (RA) stimulates the translation of the repressed mRNAs (KIT, SOHLH1, SOHLH2) in undifferentiated spermatogonia and thus induces the differentiation of the spermatogonia. Moreover, RA may activate the transcription of genes important for meiosis such as *Stra8* and *Rec8*, etc... (Busada et al. 2015).

❖ The impact of obesity on spermatogonia:

Interleukin 6 (IL-6) is a cytokine secreted by adipose tissue and macrophages; it induces inflammatory response at elevated concentrations. A recent study showed that a high level of IL-6 was detected in the serum and testis of obese mice. This may decrease the level of a zinc finger protein (Zfp637) in spermatogonia. The downregulation of Zfp637 reduces spermatogonial differentiation (Huang et al. 2016). Furthermore, obesity may induce testicular hyperthermia and the spermatogonia ( $A_{\text{dark}}$ ) are more vulnerable to heat stress due to their high mitotic activity (Ivell et al. 2007; Durairajanayagam et al. 2015). Altogether, these data highlight the drawbacks of obesity on spermatogonial survival and differentiation.

c. 2The meiotic cells

The type B spermatogonia are diploid cells made up of 23 chromosomes, each with two homologs: one is paternally and the other is maternally inherited. To avoid the doubling of chromosome number at each generation, gametogenesis involves meiotic divisions which reduce the 46 chromosomes to 23 chromosomes. As a result, the gametogenesis process produces haploid gametes containing each a single homologue of each chromosome (Morgan et al. 2007). The first phase during meiotic divisions is the differentiation of type B spermatogonia into pre-leptotene spermatocytes (leptotene from Greek leptos, thin + *tainia*, filament). This is a phase of DNA synthesis with a replication of chromosomal filaments (2 x 46 chromosomes). Following this step, the spermatocytes are transferred to the adluminal compartment of the BTB

and enter the prophase I with its five steps; leptotene, zygotene, pachytene, diplotene, and diakinesis (**Figure 29**)(Harris et al. 2014):

- **Leptotene**: the duplicated chromosomes become visible under the form of irregular filaments and close to each other.
- **Zygotene** (from Greek *zygon*, pairing + *tainia*, filament): the two homologous chromosomes stick together via the synaptonemal complex. It is a group of proteins that allows the pairing and the exchange between the homologous chromosomes.
- **Pachytene** (from Greek *pachys*, thick + *tainia*, filament): the duplicated homologous chromosomes are shorter and thicker forming a tetrad. At this step, the exchange of genetic materials between the homologous chromosomes occurs.
- **Diplotene** (from Greek *diploos*, two + *tainia*, filament): disintegration of the synaptonemal, the homologous chromosomes are just bound at a specific points termed chiasma.
- **Diakinesis** (from Greek meaning moving through): maximal condensation of the chromosomes. At the end of this phase the nuclear envelope disappears.

At the end of the prophase I, every spermatocyte contain a new recombination of paternal and maternal genetic materials. Following this phase, the spermatocytes enter into the metaphase I where the chromosomes align at the metaphase plate. Next, each of the homologous chromosomes will migrate to a pole of the cell during the anaphase I. The first round of meiosis ends by a cytokinesis (cell division), giving rise to two spermatocytes II. The generated spermatocytes II undergo a second round of cell division with a short prophase II. During this step, neither DNA replication nor a recombination of genetic materials occur. Afterwards, the chromosomes of the spermatocytes II align at the metaphase II plate. Then they proceed through anaphase II where each of the 2 copies of the chromosomes migrates to a pole of cell. Finally, the spermatocyte II undergoes a cytoplasmic division resulting in two haploid spermatids (**Figure 29**)(Hartl et al. 2011).

### c. 3 The spermiogenesis

The spermiogenesis is a complex phase of the male germline differentiation that represents the transformation of spermatids into spermatozoa. It is a transition from a round motionless cell to a flagellated, motile sperm. The sperm is a highly differentiated cell able to fertilize a female gamete and to induce embryo development (**Figure 30**).

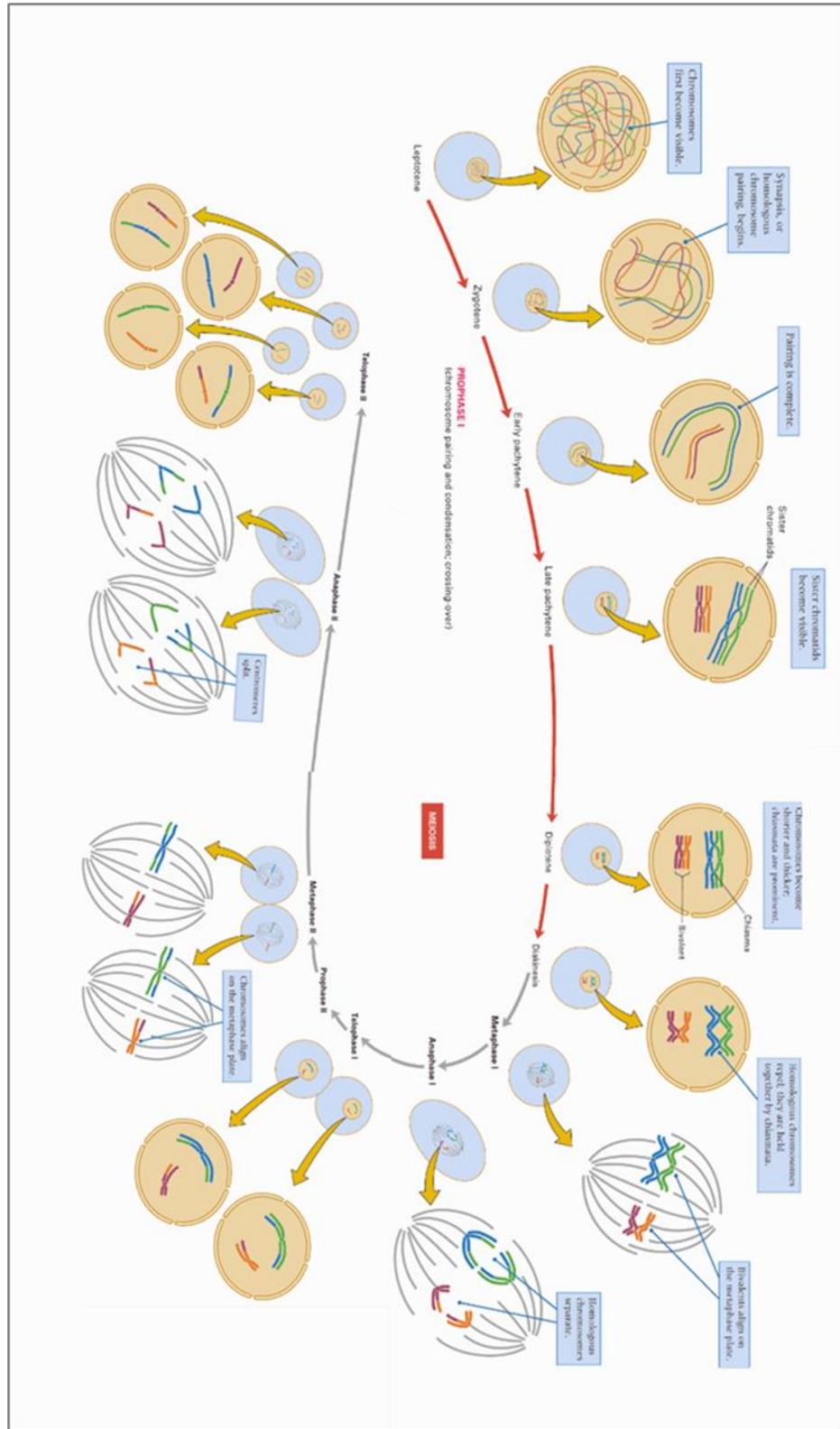


Figure 29: Illustration of the major events that occur during the meiotic division (Hartl et al. 2011)

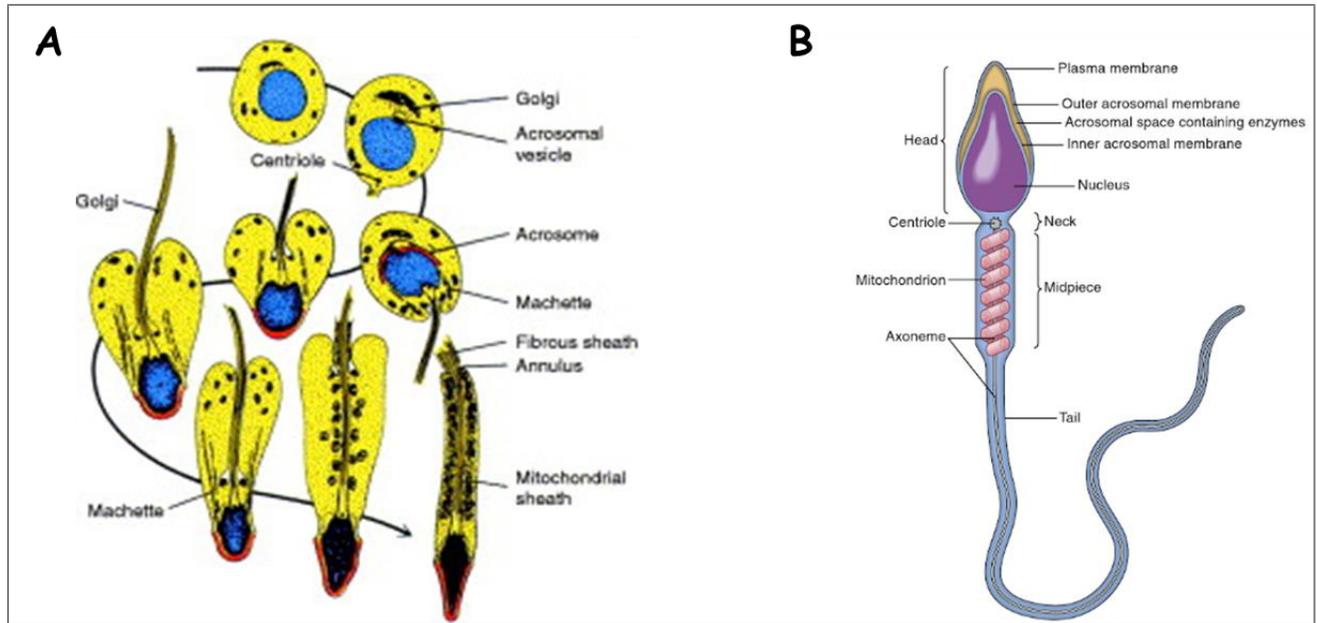
The propelling sperm flagellum formation starts in the round spermatid, when the centrosome migrates to one pole of the cell, and nucleates the tubulin proteins into microtubules. The microtubules form the motility apparatus of the sperm tail termed as axoneme. Mammalian sperm flagellum is divided into three parts(Sharma et al. 2011):

- a) The midpiece: The microtubules are surrounded by outer dense fibres (ODF) and mitochondria. The mitochondria play a central role in mature sperm metabolism, which can now metabolize different substrates (e.g., glucose, fructose, lactate, fatty acid, amino acids). The produced energy (ATP) is utilised for sperm motility, and various sperm functions (Boussouar et al. 2004; Dias et al. 2014).
- b) The principle piece: The microtubules are just surrounded by a fibrous sheath
- c) End piece: contains only a part of the microtubules

On the opposite side of the cell, the vesicles derived from the Golgi apparatus fuse together to form a cap-like structure, covering 40 to 70% of the anterior sperm head; called acrosome. The acrosome contains different hydrolytic enzymes playing an important role in the fertilization process. Moreover, intracellular forces (from the sperm cytoskeleton) and extracellular forces (from the Sertoli cells) interact to induce sperm head elongation. Altogether, these mechanisms will lead to differentiation of a sperm with an oval head and propelling flagella (Kierszenbaum et al. 2004; Krohmer et al. 2004).

One of the important spermatid cytodifferentiation features is the compaction of the nucleus. This compaction protects the paternal DNA once ejaculated outside the body. The nuclear compaction involves torsional stresses on the DNA that could be relieved by physiological activity of the topoisomerase. An absence of proper DNA strand breaks repair will lead to DNA fragmentation in the germ cells. In addition, oxidative stress and abortive apoptosis may also lead to sperm DNA fragmentation. High levels of sperm DNA fragmentation can be responsible of low fertilization rate, poor embryo quality, repeated failure of assisted reproductive technology attempts, and miscarriages (Balhorn et al. 2011; Choucair et al. 2016).

At the end of spermiogenesis a shedding of the majority of the cytoplasm takes place. Consequently there is a reduction in the mechanisms of defense against the oxidative stress. The resulting mature sperm, is characterized by a tiny amount of cytoplasm and a fluid plasma membrane rich in unsaturated fatty acids (plasmalogenes and sphingomyelins) facilitating the flexibility and the functional ability of the sperm (Atiken et al. 1994; Saraswat et al. 2014).



**Figure 30: The process of spermiogenesis.** (A) Schematic representation of the main morphological changes during spermiogenesis. (B) Gross anatomy of mature sperm (Boussouar et al. 2004; Jones et al. 2014).

#### c. 4The impact of male obesity on sperm parameters and embryo development

Several studies using rodent models of diet-induced obesity showed that male obesity decreases sperm count, impairs sperm motility and morphology; whereas one study showed no difference in sperm motility (O Mc Pherson et al. 2015). In addition, acquired obesity in rodent males increases the oxidative stress in the testis, sperm, and seminal plasma (Bakos et al. 2011; Chen et al. 2013; Duale et al. 2013; Zhao et al. 2014; Fullston et al. 2015); by itself oxidative stress in *Macaca mulatta*, mice and bull sperm may alter the pre-implantation embryo development and the health of the offspring (Burrue et al. 2014; Lane et al. 2014; De castro et al. 2015). Also, the percentage of DNA damage in the spermatids (Palmer et al. 2011) and in the mature sperm was found to be significantly higher in obese male mice compared to non-obese mice (Bakos et al. 2011; Chen et al. 2013; Duale et al. 2013; Fullston et al. 2013; Vendramini et al. 2014; Zhao et al. 2014). At the functional level, it was demonstrated that obese male mice have a negative contribution to the pre-implantation embryo development, including a higher percentage of one – cell embryo block, delayed cell cycle progression, decreased blastocyst number, and altered carbohydrates metabolism (Mitchell et al. 2010; Binder et al. 2012).

In parallel, different systematic reviews have been performed in humans to evaluate the effects of male obesity on the sperm parameters, DNA fragmentation, mitochondrial membrane potential, and fertility outcomes. Male obesity in humans is associated with high incidence of

oligozoospermia and azoospermia (Sermondade et al. 2012), reduction in the percentage of normal sperm morphology, (MacDonald et al. 2013; Campbell et al. 2015), increased percentage of sperm with DNA fragmentation, and abnormal mitochondrial membrane potential(Campbell et al. 2015).Moreover, a study on 81 men indicates that sperm ROS production is positively correlated to the BMI (Tunc et al. 2011). Regarding in vitro embryo development, three reports were published; Bakos and colleagues high spotted the significant decrease in blastulation rate with increasing BMI, whereas Colaci et al., and Schliep et al., didn't find any significant difference between obese and non-obese men for the analyzed embryologic parameters (Bakos et al. 2011; Colaci et al. 2012; Schliep et al. 2015).Furthermore, a recent meta-analysis showed that paternal obesity may reduce the rate of live birth per assisted reproductive technology (ART) cycle, and obese father have a 10% risk of facing a non-viable pregnancy(Campbell et al. 2015).

### III- Epigenetic inheritance of paternal acquired obesity

#### A- Epigenetic control of gene expression during spermatogenesis and early embryogenesis

Genetics is the study of heritable modification in the gene function due to a direct modification in the gene structure such as single mutation, translocation, insertion, and inversion of the nucleobases (cytosine, guanine, adenine, and thymine). In contrast, epigenetics (from Greek *epi-*, above and beyond) can be defined as the mitotically or meiotically heritable changes in gene expression without changes in the DNA sequence. This biological event occurs via DNA methylation/hydroxymethylation, chromatin modifications, and non-coding RNAs activity (Gunes et al. 2013; Moore et al. 2013). In fact, the majority of the cells in our body are genetically homogenous, but each cell type has its own epigenetic signature and consequently its own gene expression profile (Abdallah et al. 2009).

##### A. 1 DNA methylation

The chemical modification of the DNA by the addition of a methyl group to the cytosine nucleobase at the position C-5 was first discovered in 1948 by Rollin Hotchkiss. This chemical reaction is catalyzed by a family of DNA methyltransferases (DNMT) that transfers the methyl group from S-adenyl methionine (SAM) to the cytosine leading to 5-methylcytosine (5-mC) formation (Moore et al. 2013). SAM is a product of the methionine (amino acid) – folic acid (Vitamin B9) metabolism playing a major role in the cross-talk between food intake and DNA methylation (Figure 31) (Zhang et al. 2015).

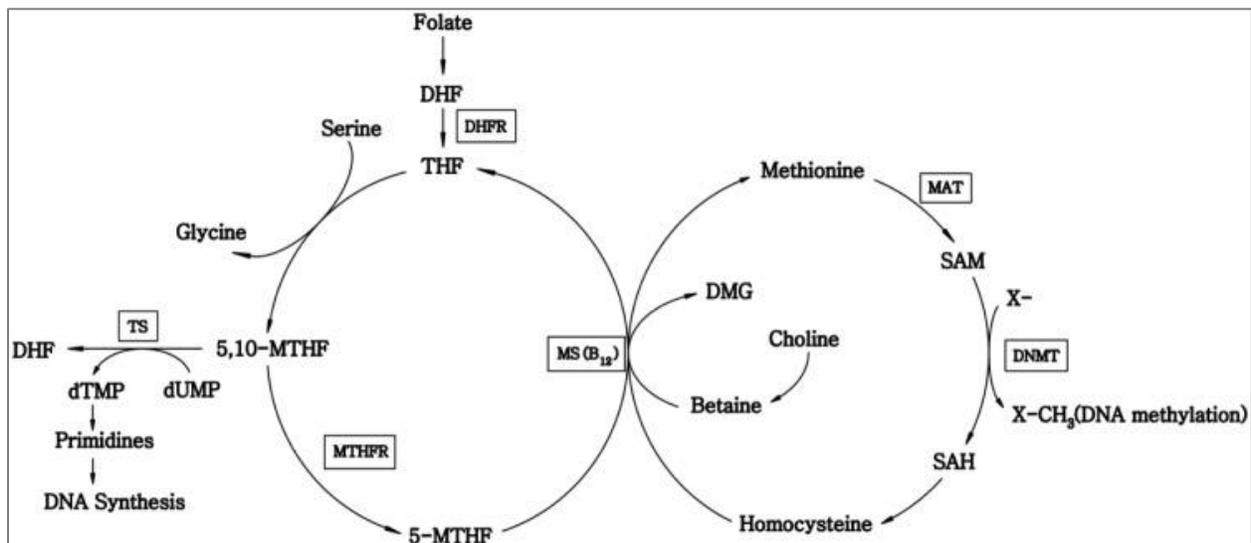


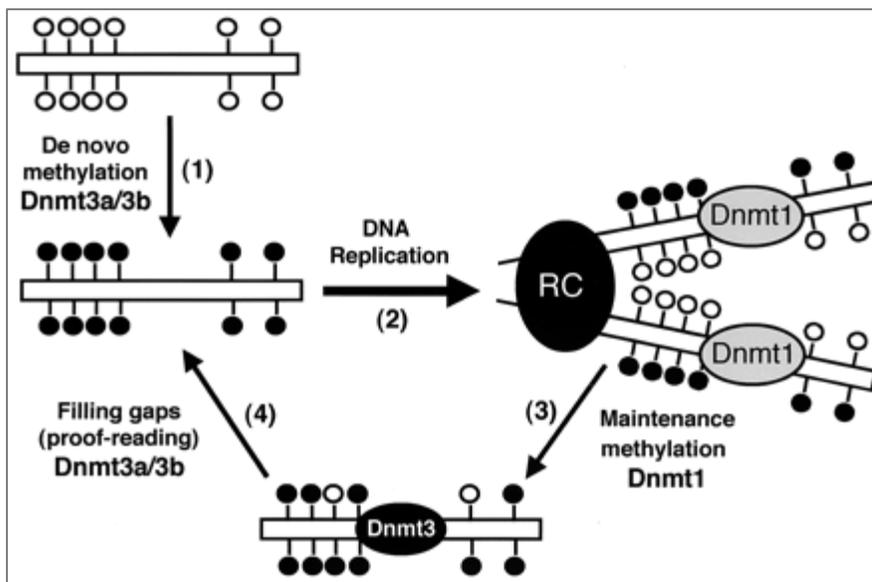
Figure 31: Flow diagram of methionine-folic acid metabolism.

This diagram shows the formation of the methyl-donor S-adenyl methionine (SAM) via the methionine metabolism. The DNA methyltransferases (DNMT) can transfer the methyl-group (X-) from SAM to the methyl acceptor molecules such as DNA, proteins, lipids, etc...MAT:

methionine adenosyltransferase, SAH: S-adenosylhomocysteine, DMG: dimethylglycine, MS: methionine synthase, DHFR: dihydrofolatereductase, DHF: dihydrofolate, THF: tetrahydrofolate. 5,10MTHF: 5,10-methylenetetrahydrofolate, TS: thymidylate synthase, dTMP: deoxythymidine monophosphate, dUMP: deoxyuridine monophosphate, MTHFR: methylenetetrahydrofolatereductase; 5-MTHF: 5-methyltetrahydrofolate(Zhang et al. 2015).

The DNMTs could be divided into three groups (**Figure 32**)(Smallwood et al. 2011; Moore et al. 2013):

- DNMT3 family is made up of 3 members DNMT3a, DNMT3b, and DNMT3L. DNMT3A and DNMT3b catalyze the methylation of the unmodified cytosine, also known as *de novo* methylation. However, DNMT3L does not have a catalytic activity, but it can regulate the activity of other DNMT3 members.
- DNMT1 acts during DNA replication and copies the methylation profile on the new synthesized DNA strand. Thus the role of DNMT1 is in the maintenance of DNA methylation.
- DNMT2 is known to methylate several tRNAs.



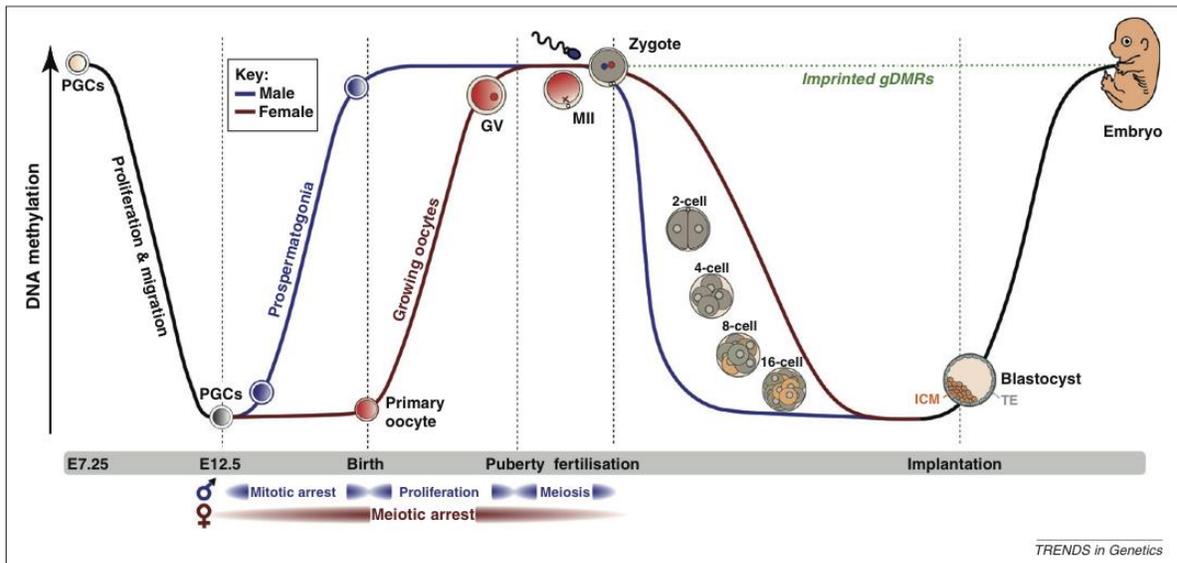
**Figure 32: The DNA methyltransferases functions.** The Dnmt3a/3b catalyzes the transfer of methyl group to the cytosine and thus induces a *de novo* methylation. During DNA replication, The Dnmt1 is associated to the replication complex (RC). It can copy the methylation from the parental DNA strand into the new synthesized strand. Moreover, Dnmt3a/3b can act as a “checkpoint” and can remethylate the new synthesized strand in case of unwanted methylation erasure(Chen et al. 2003).

The 5-mC is considered as an epigenetic mark since it is globally associated with gene repression. Generally, The DNA methylation occurs on cytosine that precedes a guanine nucleotide (CpG dinucleotide). The CpG dinucleotides are found mainly in clusters called the CpG islets. The gene silencing by DNA methylation occurs *via* two ways: by inhibiting the binding of transcriptional factors to the target sequence or by the direct binding between the 5-mC and the transcription repressors (e.g., methyl-CpG binding protein 2 (MeCP2)). In contrast, DNA methylation may activate the gene transcription; for example when it takes place on a negative regulatory element of the target gene (Abdallah et al. 2009). Furthermore, the 5-mC is found at a high level in the heterochromatin (gene-free DNA), whereas the euchromatin (gene-rich DNA) contains a lower 5-mC level. In addition, DNA methylation contributes to the transposons silencing, and to the X chromosome inactivation in female embryos. Interestingly, the DNA methylation is in the core of the genomic imprinting process. This phenomenon allows the mono-allelic expression of genes in a parent-of-origin specific manner. Alterations in the imprinted genes may lead to several pathologies (e.g., cancers) and syndromes (such as Beckwith-Wiedemann, Silver-Russel, Angelman, and Prader-Willi) (Balhorn et al. 2011).

#### A. 1.1 DNA methylation reprogramming in mouse germ cells and pre-implantation embryos

Like all the somatic cells, each of the male and female gametes contains a specific DNA methylation landscape. After fertilization, these marks should be erased and replaced in a manner to generate a totipotent zygote at each generation. This phenomenon is termed as epigenetic reprogramming (**Figure 33**).

At first, the primordial germ cells (PGCs) after their specification migrate to the gonadal ridge. During this proliferation and migration phase their DNA methylation profile is erased. Surprisingly, some genomic regions retain the 5-mC mark and escape this epigenetic reprogramming; for example several retrotransposons, and some imprinted genes retained a low levels of methylation. These findings high spot the possibility of epigenetic inheritance of parental DNA methylation. Next, *de novo* DNA methylation of the pre-spermatogonia takes place in the male gonad before birth and before the meiotic initiation. In contrast, the *de novo* DNA methylation in the female gonad occurs after birth, in the oocytes arrested in the prophase I of the meiosis. This chemical modification of the cytosine appears on unmethylated CG and on imprinted genes in a sex-dependent manner. After fertilization, a second wave of DNA demethylation arises. The mechanism by which the 5-mC is erased in paternal and maternal pronuclei is not the same and it will be discussed in the next section. At this stage, not all the genomic regions undergo DNA demethylation. The imprinted genes from the two parents maintain their 5-mC profile as a legacy to the next generation (Smallwood et al. 2011).



**Figure 33: The dynamics of DNA methylation during mouse gametogenesis and pre-implantation embryos (Smallwood et al. 2011).**

A. 1.2 The drawbacks of abnormal DNA methylation profile in gametes

In order to study the effects of abnormal DNA methylation on the gametes and embryos, several targeted studies have been performed in mice models. In summary, it was shown that mutations in the genes coding for the DNA methyltransferases enzymes may lead to dramatic changes in the phenotype (**Table 4**)(Chan et al. 2011; Jenkins et al. 2012). Moreover, the alternative way to study the impact of DNA hypomethylation on mouse sperm and embryos is the use of DNA methylation inhibitor drugs (e.g., 5-azacytidine, and 5-aza-2'-deoxycytidine). Okas et al., demonstrated that male mice treated with 5-aza-2'-deoxycytidine suffer from reduced sperm motility, reduced fertilization ability, and inability of the demethylated paternal genome to support embryo development(Oakes et al. 2007). In humans, several studies have shown that aberrant DNA methylation at some gene promoters (e.g., MTHFR: methylenetetrahydrofolate reductase), at some imprinted genes, and at repetitive elements are associated with male infertility, sperm defects and recurrent pregnancy loss (Navarro-costa et al. 2010; Wu et al. 2010; El Hajj et al. 2011; Pacheco et al. 2011). Furthermore, it was shown that global sperm DNA methylation was lower in patients with high seminal reactive oxygen species and with sperm having high DNA fragmentation index, abnormal chromatin condensation, and with reduced motility (Tunc et al. 2009; Montjean et al. 2015). Plus, advanced paternal age may differently methylate gene promoters implicated in schizophrenia, and bipolar disorder in the sperm. So, it does raise the risk of non-genetic transmission of neuropsychiatric disorders to the next generation (Frans et al. 2008; Miller et al. 2010; Jenkins et al. 2014).

**Table 4: A summary of the phenotypes derived from DNMTs genes mutations.**

Target gene	Phenotype	Reference
DNMT1 complete or partial loss of function in mice	<ul style="list-style-type: none"> <li>- Delayed development</li> <li>- Death at mid-gestation</li> <li>- Abnormal biallelic expression of imprinted genes</li> <li>- Loss of X-chromosome inactivation</li> </ul>	(Chan et al. 2011; Jenkins et al. 2012)
Deficiency of oocyte-specific DNMT1 (DNMT1o) in mice	<ul style="list-style-type: none"> <li>- Embryonic lethality</li> <li>- Abnormal methylation at imprinted loci</li> </ul>	
DNMT1 mutations in humans	<ul style="list-style-type: none"> <li>- Associated with some subtypes of colorectal cancer</li> </ul>	
DNMT3a-deficient mice	<ul style="list-style-type: none"> <li>- Survive to term</li> <li>- Death after few weeks of birth</li> <li>- Embryo are underdeveloped</li> <li>- Normal global methylation profile</li> <li>- Affected spermatogenesis</li> <li>- Decreased methylation at <i>H19</i> imprinted locus</li> </ul>	
Conditional inactivation of DNMT3a in mouse germ cells	<ul style="list-style-type: none"> <li>- Infertility due to spermatogenic arrest</li> </ul>	
DNMT3b-deficient mice	<ul style="list-style-type: none"> <li>- Mid-gestation lethality</li> <li>- Demethylation of some genomic sequences</li> </ul>	
DNMT3b mutations in humans	<ul style="list-style-type: none"> <li>- Autosomal recessive genetic disorders with immunodeficiency, centromeric instability, and facial anomalies</li> </ul>	
DNMT3L-deficient mice	<ul style="list-style-type: none"> <li>- Male and female infertility</li> <li>- Small testis</li> <li>- Extensive chromosomal mispairing</li> <li>- Abnormal methylation at specific genomic sequences</li> </ul>	

## A. 2 DNA hydroxymethylation and DNA demethylation

The 5-hydroxymethylcytosine (5-hmC) is an epigenetic mark first discovered in the T-even bacteriophage. The 5-hmC is present in several tissues in vertebrate and it is involved in several biological processes such as brain physiology, hematopoietic cells homeostasis, and cancer pathogenesis (Tan et al. 2012). Furthermore, accumulation of 5-hmC was positively correlated to an increase in the level of gene expression (Vasanthakumar et al. 2015).

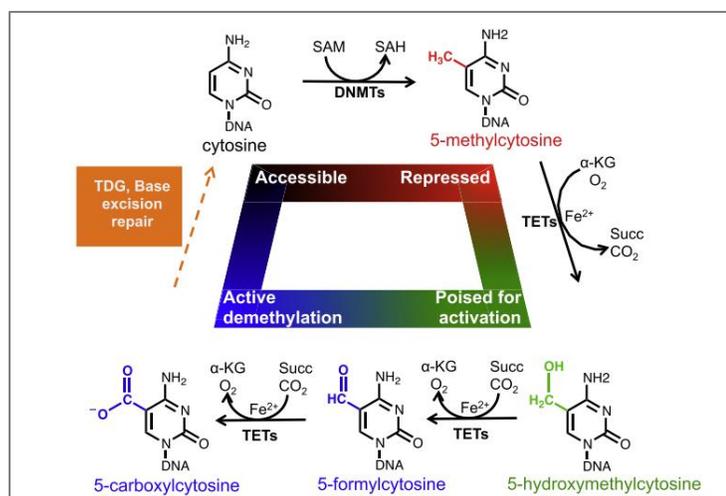
The 5-hmC is generated by the oxidation of the 5-mC, a reaction catalyzed by the TET (ten-eleven translocation) proteins. The TETs are a group of proteins having an enzymatic activity (methylcytosine deoxygenase) that depends on Fe (II) and  $\alpha$ -ketoglutarate ( $\alpha$ -KG or 2-oxoglutarate). Subsequently, TETs oxidize 5-hmC sequentially to 5-formylcytosine (5-fC), and 5-carboxylcytosine (5-CaC). The 5-hmC, 5-fC, and 5-CaC can participate to the DNA demethylation process which could occur by two ways (**Figure 34**) (Tan et al. 2012; Vasanthakumar et al. 2015):

- Passive DNA demethylation:

During DNA replication, if the 5-mC is not copied to the new synthesized strand the methylation will be passively erased. Moreover, the presence of 5-hmC, 5-fC, and 5-CaC instead of 5-mC at the time of replication, may disrupt the maintenance of DNA methylation catalyzed by DNMT1. Thus, the methylation could be passively diluted and erased during replication.

- Active DNA demethylation:

It is a replication-independent way that requires enzymatic activity. On one hand, the thymine DNA glycosylase (TDG) can recognize and excise the 5-fC and 5-CaC. Following this step, a base-excision repair (BER) pathway converts the chemically modified cytosine to cytosine. On the other hand, the 5-mC and the 5-hmC may undergo a deamination process *via* an enzyme called activation-induced deaminase (AID). Then; the deaminated cytosine could be converted to cytosine also by the BER pathway-mediated demethylation.



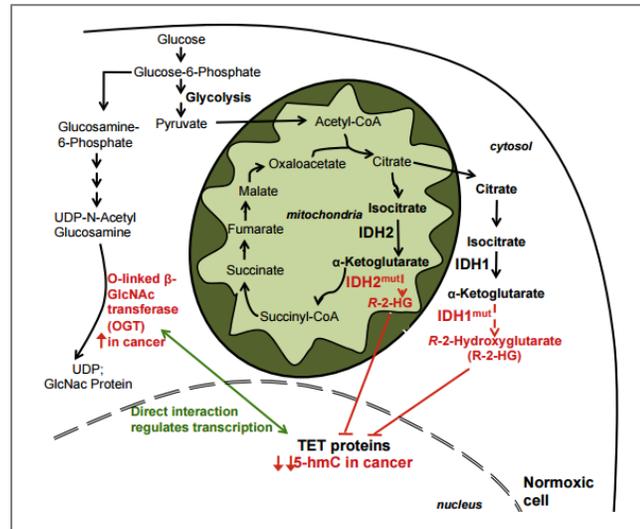
**Figure 34: Regulation of gene expression by DNA methylation and demethylation.**

DNA methylation takes place at the position C-5 of the cytosine. DNMTs transfer the methyl group from SAM to the cytosine and thus induce cytosine methylation. DNA methylation is generally associated with repressed gene transcription. In contrast, the TET enzymes oxidize the 5-mC sequentially to 5-hmC, 5-fC, and 5-CaC. These reactions require  $\alpha$ -KG, succinate, Fe (II), and oxygen. The 5-hmC is a positive regulator of gene expression and it is involved in passive and/or active DNA demethylation pathway (Vasanthakumar et al. 2015).

As discussed above, some metabolites derived from nutrient intake and cellular metabolism may regulate the TET functions. For instance, glucose could be metabolized inside the cell by several pathways to produce different metabolites that can interfere with the 5-hydroxymethylcytosine generation. On one hand, glucose metabolism through the glycolysis will generate the pyruvate. This pyruvate will enter the Krebs cycle in the mitochondria to produce the  $\alpha$ -KG which is the co-factor/substrate that regulates the TET enzyme. Interestingly, the conversion of the isocitrate to  $\alpha$ -KG is catalyzed by the isocitrate dehydrogenase (IDH), but mutations in IDH 1 and 2 can instead lead to the production of R-2-Hydroxymethylglutarate (R-2-HG). Consequently, R-2-HG produced in several human cancers inhibits TET proteins leading to a decrease in 5-hmC in cancer cells.

On the other hand, approximately 3% of the cellular glucose can enter the hexosamine biosynthesis pathway (HBP). The end-product of the HBP is the uridinediphosphate N-acetylglucosamine (UDP-GlcNAc). The latter, is transferred to the proteins and lipids via the activity of the O-GlcNAc transferase (OGT). In parallel, TETs have the ability to recruit the OGT to the chromatin in order to modify it and to subsequently modify the gene expression. In contrast, the OGT is overexpressed in some cancer types and this may alter the TET proteins expression and decrease the level of 5-hmC (Figure 35) (Vasanthakumar et al. 2015).

Above that, Delatte et al. demonstrated that oxidative stress can also decrease the global 5-hydroxymethylation pattern *in vivo* and *in vitro*. The data on SY5Y cell culture after their exposure to oxidative stress and on colon epithelial cells from mice lacking glutathione peroxidase enzyme (*GPx1/2*= major cellular antioxidant enzymes), show a global decrease in 5-hmC and a local increase in 5-hmC on specific genomic loci involved in oxidative stress response. Moreover, the depletion of TET1 in SY5Y cells sensitizes them to oxidative stress, thus highlighting the protective role of TET1 against oxidative stress (Delatte et al. 2015).



**Figure 35: The cross-talk between the glucose metabolism and 5-hydroxymethylcytosine formation in normal and cancer cells** (Vasanthakumar et al. 2015).

#### A. 2.1 5-hmC dynamics during mouse spermatogenesis and embryo development

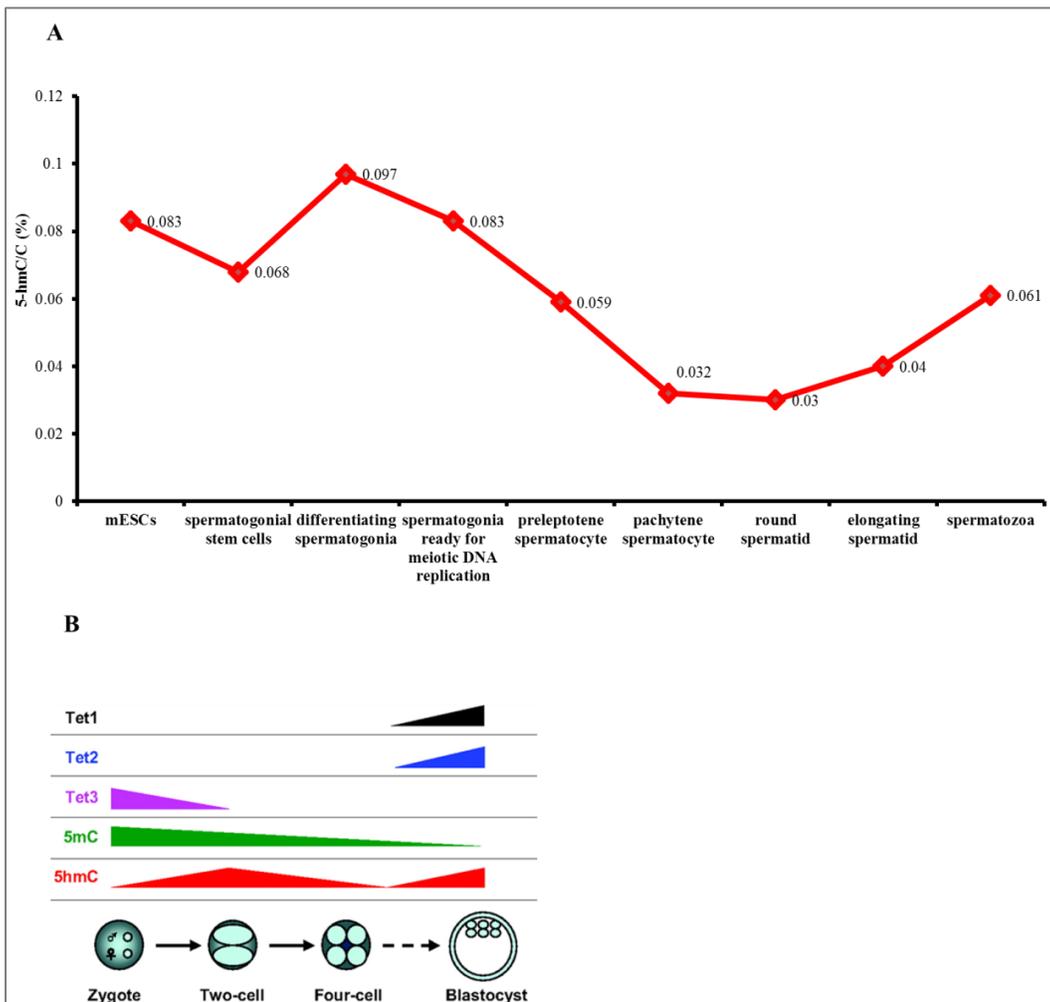
The 5-hmC is present in mouse embryonic stem cells (mESCs) and in male germ cells. It may play a role in embryo development. In contrast to the 5-mC, the global pattern of 5-hmC is different between the mouse male germ cell types. The highest percentage of 5-hmC accumulation is in the diploid germ cells (differentiating spermatogonia (0.097%) and spermatogonia ready for meiotic DNA replication (0.083)). However, the haploid germ cells have the lowest percentage ranging from 0.059% in preleptotene spermatocytes to 0.04% in elongating spermatids. Surprisingly, the mouse spermatozoa have a higher percentage of 5-hmC (0.061%) when compared to the other haploid germ cells (**Figure 36 A**). Altogether, these results indicate that the 5-hmC represents a small but dynamic fraction during mouse spermatogenesis. In parallel, the diploid germ cells exhibit an increase in TET enzymes expression (mainly *Tet3*) and this expression drops in the haploid cells (Gan et al. 2013). The dynamics of 5-hydroxymethylcytosine in mouse germ cells mirrors the level of gene expression in each cell

type. Haiyun Gan et al., found a positive correlation between 5-hmC accumulation and gene activation. For instance, the protamines (1 and 2) and the transition protein 2 gene promoters are highly hydroxymethylated and thus they are expressed in pachytene spermatocytes. In contrast, the genes of PIWI- interacting RNAs (piRNAs) are highly hydroxymethylated in preleptotene spermatocytes facilitating their expression in pachytene spermatocytes(Gan et al. 2013).

In humans, TET1-3 enzymes were shown to be expressed from pachytene spermatocytes stage until the elongated spermatids stage. However, the 5-hmC was only detected in the elongated spermatids(Ni et al. 2016). Defectively, malignant transformation in germ cells could affect the DNA methylation/hydroxymethylation status, and the expression profile of *TET1*, *TDG*, *DNMT3B*, and *DNMT3L*. For instance, some types of germ cell cancers present a global DNA hypohydroxymethylation, and certain germ cells malignant transformation could be accompanied by *de novo* DNA methylation and oxidation of 5-mC to 5-hmC , 5-fC, and 5-CaC (Nettersheim et al. 2013). Variously, the mature human spermatozoa of fertile men, express the TET1-3 enzymes at the mRNA level and at the protein level. When the level of TETs mRNAs expression has been compared between the sperm of fertile and infertile patients, it reveals that there is a positive correlation between TET expression and sperm parameters (concentration, and motility)(Ni et al. 2016).In addition, it has been shown that the human sperm present a strong DNA methylation and weak DNA hydroxymethylation profile (Efimova et al. 2015). Interestingly, the level of 5-hmC in human sperm is lower than that found in leukocytes and this level increases significantly with advanced paternal age (Jenkins et al. 2013).

The Tet3 is not only expressed in male germ line but also in mouse oocytes and zygotes. Interestingly, Xu and colleagues showed that 5-mC can be oxidized into 5-hmC exclusively in the paternal pronucleus but not in the maternal one (Gu et al. 2011; Iqbal et al. 2011; Wossidlo et al. 2011). In addition, the maternally expressed STELLA protein inhibits the DNA demethylation and 5-hmC formation in the female pronuclei (Nakamura et al. 2007; Wossidlo et al. 2011). So, this selective accumulation of the 5-hmC can play a role in the epigenetic reprogramming after fertilization. Furthermore, the tet3 expression decreases rapidly during embryo cleavage divisions, whereas the expression of the tet1 and tet2 increases in mESC after blastocyst differentiation (**Figure 36 B**)(Tan et al. 2012). It is also important to note, that homozygous mutation of tet3 in mice is lethal, highlighting the crucial role of 5-hmC in development and organogenesis(Gu et al. 2011).

In line with the studies performed on mice, Efimova et al., demonstrated that human triploid zygotes present a highly hydroxymethylated and hypomethylated paternal pronucleus but the maternal pronucleus is hypermethylated with low levels of 5-hmC. At cleavages, these embryos passively loose the 5-hmC from their chromosomes in a replication-dependent manner (Efimova et al. 2015). Curiously, the downregulation of TET3 and TET2 enzymes in the sperm of infertile men may significantly reduce the fertilization rate and the pregnancy rate respectively after intracytoplasmic sperm injection (ICSI). Altogether, these studies accentuate the role of 5-hmC and TET enzymes in epigenetic remodelling during development.



**Figure 36: The dynamics of 5-hydroxymethylcytosine during mouse spermatogenesis and embryo development.**(A) A graph showing the content in 5-hmC relative to cytosine (%) in mouse spermatogenic cells. (B) An illustration of Tet gene expression, and 5-mC, and 5-hmC dynamics during pre-implantation embryo development (Tan et al. 2012; Gan et al. 2013).

### A. 3 Chromatin modifications

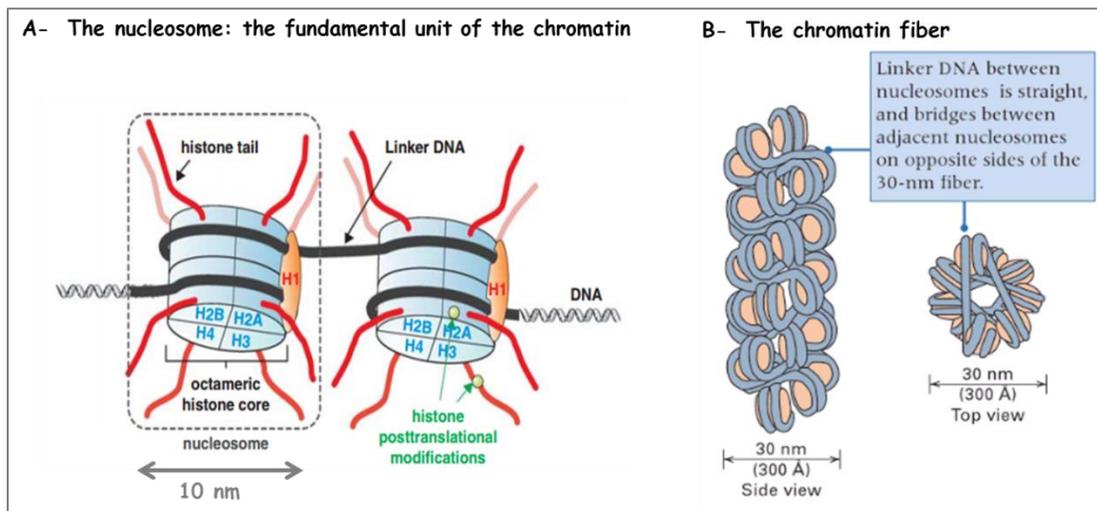
#### A. 3.1 Chromatin structure in somatic cells

The state in which the DNA is packed inside the nucleus of the cell is termed chromatin. The first level of DNA packaging corresponds to the nucleosome; which is the fundamental unit of

the chromatin (**Figure 37 A**). The nucleosome has a discoid structure of 10 nm in diameter and it is made up of:

- a) Four core histones (H3, H4, H2A, and H2B): each nucleosome contains 2 copies of each histone type. The 8 histones form together the octameric histone core.
- b) 147 base pairs (bp) of double helix DNA wrapped around the octameric histone core.
- c) Linker proteins: The histone linker H1 and the other non-histone proteins bind to the DNA while entering and exiting the nucleosome.

The nucleosomes are linked together by a linker DNA. They compact into a shorter thicker fiber called the 30 nm-chromatin fiber (**Figure B**) (Füllgrabe et al. 2011).



**Figure 37: Chromatin structure.**(A) The chromatin is composed of repeating nucleosome units. Each 10 nm-nucleosome is made up of an octameric histone core containing 2 copies of the four core histones (H3, H4, H2A, and H2B). In addition, a DNA fragment is wrapped around this octamer. The nucleosomes are linked by a linker DNA. The latter is connected to a linker histone (H1) (Füllgrabe et al. 2011). (B) A side and a top view of the 30 nm-chromatin fiber containing the compact form of the nucleosome fiber (Hartel et al. 2012).

The histones are basic proteins, positively charged with a molecular weight varying between 11 and 23 KDa (**Table 5**). The core histones have a globular structure with a protruding N-terminal tail (histone tail)(TROPP et al. 2012). Furthermore, the amino acids residues of the histones tail are subject to post-translational modifications (PTMs) by the histone- modifying enzymes (e.g., acetyltransferases, deacetylases, lysine methyltransferases, and etc...). The post-translational modifications such as acetylation, methylation, phosphorylation, and ubiquitylation, may disrupt the contact between the nucleosomes and/or recruit non-histones proteins (e.g. acetylation is recognized by bromodomains) to the chromatin. Thus, PTMs play a cardinal role in the creation of a global chromatin environment: silent heterochromatin and active euchromatin.

Grossly speaking, the heterochromatin condenses and protects the DNA during cell divisions, helps in the maintenance of the inactive X chromosomes, etc...whereas; the euchromatin or open chromatin regulates the flexibility and the biological tasks of the DNA (e.g., DNA repair, replication, transcription) (**Table 6**) (Kouzarides et al. 2007).

**Table 5: The biochemical properties of the histones (TROPP et al. 2012)**

<b>Histones</b>	<b>Molecular weight (KDa)</b>	<b>% lysine</b> ( basic amino acid, positively charged)	<b>% arginine</b> ( basic amino acid, positively charged)	<b>% lysine + arginine</b>
<b>H1</b>	~ 21	29	1.5	30.5
<b>H2A</b>	14.5	11	9.5	20.5
<b>H2B</b>	13.7	16	6.5	22.5
<b>H3</b>	15.3	10	13.5	23.5
<b>H4</b>	11.3	11	14	25

**Table 6: The different types and functions of the post-translational modifications of histones (Kouzarides et al. 2007)**

<b>Chromatin modifications</b>	<b>Residues modified</b>	<b>Function regulated</b>
Acetylation (-ac)	Lysine ( <b>K</b> )	Transcription, repair, replication, condensation
Methylation (-me)	Lysine ( <b>K</b> ), mono, di, or tri methylation	Transcription, repair
	Arginine ( <b>R</b> ), mono, or dimethylation	Transcription
Phosphorylation	Serine ( <b>S</b> ) and Threonine ( <b>T</b> )	Transcription, repair, condensation
Ubiquitylation	Lysine	Transcription, repair
Sumoylation	Lysine	Transcription
ADP ribosylation	Glutamic acid	Transcription
Deimination	Arginine	Transcription
Proline isomerisation	Proline	Transcription

### A. 3.2 Chromatin remodelling during spermatogenesis

#### a) Spermatogonia

Spermatogonia possess a nucleosome-based chromatin. The maintenance of their self-renewal capacity and/or the activation of genes implicated in meiosis and differentiation may be influenced by histones modifications (**Table 7**). It was demonstrated that, the histone H3 is expressed in mouse spermatogonial cells and is subject to several modifications at its lysine residues. On one hand, an important acetylation occurs at histones H3 (H3K9ac, H3K18ac, H3K23ac), which is consistent in general with an active chromatin state. On the other hand, the site of H3 methylation may influence the activation, repression or bivalency of the spermatogonial chromatin. For instance, H3 methylation at lysine 27 (H3K27me3) is correlated with gene silencing, and it represses the meiotic genes (e.g., aldehyde hydrogenase 2 and STRA8) in undifferentiated spermatogonia. Fascinatingly, 2 sets of spermatogonial genes, implicated in embryo development and germline specification, are selectively packaged in regions of bivalent chromatin (**Table 7 and 8**). The bivalent chromatin combines both repressive (H3K27me3) and activating (H3K4me3) H3 methylation and it is accompanied by an underlying DNA hypomethylation in the germline cycle (Bernstein et al. 2006; Song et al. 2011; Hammoud et al. 2014).

**Table 7: Table summarizing some of the epigenetic events that occur during spermatogonia differentiation from type A undifferentiated to type A differentiated in a mouse model (Hammoud et al. 2014)**

		Type A <sub>undiff</sub>	Type A <sub>diff</sub>	function
RNA expression profile	KIT	repressed	+	Regulates spermatogonial differentiation through PI3K/AKT signalling pathway
	STRA8	repressed	+	DNA replication and meiosis initiation
	SOHLH1, SOHLH2	repressed	+	Spermatogial differentiation
	Multiple aldehyde dehydrogenase		+	Involved in retinoic acid metabolism and thus meiotic entry
	Colony stimulating factor 1 receptor	+	-	Stimulates self-renewal of SSC
	GFra1	+	-	Receptor for GDNF secreted by Sertoli cells that stimulates SSC self-renewal
Chromatin status	Nucleosome-based chromatin	+	+	The differentiated spermatogonia start to express testis specific histones variants (TH3, TH2B, TH2A). Some histones variants are integrated into the pacytene spermatocytes just before meiosis.
Histones modifications	H3K27me3 ( associated with gene silencing)	+	-	The promoters of aldehyde hydrogenase 2 and STRA8 genes in A <sub>diff</sub> loose the H3K27me3 modification and consequently the chromatin becomes active
	H3K4me3 +/- H3K27me3 - ( alternative repression mode)	+		This histone modification is present on meiotic genes in A <sub>undiff</sub> spermatogonia and inhibits their transcription
DNA methylation	Syce3	+	Lost	Facilitates homologous chromosomes pairing
	Tex12	+	Lost	Meiotic DNA repair
	Hormad1	+	Lost	Meiosis checkpoint protein
	CCIN	+	Lost	Implicated in sperm shaping
	AnKrd7	+	Lost	Important for meiosis
	Keng4	-	+	Potassium channels
	Kenmb2	-	+	

**Table 8: A comparison between the epigenetic regulation in the embryonic stem cells and the spermatogonia (Hammoud et al. 2014)**

		Embryonic stem cells ESCs	Spermatogonia	function
Promoters of genes containing the two modified histone (H3K4me3 + H3K27me3) with underlying hypomethylated DNA	Hox-, Fox-, Sox-, Tbx-, and Gata-family	+	+	Key genes for embryo development
	Zinc finger clusters	-	+	germline specification
	protocadherins	-	+	
	Wnt pathway	-	+	
	PRDM family	-	+	
	Toll-like receptors	-	+	
DNA methylation profile	Dppa3, Stra8, Piwil, Mov10, SCP-family, Tex-family, Rnf7, Sohlh, Tdrd1, Catsper-family, Nobox, Adad, and Spag	methylated	demethylated	These genes are implicated in germ cells development and they are repressed by DNA methylation in the ESCs
	Small nucleolar RNAs ( snoRNAs) and other genes involved in ESC self-renewal	demethylated	methylated	

b) Meiotic cells

The differentiated spermatocytes also possess a nucleosome-based chromatin. The spermatocytes undergo 2 phases of meiotic divisions where the exchange of genetic materials and segregation of the chromosomes take place. During this step the transcription is globally silenced. This may explain the low levels of H3K9ac, H3K18ac, H3K23ac, and H3K4me3 in the chromatin of mouse germ cells from leptotene to pachytene stage. In mouse diplotene spermatocytes, the chromatin is active once again and presents activating histone modifications such as H3K18ac, H3K23ac, and H3K4me3. Furthermore, each diplotene spermatocyte will divide to give rise to two spermatids. This cell division is under the control of histone H3 phosphorylation at serine 10 (H3S10phos). Interestingly, a set of transcribed genes (e.g., piRNAs) in spermatocytes and spermatids present atypical promoters made up of low methylated CG content, high levels of 5-hmC, H3K4me3, H3K9ac, and H3K27ac (Song et al. 2011; Hammoud et al. 2014).

The murine spermatogenesis is not only regulated by H3 modifications but also by histones H4 modifications. In addition, the H3 and H4 acetylation follow the same pattern during meiosis. However, the H4me3 remains relatively high in pachytene spermatocytes chromatin. H4me3 may be a negative regulator of genes transcription at this level of differentiation (Shirakata et al. 2014).

### c) Spermatids and spermiogenesis

The post-meiotic phase is characterized by fascinating cytodifferentiation and progressive chromatin condensation with inactivation of the genome. The produced sperm is metabolically active and transcriptionally quiescent until it enters the oocyte and combines with the maternal genome. The chromatin remodelling during spermiogenesis involves the replacement of nucleosome-based chromatin by nucleoprotamine-based chromatin. This replacement may occur *via* the following mechanisms (**Figure 38**) :

#### c. 1 The incorporation of histones variants into the chromatin

Histones variants are histones counterpart which are also expressed in the germ cells of mice and humans. Among these histone variants we can enumerate H2A variants ( in mice : H2AL1, H2AL2, H2AL3/ in humans: H2A.Bbd), H2B variants (in mice:H2BL1, H2BL2, testis specific H2B (TH2B)/ in humans : hTSH2B, H2BFWT), H3 variants ( H3.3, testis specific H3 (H3t) in mice and H3T in humans), and testis specific H1 variants ( in mice: H1t, H1t2, Hils1/ in humans: H1T, H1T2, HILS1). Some of these variants appear very early in spermatogenesis ( TH3 is expressed in spermatogonia), and they are incorporated in the chromatin of pachytene spermatocytes (TH2A, TH2B). It was shown that histone variants-containing nucleosomes may create a less stable and open chromatin state; therefore, facilitating nucleosome disassembly and histone displacement in germ cells(Gaucher et al. 2009; Balhorn et al. 2011; Montellier et al. 2013; Yuen et al. 2014).

#### c. 2 Histones hyperacetylation

The elongating spermatids hyperacetylate their histones in the absence of transcription and replication. Furthermore, this acetylation mark is recognized by the double bromodomain factor of the BET family (Brdt) that is expressed in mouse meiotic and post-meiotic germ cells. On one hand, Brdt activates the expression of specific genes during meiosis; and on the other hand, Brdt bromodomain (BD1) binds to hyperacetylated histones in elongating spermatids facilitating their removal. It was shown that Brdt interacts with spermatoproteasomes to induce the removal and degradation of hyperacetylated histones. This could explain how the histone acetylation mark gradually disappears during the replacement of histones by non-histones proteins (Hazzouri et al. 2000; Gaucher et al. 2012; Qian et al. 2013).

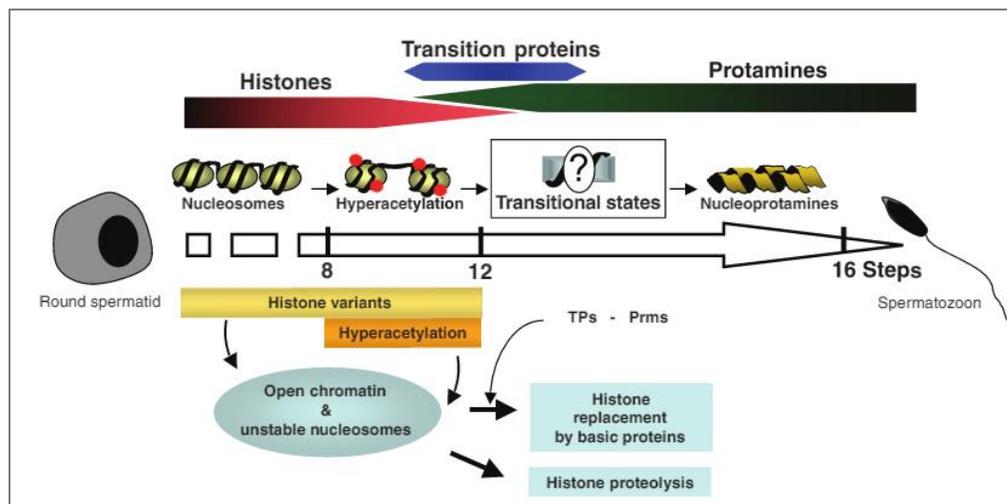
Beside histone acetylation, cell metabolism and some chromatin modifying enzymes (e.g., sirtuins, and CREB-binding protein/EP/300) may also induce different histones modifications such as, butyrylation, propionylation, crotonylation, and etc...(Rousseaux et al. 2014).Recently, Goudarzi and colleagues demonstrated that H4K5/K8 acetyl/butyryl are enriched at specific genes in spermatogenic cells. However, Brdt is not able to bind the butyrylated histones and consequently the butyrylated histones can survive the wave of histones acetylation, removal and degradation. It has been hypothesized, that this type of modification will create a less compact structure in specific regions of the sperm chromatin (Goudarzi et al. 2016).

### c. 3 Replacement of histones by transition proteins

At the mid-stage spermatids, the removed histones are replaced by transition proteins (TPs) which remain in contact with the DNA for a short period. The predominant ones are TP1 and TP2. The TP1 has a molecular weight of 6.2 KDa, containing ~ 20% arginine, ~ 20% lysine, and lacks cysteine. However, the molecular weight of TP2 is 13 KDa, composed of ~ 10% arginine, ~ 10 % lysine, and 5% cysteine. So, TPs are smaller and more basic than histones. Their biochemical properties allow them to compact the chromatin, to prepare for the termination of the transcription, and to facilitate the DNA strand breaks repair(Yu et al. 2000; Balhorn et al. 2011).

### c. 4 Replacement of TP by protamine

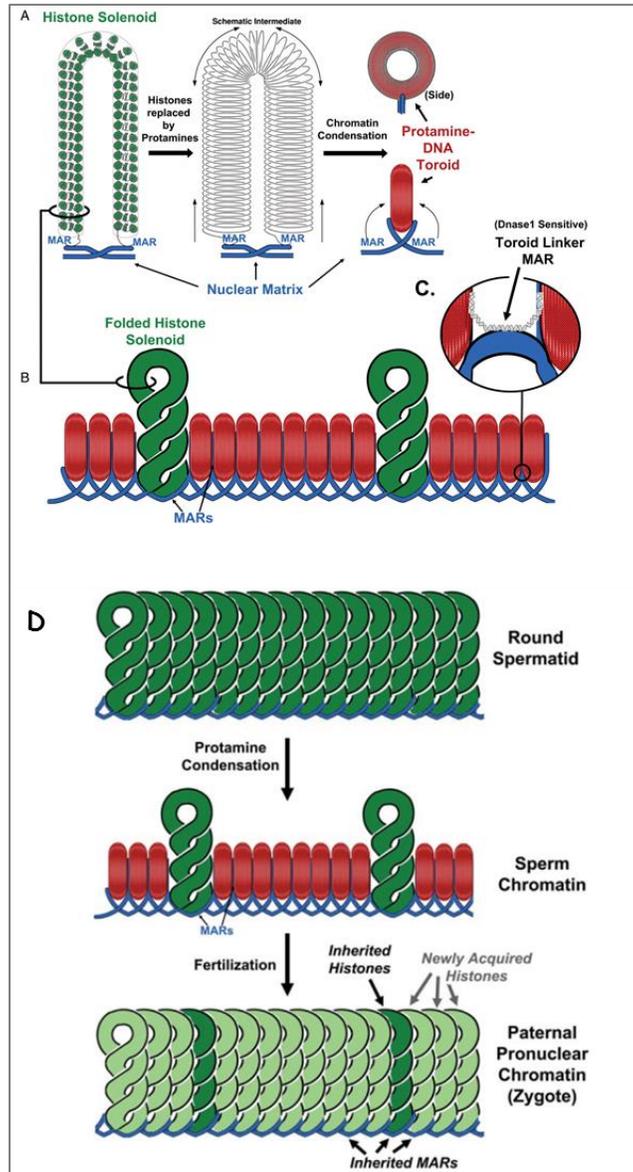
Elongating spermatids start to express small proteins (5-8 KDa) termed as protamine. In the late-steps of spermiogenesis the protamines replace the TPs in the chromatin. The major two types of protamines in humans are P1 and P2. They are characterized by the high content of positively charged amino acids, arginine (~ 50 %), cysteine (~10-13%), and histidine (~ 5-15%). Due to this unique composition, the protamines are highly basic and therefore, they strongly interact with the negatively charged DNA to induce high level of chromatin compaction. Moreover, disulphide bridges between the thiol (-SH) groups of the different cysteine add another level of stabilization to the nucleoprotamine-based chromatin. This high level of condensation is important to protect the sperm DNA during the transit in the female tract, to create a hydrodynamic nucleus, and to control epigenetic reprogramming(Gusse et al. 1986; Aoki et al. 2003; Bjorndahl et al. 2010; Hammadeh et al. 2010; Balhorn et al. 2011).



**Figure 38: The major steps of spermatid chromatin remodelling during spermiogenesis(Gaucher et al. 2009).**

d) Sperm chromatin

The mature sperm is characterized by a highly packed chromatin, playing an important role in fertility and embryo development. The sperm chromatin could be divided into 3 regions (**Figure 39**):



**Figure 39:** The structure of the sperm chromatin (Wared et al. 2010).

#### d.1 Nucleoprotamine-bound sperm chromatin

The majority of sperm DNA (negatively charged) is bound to the small basic protamines (positively charged) causing DNA to compact into toroids. The toroids are packaged together side to side into a highly condensed chromatin. Moreover, they are attached to the sperm nuclear matrix by their linker DNA. The vast majority of the DNA is hidden within the toroids, and it is protected from nuclease digestion. As mentioned earlier, the cysteine residues of the protamines can form disulfide bridges between them *via* the thiol (-SH) groups, increasing the stability of this chromatin. At the functional level, this complex and highly compacted structure, represses the transcription during spermiogenesis and protects the paternal genome during its journey in the female tract. Moreover, paternal protamines are replaced in the first 2-4 hours after fertilization by maternal histones, rendering the chromatin accessible to transcription (Ward et al. 2010).

In contrast, a recent meta-analysis showed that abnormal sperm protamination was associated with male infertility and sperm DNA damage. The normal ratio of P1-P2 is approximately 1. However, this ratio could be affected by several internal or external factors such as thermal stress and cigarette smoking (Love et al. 1999; EVENSON et al. 2000; IUCHI et al. 2003; Hammadeh et al. 2010; Ni et al. 2016).

#### d.2 Nucleosome-bound sperm chromatin

Even though the majority of histones are replaced by protamines, a small percentage from 5-10% of the human sperm genome retains the paternal histones. In the mature sperm of mice and humans the retained histones and their modifications (e.g., H3K4me3, H4K27me3) are not randomly distributed, and they are not replaced by the maternal histones after fertilization. Interestingly, the retained nucleosomes were found at the promoters of different genes of developmental importance in the embryo (such as Hox-, Fox-, Sox-, gata family genes and non-coding RNAs (micro-RNAs and long non-coding RNAs)). Moreover, the retained histones are generally associated with an underlying DNA hypomethylation. Altogether, these findings highlight the possible role of nucleosome-bound sperm chromatin as a paternally inherited epigenetic regulator (Ward et al. 2010; Jenkins et al. 2012; Hammoud et al. 2014). In parallel, several studies in mice have demonstrated that alterations in histone methylation and acetylation status resulted in varying degree of fertility loss (FENIC et al. 2004; LEE et al. 2005; Okada et al. 2007; FENIC et al. 2008; GLASER et al. 2009). In addition, H3K4ac was shown to be erased in the sperm of infertile men; whereas H4K12ac and H4K8ac were significantly higher in the sperm of smokers (Kim et al. 2015; Vieweg et al. 2015). This will open the question on the drawbacks of these alterations on embryogenesis and on the offspring.

### d.3 Matrix attachment regions (MARs)

Sperm toroids and linker DNA sequences are attached to a proteinaceous structure, termed nuclear matrix. Several researchers tried to identify the function (s) of this matrix and it was suggested that it is important for post-fertilization events. After fertilization, MARs is required for paternal DNA replication and may act as a checkpoint for sperm DNA integrity (Wared et al. 2010).

## A. 4 Sperm RNAs

Mammalian sperm is a transcriptionally inactive cell, with a reduced cytoplasm. However, RNA populations were detected in the sperm cells: microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs), Piwi-interacting RNAs (piRNAs). Those ncRNAs may regulate the gene expression by several means. Interestingly, sperm ncRNAs could play an important role in embryogenesis (Liu et al. 2012) and in inheritance (Rassoulzadegan et al. 2006; Wagner et al. 2008; Grandjean et al. 2009).

For instance, the microinjection of mouse spermatozoa with an aberrant sperm-borne miRNA and endo-siRNA content into a mature mouse egg, can achieve fertilization but alter the developmental potential of the embryo (Yuan et al. 2016). Also, the sperm-borne microRNA-34c is only detected in spermatozoa and zygotes and it is not expressed in the oocytes. Microinjection of microRNA-34c inhibitor into the cytoplasm of fertilized mouse zygote alters the DNA synthesis of the one-cell embryo and inhibits the first cleavage division (Liu et al. 2012).

Moreover, the changing environment such as psychological stress and tobacco intake may modify the level of expression of these RNAs and induce behavioral and physiological changes in the progeny (Lane et al. 2014; Bohacek et al. 2015). Microinjection of sperm RNAs derived from male sperm exposed to postnatal trauma into the zygote, reproduces the paternal phenotype in the offspring (Gapp et al. 2014). Furthermore, cardiac hypertrophy, abnormal adult growth, and fur depigmentation may be induced by microinjection of miR-1, miR-124 and miR-221 respectively into a mouse fertilized oocytes (Rassoulzadegan et al. 2006; Wagner et al. 2008; Grandjean et al. 2009).

## B- Transgenerational inheritance of paternal acquired obesity

### B. 1 Overview

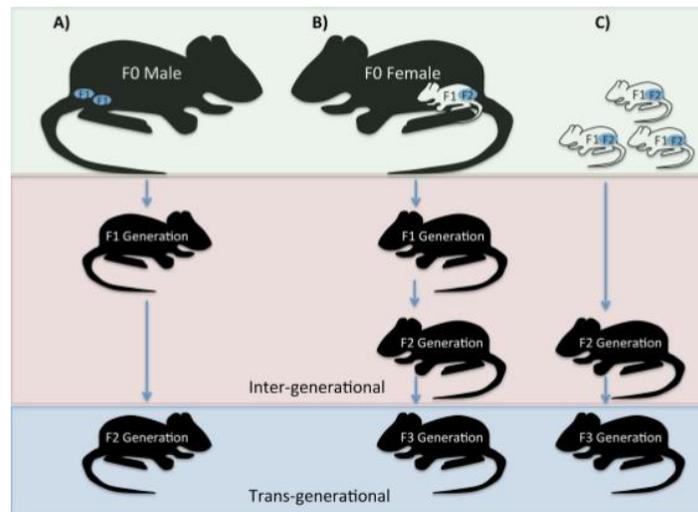
Mendelian inheritance focuses on the heritable changes in the phenotype due to changes in the primary DNA sequence (mutations). In contrast, heritable changes in the phenotype could be associated to modifications in the epigenetic marks (epimutations) without any change in the DNA sequence. This epigenetic inheritance may occur in two ways (**Figure 40**):

- Gametic epigenetic inheritance occurs *via* the gamete epigenome ( DNA chemical modifications, histones modifications, and non-coding RNAs):increasing evidence suggests that information from parental environment remains in the gamete epigenome and could be transmitted to the offspring (wei et al. 2014).
- Non-gametic epigenetic inheritance involves the effects of enviromental exposure on the adults that may modify the phenotype of the next generation. For instance, the uterine exposure at the time of conception to the seminal fluid regulates the postnatal body homeosatsis and the behaviorof the offspring. In addition, in-utero exposure of the developing embryo to different molecules, or the exposure of the newborn to the milk during lactaion may modulate the health of the offspring(Danxinger et al. 2012; Lane et al. 2014; Vickers et al. 2014).

When studying such influence of ancestral enviroments on the future generations, it is important to distinguish between the intergenerational and the transgenerational transmission. The inheritance of the acquired phenotype by the immediately succeeding generation (F1), coming from the exposed gametes could be termed as “ intergenerational inheritance”. However, the apearance of the phenotype in the grandchildren and the next generations (F2, F3), coming from the unexposed gametes may be termed as “transgenerational inheritance” (**Figure 40**)(Dias et al. 2014; Terashima et al. 2015).

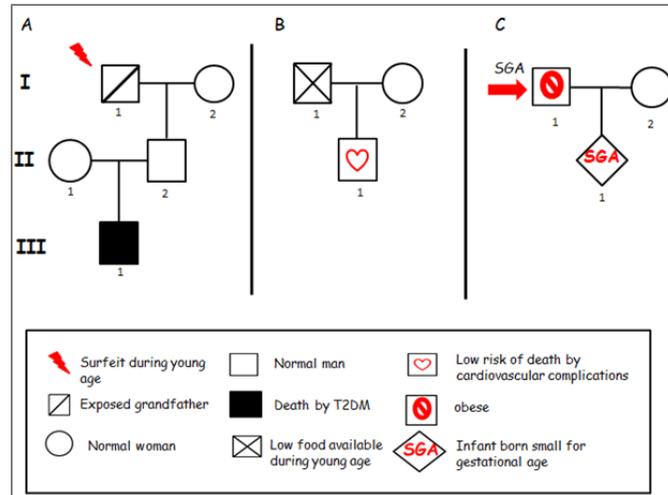
A new area of research in andrology, shed the light on the long term consequences of paternal health, at the time of conception, on the health of the offspring. For example, paternal age, smoking, and exposure to toxic chemicals may increase the risk of neuropsychiatric disorders, metabolic alterations, respiratory tract infection, and cancer in the offspring (Sorahan et al. 1997; Chang et al. 2006; Toschke et al. 2007; Fernandez-Gonzalez et al. 2008; Lin et al. 2008; Sung et al. 2008; Sartorius et al. 2010). In addition, accumulating epidemiological studies in humans suggest that paternal body mass index (BMI) may influence the health of the next generation (Lane et al. 2014). As reported by G kaati et al., when paternal grandfather experienced a surfeit of food at the age of 8-12 years old, the risk of grandson’s death by T2DM had increased by 4 folds (**Figure 41 A**). Plus, the risk of death by cardiovascular complications was reduced when there was low food availability during father’s young age (**Figure 41 B**) (Kaati et al. 2002). Moreover, a father born small for gestational age (SGA) had a higher risk of developing obesity

and giving birth to a (SGA) baby (**Figure 41 C**) (McCowan et al. 2011). Furthermore, the incidence of obesity and diabetes in the grandchildren, coming from sons who were exposed *in utero* to the Dutch famine, were very high (Veenendaal et al. 2013). Also, paternal obesity was associated with hypo methylation of *Insulin-like Growth Factor 2* (IGF2) gene in newborns' cord blood. By itself IGF2 has been linked to an increased risk of developing cancers (Soubry et al. 2013). This finding, opened the possibility of the presence of an epigenetic pathway for the transmission of this acquired pathology.



**Figure 40: Scheme showing different modes of non-genetic inheritance.**

(A) The exposed gametes from the paternal ancestor will lead to an intergenerational inheritance in (F1). The inheritance of the phenotype by offspring (F2) generated from the F1 (unexposed gametes) is termed as transgenerational inheritance. (B) In utero-exposure can lead to an intergenerational inheritance in F1 and F2 since they are produced from exposed gametes, whereas the persistence of the phenotype in F3 is termed as transgenerational inheritance. (C) A peri-natal exposure (F1) may alter the phenotype and its exposed gametes will lead to an intergenerational inheritance of the phenotype in F2. However, if the phenotype appears in the F3 generation, it is called a transgenerational inheritance (Dias et al. 2014).



**Figure 41: Pedigree pattern of acquired pathologies.**

(A) The grandson's (III-1) risk of death by T2DM was increased by 4 folds when his grandfather ( I-1) was exposed to a surfeit during his young age.(B) Low food availability during father's young age ( I-1) has reduced the risk of death of his son (II-1) by cardiovascular complications. (C) A father (I-1) born small for gestational age (SGA) had a high risk to become obese and to give birth to a SGA infant (II-1) (Kaati et al. 2002; McCowan et al. 2011).

## B. 2 Possible mechanisms of intergenerational and transgenerational inheritance of paternal acquired obesity

In order to understand the molecular mechanisms underlying the above described results in humans, animal models were developed. Ng Sheau-Fang and colleagues showed that male rats fed on a high fat diet (HFD), gave birth to females (F1) intolerant to glucose with abnormal insulin secretion. These metabolic alterations could be explained by the histopathological changes in the pancreatic islets of these females. At the molecular level, a key islet gene *Il13ra2* (interleukin 13 receptor subunit alpha 2) was hypomethylated and its messenger RNA was upregulated in the pancreatic islets of the F1 offspring. Plus, transcriptomic analysis of the retroperitoneal fat and the pancreas of the female offspring showed deregulation in genes involved in mitochondrial and cellular stress response. Altogether, these findings indicate a possible role of epigenetics in the intergenerational inheritance of the paternal acquired phenotype (Ng et al. 2010; Ng et al. 2014) .

In the same context, a non-genetic prediabetes mouse model has been generated with injection of low doses of streptozotocin which destroys the pancreatic  $\beta$ -cells. The generated prediabetic male mice fathered offspring (F1) with glucose intolerance and insulin resistance. Excitingly, the same phenotype was transmitted to the second generation (F2). In addition, the expression

pattern of 402 genes in the pancreas of these offspring was altered when compared to a control group; among them the level of expression of phosphatidylinositol 3 kinase catalytic and regulatory subunits (*PiK3ca* and *PiK3r1*) was lower in the two subsequent generations of mice. Of particular interest, the DNA methylation profile in the sperm of the treated father overlapped with that of pancreatic islets in the offspring. Most interestingly, the *PiK3ca* and *PiK3r1* genes were hypermethylated in the sperm and in the pancreas of the offspring (F1 and F2). These results confirm the hypothesis that non-genetic metabolic alteration could be intergenerationally and transgenerationally inherited *via* the DNA methylation (Wei et al. 2014).

In parallel, the model of diet-induced obesity in male mice (C57BL6) was used in different publications trying to decipher the mystery behind this kind of inheritance. The male acquired obesity in mice may result in fetal growth restriction characterized by reduced fetal and placental weights. The fetal growth restriction is associated with an increased risk of developing obesity and diabetes later in life. The molecular analysis of the placentas showed that peroxisome proliferator-activated receptor alpha (*Ppara*) and caspase-12 (*Casp12*) mRNAs expression were significantly deregulated in male placentas from obese fathers when compared to a normal father, whereas the global DNA methylation was only increased in female placentas (Binder et al. 2015).

Furthermore, the paternal acquired obesity in mice alters the total body weight and the glucose homeostasis in the female offspring (F1) with a lesser severity in the males. These phenotypes were also transmitted to the second generation (F2) but in a sex-specific manner. At the epigenetic level, the elongated spermatids of the grandfather's testes were significantly hypomethylated when detected by immunohistochemistry on testis sections. Moreover, 4 microRNAs were found to be deregulated in the testis and in the sperm of the obese grandfathers (miR133b-3p, miR-304-5p, miR196a-5p, miR205); these miRNAs may be implicated in the regulation of pre-implantation mouse embryo development (Fullston et al. 2013).

Fascinatingly, Grandjean and colleagues has showed that microinjection of testis or sperm RNAs from obese male mice into the pronucleus of a naïve zygote induces metabolic disturbances in the offspring. Deep sequencing analysis of these microinjected RNAs allows the identification of 13 miRNAs and 190 piRNAs differentially expressed in the testis of obese mice. Among these RNAs, the miR19b was one of the most deregulated micro-RNAs. Microinjection of the miR-19b into the pronucleus of a naïve zygote also induces alterations in the body weight of the offspring. These results indicate that the testicular RNAs could be a vector of transmission of paternal acquired obesity (Grandjean et al. 2015). In parallel, Binder et al. found an increase in the level of expression of cytochrome c oxidase subunit IV isoform I (*Cox4il*) mRNA in sperm cells of obese mice. *Cox4il* is a nuclear encoded gene, which may regulate the mitochondrial function in the embryos, emphasizing the role of sperm RNAs in the regulation of embryo development (Binder et al. 2015).

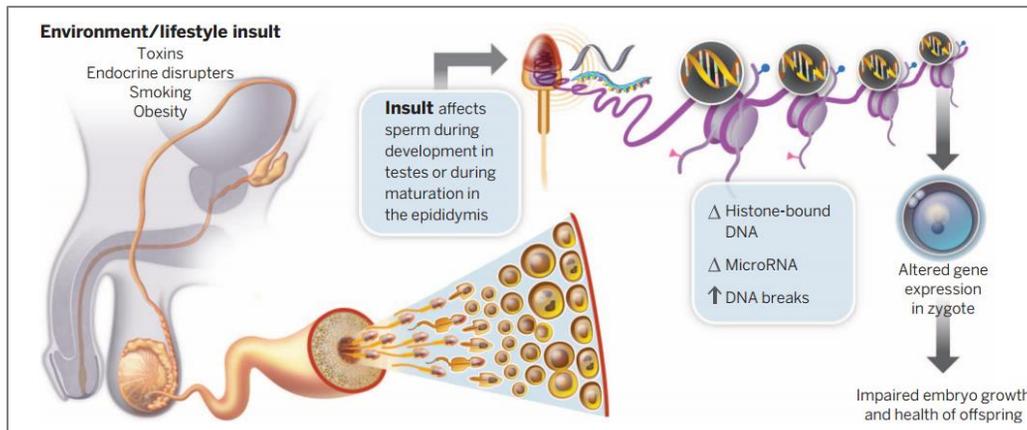
Not only the DNA methylation, and the sperm RNAs but also the sperm chromatin is considered as a potential candidate. On one hand, Terashima et al. demonstrated that paternal acquired obesity may alter the expression of 7 genes in the liver of the offspring. In addition, the sperm of these obese fathers showed a higher H3 and H3K4me1 retention at specific genes implicated in development and cell fate decision. On the other hand, the histone deacetylases (HDAC) class III or Sirtuin (SIRT1-7) proteins are regulated by caloric intake. Of particular interest, SIRT6 is expressed in the mouse elongating spermatids and could play the role of ADP-ribosyltransferase and H3 deacetylase (H3K9 and H3K56). Palmer et al. found that the protein level of SIRT6 is significantly decreased in the spermatids of male mice on high fat diet (HFD) when compared to a control group. Consequently, the percentage of spermatids that stain positive to the H3K9ac antibody was higher in the HFD group compared with those on control diet (Palmer et al. 2011). Moreover, the offspring of male mice raised on a low protein diet overexpressed in their hepatocytes genes involved in lipid metabolism. This low-protein diet was associated with a decrease in the H3K27me3 retention in the sperm at the promoters of *Maoa* (Monoamine oxidase) and *Eftud1* (Elongation Factor Like GTPase 1) (Carone et al. 2010). These findings clearly show that sperm chromatin could be modulated by the dietary conditions and could transfer epigenetic information to the next generation.

Interestingly, other physiological processes were showed to be affected by the paternal obesity. For instance it may compromise the reproductive health of two subsequent generations of mice (Fullston et al. 2012; Fullston et al. 2015), and it may increase the susceptibility of malignant transformation in the mammary tissue of the female offspring (Fontelles et al. 2016).

In humans, a recent study showed that obesity can remodel the human sperm epigenome; thus, playing a crucial role in the sperm-dependent non-genetic inheritance of acquired obesity. Donkin et al. found that obese men have a different sperm DNA methylation profile for genes implicated in the central nervous system function and metabolism when compared to normal weight men. Plus, the sperm transcriptome analysis showed deregulation in the Piwi-interacting RNAs (piRNAs) expression pattern in the sperm of obese patients (Donkin et al. 2016).

Besides the gametic pathway, the seminal plasma was hypothesized to participate in the inheritance of the paternal obesity. On one hand, differences in the seminal plasma composition between obese and non-obese mice were detected. Similar differences in the composition were also seen in humans (Binder et al. 2015). On the other hand, the metabolism of embryos derived from *in vivo* mating (where the seminal plasma may interfere) of an obese male mice and normal female mice showed a higher glycolytic rate than normal (Binder et al. 2012). However, the embryos derived from *in vitro* fertilization using sperm cells without seminal fluid from obese male mice, and oocytes from normal female, showed normal glycolytic rate (Binder et al. 2012). Inappropriate early increase in the glycolytic rate negatively impacts the embryo development (Leese et al. 2002). Therefore, the suboptimal plasma composition associated with male obesity may have drawbacks on the uterine environment, embryo physiology, and offspring health (Binder et al. 2015).

To sum up, all these studies suggested that paternal life style could affect the seminal fluid and the sperm epigenome (DNA modifications, chromatin composition, and RNAs content), thus playing a possible role in the transfer of non-genetic information from one generation to another (Figure 42).



**Figure 42: The impact of the lifestyle on the non-genetic paternal contribution to the embryogenesis(Lane et al. 2014).**

#### IV- Research aims

Despite what is known about the drawbacks of male obesity on the reproductive system and on the offspring health, several significant questions remained unanswered. The majority of the studies in humans assessed the impact of obesity on the raw semen containing heterogeneous populations of sperm (motile and non-motile spermatozoa). Here we aimed to evaluate the effect of paternal obesity on the molecular composition of the motile spermatozoa which are the only able to fertilize the oocyte. Next, using this fraction of sperm we analyzed to which extent paternal obesity influences the preimplantation embryo morphokinetics.

Furthermore, we intended to evaluate the adaptive and evolutionary potential of non-genetic heritable mechanisms in experimentally controlled animal models. Using a high fat diet (HFD)-induced obesity mouse model, we examined how feeding male mice with a high fat diet for multiple generations impacts the phenotype of the resulting mice. We focused mainly on the evolution of obesity-associated complications such as glomerulopathy and infertility.

These studies should add more insights into our current knowledge on the epigenetic signature transferred by the sperm of obese men to the oocyte during fertilization and into how biological systems respond and adapt to continuous exposure to a HFD stress.

## V- Bibliography

1. Abd-Allah, Adel RA, et al. "Pro-inflammatory and oxidative stress pathways which compromise sperm motility and survival may be altered by L-carnitine." *Oxidative medicine and cellular longevity* 2.2 (2009): 73-81.
2. Abdalla, Hany, Yusuke Yoshizawa, and Shinichi Hoshi. "Active demethylation of paternal genome in mammalian zygotes." *Journal of Reproduction and Development* 55.4 (2009): 356-360.
3. Albrecht, Martin. "Insights into the nature of human testicular peritubular cells." *Annals of Anatomy-AnatomischerAnzeiger* 191.6 (2009): 532-540.
4. Al-Quwaidhi, A. J., et al. "Trends and future projections of the prevalence of adultobesity in Saudi Arabia, 1992-2022/Tendances et projections de la prévalence de l'obésité chez l'adulte en Arabie saoudite, 1992-2022." *Eastern Mediterranean Health Journal* 20.10 (2014): 589.
5. Alsaad, K. O., and A. M. Herzenberg. "Distinguishing diabetic nephropathy from other causes of glomerulosclerosis: an update." *Journal of clinical pathology* 60.1 (2007): 18-26.
6. G Alves, Marco, et al. "Impact of diabetes in blood-testis and blood-brain barriers: resemblances and differences." *Current diabetes reviews* 8.6 (2012): 401-412.
7. Amrolia, P., et al. "An investigation of glucose uptake in relation to steroidogenesis in rat testis and tumour Leydig cells." *Biochemical Journal* 249.3 (1988): 925-928.
8. Aoki, Vincent W., and Douglas T. Carrell. "Human protamines and the developing spermatid: their structure, function, expression and relationship with male infertility." *Asian journal of andrology* 5.4 (2003): 315-324.
9. Aquila, Saveria, et al. "Autocrine regulation of insulin secretion in human ejaculated spermatozoa." *Endocrinology* 146.2 (2005): 552-557.
10. Aitken, John, and Helen Fisher. "Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk." *Bioessays* 16.4 (1994): 259-267.

11. Auger, J. (2009). Preface. In J. Auger, *Exploration de la fonction de reproduction: versant masculin* (Vol. 42). Paris: Bioforma: formation continue des biologistes.
12. Bacon, Constance G., et al. "A prospective study of risk factors for erectile dysfunction." *The Journal of urology* 176.1 (2006): 217-221.
13. Bagchi, Mandrita. *Role of Vascular Endothelial Growth Factor Signaling in Brown Adipocyte Survival, Proliferation and Function*. Diss. 2013.
14. Bakos, Hassan Waleed. *The Effect of Paternal Obesity on Sperm Function, Embryo Development and Subsequent Pregnancy Outcomes*. Diss. The University of Adelaide, 2010.
15. Bakos, Hassan W., et al. "Paternal body mass index is associated with decreased blastocyst development and reduced live birth rates following assisted reproductive technology." *Fertility and sterility* 95.5 (2011): 1700-1704.
16. Bakos, Hassan W., et al. "Paternal body mass index is associated with decreased blastocyst development and reduced live birth rates following assisted reproductive technology." *Fertility and sterility* 95.5 (2011): 1700-1704.
17. Bakos, H. W., et al. "The effect of paternal diet-induced obesity on sperm function and fertilization in a mouse model." *International journal of andrology* 34.5pt1 (2011): 402-410.
18. Balhorn, Rod. "Sperm chromatin: an overview." *Sperm Chromatin*. Springer New York, 2011. 3-18.
19. Ballester, Joan, et al. "Evidence for a functional glycogen metabolism in mature mammalian spermatozoa." *Molecular reproduction and development* 56.2 (2000): 207-219.
20. Bauché, Françoise, Marie-Hélène Fouchard, and Bernard Jégou. "Antioxidant system in rat testicular cells." *FEBS letters* 349.3 (1994): 392-396.
21. Bernstein, Bradley E., et al. "A bivalent chromatin structure marks key developmental genes in embryonic stem cells." *Cell* 125.2 (2006): 315-326.

22. Berridge, Michael J. "Calcium signalling remodelling and disease." *Biochemical Society Transactions* 40.2 (2012): 297-309.
23. Berridge, M.J. (2012) *Cell Signalling Biology*; doi:10.1042/csb0001007
24. Binder, Natalie K., Megan Mitchell, and David K. Gardner. "Parental diet-induced obesity leads to retarded early mouse embryo development and altered carbohydrate utilisation by the blastocyst." *Reproduction, Fertility and Development* 24.6 (2012): 804-812.
25. Binder, Natalie K., Natalie J. Hannan, and David K. Gardner. "Paternal diet-induced obesity retards early mouse embryo development, mitochondrial activity and pregnancy health." *PLoS One* 7.12 (2012): e52304.
26. Binder, Natalie K., et al. "Male obesity is associated with changed spermatozoa Cox4i1 mRNA level and altered seminal vesicle fluid composition in a mouse model." *Molecular human reproduction* (2015): gav010.
27. Binder, Natalie K., et al. "Paternal obesity in a rodent model affects placental gene expression in a sex-specific manner." *Reproduction* 149.5 (2015): 435-444.
28. Björndahl, Lars, and Ulrik Kvist. "Human sperm chromatin stabilization: a proposed model including zinc bridges." *Molecular human reproduction* 16.1 (2010): 23-29.
29. Blundell, John E., and John Cooling. "Routes to obesity: phenotypes, food choices and activity." *British journal of nutrition* 83.S1 (2000): S33-S38.
30. Blundell, J. E., et al. "The biology of appetite control: Do resting metabolic rate and fat-free mass drive energy intake?." *Physiology & behavior* 152 (2015): 473-478.
31. Bohacek, Johannes, and Isabelle M. Mansuy. "Molecular insights into transgenerational non-genetic inheritance of acquired behaviours." *Nature Reviews Genetics* (2015).
32. Boussouar, Fayçal, and Mohamed Benahmed. "Lactate and energy metabolism in male germ cells." *Trends in Endocrinology & Metabolism* 15.7 (2004): 345-350.

33. Brooker, C. (1998). chapitre 20 la reproduction. In C. Brooker, *Le corps humain: Étude, structure et fonction* (Vol. second edition). Bruxelles: de boeck.
34. Burrue, Victoria, et al. "Abnormal early cleavage events predict early embryo demise: sperm oxidative stress and early abnormal cleavage." *Scientific reports* 4 (2014).
35. Busada, Jonathan T., and Christopher B. Geyer. "The role of retinoic acid (RA) in spermatogonial differentiation." *Biology of reproduction* 94.1 (2016): 10.
36. Campbell, Jared M., et al. "Paternal obesity negatively affects male fertility and assisted reproduction outcomes: a systematic review and meta-analysis." *Reproductive biomedicine online* 31.5 (2015): 593-604.
37. Carone, Benjamin R., et al. "Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals." *Cell* 143.7 (2010): 1084-1096.
38. Caroppo, Ettore. "Male reproductive medicine: anatomy and physiology." *An Introduction to Male Reproductive Medicine* (2011): 1.
39. Chagnac, Avry, et al. "Glomerular hemodynamics in severe obesity." *American Journal of Physiology-Renal Physiology* 278.5 (2000): F817-F822.
40. Chan, Donovan, and Jacquetta Trasler. "The sperm epigenome." *Sperm Chromatin*. Springer New York, 2011. 95-106.
41. Chang, Jeffrey S., et al. "Parental smoking and the risk of childhood leukemia." *American journal of epidemiology* 163.12 (2006): 1091-1100.
42. Chen, Jing, et al. "The metabolic syndrome and chronic kidney disease in US adults." *Annals of internal medicine* 140.3 (2004): 167-174.
43. Chen, Taiping, et al. "Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b." *Molecular and cellular biology* 23.16 (2003): 5594-5605.

44. Chen, X. L., L. Z. Gong, and J. X. Xu. "Antioxidative activity and protective effect of probiotics against high-fat diet-induced sperm damage in rats." *animal* 7.02 (2013): 287-292.
45. Choucair, Fadi B., et al. "High level of DNA fragmentation in sperm of Lebanese infertile men using Sperm Chromatin Dispersion test." *Middle East Fertility Society Journal* (2016).
46. Chughtai, Bilal, et al. "A neglected gland: a review of Cowper's gland." *International journal of andrology* 28.2 (2005): 74-77.
47. Colaci, Daniela S., et al. "Men's body mass index in relation to embryo quality and clinical outcomes in couples undergoing in vitro fertilization." *Fertility and sterility* 98.5 (2012): 1193-1199.
48. Costanian, Christy, et al. "Prevalence, correlates and management of type 2 diabetes mellitus in Lebanon: findings from a national population-based study." *Diabetes research and clinical practice* 105.3 (2014): 408-415.
49. D'Agati, Vivette D., et al. "Obesity-related glomerulopathy: clinical and pathologic characteristics and pathogenesis." *Nature Reviews Nephrology* (2016).
50. Daxinger, Lucia, and Emma Whitelaw. "Understanding transgenerational epigenetic inheritance via the gametes in mammals." *Nature Reviews Genetics* 13.3 (2012): 153-162.
51. Datta, B. (2004). Pathology of the kidney and urinary tract. In B. Datta, *Textbook of pathology* (Vol. second edition). New delhi: JAYPEE BROTHERS.
52. de Castro, Letícia S., et al. "Sperm oxidative stress is detrimental to embryo development: a dose-dependent study model and a New and more sensitive oxidative status evaluation." *Oxidative medicine and cellular longevity* 2016 (2015).
53. De Lorenzo, Antonino, et al. "New obesity classification criteria as a tool for bariatric surgery indication." *World journal of gastroenterology* 22.2 (2016): 681.
54. De Vries, Aiko PJ, et al. "Fatty kidney: emerging role of ectopic lipid in obesity-related renal disease." *The Lancet Diabetes & Endocrinology* 2.5 (2014): 417-426.

55. Deji, Naoko, et al. "Structural and functional changes in the kidneys of high-fat diet-induced obese mice." *American Journal of Physiology-Renal Physiology* 296.1 (2009): F118-F126.
56. Delatte, Benjamin, et al. "Genome-wide hydroxymethylcytosine pattern changes in response to oxidative stress." *Scientific reports* 5 (2015).
57. Despopoulos, A. (2003). *Color atlas of physiology*. Thieme.
58. Dias, Brian G., and Kerry J. Ressler. "Experimental evidence needed to demonstrate inter- and trans-generational effects of ancestral experiences in mammals." *BioEssays* 36.10 (2014): 919-923.
59. Dias, Tânia R., et al. "Sperm glucose transport and metabolism in diabetic individuals." *Molecular and cellular endocrinology* 396.1 (2014): 37-45.
60. Discacciati, A., N. Orsini, and A. Wolk. "Body mass index and incidence of localized and advanced prostate cancer—a dose–response meta-analysis of prospective studies." *Annals of Oncology* (2012): mdr603.
61. Dissanayake, D. M. A. B., et al. "Relationship between seminal plasma zinc and semen quality in a subfertile population." *Journal of human reproductive sciences* 3.3 (2010): 124.
62. Donkin, Ida, et al. "Obesity and bariatric surgery drive epigenetic variation of spermatozoa in humans." *Cell metabolism* 23.2 (2016): 369-378.
63. Duale, N., et al. "Impaired sperm chromatin integrity in obese mice." *Andrology* 2.2 (2014): 234-243.
64. Durairajanayagam, Damayanthi, Ashok Agarwal, and Chloe Ong. "Causes, effects and molecular mechanisms of testicular heat stress." *Reproductive biomedicine online* 30.1 (2015): 14-27.
65. Eckel, Robert H., Scott M. Grundy, and Paul Z. Zimmet. "The metabolic syndrome." *The Lancet* 365.9468 (2005): 1415-1428.
66. Efimova, Olga A., et al. "Chromosome hydroxymethylation patterns in human zygotes and cleavage-stage embryos." *Reproduction* 149.3 (2015): 223-233.

- 67.Ehmcke, Jens, Joachim Wistuba, and Stefan Schlatt. "Spermatogonial stem cells: questions, models and perspectives." *Human Reproduction Update* 12.3 (2006): 275-282.
- 68.Eisenberg, Michael L., et al. "The relationship between male BMI and waist circumference on semen quality: data from the LIFE study." *Human reproduction* 29.2 (2014): 193-200.
- 69.El Hajj, N., et al. "Methylation status of imprinted genes and repetitive elements in sperm DNA from infertile males." *Sexual Development* 5.2 (2011): 60-69.
- 70.Engeli, Stefan, et al. "Weight loss and the renin-angiotensin-aldosterone system." *Hypertension* 45.3 (2005): 356-362.
- 71.Esposito, Katherine, et al. "Effect of lifestyle changes on erectile dysfunction in obese men: a randomized controlled trial." *Jama* 291.24 (2004): 2978-2984.
- 72.Evenson, DONALD P., et al. "Characteristics of human sperm chromatin structure following an episode of influenza and high fever: a case study." *Journal of andrology* 21.5 (2000): 739-746.
- 73.Fan, Yong, et al. "Diet-induced obesity in male C57BL/6 mice decreases fertility as a consequence of disrupted blood-testis barrier." *PloS one* 10.4 (2015): e0120775.
- 74.Fenic, Irina, et al. "In Vivo Effects of Histone-Deacetylase Inhibitor Trichostatin-A on Murine Spermatogenesis." *Journal of andrology* 25.5 (2004): 811-818.
- 75.Fenic, Irina, et al. "In Vivo Application of Histone Deacetylase Inhibitor Trichostatin-A Impairs Murine Male Meiosis." *Journal of andrology* 29.2 (2008): 172-185.
- 76.Fernández-Gonzalez, Raúl, et al. "Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring." *Biology of Reproduction* 78.4 (2008): 761-772.
- 77.Flenkenthaler, Florian, et al. "Secretome analysis of testicular peritubular cells: a window into the human testicular microenvironment and the spermatogonial stem cell niche in man." *Journal of proteome research* 13.3 (2014): 1259-1269.

78. Fontelles, Camile Castilho, et al. "Paternal overweight is associated with increased breast cancer risk in daughters in a mouse model." *Scientific Reports* 6 (2016).
79. Frans, Emma M., et al. "Advancing paternal age and bipolar disorder." *Archives of general psychiatry* 65.9 (2008): 1034-1040.
80. Füllgrabe, J., E. Kavanagh, and B. Joseph. "Histone onco-modifications." *Oncogene* 30.31 (2011): 3391-3403.
81. Fullston, T., et al. "Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice." *Human reproduction* 27.5 (2012): 1391-1400.
82. Fullston, T., et al. "Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice." *Human reproduction* 27.5 (2012): 1391-1400.
83. Fullston, Tod, et al. "Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete penetrance to the F2 generation and alters the transcriptional profile of testis and sperm microRNA content." *The FASEB Journal* 27.10 (2013): 4226-4243.
84. Fullston, Tod, et al. "Paternal obesity induces metabolic and sperm disturbances in male offspring that are exacerbated by their exposure to an "obesogenic" diet." *Physiological reports* 3.3 (2015): e12336.
85. Fullston, Tod, et al. "Paternal obesity induces metabolic and sperm disturbances in male offspring that are exacerbated by their exposure to an "obesogenic" diet." *Physiological reports* 3.3 (2015): e12336.
86. Gan, Haiyun, et al. "Dynamics of 5-hydroxymethylcytosine during mouse spermatogenesis." *Nature communications* 4 (2013).
87. Gapp, Katharina, et al. "Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice." *Nature neuroscience* 17.5 (2014): 667-669.
88. Gaucher, Jonathan, et al. "From meiosis to postmeiotic events: the secrets of histone disappearance." *FEBS journal* 277.3 (2010): 599-604.

89. Gaucher, Jonathan, et al. "Bromodomain-dependent stage-specific male genome programming by Brdt." *The EMBO journal* 31.19 (2012): 3809-3820.
90. Gauthier, Marie-Soleil, and Neil B. Ruderman. "Adipose tissue inflammation and insulin resistance: all obese humans are not created equal." *Biochemical Journal* 430.2 (2010): e1-e4.
91. Gee, M. (2008). Weight management. In K. a.-S. Mahan, *Krause's food and nutrition therapy* (Vol. 12). SAUNDER ELSEVIER.
92. Glaser, Stefan, et al. "The histone 3 lysine 4 methyltransferase, Mll2, is only required briefly in development and spermatogenesis." *Epigenetics & chromatin* 2.1 (2009): 1.
93. Golay, A., and J. Ybarra. "Link between obesity and type 2 diabetes." *Best Practice & Research Clinical Endocrinology & Metabolism* 19.4 (2005): 649-663.
94. Goudarzi, Afsaneh, et al. "Dynamic Competing Histone H4 K5K8 Acetylation and Butyrylation Are Hallmarks of Highly Active Gene Promoters." *Molecular cell* 62.2 (2016): 169-180.
95. Grandjean, Valérie, et al. "The miR-124-Sox9 paramutation: RNA-mediated epigenetic control of embryonic and adult growth." *Development* 136.21 (2009): 3647-3655.
96. Grandjean, Valérie, et al. "RNA-mediated paternal heredity of diet-induced obesity and metabolic disorders." *Scientific reports* 5 (2015).
97. Griffin, Karen A., Holly Kramer, and Anil K. Bidani. "Adverse renal consequences of obesity." *American Journal of Physiology-Renal Physiology* 294.4 (2008): F685-F696.
98. Gu, Tian-Peng, et al. "The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes." *Nature* 477.7366 (2011): 606-610.
99. Güneş, Sezgin, and Tuba Kulaç. "The role of epigenetics in spermatogenesis." *Turkish journal of urology* 39.3 (2013): 181.

100. Guo, Kai-Ying, et al. "Effects of obesity on the relationship of leptin mRNA expression and adipocyte size in anatomically distinct fat depots in mice." *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 287.1 (2004): R112-R119.
101. Gusse, Michel, et al. "Purification and characterization of nuclear basic proteins of human sperm." *Biochimica et Biophysica Acta (BBA)-General Subjects* 884.1 (1986): 124-134.
102. Hai, Yanan, et al. "The roles and regulation of Sertoli cells in fate determinations of spermatogonial stem cells and spermatogenesis." *Seminars in cell & developmental biology*. Vol. 29. Academic Press, 2014.
103. Hales, D. (2002). Testicular macrophage modulation of leydig cell steroidogenesis. 57.
104. Hammadeh, M. E., et al. "Protamine contents and P1/P2 ratio in human spermatozoa from smokers and non-smokers." *Human reproduction* (2010): deq226.
105. Hammoud, Saher Sue, et al. "Chromatin and transcription transitions of mammalian adult germline stem cells and spermatogenesis." *Cell Stem Cell* 15.2 (2014): 239-253.
106. Harris, Peter, Sue Nagy, and Nicholas Vardaxis. *Mosby's Dictionary of Medicine, Nursing and Health Professions-Australian & New Zealand Edition*. Elsevier Health Sciences, 2014.
107. Hartel, D. et al. (2012). Molecular organization of chromosomes. In *Genetics: analysis of genes and genomes* (Vol. Eighth edition). Jones and Bartlett learning.
108. Hartl, D. (2011). The chromosomal Basis of heredity. In D. Hartl, *Essential genetics: A genomics perspective*. Sudbury: Jones and Bartlett publisher.
109. Hazzouri, Mira, et al. "Regulated hyperacetylation of core histones during mouse spermatogenesis: involvement of histone-deacetylases." *European journal of cell biology* 79.12 (2000): 950-960.
110. Helal, I. (2012). Glomerular hyperfiltration: definitions, mechanisms and clinical implications. 8.
111. Heitzmann, Dirk, and Richard Warth. "Physiology and pathophysiology of potassium channels in gastrointestinal epithelia." *Physiological Reviews* 88.3 (2008): 1119-1182.
112. Hicks, J. J. (1973). Insulin regulation of spermatozoa metabolism. 92 (833-839).
113. Hinrichsen, Klaus V. *Slides on human embryology*. Springer Science & Business Media, 1986.

114. Huang, Guizhen, et al. "IL-6 mediates differentiation disorder during spermatogenesis in obesity-associated inflammation by affecting the expression of Zfp637 through the SOCS3/STAT3 pathway." *Scientific Reports* 6 (2016).
115. Huleihel, Mahmoud, and Eitan Lunenfeld. "Regulation of spermatogenesis by paracrine/autocrine testicular factors." *Asian journal of andrology* 6.3 (2004): 259-268.
116. Idelman, Simon, and Jean Verdeti. *Endocrinologie et communications cellulaires*. EDP sciences, 2001.
117. Iqbal, Khursheed, et al. "Reprogramming of the paternal genome upon fertilization involves genome-wide oxidation of 5-methylcytosine." *Proceedings of the National Academy of Sciences* 108.9 (2011): 3642-3647.
118. Iuchi, Yoshihito, et al. "Concerted changes in the YB2/RYB-a protein and protamine 2 messenger RNA in the mouse testis under heat stress." *Biology of reproduction* 68.1 (2003): 129-135.
119. Ivell, Richard. "Lifestyle impact and the biology of the human scrotum." *Reproductive biology and endocrinology* 5.1 (2007): 1
120. Jefferson, J. A., S. J. Shankland, and R. H. Pichler. "Proteinuria in diabetic kidney disease: a mechanistic viewpoint." *Kidney international* 74.1 (2008): 22-36.
121. Jenkins, Timothy G., and Douglas T. Carrell. "The sperm epigenome and potential implications for the developing embryo." *Reproduction* 143.6 (2012): 727-734.
122. Jenkins, Timothy G., et al. "Paternal aging and associated intraindividual alterations of global sperm 5-methylcytosine and 5-hydroxymethylcytosine levels." *Fertility and sterility* 100.4 (2013): 945-951.
123. Jenkins, Timothy G., et al. "Age-associated sperm DNA methylation alterations: possible implications in offspring disease susceptibility." *PLoS Genet* 10.7 (2014): e1004458.
124. Jensen, Jørgen, et al. "The role of skeletal muscle glycogen breakdown for regulation of insulin sensitivity by exercise." *Frontiers in physiology* 2 (2011): 112.

125. Jones, R. (2014). Transport of the sperm and ovum in the oviduct. In R. Jones, *Human reproductive biology* (Vol. fourth edition). Colorado: Elsevier.
126. Jones, R. et al. (2013). *Human reproductive biology*. (p 102).
127. Julia, Chantal, and Serge Hercberg. "Épidémiologie de l'obésité en France." *Revue du Rhumatisme Monographies* 83.1 (2016): 2-5.
128. Kaati, G. (2002). Cardiovascular and diabetes mortality determined by nutrition during parents' and grandparents' slow growth period. *IO* (11).
129. Kierszenbaum, Abraham L., and Laura L. Tres. "The acrosome-acroplaxome-manchette complex and the shaping of the spermatid head." *Archives of histology and cytology* 67.4 (2004): 271-284.
130. Kim, Seul Ki, ByungChulJee, and Seok Hyun Kim. "Histone methylation and acetylation in ejaculated human sperm: effects of swim-up and smoking." *Fertility and sterility* 103.6 (2015): 1425-1431.
131. Kouzarides, Tony. "Chromatin modifications and their function." *Cell* 128.4 (2007): 693-705.
132. Krohmer, R. (2004). Glossary. In R. Krohmer, *The reproductive system*. New York: Chelsea house publishers: an imprint of infobase publishing.
133. Vignera, Sandro, et al. "Negative effect of increased body weight on sperm conventional and nonconventional flow cytometric sperm parameters." *Journal of andrology* 33.1 (2012): 53-58.
134. Lampiao, Fanuel, and Stefan S. Du Plessis. "Insulin and leptin enhance human sperm motility, acrosome reaction and nitric oxide production." *Asian journal of andrology* 10.5 (2008): 799-807.
135. Lane, Michelle, et al. "Oxidative stress in mouse sperm impairs embryo development, fetal growth and alters adiposity and glucose regulation in female offspring." *PLoS One* 9.7 (2014): e100832.
136. Lane, Michelle, et al. "Oxidative stress in mouse sperm impairs embryo development, fetal growth and alters adiposity and glucose regulation in female offspring." *PLoS One* 9.7 (2014): e100832.

- 137.Lane, Michelle, Rebecca L. Robker, and Sarah A. Robertson. "Parenting from before conception." *Science* 345.6198 (2014): 756-760.
- 138.Larsen, S. H., G. Wagner, and Berit Lilienthal Heitmann. "Sexual function and obesity." *International Journal of Obesity* 31.8 (2007): 1189-1198.
- 139.Lee, Min Gyu, et al. "An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation." *Nature* 437.7057 (2005): 432-435.
- 140.Leese, Henry J. "Quiet please, do not disturb: a hypothesis of embryo metabolism and viability." *Bioessays* 24.9 (2002): 845-849.
- 141.Leichtmann-Bardoogo, Yael, et al. "Compartmentalization and regulation of iron metabolism proteins protect male germ cells from iron overload." *American Journal of Physiology-Endocrinology and Metabolism* 302.12 (2012): E1519-E1530.
- 142.Leisegang, Kristian, et al. "Obesity is associated with increased seminal insulin and leptin alongside reduced fertility parameters in a controlled male cohort." *Reproductive Biology and Endocrinology* 12.1 (2014): 1.
- 143.Lin, Ching-Chun, et al. "Increased risk of death with congenital anomalies in the offspring of male semiconductor workers." *International journal of occupational and environmental health* (2013).
- 144.Liu, Wei-Min, et al. "Sperm-borne microRNA-34c is required for the first cleavage division in mouse." *Proceedings of the National Academy of Sciences* 109.2 (2012): 490-494.
- 145.Lotti, F., et al. "Elevated body mass index correlates with higher seminal plasma interleukin 8 levels and ultrasonographic abnormalities of the prostate in men attending an andrology clinic for infertility." *Journal of endocrinological investigation* 34.10 (2011): e336-e342.
- 146.Love, Charles C., and Robert M. Kenney. "Scrotal heat stress induces altered sperm chromatin structure associated with a decrease in protamine disulfide bonding in the stallion." *Biology of reproduction* 60.3 (1999): 615-620.

- 147.Lu, Zhao-Hui, et al. "Saturated free fatty acids, palmitic acid and stearic acid, induce apoptosis by stimulation of ceramide generation in rat testicular Leydig cell." *Biochemical and biophysical research communications* 303.4 (2003): 1002-1007.
- 148.Macdonald, A. A., A. W. Stewart, and C. M. Farquhar. "Body mass index in relation to semen quality and reproductive hormones in New Zealand men: a cross-sectional study in fertility clinics." *Human Reproduction* 28.12 (2013): 3178-3187.
- 149.Martini, Ana C., et al. "Overweight and seminal quality: a study of 794 patients." *Fertility and sterility* 94.5 (2010): 1739-1743.
- 150.McCowan, Lesley ME, et al. "Paternal contribution to small for gestational age babies: a multicenter prospective study." *Obesity* 19.5 (2011): 1035-1039.
- 151.McLachlan, R. I., et al. "Histological evaluation of the human testis—approaches to optimizing the clinical value of the assessment: mini review." *Human Reproduction* 22.1 (2007): 2-16.
- 152.McPherson, Nicole O., and Michelle Lane. "Male obesity and subfertility, is it really about increased adiposity?." *Asian journal of andrology* 17.3 (2014): 450-458.
- 153.Melvin, James E., et al. "Regulation of fluid and electrolyte secretion in salivary gland acinar cells." *Annu. Rev. Physiol.* 67 (2005): 445-469.
- 154.Mergenthaler, Philipp, et al. "Sugar for the brain: the role of glucose in physiological and pathological brain function." *Trends in neurosciences* 36.10 (2013): 587-597.
- 155.Michalakis, Konstantinos, et al. "The complex interaction between obesity, metabolic syndrome and reproductive axis: a narrative review." *Metabolism* 62.4 (2013): 457-478.
- 156.Miller, Brian, et al. "Meta-analysis of paternal age and schizophrenia risk in male versus female offspring." *Schizophrenia bulletin* 37.5 (2011): 1039-1047.
- 157.Mitchell, Megan, Hassan W. Bakos, and Michelle Lane. "Paternal diet-induced obesity impairs embryo development and implantation in the mouse." *Fertility and sterility* 95.4 (2011): 1349-1353.

158. Moehlecke, Milene, et al. "Determinants of body weight regulation in humans." *Archives of endocrinology and metabolism AHEAD* (2016): 0-0.
159. Møller, Henrik, et al. "Prostate cancer incidence, clinical stage and survival in relation to obesity: a prospective cohort study in Denmark." *International Journal of Cancer* 136.8 (2015): 1940-1947.
160. Montagnon, D., A. Clavert, and C. Cranz. "Fructose, proteins and coagulation in human seminal plasma." *Andrologia* 14.5 (1982): 434-439.
161. Montellier, Emilie, et al. "Chromatin-to-nucleoprotamine transition is controlled by the histone H2B variant TH2B." *Genes & development* 27.15 (2013): 1680-1692.
162. Montjean, D., et al. "Sperm global DNA methylation level: association with semen parameters and genome integrity." *Andrology* 3.2 (2015): 235-240.
163. Moore, Lisa D., Thuc Le, and Guoping Fan. "DNA methylation and its basic function." *Neuropsychopharmacology* 38.1 (2013): 23-38.
164. Morgan, David Owen. *The cell cycle: principles of control*. New Science Press, 2007.
165. Nakamura, Toshinobu, et al. "PGC7/Stella protects against DNA demethylation in early embryogenesis." *Nature cell biology* 9.1 (2007): 64-71.
166. Nasreddine, Lara, et al. "Trends in overweight and obesity in Lebanon: evidence from two national cross-sectional surveys (1997 and 2009)." *BMC public health* 12.1 (2012): 1.
167. Navarro-Costa, Paulo, et al. "Incorrect DNA methylation of the DAZL promoter CpGisland associates with defective human sperm." *Human reproduction* 25.10 (2010): 2647-2654.
168. Neto, Filipe Tenorio Lira, et al. "Spermatogenesis in humans and its affecting factors." *Seminars in cell & developmental biology*. Academic Press, 2016.
169. Nettersheim, Daniel, et al. "Analysis of TET expression/activity and 5mC oxidation during normal and malignant germ cell development." *PLoS One* 8.12 (2013): e82881.

170. Ng, Marie, et al. "Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013." *The Lancet* 384.9945 (2014): 766-781.
171. Ng, Sheau-Fang, et al. "Chronic high-fat diet in fathers programs [bgr]-cell dysfunction in female rat offspring." *Nature* 467.7318 (2010): 963-966.
172. Ng, Sheau-Fang, et al. "Paternal high-fat diet consumption induces common changes in the transcriptomes of retroperitoneal adipose and pancreatic islet tissues in female rat offspring." *The FASEB Journal* 28.4 (2014): 1830-1841.
173. Ni, Kai, et al. "TET enzymes are successively expressed during human spermatogenesis and their expression level is pivotal for male fertility." *Human Reproduction* (2016): dew096.
174. Ni, K., et al. "The impact of sperm protamine deficiency and sperm DNA damage on human male fertility: a systematic review and meta-analysis." *Andrology* (2016).
175. McPherson, Nicole O., and Michelle Lane. "Male obesity and subfertility, is it really about increased adiposity?." *Asian journal of andrology* 17.3 (2014): 450-458.
176. Oakes, Christopher C., et al. "Adverse effects of 5-aza-2'-deoxycytidine on spermatogenesis include reduced sperm function and selective inhibition of de novo DNA methylation." *Journal of Pharmacology and Experimental Therapeutics* 322.3 (2007): 1171-1180.
177. Ogden, Cynthia L., et al. "Prevalence of childhood and adult obesity in the United States, 2011-2012." *Jama* 311.8 (2014): 806-814.
178. Okada, Yuki, et al. "Histone demethylase JHDM2A is critical for Tnp1 and Prml transcription and spermatogenesis." *Nature* 450.7166 (2007): 119-123.
179. Oliveira, P. F., et al. "Effect of insulin deprivation on metabolism and metabolism-associated gene transcript levels of in vitro cultured human Sertoli cells." *Biochimica et Biophysica Acta (BBA)-General Subjects* 1820.2 (2012): 84-89.

180. Owen, Derek H., and David F. Katz. "A review of the physical and chemical properties of human semen and the formulation of a semen simulant." *Journal of andrology* 26.4 (2005): 459-469.
181. Pacheco, Sara E., et al. "Integrative DNA methylation and gene expression analyses identify DNA packaging and epigenetic regulatory genes associated with low motility sperm." *PLoS One* 6.6 (2011): e20280.
182. Palmer, Nicole O., et al. "SIRT6 in mouse spermatogenesis is modulated by diet-induced obesity." *Reproduction, Fertility and Development* 23.7 (2011): 929-939.
183. Palmer, Nicole O., et al. "Impact of obesity on male fertility, sperm function and molecular composition." *Spermatogenesis* 2.4 (2012): 253-263.
184. Parker, Helen E., Fiona M. Gribble, and Frank Reimann. "The role of gut endocrine cells in control of metabolism and appetite." *Experimental physiology* 99.9 (2014): 1116-1120.
185. Paz, Gedalia, et al. "Immunoreactive insulin in serum and seminal plasma of diabetic and nondiabetic men and its role in the regulation of spermatozoal activity." *Fertility and sterility* 28.8 (1977): 836-840.
186. Pettitt, David J., et al. "Comparison of two waist circumference measurement protocols: the SEARCH for diabetes in youth study." *Pediatric obesity* 7.6 (2012): e81-e85.
187. Pitteloud, Nelly, et al. "Increasing insulin resistance is associated with a decrease in Leydig cell testosterone secretion in men." *The Journal of Clinical Endocrinology & Metabolism* 90.5 (2005): 2636-2641.
188. Proietto, Joseph, and Louise A. Baur. "10: Management of obesity." *Medical journal of Australia* 180.9 (2004): 474-481.
189. Qian, Min-Xian, et al. "Acetylation-mediated proteasomal degradation of core histones during DNA repair and spermatogenesis." *Cell* 153.5 (2013): 1012-1024.
190. Quayle, A. J., et al. "Immunosuppression by seminal prostaglandins." *Clinical and experimental immunology* 75.3 (1989): 387.

191. Ramathal, Cyril, Paul Turek, and Renee Reijo Pera. *Embryonic stem cells and the germ cell lineage*. INTECH Open Access Publisher, 2011.
192. Ramathal, Cyril, Paul Turek, and Renee Reijo Pera. *Embryonic stem cells and the germ cell lineage*. INTECH Open Access Publisher, 2011.
193. Rassoulzadegan, Minoo, et al. "RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse." *Nature* 441.7092 (2006): 469-474.
194. Rato, Luís, et al. "Tubular fluid secretion in the seminiferous epithelium: ion transporters and aquaporins in Sertoli cells." *The Journal of membrane biology* 236.2 (2010): 215-224.
195. Rato, L., et al. "High-energy diets: a threat for male fertility?." *Obesity Reviews* 15.12 (2014): 996-1007.
196. Rato, L., et al. "High-energy diets may induce a pre-diabetic state altering testicular glycolytic metabolic profile and male reproductive parameters." *Andrology* 1.3 (2013): 495-504.
197. Reidy, Kimberly, et al. "Molecular mechanisms of diabetic kidney disease." *The Journal of clinical investigation* 124.6 (2014): 2333-2340.
198. Robertson, Sarah A. "Seminal plasma and male factor signalling in the female reproductive tract." *Cell and tissue research* 322.1 (2005): 43-52.
199. Rousseaux, Sophie, and Saadi Khochbin. "Histone acylation beyond acetylation: terra incognita in chromatin biology." *Cell J* 17.1 (2015): 1-6.
200. Shafik, A., and S. Olfat. "Lipectomy in the treatment of scrotal lipomatosis." *British journal of urology* 53.1 (1981): 55-61.
201. Saraswat, S., S. Kharche, and S. Jindal. "Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects." *Iran J Appl Anim Sci* 4 (2014): 247-255.

- 202.Sartorius, Gideon A., and EberhardNieschlag. "Paternal age and reproduction." *Human reproduction update* (2009): dmp027.
- 203.Schliep, Karen C., et al. "Effect of male and female body mass index on pregnancy and live birth success after in vitro fertilization." *Fertility and sterility* 103.2 (2015): 388-395.
- 204.Schwartz, Michael W., et al. "Cooperation between brain and islet in glucose homeostasis and diabetes." *Nature* 503.7474 (2013): 59-66.
- 205.Sermondade, N., et al. "BMI in relation to sperm count: an updated systematic review and collaborative meta-analysis." *Human reproduction update* (2012): dms050.
- 206.Sharma, Kumar. "Obesity, oxidative stress, and fibrosis in chronic kidney disease." *Kidney international supplements* 4.1 (2014): 113-117.
- 207.Sharma, Rakesh, and Ashok Agarwal. "Spermatogenesis: an overview." *Sperm Chromatin*. Springer New York, 2011. 19-44.
- 208.Sharma, Virvikram, and P. L. Sharma. "Role of different molecular pathways in the development of diabetes-induced nephropathy." *Journal of Diabetes & Metabolism* 2013 (2013).
- 209.Shirakata, Yoshiki, et al. "Histone h4 modification during mouse spermatogenesis." *The Journal of reproduction and development* 60.5 (2014): 383.
- 210.Siddiqui, Athar H., et al. "Recombinant vascular endothelial growth factor 121 attenuates autoantibody-induced features of pre-eclampsia in pregnant mice." *American journal of hypertension* 24.5 (2011): 606-612.
- 211.Smallwood, Sébastien A., and Gavin Kelsey. "De novo DNA methylation: a germ cell perspective." *Trends in Genetics* 28.1 (2012): 33-42.
- 212.Song, Ning, et al. "Immunohistochemical analysis of histone H3 modifications in germ cells during mouse spermatogenesis." *Actahistochemicaetcytochemica* 44.4 (2011): 183-190.

- 213.Sorahan, T., et al. "Childhood cancer and parental use of tobacco: deaths from 1971 to 1976." *British journal of cancer* 76.11 (1997): 1525.
- 214.Soubry, Adelheid, et al. "Paternal obesity is associated with IGF2 hypomethylation in newborns: results from a Newborn Epigenetics Study (NEST) cohort." *BMC medicine* 11.1 (2013): 1.
- 215.Stensel, David. "Exercise, appetite and appetite-regulating hormones: implications for food intake and weight control." *Annals of Nutrition and Metabolism* 57.Suppl. 2 (2011): 36-42.
- 216.Strazzullo, Pasquale, et al. "Altered renal sodium handling in men with abdominal adiposity: a link to hypertension." *Journal of hypertension* 19.12 (2001): 2157-2164.
- 217.Strünker, Timo, et al. "The CatSper channel mediates progesterone-induced Ca<sup>2+</sup> influx in human sperm." *Nature* 471.7338 (2011): 382-386.
- 218.Sung, Tzu-I., Jung-Der Wang, and Pau-Chung Chen. "Increased risks of infant mortality and of deaths due to congenital malformation in the offspring of male electronics workers." *Birth Defects Research Part A: Clinical and Molecular Teratology* 85.2 (2009): 119-124.
- 219.Suzuki, Hitomi, et al. "SOHLH1 and SOHLH2 coordinate spermatogonial differentiation." *Developmental biology* 361.2 (2012): 301-312.
- 220.Tan, Li, and YujiangGeno Shi. "Tet family proteins and 5-hydroxymethylcytosine in development and disease." *Development* 139.11 (2012): 1895-1902.
- 221.Terashima, Minoru, et al. "Effect of high fat diet on paternal sperm histone distribution and male offspring liver gene expression." *Epigenetics* 10.9 (2015): 861-871.
- 222.Tervaert, Thijs W. Cohen, et al. "Pathologic classification of diabetic nephropathy." *Journal of the American Society of Nephrology* 21.4 (2010): 556-563.
- 223.Thomas, Stephanie, et al. "Seminal plasma adipokine levels are correlated with functional characteristics of spermatozoa." *Fertility and sterility* 99.5 (2013): 1256-1263.

224. Toschke, Audre M., et al. "Paternal smoking is associated with a decreased prevalence of type 1 diabetes mellitus among offspring in two national British birth cohort studies (NCDS and BCS70)." *Journal of perinatal medicine* 35.1 (2007): 43-47.
225. Traish, Abdulmageed M., Robert J. Feeley, and Andre Guay. "Mechanisms of obesity and related pathologies: androgen deficiency and endothelial dysfunction may be the link between obesity and erectile dysfunction." *FEBS journal* 276.20 (2009): 5755-5767.
226. TROPP, B. (2012). Chromosome structure. In *Molecular biology from genes to proteins* (Vol. fourth edition). Jones and Bartlett.
227. Tunc, Ozlem, and Kelton Tremellen. "Oxidative DNA damage impairs global sperm DNA methylation in infertile men." *Journal of assisted reproduction and genetics* 26.9-10 (2009): 537-544.
228. Tunc, O., H. W. Bakos, and K. Tremellen. "Impact of body mass index on seminal oxidative stress." *Andrologia* 43.2 (2011): 121-128.
229. Ueda, Hirotaka, et al. "The roles of salivary secretion, brain-gut peptides, and oral hygiene in obesity." *Obesity research & clinical practice* 7.5 (2013): e321-e329.
230. Umul, M., et al. "Effect of increasing paternal body mass index on pregnancy and live birth rates in couples undergoing intracytoplasmic sperm injection." *Andrologia* 47.3 (2015): 360-364.
231. Valensi, P., et al. "Microalbuminuria in obese patients with or without hypertension." *International journal of obesity and related metabolic disorders: journal of the International Association for the Study of Obesity* 20.6 (1996): 574-579.
232. Vasanthakumar, Aparna, and Lucy A. Godley. "5-hydroxymethylcytosine in cancer: significance in diagnosis and therapy." *Cancer genetics* 208.5 (2015): 167-177.
233. Veenendaal, Marjolein VE, et al. "Transgenerational effects of prenatal exposure to the 1944-45 Dutch famine." *BJOG: An International Journal of Obstetrics & Gynaecology* 120.5 (2013): 548-554.
234. Vendramini, V., et al. "Reproductive Function of the Male Obese Zucker Rats Alteration in Sperm Production and Sperm DNA Damage." *Reproductive Sciences* (2013): 1933719113493511.

235. Vickers, Mark H. "Early life nutrition, epigenetics and programming of later life disease." *Nutrients* 6.6 (2014): 2165-2178.
236. Vieweg, Markus, et al. "Methylation analysis of histone H4K12ac-associated promoters in sperm of healthy donors and subfertile patients." *Clinical epigenetics* 7.1 (2015): 1.
237. vanVliet-Ostapchouk, Jana V., et al. "The prevalence of metabolic syndrome and metabolically healthy obesity in Europe: a collaborative analysis of ten large cohort studies." *BMC endocrine disorders* 14.1 (2014): 1.
238. VILLARROEL-ESPÍNDOLA, F., et al. "The Many Faces of Carbohydrate Metabolism in Male Germ Cells: From Single Molecules to Active Polymers." *Int. J. Med. Surg. Sci* 2.4 (2015): 603-619.
239. Wagner, Kay D., et al. "RNA induction and inheritance of epigenetic cardiac hypertrophy in the mouse." *Developmental cell* 14.6 (2008): 962-969.
240. Wagner, Márcia Santos, Simone MagagninWajner, and Ana Luiza Maia. "The role of thyroid hormone in testicular development and function." *Journal of Endocrinology* 199.3 (2008): 351-365.
241. Wang, Zengjun, et al. "AB073. Semen liquefaction molecular pathways." *Translational Andrology and Urology* 4.Suppl 1 (2015).
242. Ward, W. Steven. "Function of sperm chromatin structural elements in fertilization and development." *Molecular human reproduction* 16.1 (2010): 30-36.
243. Wei, Yanchang, HeideSchatten, and Qing-Yuan Sun. "Environmental epigenetic inheritance through gametes and implications for human reproduction." *Human reproduction update* (2014): dmu061.
244. Wei, Yanchang, et al. "Paternally induced transgenerational inheritance of susceptibility to diabetes in mammals." *Proceedings of the National Academy of Sciences* 111.5 (2014): 1873-1878.
245. Weinbauer, Gerhard F., et al. "Physiology of testicular function." *Andrology*. Springer Berlin Heidelberg, 2010. 11-59.

246. Consultation, WHO Expert. "Waist circumference and waist-hip ratio." *Report of a WHO Expert Consultation. Geneva: World Health Organization* (2008): 8-11.
247. Wossidlo, Mark, et al. "5-Hydroxymethylcytosine in the mammalian zygote is linked with epigenetic reprogramming." *Nature communications* 2 (2011): 241.
248. Wozniak, Susan E., et al. "Adipose tissue: the new endocrine organ? A review article." *Digestive diseases and sciences* 54.9 (2009): 1847-1856.
249. Wu, Q., and M. Suzuki. "Parental obesity and overweight affect the body-fat accumulation in the offspring: the possible effect of a high-fat diet through epigenetic inheritance." *Obesity reviews* 7.2 (2006): 201-208.
250. Wu, Wei, et al. "Idiopathic male infertility is strongly associated with aberrant promoter methylation of methylenetetrahydrofolate reductase (MTHFR)." *PLoS One* 5.11 (2010): e13884.
251. Yu, Y. Eugene, et al. "Abnormal spermatogenesis and reduced fertility in transition nuclear protein 1-deficient mice." *Proceedings of the National Academy of Sciences* 97.9 (2000): 4683-4688.
252. Yuan, Shuiqiao, et al. "Sperm-borne miRNAs and endo-siRNAs are important for fertilization and preimplantation embryonic development." *Development* 143.4 (2016): 635-647.
253. Yuen, Benjamin TK, et al. "Histone H3. 3 regulates dynamic chromatin states during spermatogenesis." *Development* 141.18 (2014): 3483-3494.
254. Zhang, Donghong, et al. "Elevated homocysteine level and folate deficiency associated with increased overall risk of carcinogenesis: meta-analysis of 83 case-control studies involving 35,758 individuals." *PloS one* 10.5 (2015): e0123423.
255. Zhang, Erhong, et al. "GLP-1 Receptor Agonist Exenatide Attenuates the Detrimental Effects of Obesity on Inflammatory Profile in Testis and Sperm Quality in Mice." *American Journal of Reproductive Immunology* 74.5 (2015): 457-466.

256. Zhao, Jian, et al. "Leptin level and oxidative stress contribute to obesity-induced low testosterone in murine testicular tissue." *Oxidative medicine and cellular longevity* 2014 (2014).
257. Zirkin, Barry R., and Haolin Chen. "Regulation of Leydig cell steroidogenic function during aging." *Biology of reproduction* 63.4 (2000): 977-981.

## **Chapter 2 Selective physiological and molecular alterations in the motile sperm from obese men**

### **I- Abstract**

The prevalence of excessive weight or obesity is elevated among young men of reproductive age and its impact on male reproduction is not entirely elucidated. Accumulated evidence suggests that information from paternal environment such as acquired obesity remains in the male gamete and can modulate the phenotype of the offspring. Here, we aimed to assess the drawbacks of obesity on the molecular composition of the motile sperm and on the pre-implantation embryo morphokinetics.

The semen samples were obtained from 96 men attending the A-clinic fertility center, Lebanon. Patients were categorized into three groups according to their body mass index (BMI) and waist circumference (WC): normal weight ( $18 < \text{BMI} < 24 \text{ kg/m}^2$  and  $\text{WC} < 90 \text{ cm}$ ), overweight ( $25 < \text{BMI} < 29.9 \text{ kg/m}^2$  and  $\text{WC} < 102 \text{ cm}$ ), and obese ( $\text{BMI} > 30 \text{ kg/m}^2$  and  $\text{WC} > 103 \text{ cm}$ ).

Our results showed that the motile sperm of obese men had higher levels of retained histones ( $p < 0.001$ ), elevated percentage of decompacted chromatin ( $p < 0.001$ ), hypomethylated DNA ( $p < 0.05$ ), and hypohydroxymethylated DNA ( $p < 0.001$ ) as compared to the motile spermatozoa of normal weight men. In addition, the time of pronuclei appearance ( $p < 0.001$ ) and fading ( $p < 0.05$ ) were significantly shorter in the zygotes derived from an obese father when compared to a normal weight one. Subsequently, the embryonic cell cycles CC1 ( $p < 0.05$ ), CC2 ( $p < 0.05$ ), and CC3 ( $p < 0.05$ ) were significantly delayed in the cleavage embryos of the obese group as compared to the normal one. After the activation of the embryonic genome, there was a significant reduction in the compaction rate ( $p < 0.05$ ), in the blastulation rate ( $p < 0.05$ ), and in the quality of the trophectoderm layer of the blastocyst ( $p < 0.04$ ) in the obese group rather than the normal ones.

In conclusion, we demonstrated that male obesity may affect the molecular composition of the motile sperm and may alter the pre-implantation embryo morphokinetics.

## II- Introduction

Obesity or excessive fatness has exceeded the stage of dietary measures and has reached alerting levels worldwide (Nasreddine and Naja et al. 2012; AL-Quwaidhi et al. 2014; Ogden et al. 2014; Vilet-Ostaptchouk et al. 2014). Moreover, it is one of the leading factors in the development of several metabolic pathologies such as type 2 diabetes mellitus (T2DM), cardiovascular diseases, respiratory diseases, hypertension, and infertility (Golay et al. 2005; Fulleston et al. 2012; Costanian et al. 2014) .

Surprisingly, there is a tremendous increase in the prevalence of obesity in young men of reproductive age (Ng et al. 2014; McPherson et al. 2015). Different systematic reviews have summed up the effects of human male obesity on sperm parameters, DNA fragmentation, mitochondrial membrane potential, and fertility outcomes. Male obesity in humans may increase the incidence of oligozoospermia and azoospermia (Sermondade et al. 2012), reduce the percentage of normal sperm morphology, (MacDonald et al. 2013; Campbell et al. 2015), and increase the percentage of sperm with DNA fragmentation and altered mitochondrial membrane potential (Campbell et al. 2015). Moreover, a study on 81 men has indicated that the production of reactive oxygen species by the sperm is positively correlated to the BMI (Tunc et al. 2011). Regarding the effect of paternal obesity on pre-implantation embryo development, three reports have been published; Bakos and colleagues high spotted the significant decrease in blastulation rate with increasing BMI, whereas Colaci et al., and Schliep et al., didn't find any significant difference between obese and non-obese men for the analyzed embryologic parameters (Bakos et al. 2011; Colaci et al. 2012; Schliep et al. 2015) . Furthermore, a recent meta-analysis showed that paternal obesity may reduce the rate of live birth per assisted reproductive technology (ART) cycle, and increase the risk of facing a non-viable pregnancy (Campbell et al. 2015).

Accumulated evidence suggests that information from paternal environment such as acquired obesity remains in the male gamete and can modulate the phenotype of the offspring. This kind of inheritance is independent of changes in the inherited DNA sequence (wei et al. 2014). As reported by G kaati et al., the nutritional status of paternal grandfather may increase the risk of health complications in the grandson (Kaati et al. 2002). Further, a father born small for gestational age (SGA) has an elevated risk of developing obesity and giving birth to (SGA) babies (McCowan et al. 2011). In addition, the incidence of obesity and diabetes in the grandchildren, coming from sons who were exposed *in utero* to the Dutch famine, were very high (Veenend aal et al. 2013).

Interestingly, it was shown in humans, and in rodent model of diet-induced obesity that excessive fatness can remodel the sperm epigenome; thus, playing a crucial role in the sperm-dependent non-genetic inheritance of acquired obesity (McPherson et al. 2015; Donkin et al. 2016). At the molecular level, the sperm epigenome is made up of a protamine-rich chromatin that will be replaced after fertilization by maternal histones (Wared et al. 2010), a small amount of paternally

inherited histones (1-10%), highly compacted DNA with possible chemical modifications, and a small amount of RNAs (McPherson et al. 2015).

Acquired obesity in male mice, induces alterations in DNA methylation of phosphatidylinositol-4,5-bisphosphate 3-kinase gene (PI3K), which is implicated in insulin signalling (Wei et al. 2014). It also increases the level of expression of cytochrome C oxidase subunit IV isoform I (*Cox4il*) mRNA in sperm cells. *Cox4il* is a nuclear encoded gene that can regulate the mitochondrial function in embryos (Binder et al. 2015). Moreover, Fullston and colleagues have shown that the high fat diet (HFD) may induce a global DNA hypomethylation of late elongated spermatids and can alter the sperm micro-RNAs (miRNAs) content (miR133b-3p, miR-304-5p, miR196a-5p, miR205) in obese male mice. These miRNAs may be implicated in the regulation of pre-implantation mouse embryo development (Fullston et al. 2013). Moreover, microinjection of testis and sperm RNAs from obese mice into one cell mouse embryo has led to the development of obesity and T2DM in the offspring (Grandjean et al. 2015); highlighting the role of sperm RNAs in the inheritance of the paternal acquired obesity. Not only the DNA methylation, and the sperm RNAs but also the sperm chromatin is considered as a potential candidate; sperm cells from HFD-fed mice were found to have high histones retention and particularly H3K4me1 retention at specific genes implicated in development and cell fate decision (Terashima et al. 2015). In addition, Palmer et al, have found that the percentage of spermatids that stains positive to the H3K9ac antibody was higher in the HFD male mice as compared to those on control diet (Palmer et al. 2011).

In line with the studies performed on mice, La Vignera and colleagues have shown that obese men had a higher percentage of sperm with decondensed chromatin when assessed by toluidine blue staining (La Vignera et al. 2012). When compared to normal weight men, obese men's sperm DNA has presented different methylation profile in imprinted regions (Soubry et al. 2016) as well as in genes implicated in the function and metabolism of the central nervous system (Donkin et al. 2016). Also, deregulation in sperm Piwi-interacting RNAs (piRNAs) expression has been detected in obese subject (Donkin et al. 2016).

In the light of these results, we aimed to assess the quality of human sperm chromatin and the underlying DNA methylation/hydroxymethylation status in obese men before and after swim-up procedure. Next, we analyzed the effect of the motile-sperm enriched fraction derived from obese father on the pre-implantation embryo morphokinetics.

### **III- Materials and Methods**

#### **a) Study population**

Semen samples were obtained from a total of 96 men attending the A-clinic fertility center at Mount Lebanon hospital - Hazmieh, Lebanon between January 2016 and October 2016. In - person interviews were conducted to complete a questionnaire about the age, the length of the sexual abstinence, current or previous disease status including urogenital ones, and habits such as smoking and alcohol intake. All the patients suffering from andrological disorders or recent fever as well as those undertaking any treatment that may alter the spermatogenesis or using a frozen sperm cycle were excluded from the study (**Figure 1**). For the pre-implantation embryo morphokinetics analysis, all the couples where the female partner was <38 years at the time of oocyte collection (Bakos et al. 2011) and undergoing an intracytoplasmic sperm injection (ICSI) cycle with a time-lapse embryo monitoring were included in the study. An informed consent was obtained from each patient

#### **b) Anthropometric measurements**

Anthropometric measurements were performed by a trained staff. Weight was measured in kilograms using a weighing scale. Height was measured in centimetres. The BMI was calculated as weight in kilograms divided by the squared height in meters, and it was categorized as follows:  $18 < \text{BMI} < 24.9 \text{ kg/m}^2$  (normal weight),  $25 < \text{BMI} < 29.9 \text{ kg/m}^2$  (overweight) and  $\text{BMI} > 30 \text{ kg/m}^2$  (obese) (Einsenberget al. 2014).

Abdominal fat accumulation might be a risk factor for several pathologies and it has drawbacks on male fertility. Thus, waist circumference (WC) was measured with a standardized tape measure, which was placed over the skin or light clothing while the participant was standing. Two measurements were usually taken followed by a third one when the difference between two measurements was 0.5 cm or above (Einsenberget al. 2014).

#### **c) Semen analysis**

A semen sample was produced on-site by masturbation into a sterile plastic specimen cup. All subjects underwent semen analysis. The semen parameters analyzed included liquefaction, viscosity, seminal pH, sperm concentration ( $10^6$  sperm/mL), total progressively motile sperm per ejaculate, and sperm morphology according to the World Health Organization criteria (WHO 2010). Each sample was evaluated by 2 technicians, and readings were averaged between the two evaluations after calculation of error.

#### **d) Sperm isolation using swim-up technique**

Motile-sperm enriched fraction was prepared using a swim-up technique which selects a population of spermatozoa with the lowest DNA fragmentation rate (Volpes et al. 2016). At first, 1 ml of semen was placed in a tube and was overlaid by 1 ml of culture medium (Sperm Medium, COOK medical, Australia). The tube was incubated for 45 min (37 °C and under 5% CO<sub>2</sub>). The supernatant was aspirated and transferred to an empty tube. After the semen processing using the swim-up technique, replicate measurements of sperm concentration, sperm motility, and sperm morphology were performed on the supernatant according to the World Health Organization criteria (WHO 2010).

#### **e) Sperm morphology evaluation**

In order to observe sperm morphology and vacuolization, dried smears were stained using the SpermScan Kit (RAL diagnostics). The staining was performed according to the manufacture protocol. Briefly, the smears were fixed in the SPERMO FIX-RAL solution. Next, the sperm cytoplasm was stained using the SPERMO EOSINE-RAL, and the nucleus was stained in the SPERMO BLEU-RAL. On the dried stained smears at least 200 sperms were counted and classified as normal or abnormal morphology according to Kruger's strict criteria. Head defects: large or small, tapered, amorphous, vacuolated (more than two vacuoles or >20% of the head area occupied by unstained vacuolar areas), small or large acrosomal areas. Mid-piece defects: Bent, Cytoplasmic residues. Tail defects: Coiled, multiple (WHO 2010).

#### **f) Sperm membrane integrity assessment**

Sperm membrane integrity was assessed using the eosin-nigrosin staining. Briefly, stained smears were evaluated under microscopy. The results were expressed as the percentage of stained or pink sperm (altered head membrane) after examination of 200 sperms (WHO 2010).

#### **g) Sperm chromatin structure assessment**

Chromatin structure was assessed using the toluidine blue (TB) method. Thin smears were prepared from the raw semen and from the motile-sperm enriched fraction on pre-cleaned defatted slides and then air dried for 30 min. Next, dried smears were fixed with freshly prepared 96% ethanol:acetone (1:1) at 4°C for 30 min and air dried. Hydrolysis was performed with 0.1 N HCl at 4°C for 5 min followed by three changes of distilled water, 2 min each. TB (0.05% in 50% McIlvain's citrate phosphate buffer at pH 3.5, Gurr-BDH Chemicals Ltd, Poole, UK) was applied for 5 min. Next, slides were then rinsed briefly in distilled water then air dried and evaluated at high magnification under immersion oil. The TB working solution was prepared

monthly from a stock solution of 1% TB in distilled water and stored at 4°C. The stock solution can be stored at 4°C up to 1 year. Two hundred randomly selected sperms per sample were examined under high magnification. The cells were classified into two groups: dark violet cells (TB positive cells; abnormal chromatin structure) and, light blue cells (TB negative cells; normal chromatin structure) (Tsarev et al. 2009).

#### **h) Histone retention assessment**

Nuclear maturity was evaluated using the aniline blue staining which discriminates between lysine-rich histones and arginine/cysteine-rich protamine. Slides were prepared by smearing 5 µl of either raw semen or motile-sperm enriched fraction on glass slides. The slides were air-dried and fixed for 30 minutes in 3% glutaraldehyde in phosphate-buffered saline (PBS). The smear was dried and stained for 5 minutes in 5% aqueous aniline blue solution (pH 3.5). Sperm heads containing high percentage of histones stain blue and those with normal histones content do not take up the stain. The percentage of spermatozoa stained with aniline blue was determined by counting 200 spermatozoa per slide under bright field microscopy (Le Lannou et al. 1988).

#### **i) Sperm DNA extraction**

The sperm pellets prior to freezing were subject to an osmotic shock and flash freeze shock in liquid nitrogen for cellular lysis. In order to characterize the DNA modifications in the motile-sperm enriched fraction, the sperm derived from the supernatant of the swim-up technique was selected for DNA extraction. Each sample was thawed on ice and sperm DNA was subsequently extracted. Sperm DNA was extracted using a detergent-based lysis followed by an in-column purification using the QIAamp® DNA Mini Kit (#51304; Qiagen, The Netherlands). Briefly, thawed pellets were lysed in Buffer RLT (#79216; Qiagen, The Netherlands) with  $\beta$ -mercaptoethanol (2%) to a final volume of 500 µL. Lysates were combined with equal volumes of Buffer AL and 100% ethanol, loaded onto the spin columns, and the columns were centrifuged at  $6000 \times g$  for 1 min to bind DNA. Wash and elution steps followed the manufacturer's protocol, including 3 separate 200 µL elutions to maximize yield. DNA yields and quality were determined using the Nanodrop 2000 Spectrophotometer (#E112352; Thermo Scientific, Somerset, NJ).

#### **j) Sperm Global DNA 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) measurements**

We used Methylated DNA Quantification Kit (Colorimetric) (ab117128; abcam, USA) and Hydroxymethylated DNA Quantification kit (Colorimetric) (ab117130; abcam, USA) for the quantification of 5-mC and 5-hmC, respectively. These enzyme-linked immunosorbent assay (ELISA) kits are highly sensitive and accurate. The assays were performed according to the manufacturer's recommendations. Briefly, 100ng of genomic DNA were used for 5-mC or 5-

hmC quantification, respectively. It is important to note that each sample was run in duplicate. After incubation with the input DNA, the wells were washed, and a capture antibody was applied to each well, after which the wells were again washed and detection antibody applied. Use of enhancer solution and development solution created a color change proportional to the quantity of 5-mC or 5-hmC, and the samples were read on an automated plate reader at 450 nm absorbance. The relative methylation and hydroxymethylation percentages were calculated using a simple formula provided by the manufacturer.

#### **k) Ovarian stimulation, oocytes retrieval, and ICSI**

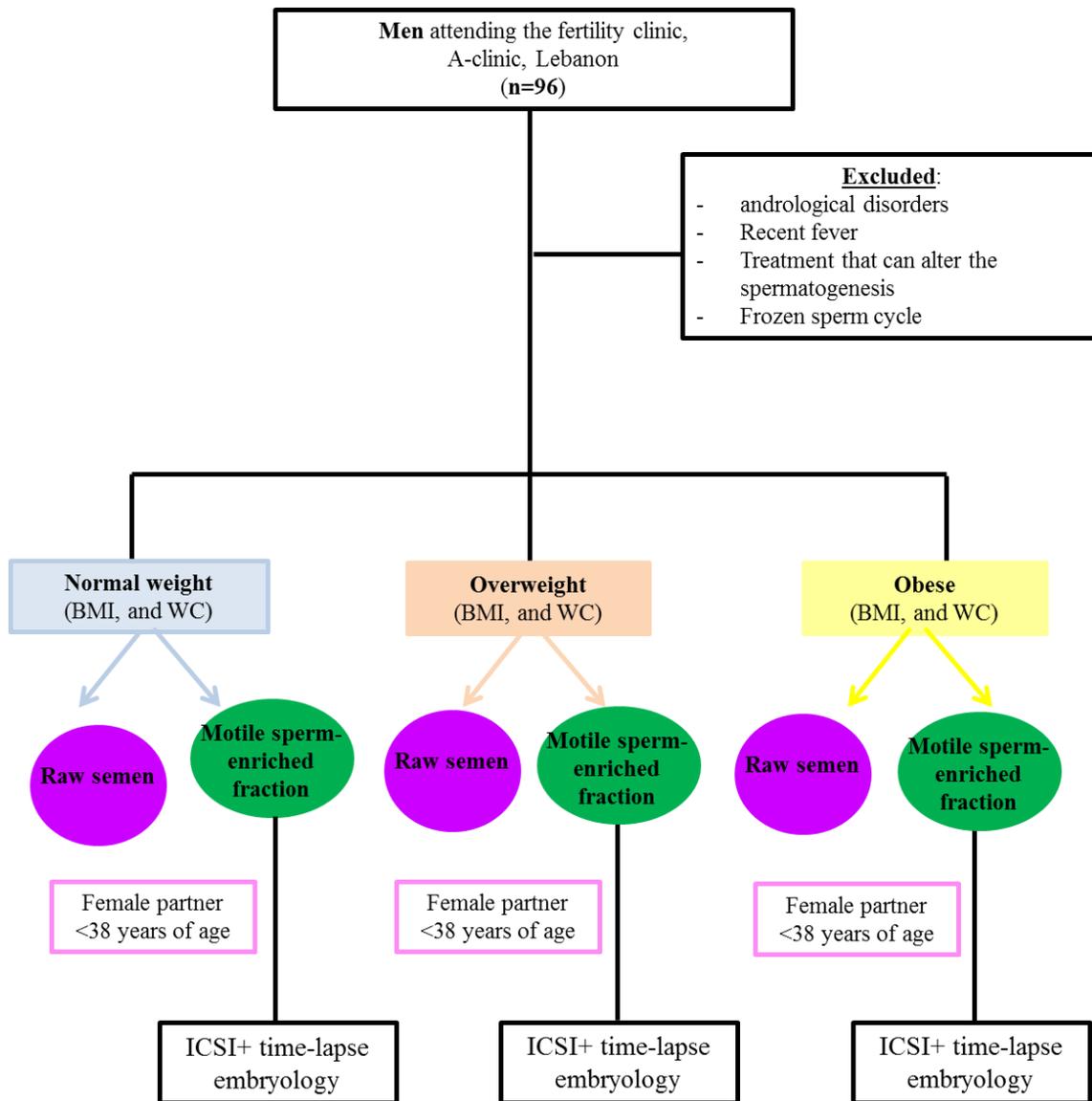
All the women in this study underwent a controlled ovarian stimulation with antagonist protocol. Moreover, recombinant human chorionic gonadotrophin (hCG) was administered when at least three follicles were >17mm. Oocytes retrieval was performed 36 hours post-hCG administration. The collected oocytes were cultured for 3 hours in Global fertilization medium (Life Global, Canada). ICSI was performed at 39 hours post hCG administration with sperm-motile enriched fraction post swim-up technique. Then, the zygotes were placed inside a pre-equilibrated embryoslide (Embryoslide, Vitrolife) containing 12 wells, each filled with 25  $\mu$ l of Global medium and overlaid with 1.2 ml of culture oil (Life Global, Canada). The pre-equilibration step was performed overnight at 37 °C under 5% CO<sub>2</sub> (Bellver et al. 2013).

#### **l) Embryo culture and evaluation of time-lapse images**

Embryoslides containing the zygotes were placed in the Embryoscope (Vitrolife) immediately after ICSI and cultured for 5 days with 5% CO<sub>2</sub> and at 37°C. Images were acquired for every embryo each 10 min at seven different focal plans. Embryo quality and morphokinetics were analyzed using the Embryo viewer software (Vitrolife). We determined the precise timing of pronuclei appearance (tPNa), pronuclei fading (tPNf), duration of the embryonic first cell cycle (CC1=time to 2 cells (t2)-tPNf), second embryonic cell cycle (CC2= time to 4 cells t4 – t2), and third embryonic cell cycle (CC3= time to 8 cells – t4) (Bellver et al. 2013). Moreover, we calculated the fertilization rate, compaction rate, and blastulation rate per cycle (Bakos et al. 2011). In order to assess the effect of paternal obesity on blastocyst quality, we used the Gardner and Schoolcraft grading system using numerical grades for the degree of blastocyst expansion, trophectoderm (TE), and inner cell mass (ICM) quality (Hardarson et al. 2012).

#### **m) Statistical analysis**

All the data were statistically analyzed and graphically represented using Microsoft Office Excel software. The error bars represent the standard error of the mean (SEM). Two-tailed Student's t-test was used for the analysis of statistical significance between two groups. Also, one-way ANOVA was used for the analysis of statistical significance between several groups. A p value <0.05 was considered statistically significant.



**Figure 43: The study design.**

## IV- Results

### a) Characteristics of the cohort

In this study, patients were characterized into three groups according to the paternal BMI and WC (**Table 1**). There were no statistically significant differences in the paternal age, tobacco intake, alcohol consumption, and abstinence days ( $p>0.05$ ) across the groups (**Table 1**). In parallel, there were no differences in the maternal BMI, age, and number of MII oocytes/per cycle ( $p>0.05$ ) (**Table 4**). For all the three groups, the percentage of females with polycystic ovary, endometriosis, and tubal/pelvic disease was similar ( $p>0.05$ ) (**Table 4**).

### b) The effect of paternal obesity on the macroscopic semen parameters

For all males, assessment of the macroscopic semen characteristics was performed prior to any treatment (**Table 2**). The semen volume for the obese group was significantly lower than overweight group ( $p<0.05$ ). Moreover, there was a statistically significant delayed liquefaction in the semen of the obese group when compared to the normal weight ( $p<0.05$ ) and overweight ( $p<0.05$ ) groups. In addition, a significant increase in semen viscosity was detected in the obese group when compared to the normal weight group ( $p<0.05$ ). However, there was no effect of paternal obesity neither on the appearance nor on the pH of this biological fluid.

### c) Description of the motile sperm-enriched fraction according to the paternal BMI and WC

Sperm parameters before and after swim-up are listed in **Table 3 (Figure 1)**. When compared to the normal weight group, the raw semen of the obese men had a statistically significant lower sperm concentration ( $60.6 \times 10^6/\text{ml} \pm 4.80$  vs  $41 \times 10^6/\text{ml} \pm 4.24$ ) ( $p<0.05$ ), lower percentage of progressive motile sperm (PR) ( $33.1 \pm 1.125$  vs  $22.57 \pm 1.48$ ) ( $p<0.001$ ), lower percentage of non-progressive motile sperm ( $30.6 \pm 1.24$  vs  $26.12 \pm 1.65$ ) ( $p<0.05$ ), higher percentage of non-motile sperm ( $36.2 \pm 1.64$  vs  $48.8 \pm 2.73$ ) ( $p<0.001$ ), reduction in the percentage of sperm with normal morphology ( $11 \pm 0.85$  vs  $6.56 \pm 1.40$ ) ( $p<0.05$ ), and higher percentage of vacuolated sperm ( $13 \pm 1.39$  vs  $23.75 \pm 2.35$ ) ( $p<0.05$ ).

Moreover, The raw semen of the overweight group showed lower sperm concentration ( $p<0.001$ ), lower percentage of motile sperm ( $p<0.001$ ), higher percentage of non-motile sperm ( $p<0.001$ ), higher percentage of sperm with altered membrane integrity ( $p<0.05$ ), and lower normal form ( $p<0.05$ ) as compared to the normal weight group.

Furthermore, the motile sperm-enriched fraction from obese men had statistically significant differences when compared to that of normal weight group. After capacitation, obese men's spermatozoa showed lower percentage of progressive motile sperm ( $p<0.05$ ), higher percentage of non-progressive motile sperm ( $p<0.001$ ), and higher percentage of sperm vacuolization ( $p<0.01$ ). It is important to note that post swim-up technique there is improvement in the majority of the assessed parameters except of sperm vacuolization which had a dramatic increase.

**Table 1: Characteristics of the study population according to the paternal BMI and WC**

<b>Paternal characteristics (n=96)</b>	<b>Normal weight (n=30)</b>	<b>Overweight (n=32)</b>	<b>Obese (n=34)</b>
<b>Body mass index (BMI) (kg/m<sup>2</sup>)</b>	23.72 ± 0.2	27.62 ± 0.2 <sup>c</sup>	33.98 ± 0.8 <sup>c,f</sup>
<b>Waist circumference (WC) (cm)</b>	87.2 ± 1.6	99.18 ± 0.96 <sup>c</sup>	113.41 ± 2.96 <sup>c,f</sup>
<b>Age (years)</b>	35.5 ± 1.33	38.8 ± 1.28	39.2 ± 1.23
<b>Smokers (%)</b>	43.3	43.7	45.45
<b>Alcoholics (%)</b>	10	9.37	8.82
<b>Abstinence (days)</b>	4.4 ± 0.35	4.12 ± 0.47	5.12 ± 0.53

Note: Results are expressed as mean ± SEM for the BMI, WC, Age, and abstinence; and as mean percentage for the tobacco intake and alcohol consumption.

Superscripts a (p<0.05), b (p<0.01), c (p<0.001) denote statistically significant differences when overweight and obese groups were compared to the normal weight group.

Superscripts d (p<0.05), e (p<0.01), f (p<0.001) denote statistically significant differences when the overweight group was compared to the obese group.

**Table 9: Comparison between the macroscopic semen parameters according to the paternal BMI and WC levels**

<b>Macroscopic characteristics of the semen</b>	<b>Normal weight (n=30)</b>	<b>Overweight (n=32)</b>	<b>Obese (n=34)</b>
<b>Volume (ml)</b>	3.57 ± 0.24	4 ± 0.29	<b>3.29 ± 0.21<sup>d</sup></b>
<b>Appearance</b> 0= transparent 1= Yellowish 2= opaque	1.6 ± 0.08	1.5 ± 0.085	1.5 ± 0.085
<b>Liquefaction (minutes)</b>	21.6 ± 0.92	23.43 ± 1.79	<b>28.29 ± 2.11<sup>a,d</sup></b>
<b>Viscosity</b> 0= non viscous 1= viscous 2= high viscosity	0.06 ± 0.04	0.18 ± 0.09	<b>0.35 ± 0.10<sup>a</sup></b>
<b>pH</b>	8.21 ± 0.08	8.28 ± 0.09	8.11 ± 0.07

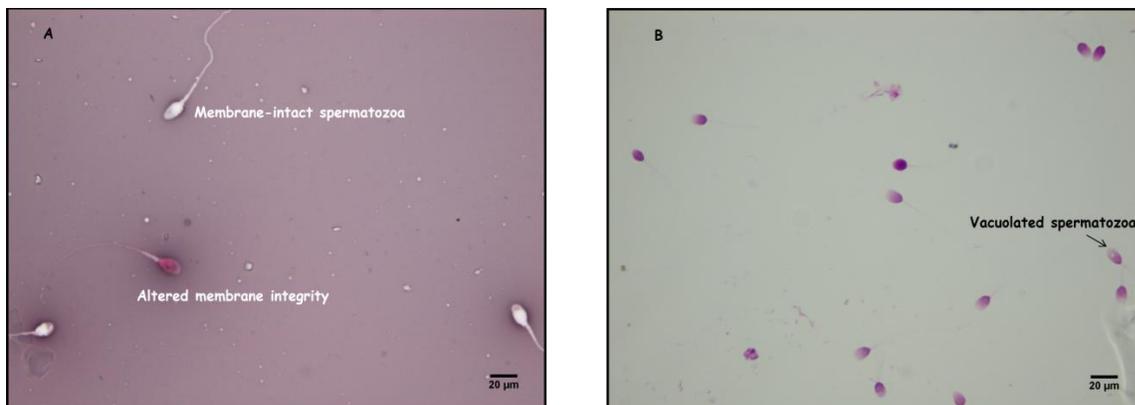
Note: Data expressed as mean ± SEM. Superscripts a (p<0.05), b (p<0.01), c (p<0.001) denote statistically significant differences when overweight and obese groups were compared to the normal weight group.

Superscripts d (p<0.05), e (p<0.01), f (p<0.001) denote statistically significant differences when the overweight group was compared to the obese group.

**Table 3: Comparison between the sperm parameters in the raw semen and in the motile-enriched fraction post swim-up technique according to paternal BMI and WC levels .**

	Raw semen			Swim-up technique		
	Normal (n=30)	Overweight (n=32)	Obese (n=34)	Normal (n=30)	Overweight (n=32)	Obese (n=34)
Concentration ( $\times 10^6$ /ml)	60.6 $\pm 4.80$	32.9 $\pm 3.76^c$	41 $\pm 4.24^a$	34.13 $\pm 4.11^i$	14.6 $\pm 2.57^{a,g}$	20.59 $\pm 3.88^g$
PR motility (%)	33.1 $\pm 1.125$	23.04 $\pm 1.552^c$	22.57 $\pm 1.48^c$	85.13 $\pm 2.74^i$	75.06 $\pm 4.28^i$	72.12 $\pm 4.65^{a,i}$
Non-PR motility (%)	30.6 $\pm 1.24$	24.6 $\pm 1.79$	26.12 $\pm 1.65^a$	8.4 $\pm 1.54^i$	18.8 $\pm 4.40$	22.5 $\pm 4.29^c$
Non-motile (%)	36.2 $\pm 1.65$	50.7 $\pm 3.06^c$	48.8 $\pm 2.73^c$	6.46 $\pm 1.37^i$	6.37 $\pm 2.20^i$	5.18 $\pm 2.37^i$
Altered membrane integrity (%)	24.4 $\pm 2.04$	32.3 $\pm 2.79^a$	28.9 $\pm 2.62$	6.6 $\pm 0.90^i$	8.19 $\pm 1.45^i$	8.9 $\pm 1.97^i$
Normal morphology (%)	11 $\pm 0.85$	7.28 $\pm 1.14^a$	6.56 $\pm 1.40^a$	10.55 $\pm 0.86$	9.7 $\pm 1.40$	7.46 $\pm 1.53$
Vacuolization (%)	13 $\pm 1.39$	14.89 $\pm 1.84$	23.75 $\pm 2.35^a$	25.17 $\pm 2.03^g$	29.17 $\pm 2.60^g$	40.26 $\pm 3.35^{b,i}$

Data expressed as mean  $\pm$  SEM. Superscripts denote statistically significant differences: a (p<0.05), b (p<0.01), c (p<0.001) when the overweight and obese were compared to normal group; d (p<0.05), e(p<0.01), f (p<0.001) when the obese group was compared to overweight one; g (p<0.05), h (p<0.01), i (p<0.001) when the motile-enriched fraction post swim-up was compared to the raw semen.

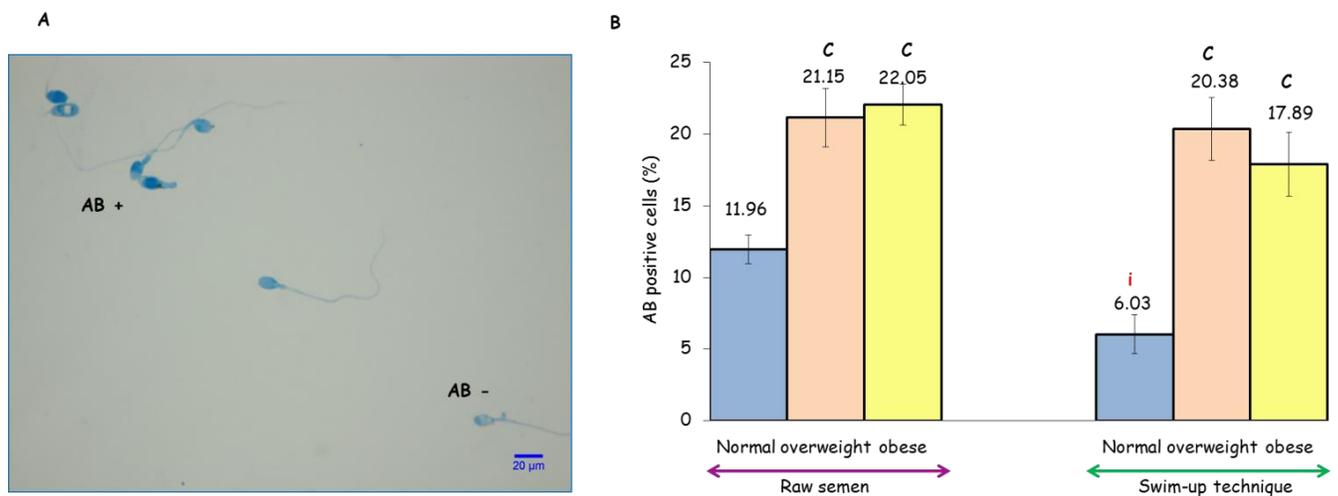


**Figure 2: Cytological assessment of sperm membrane integrity and morphology.**

(A)Figure showing the status of the sperm membrane integrity as assessed by the eosin-nigrosin staining. Unstained (white) spermatozoa has an intact membrane, stained (pink) spermatozoa has a loss in the membrane integrity. (B) The arrow represents a spermatozoon with a vacuole stained by the SpermScan Kit. Scale bar= 20µm. Magnification x100.

**d) Higher histones retention in motile sperm-enriched fraction of obese men**

In the raw semen, the percentage of sperm with high histones retention (AB+) is significantly higher in the overweight and obese groups in comparison to the normal weight group ( $p < 0.001$ ). Interestingly, when the percentage of AB+ cells before and after swim-up was compared, a significant decrease ( $p < 0.05$ ) was observed in the normal weight group, whereas no difference was seen in the overweight ( $p > 0.05$ ) and obese ( $p > 0.05$ ) groups. Consequently, the motile-enriched fractions of obese and overweight men present a significantly higher percentage of AB+ cells when compared to that of the normal weight group ( $p < 0.001$ ) (**Figure 3**).



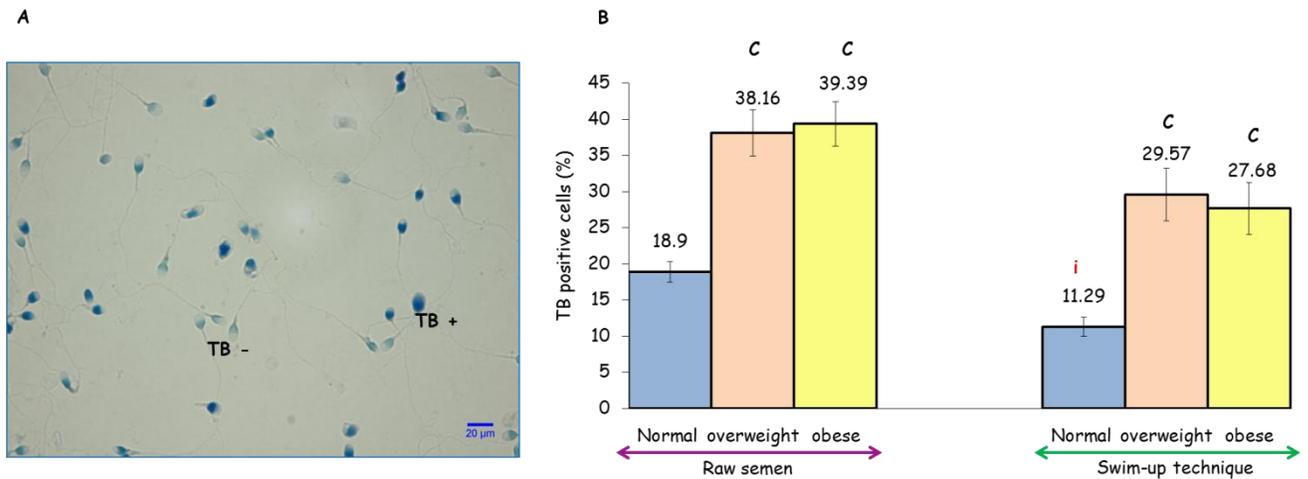
**Figure 44: Sperm histones retention as assessed by the aniline blue staining (AB).**

(A) Light blue sperm heads show normal histones retention (AB-) and abnormal dark blue sperm heads show abnormal histones retention (AB+). Scale bar= 20µm. Magnification x100.

(B) Histogram presenting the percentage of AB+ cells in the raw semen, and in the motile-enriched fraction post swim-up technique of the normal weight, overweight, and obese groups. Superscripts denote statistically significant differences: a ( $p < 0.05$ ), b ( $p < 0.01$ ), c ( $p < 0.001$ ) when the overweight and obese were compared to the normal group and g ( $p < 0.05$ ), h ( $p < 0.01$ ), i ( $p < 0.001$ ) when the motile-enriched fraction post swim-up was compared to the raw semen.

**e) Altered sperm chromatin integrity in motile sperm-enriched fraction of obese men**

In the raw semen, the percentage of sperm with altered chromatin integrity (TB+) is significantly higher in the overweight and obese groups when compared to the normal weight group ( $p < 0.001$ ). The levels of TB+ cells were significantly decreased in the swim-up fraction of normal weight men ( $p < 0.05$ ), whereas this decrease was not observed in the overweight and obese groups ( $p > 0.05$ ). Therefore, the obese and overweight men's motile spermatozoa present a significantly higher percentage of TB+ cells ( $p < 0.001$ ) (**Figure 4**).



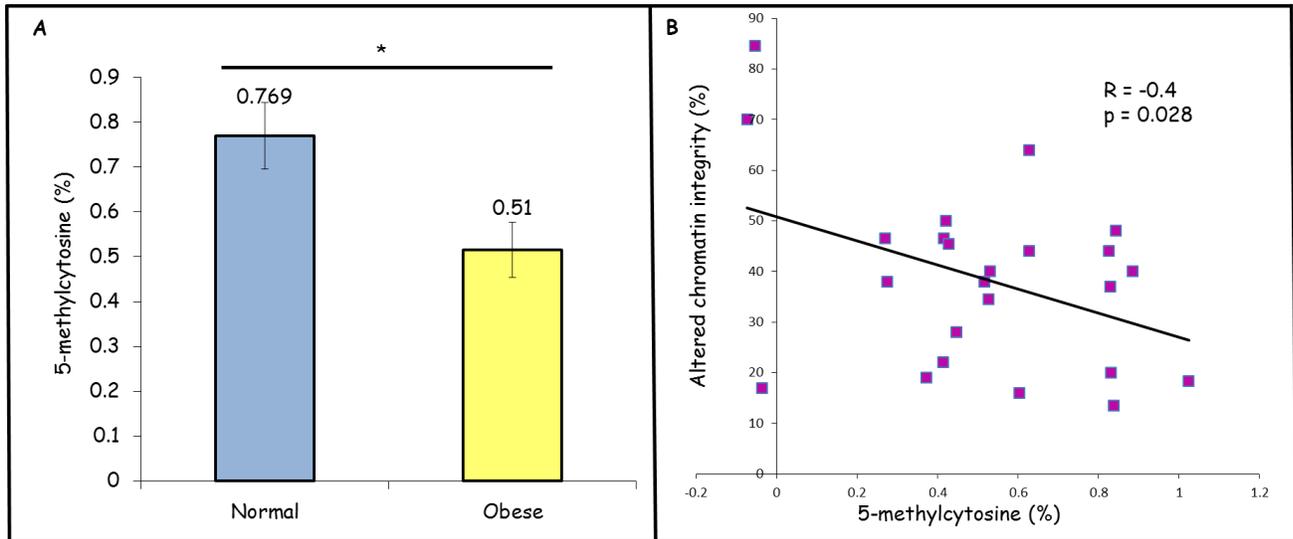
**Figure 4: Sperm chromatin integrity as assessed by the toluidine blue staining (TB).**

(A) Light blue sperm heads show normal chromatin integrity (TB-) and abnormal dark purple sperm heads show abnormal chromatin integrity (TB+). Scale bar= 20µm. Magnification x100.

(B) Histogram presenting the percentage of TB+ cells in the raw semen and in the motile-enriched fraction post swim-up technique of the normal weight, overweight, and obese groups. Superscripts denote statistically significant differences: a ( $p < 0.05$ ), b ( $p < 0.01$ ), c ( $p < 0.001$ ) when the overweight and obese were compared to the normal group; g ( $p < 0.05$ ), h ( $p < 0.01$ ), i ( $p < 0.001$ ) when the motile-enriched fraction post swim-up was compared to the raw semen.

### f) Global DNA hypomethylation in the motile sperm of obese men

Analysis of the global methylation (5-methylcytosine) of sperm DNA showed a statistically significant decrease in the methylation status of obese men's sperm DNA ( $p < 0.05$ ). Moreover, the levels of 5-methylcytosine were negatively correlated with the percentage of the altered chromatin structure ( $r = -0.4$ ;  $p = 0.028$ )(figure 5).



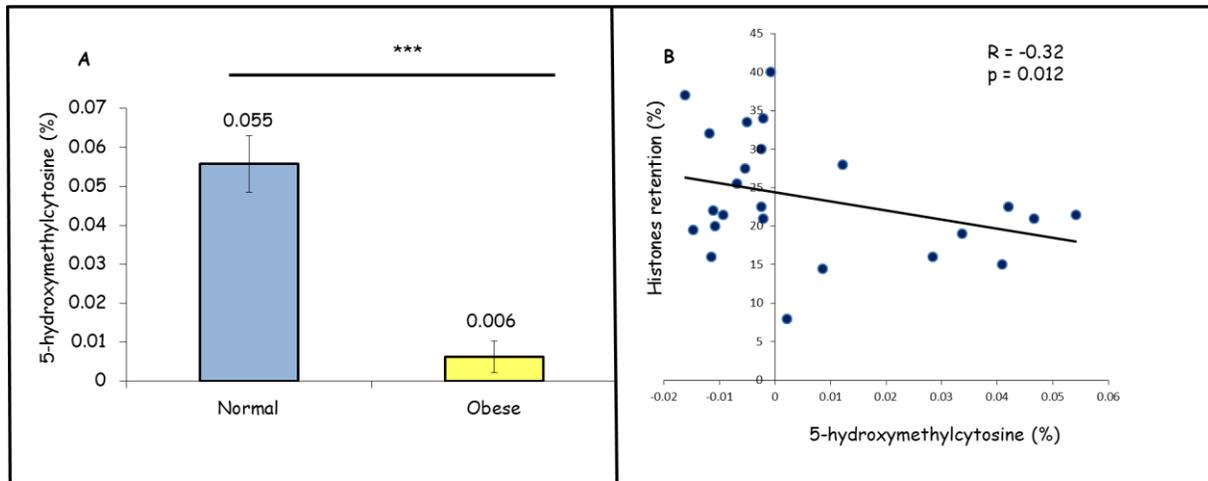
**Figure 5: Assessment of the global sperm DNA methylation status.**

(A) Effect of paternal obesity on the global sperm DNA methylation status (5-methylcytosine).<sup>\*</sup> Statistically significant difference with a ( $p < 0.05$ ).

(B) Correlation between sperm altered chromatin integrity and the sperm DNA methylation status. Statistically significant negative correlation ( $R = -0.4$ ,  $p = 0.028$ ) was found between these 2 parameters.

### g) Global DNA hydroxymethylation in the motile sperm of obese men

Global hydroxymethylation (5-hydroxymethylcytosine) of DNA extracted from spermatozoa differed significantly between normal weight and obese men ( $p < 0.001$ ). Interestingly, the levels of 5-hydroxymethylcytosine were negatively correlated with the percentage of sperm histones retention ( $r = -0.32$ ;  $p = 0.012$ )(figure 6).



**Figure 45: Assessment of the global sperm DNA hydroxymethylation status.**

**(A) Effect of paternal obesity on the global sperm DNA hydroxymethylation status (5-hydroxymethylcytosine).** \*\*\*Statistically significant difference with a ( $p < 0.001$ ). **(B) Correlation between sperm histones retention and the underlying DNA hydroxymethylation status.** Statistically significant negative correlation ( $R = -0.32$ ,  $p = 0.012$ ) was found between these 2 parameters.

#### **h) The effect of paternal obesity on pre-implantation embryo morphokinetics**

The time of pronuclei appearance (tPNa) was significantly shorter in the zygotes generated from obese men when compared to that of normal weight ( $p < 0.001$ ) and overweight ( $p < 0.01$ ) groups. In addition, statistically significant differences were detected in the mean duration of pronuclei fading (tPNf) between overweight and normal weight groups ( $p < 0.05$ ) and between obese and normal weight groups ( $p < 0.01$ ) (**Table 5**). However, the duration of the first embryonic cell cycle (CC1) was significantly longer in the overweight ( $p < 0.05$ ) and obese ( $p < 0.05$ ) groups in comparison with the CC1 of the normal weight group. Moreover, the second embryonic cell cycle (CC2) was significantly delayed in the obese group in comparison with the overweight group ( $p < 0.05$ ). Also, the third embryonic cell cycle (CC3) was significantly delayed in the obese group ( $p < 0.05$ ) (**Table 5**) (**Figure 7**).

#### **i) The effect of paternal obesity on embryo quality**

The embryo compaction rate and blastulation rate were significantly affected by paternal BMI and WC (**Table 6**). To establish whether paternal obesity may affect the blastocyst quality, the grade of day 5 blastocysts was scored as previously described by Schoolcraft and colleagues (Balaban et al. 2011). There were no statistically significant differences in the blastocyst expansion degree, neither in the inner cell mass (ICM) morphology across the groups. In contrast, a significant decrease in the trophectoderm (TE) morphology was detected in the obese group ( $p < 0.05$ ) (**Table 6**) (**Figure 8**).

**Table 4: The diagnosis of female infertility according to the paternal BMI and WC**

Paternal characteristics / Maternal characteristics	Normal weight (n=71 oocytes)	Overweight (n=73 oocytes)	Obese (n=80 oocytes)	P value
<b>Body mass index (BMI) (kg/m<sup>2</sup>)</b>	22.84 ± 1.16	23.81 ± 0.13	22.41 ± 0.81	p>0.05
<b>Age (years)</b>	29.91 ± 1.69	29.90 ± 1.94	30 ± 1.35	p>0.05
<b>Number of MII oocytes</b>	8.87 ± 1.63	7.3 ± 1.14	6.66 ± 0.72	p>0.05
<b>Polycystic ovary (%)</b> 0= No 1= Yes	12.5	20	22	p>0.05
<b>Endometriosis (%)</b> 0= No 1= Yes	0	11.1	0	p>0.05
<b>Tubal/pelvic disease (%)</b> 0= NO 1= Yes	0	20	0	p>0.05
<b>Other (%)</b> 0= NO 1= Yes	25	33.33	40	p>0.05

Note: Data expressed as mean ± SEM for BMI, age, and number of MII oocytes; Data expressed as mean percentage for the polycystic ovary, endometriosis, tubal/pelvic disease, and others. There is no statistically significant difference between groups (p>0.05).

**Table 5: The effect of obesity on the morphokinetic parameters of pre-implantation embryo development**

Paternal characteristics	Normal weight (n=71 oocytes)	Overweight (n=73 oocytes)	Obese (n=80 oocytes)	p value
tPB extrusion (hours)	2.63 ± 0.103	2.40 ± 0.102	2.47 ± 0.103	p>0.05
tPNa (hours)	7.53 ± 0.132	7.30 ± 0.168	<b>6.60 ± 0.166</b> <sup>c, e</sup>	<b>p = 0.0003</b>
tPNf (hours)	25.09 ± 0.45	<b>23.38 ± 0.504</b> <sup>a</sup>	<b>23.35 ± 0.35</b> <sup>b</sup>	<b>p = 0.01</b>
CC1 (t2-tPNf) (hours)	2.5 ± 0.06	<b>3.1 ± 0.217</b> <sup>a</sup>	<b>3.25 ± 0.259</b> <sup>a</sup>	<b>p = 0.04</b>
CC2 (t4-t2) (hours)	13.04 ± 0.55	12.33 ± 0.52	<b>14.70 ± 0.77</b> <sup>d</sup>	<b>P = 0.04</b>
CC3 (t8-t4) (hours)	22.11 ± 1.02	23.78 ± 1.51	<b>26.74 ± 1.73</b> <sup>a</sup>	p>0.05

Note: Data is expressed as mean ± SEM for the time of polar body extrusion (tPB), time of pronuclei appearance (tPNa), time of pronuclei fading (tPNf), CC1 (duration of the first cell cycle), CC2 (duration of the second cell cycle), CC3 (duration of the third cell cycle). t2-t8 (timing of 2 to 8 cell-stages).

Superscript a(p<0.05), b(p<0.01), c(p<0.001) for significant differences between the overweight/obese group and the normal weight group.

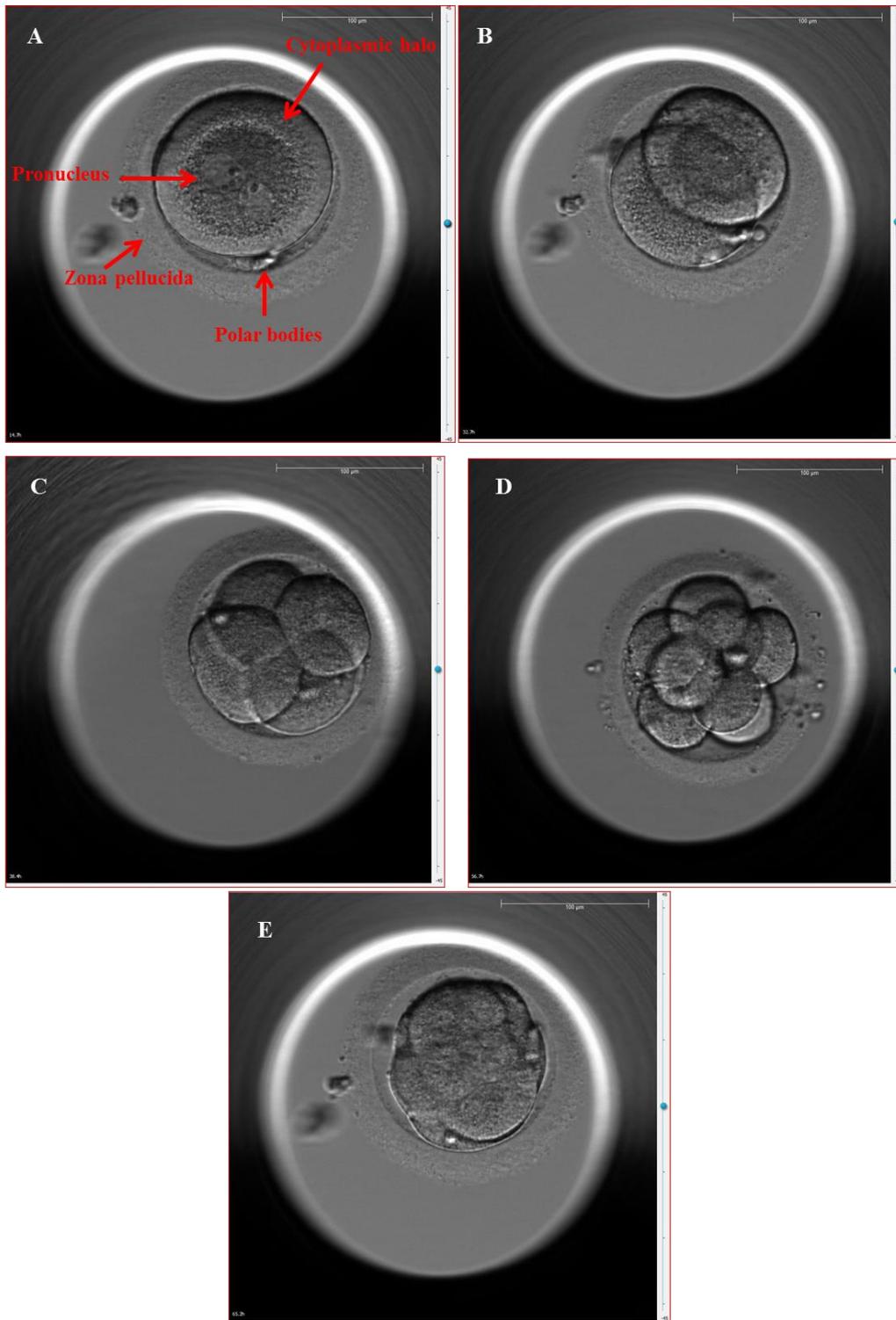
Superscript d(p<0.05), e (p<0.01), f (p<0.001) for significant differences between the overweight group and the obese group.

**Table 10: The effect of paternal obesity on the fertilization rate and the embryo development on day 4 and 5 of culture**

Paternal characteristics	Normal weight (n=71 oocytes)	Overweight (n=73 oocytes)	Obese (n=80 oocytes)	p value
<b>Fertilization rate (%)</b>	90.5	74.28	84.225	p>0.05
<b>Compaction rate (%)</b>	89.26 ± 3.10	<b>51.57 ± 9.51<sup>a</sup></b>	<b>59.35 ± 7.43<sup>a</sup></b>	<b>p = 0.02</b>
<b>Blastulation rate (%)</b>	74.41 ± 5.52	<b>31.98 ± 7.51<sup>b</sup></b>	<b>36.52 ± 8.33<sup>b</sup></b>	<b>p = 0.009</b>
<b>Expansion at 115 h</b>	3.34 ± 0.19	2.94 ± 0.206	3.07 ± 0.19	p > 0.05
<b>TE at 115h</b>	1.22 ± 0.08	1.45 ± 0.106	<b>1.6 ± 0.103<sup>a</sup></b>	<b>p = 0.04</b>
<b>ICM at 115h</b>	1.23 ± 0.09	1.54 ± 0.17	1.22 ± 0.09	p > 0.05

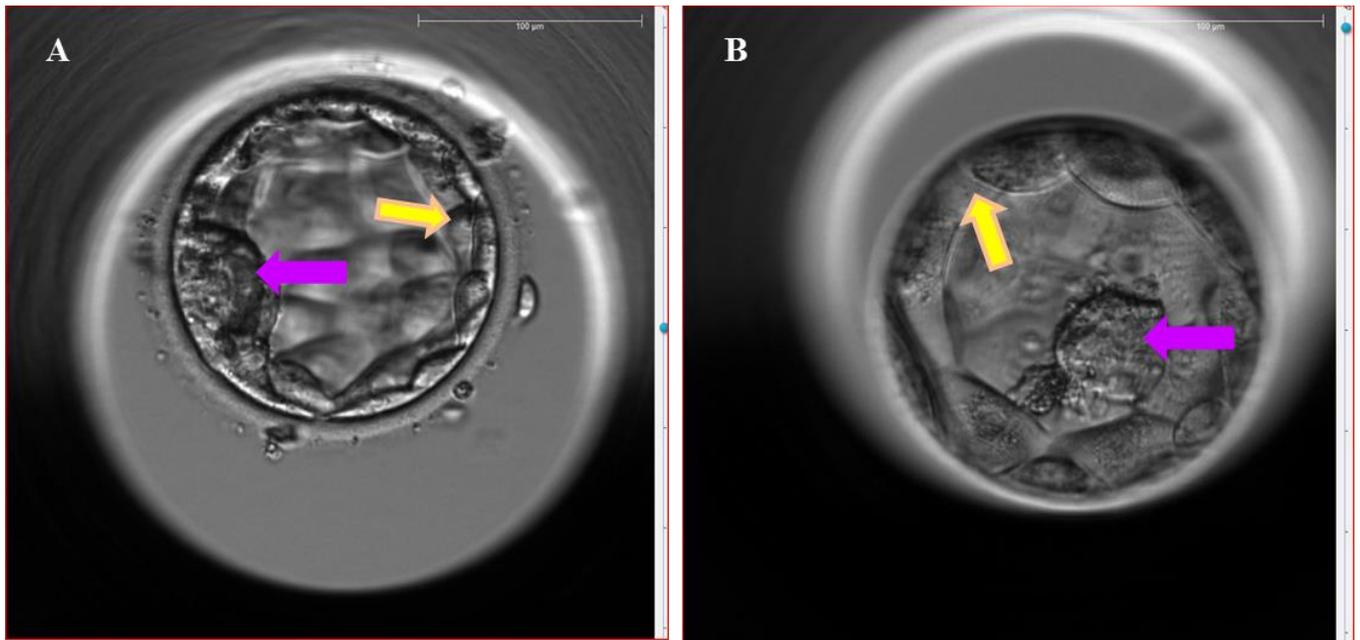
Note: Data is expressed as mean ± SEM for the blastocyst expansion (from 1 to 4 according to the degree of expansion), the TE (trophectoderm A=1, B=2, C=3), ICM (inner cell mass A=1, B=2, C=3); and as mean percentage for the fertilization, compaction, and blastulation rate.

Superscript a(p<0.05), b(p<0.01), c(p<0.001) for significant differences between the overweight/obese group and the normal weight group. Superscript d(p<0.05), e (p<0.01), f (p<0.001) for significant differences between the overweight group and the obese group.



**Figure 7: The different stages of pre-implantation embryo development.**

(A) Fertilized oocyte presenting, two polar bodies, two pronuclei, a cytoplasmic halo, and enclosed in the zona pellucida. (B) embryo with 2 blastomeres. (C) embryo with 4 blastomeres. (D) embryo with 8 blastomeres. (E) embryo during compaction. Scale bar = 100 µm.



**Figure 8: Figure showing two blastocysts.**

(A) Normal expanded blastocyst with grade A inner cell mass (purple arrow) made up of many cells which are tightly packed, and grade A trophoblast (yellow arrow) containing many cells forming a cohesive layer. (B) Expanded blastocyst with grade A inner cell mass (purple arrow) and grade B trophoblast (yellow arrow) characterized by the presence of only a few cells forming a loose epithelium. Scale bar = 100 µm.

## V- Discussion

Recent years have witnessed extensive research on the long-term consequences of paternal health (at the time of conception) on the development of diseases in the offspring. For instance, paternal age, smoking, and exposure to toxic chemicals may increase the risk of neuropsychiatric disorders, metabolic alterations, respiratory tract infection, and cancer in the offspring (Sorahan et al. 1997; Chang et al. 2006; Toschke et al. 2007; Fernandez-Gonzalez et al. 2008; Lin et al. 2008; Sung et al. 2008; Sartorius et al. 2010). In addition, accumulating epidemiological studies in humans suggest that paternal body mass index (BMI) may influence the health of the next generation (Lane et al. 2014). Interestingly, it was demonstrated that epimutations may be present in the sperm of obese men (Soubry et al. 2016), smokers (Kim et al. 2015; Vieweg et al. 2015) and in the sperm of aged fathers (Frans et al. 2008; Miller et al. 2010; Jenkins et al. 2014). Hence, understanding the molecular mechanisms underlying the contribution of sperm epigenome to early embryogenesis and to body homeostasis later in life is of paramount importance.

Here, we aimed to assess the chromatin composition in the motile sperm of obese men and its impact on pre-implantation embryo morphokinetics. Sperm chromatin is highly packed playing a crucial role in fertilization and embryo development. Approximately, 90% of the DNA is tightly condensed by small basic protamins; thus, inhibiting any transcriptional activity and protecting the sperm during the transit into the female tract(Wared et al. 2010). Moreover, a recent meta-analysis showed that abnormal sperm protamination was associated with male infertility and sperm DNA damage(Ni et al. 2016). In contrast, the remaining 10% of the DNA are bound to histones controlling the activation of paternal genes implicated in early embryo development. Fascinatingly, the paternal protamins are replaced by maternal histones 2-4 hours after fertilization; whereas, the retained histones are paternally inherited and they are not replaced (Wared et al. 2010).

The present study shows that the raw semen of obese and overweight men had a significantly lower concentration of spermatozoa and lower percentage of progressive and non-progressive motile sperm when compared to that of the normal weight men; whereas, the percentage of gametes with impaired motility, abnormal morphology, and vacuolisation was significantly higher.

In order to select the motile sperm able to fertilize the oocyte and to achieve embryo development, the semen was processed using the swim-up technique. After the incubation period, the assessment of the swim-up fraction showed an improvement in the majority of the analyzed parameters (the three types of motility, and the membrane integrity), however the spermatozoa were subject to a dramatic increase in the vacuolisation. In parallel, the comparison between the swim-up fraction of the three groups showed a significant decrease in the concentration and in the progressive motility, and a significant increase in the percentage of non-progressive and vacuolised sperm in the obese group. These findings indicate that on one hand, the swim-up technique may improve to a certain extent the percentage of motile sperm in obese men prior to ART. On the other hand, longer exposure of the gametes to the seminal plasma of obese patients during the semen processing significantly increases the sperm vacuolization. It has been demonstrated that male obesity may alter the concentrations of some seminal fluid components, raising the question of the possible effects on sperm physiology(Binder et al. 2015). The drawbacks of sperm vacuoles on the embryo development and pregnancy outcome are still debatable. Some authors have considered them as cytologic variations without pathological significance, but other scientists have shown that vacuoles are present in sperm with fragmented DNA, impaired mitochondrial physiology, and abnormal karyotype (Garolla et al. 2015).

Furthermore, the evaluation of histone levels in the sperm chromatin of obese men was assessed using the aniline blue staining. Our results indicate that the percentage of motile sperm with high histones retention is significantly reduced in the swim-up fraction of the normal weight subjects when compared to the raw semen. However, the percentage of sperm with abnormal histones retention is significantly elevated in the raw semen of obese men and it is not reduced after the swim-up procedure. These findings indicate that male obesity may increase the probability of

transferring abnormal levels of paternal histones to the new forming zygote. Our result is consistent with the result in a mice model of diet-induced obesity where the sperm derived from obese father presented higher level of histone (H3) retention at genes involved in development and cell fate decisions. The offspring of these mice had alterations in the liver gene expression (Terashima et al. 2015).

Moreover, the swim-up technique was able to select from the raw semen of normal weight men a sperm population with a lower percentage of altered chromatin integrity. Unfortunately, obese and overweight subjects had a statistically significant increase in the percentage of gametes with altered chromatin integrity in the raw semen and in the swim-up fraction. The toluidine blue protocol allowed the staining of the sperm histones and the phosphate residues of the DNA fragments at the same time; thus helped in the assessment of the chromatin status (Tsarev et al. 2009). Our results showed that motile sperm of obese men had high levels of sperm DNA fragmentation and decondensed chromatin which may be responsible of low fertilization rate, poor embryo quality, repeated failure of assisted reproductive technology attempts, and miscarriages (Balhorn et al. 2011; Choucair et al. 2016). These data are consistent with the findings of a recent systematic review and meta-analysis where the authors concluded that obese men had higher percentage of sperm DNA fragmentation and reduced fertility potential (Campbell et al. 2015).

The motile sperm-enriched fraction post swim-up was selected for the quantification of global DNA methylation and hydroxymethylation. The 5-mC is an epigenetic mark globally associated with gene repression (Abdallah et al. 2009). The levels of 5-methylcytosine were significantly lower in the sperm of obese patients than normal weight ones. Also, we found a negative correlation between the sperm DNA methylation and the altered chromatin integrity. Several studies showed that global sperm DNA methylation was lower in patients with high seminal reactive oxygen species and with sperm having high DNA fragmentation index, abnormal chromatin condensation, and with reduced motility (Tunc et al. 2009; Montjean et al. 2015). In parallel, Okas et al., demonstrated that male mice treated with DNA methylation inhibitor drug, suffered from reduced sperm motility and inability of the demethylated paternal genome to support embryo development (Oakes et al. 2007).

In addition, we found that the DNA in the motile sperm of obese subjects was significantly hypohydroxymethylated when compared to the normal weight group. Plus, the levels of sperm histone retention were negatively correlated to the 5-hmC levels. The latter is also considered as an epigenetic mark that can regulate gene expression, and it is generated from the oxidation of the 5-mC. This reaction is catalyzed by the TET (ten-eleven translocation) proteins (Vasanthakumar et al. 2015). Recently, it has been demonstrated that mature human spermatozoa of fertile men, express the TET1-3 enzymes at the mRNA and protein levels. Also, a positive correlation exists between TET expression profile and sperm parameters. Curiously, the downregulation of TET3 and TET2 enzymes in the sperm of infertile men may reduce the fertilization rate and the pregnancy rate respectively after ART (Ni et al. 2016). Interestingly,

some metabolites generated from cellular metabolism can regulate the TET functions. For instance, altered glucose metabolism in cancer cells may inhibit the TET activity leading to a decrease in DNA hydroxymethylation (Vasanthakumar et al. 2015). Moreover, Delatte et al. demonstrated that oxidative stress could also decrease the global 5-hydroxymethylcytosine pattern *in vivo* and *in-vitro* (Delatte et al. 2015). Since obesity may affect the testicular metabolism and the oxidative stress status (Dias et al. 2014; Rato et al. 2013), it will be important to study the molecular mechanisms by which the testis environment in obese patients modifies the sperm global DNA hydroxymethylation pattern.

After fertilization, the incorporated sperm undergoes biochemical modifications involving the removal of sperm nuclear envelope, decondensation of the chromatin through the reduction of the disulfide bonds between protamines, replacement of paternal protamines by maternal histones, and formation of the pronuclear envelope (Swain et al. 2008). In humans, the DNA in the paternal pronucleus (PN) is hypomethylated and highly hydroxymethylated; whereas the maternal pronucleus is hypermethylated with low levels of 5-hmC (Efimova et al. 2015). Our morphokinetic study showed an earlier PN appearance and earlier PN fading in the obese group than in the normal weight one. On one hand, the abnormal level of histones, the decondensed chromatin, and the hypomethylated DNA in the sperm of obese patients could be the cause of early PN formation and fading. On the other hand, an early PN fading was shown to be associated decreases live birth rate (Azzarello et al. 2012). Our finding is in line with the result of a recent meta-analysis showing that paternal obesity may decrease the live birth rate per ART cycle (Campbell et al. 2015).

Following the pronuclei fading, the fertilized oocyte undergoes rapid cell division termed cleavage, transforming the unicellular zygote to a multicellular embryo. At the third day of development, human embryos activate their embryonic genome and consequently the paternal and maternal genome could be expressed. During the first three embryonic cell cycles, the blastomeres are separated from each others. After that, the activation of the embryonic genome intercellular junctions are established between them forming a compact embryo. Next, the compacted embryo differentiate into a blastocyst containing an expanded blastocoel, an inner cell mass that will give rise to the fetal tissues, and a trophoctoderm layer which will develop into the placenta (Hartshorne et al. 2000; Hardarson et al. 2012). Here, we show that paternal obesity significantly delays the duration of early embryonic cell cycles, leading to a statistically significant decrease in the compaction rate, blastulation rate, and in the quality of the trophoctoderm layer. Similar to other studies, delayed cleavage intervals are associated with lower embryos developing to expanded blastocysts, and lower ability to implant in the endometrium (Dal Canto et al. 2012). Recently, the paternal influence on the early embryonic development prior to embryonic genome activation is more investigated. Simon and colleagues demonstrated that sperm DNA fragmentation might affect the embryo quality at all the developmental stages and may reduce the pregnancy rate (Simon et al. 2014). In parallel, animal studies on *Macaca mulatta*, mice and bull showed that male factors and specifically oxidative

stress in the sperm might alter the cleavage divisions of the pre-implantation embryo and the health of the offspring (Burrue1 et al. 2014; Lane et al. 2014; De castro et al. 2015). Moreover, male mice with acquired obesity might negatively impact the early cleavage divisions, the carbohydrate metabolism in the blastocysts, the gene expression in the male placentas, global DNA hypermethylation of the female placentas, and might result in a fetal growth restriction (FGR) (Binder et al. 2012; Binder et al. 2012; Binder et al. 2015). The FGR increases the risk of developing obesity, and its associated co-morbidities later in life (Kanaka-Gantenbein et al. 2010).

In conclusion, this study identified alterations in the levels of sperm nucleoproteins in the swim-up fraction of obese men. Moreover, paternal obesity was found to hypomethylate and hypohydroxymethylate the sperm DNA probably inducing epigenetic modifications in the embryos and the offspring. Altogether, these differences resulted in an abnormal embryo morphokinetic and reduced blastocyst quality.

## VI- Bibliography

1. Abdalla, Hany, Yusuke Yoshizawa, and Shinichi Hochi. "Active demethylation of paternal genome in mammalian zygotes." *Journal of Reproduction and Development* 55.4 (2009): 356-360.
2. Al-Quwaidhi, A. J., et al. "Trends and future projections of the prevalence of adult obesity in Saudi Arabia, 1992-2022/Tendances et projections de la prévalence de l'obésité chez l'adulte en Arabie saoudite, 1992-2022." *Eastern Mediterranean Health Journal* 20.10 (2014): 589.
3. Azzarello, A., T. Hoest, and A. L. Mikkelsen. "The impact of pronuclei morphology and dynamicity on live birth outcome after time-lapse culture." *Human reproduction* (2012): des210.
4. Bakos, Hassan W., et al. "Paternal body mass index is associated with decreased blastocyst development and reduced live birth rates following assisted reproductive technology." *Fertility and sterility* 95.5 (2011): 1700-1704.
5. Balaban, Başak, et al. "The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting." *Human Reproduction* 26.6 (2011): 1270-1283.
6. Balhorn, Rod. "Sperm chromatin: an overview." *Sperm Chromatin*. Springer New York, 2011. 3-18.
7. Bartlett, Zane. *A History of Cellular Senescence and Its Relation to Stem Cells in the Twentieth and Twenty-First Centuries*. Diss. ARIZONA STATE UNIVERSITY, 2015.
8. Bellver, J., et al. "Similar morphokinetic patterns in embryos derived from obese and normoweight infertile women: a time-lapse study." *Human Reproduction* 28.3 (2013): 794-800.
9. Binder, Natalie K., et al. "Male obesity is associated with changed spermatozoa Cox4i1 mRNA level and altered seminal vesicle fluid composition in a mouse model." *Molecular human reproduction* (2015): gav010.
10. Binder, Natalie K., et al. "Paternal obesity in a rodent model affects placental gene expression in a sex-specific manner." *Reproduction* 149.5 (2015): 435-444.
11. Binder, Natalie K., Megan Mitchell, and David K. Gardner. "Parental diet-induced obesity leads to retarded early mouse embryo development and altered carbohydrate utilisation by the blastocyst." *Reproduction, Fertility and Development* 24.6 (2012): 804-812.

12. Binder, Natalie K., Natalie J. Hannan, and David K. Gardner. "Paternal diet-induced obesity retards early mouse embryo development, mitochondrial activity and pregnancy health." *PLoS One* 7.12 (2012): e52304.
13. Burruel, Victoria, et al. "Abnormal early cleavage events predict early embryo demise: sperm oxidative stress and early abnormal cleavage." *Scientific reports* 4 (2014).
14. Campbell, Jared M., et al. "Paternal obesity negatively affects male fertility and assisted reproduction outcomes: a systematic review and meta-analysis." *Reproductive biomedicine online* 31.5 (2015): 593-604.
15. Chang, Jeffrey S., et al. "Parental smoking and the risk of childhood leukemia." *American journal of epidemiology* 163.12 (2006): 1091-1100.
16. Choucair, Fadi B., et al. "High level of DNA fragmentation in sperm of Lebanese infertile men using Sperm Chromatin Dispersion test." *Middle East Fertility Society Journal* (2016).
17. Colaci, Daniela S., et al. "Men's body mass index in relation to embryo quality and clinical outcomes in couples undergoing in vitro fertilization." *Fertility and sterility* 98.5 (2012): 1193-1199.
18. Costanian, Christy, et al. "Prevalence, correlates and management of type 2 diabetes mellitus in Lebanon: findings from a national population-based study." *Diabetes research and clinical practice* 105.3 (2014): 408-415.
19. Dal Canto, Mariabeatrice, et al. "Cleavage kinetics analysis of human embryos predicts development to blastocyst and implantation." *Reproductive biomedicine online* 25.5 (2012): 474-480.
20. de Castro, Letícia S., et al. "Sperm oxidative stress is detrimental to embryo development: a dose-dependent study model and a New and more sensitive oxidative status evaluation." *Oxidative medicine and cellular longevity* 2016 (2015).
21. Delatte, Benjamin, et al. "Genome-wide hydroxymethylcytosine pattern changes in response to oxidative stress." *Scientific reports* 5 (2015).
22. Dias, Tânia R., et al. "Sperm glucose transport and metabolism in diabetic individuals." *Molecular and cellular endocrinology* 396.1 (2014): 37-45.
23. Donkin, Ida, et al. "Obesity and bariatric surgery drive epigenetic variation of spermatozoa in humans." *Cell metabolism* 23.2 (2016): 369-378.
24. Donkin, Ida, et al. "Obesity and bariatric surgery drive epigenetic variation of spermatozoa in humans." *Cell metabolism* 23.2 (2016): 369-378.

25. Efimova, Olga A., et al. "Chromosome hydroxymethylation patterns in human zygotes and cleavage-stage embryos." *Reproduction* 149.3 (2015): 223-233.
26. Eisenberg, Michael L., et al. "The relationship between male BMI and waist circumference on semen quality: data from the LIFE study." *Human reproduction* 29.2 (2014): 193-200.
27. Fernández-Gonzalez, Raúl, et al. "Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring." *Biology of Reproduction* 78.4 (2008): 761-772.
28. Frans, Emma M., et al. "Advancing paternal age and bipolar disorder." *Archives of general psychiatry* 65.9 (2008): 1034-1040.
29. Fullston, T., et al. "Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice." *Human reproduction* 27.5 (2012): 1391-1400.
30. Fullston, Tod, et al. "Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete penetrance to the F2 generation and alters the transcriptional profile of testis and sperm microRNA content." *The FASEB Journal* 27.10 (2013): 4226-4243.
31. Garolla, Andrea, et al. "Molecular karyotyping of single sperm with nuclear vacuoles identifies more chromosomal abnormalities in patients with testiculopathy than fertile controls: implications for ICSI." *Human Reproduction* (2015): dev202.
32. Golay, A., and J. Ybarra. "Link between obesity and type 2 diabetes." *Best Practice & Research Clinical Endocrinology & Metabolism* 19.4 (2005): 649-663.
33. Grandjean, Valérie, et al. "RNA-mediated paternal heredity of diet-induced obesity and metabolic disorders." *Scientific reports* 5 (2015).
34. Hartshorne, Geraldine. "The embryo." *Human Reproduction* 15.suppl 4 (2000): 31-41.
35. Jenkins, Timothy G., et al. "Age-associated sperm DNA methylation alterations: possible implications in offspring disease susceptibility." *PLoS Genet* 10.7 (2014): e1004458.
36. Kaati, Gunnar, Lars O. Bygren, and SorenEdvinsson. "Cardiovascular and diabetes mortality determined by nutrition during parents' and grandparents' slow growth period." *European Journal of Human Genetics* 10.11 (2002): 682-688.
37. Kanaka-Gantenbein, Christina. "Fetal origins of adult diabetes." *Annals of the New York Academy of Sciences* 1205.1 (2010): 99-105.

38. Kim, Seul Ki, ByungChulJee, and Seok Hyun Kim. "Histone methylation and acetylation in ejaculated human sperm: effects of swim-up and smoking." *Fertility and sterility* 103.6 (2015): 1425-1431.
39. Lane, Michelle, et al. "Oxidative stress in mouse sperm impairs embryo development, fetal growth and alters adiposity and glucose regulation in female offspring." *PLoS One* 9.7 (2014): e100832.
40. Lane, Michelle, Rebecca L. Robker, and Sarah A. Robertson. "Parenting from before conception." *Science* 345.6198 (2014): 756-760.
41. Le Lannou, D., and Y. Blanchard. "Nuclear maturity and morphology of human spermatozoa selected by Percoll density gradient centrifugation or swim-up procedure." *Journal of reproduction and fertility* 84.2 (1988): 551-556.
42. Lin, Ching-Chun, et al. "Increased risk of death with congenital anomalies in the offspring of male semiconductor workers." *International journal of occupational and environmental health* (2013).
43. Macdonald, A. A., A. W. Stewart, and C. M. Farquhar. "Body mass index in relation to semen quality and reproductive hormones in New Zealand men: a cross-sectional study in fertility clinics." *Human Reproduction* 28.12 (2013): 3178-3187.
44. McCowan, Lesley ME, et al. "Paternal contribution to small for gestational age babies: a multicenter prospective study." *Obesity* 19.5 (2011): 1035-1039.
45. McPherson, Nicole O., and Michelle Lane. "Male obesity and subfertility, is it really about increased adiposity?." *Asian journal of andrology* 17.3 (2014): 450-458.
46. Miller, Brian, et al. "Meta-analysis of paternal age and schizophrenia risk in male versus female offspring." *Schizophrenia bulletin* 37.5 (2011): 1039-1047.
47. Montjean, D., et al. "Sperm global DNA methylation level: association with semen parameters and genome integrity." *Andrology* 3.2 (2015): 235-240.
48. Nasreddine, Lara, et al. "Trends in overweight and obesity in Lebanon: evidence from two national cross-sectional surveys (1997 and 2009)." *BMC public health* 12.1 (2012): 1.
49. Ng, Marie, et al. "Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013." *The Lancet* 384.9945 (2014): 766-781.
50. Ni, K., et al. "The impact of sperm protamine deficiency and sperm DNA damage on human male fertility: a systematic review and meta-analysis." *Andrology* (2016).

51. Oakes, Christopher C., et al. "Adverse effects of 5-aza-2'-deoxycytidine on spermatogenesis include reduced sperm function and selective inhibition of de novo DNA methylation." *Journal of Pharmacology and Experimental Therapeutics* 322.3 (2007): 1171-1180.
52. Ogden, Cynthia L., et al. "Prevalence of childhood and adult obesity in the United States, 2011-2012." *Jama* 311.8 (2014): 806-814.
53. Palmer, Nicole O., et al. "SIRT6 in mouse spermatogenesis is modulated by diet-induced obesity." *Reproduction, Fertility and Development* 23.7 (2011): 929-939.
54. Rato, L., et al. "High-energy diets may induce a pre-diabetic state altering testicular glycolytic metabolic profile and male reproductive parameters." *Andrology* 1.3 (2013): 495-504.
55. Schliep, Karen C., et al. "Effect of male and female body mass index on pregnancy and live birth success after in vitro fertilization." *Fertility and sterility* 103.2 (2015): 388-395.
56. Sermondade, N., et al. "BMI in relation to sperm count: an updated systematic review and collaborative meta-analysis." *Human reproduction update* (2012): dms050.
57. Simon, L., et al. "Paternal influence of sperm DNA integrity on early embryonic development." *Human Reproduction* (2014): deu228.
58. Sorahan, T., et al. "Childhood cancer and parental use of tobacco: deaths from 1971 to 1976." *British journal of cancer* 76.11 (1997): 1525.
59. Soubry, Adelheid, et al. "Obesity-related DNA methylation at imprinted genes in human sperm: Results from the TIEGER study." *Clinical epigenetics* 8.1 (2016): 1.
60. Soubry, Adelheid, et al. "Obesity-related DNA methylation at imprinted genes in human sperm: Results from the TIEGER study." *Clinical epigenetics* 8.1 (2016): 1.
61. Sung, Tzu-I., Jung-Der Wang, and Pau-Chung Chen. "Increased risks of infant mortality and of deaths due to congenital malformation in the offspring of male electronics workers." *Birth Defects Research Part A: Clinical and Molecular Teratology* 85.2 (2009): 119-124.
62. Swain, Jason E., and Thomas B. Pool. "ART failure: oocyte contributions to unsuccessful fertilization." *Human Reproduction Update* 14.5 (2008): 431-446.
63. Terashima, Minoru, et al. "Effect of high fat diet on paternal sperm histone distribution and male offspring liver gene expression." *Epigenetics* 10.9 (2015): 861-871.

64. Toschke, Audre M., et al. "Paternal smoking is associated with a decreased prevalence of type 1 diabetes mellitus among offspring in two national British birth cohort studies (NCDS and BCS70)." *Journal of perinatal medicine* 35.1 (2007): 43-47.
65. Tsarev, I., et al. "Evaluation of male fertility potential by Toluidine Blue test for sperm chromatin structure assessment." *Human reproduction* 24.7 (2009): 1569-1574.
66. Tunc, O., H. W. Bakos, and K. Tremellen. "Impact of body mass index on seminal oxidative stress." *Andrologia* 43.2 (2011): 121-128.
67. Tunc, Ozlem, and Kelton Tremellen. "Oxidative DNA damage impairs global sperm DNA methylation in infertile men." *Journal of assisted reproduction and genetics* 26.9-10 (2009): 537-544.
68. vanVliet-Ostaptchouk, Jana V., et al. "The prevalence of metabolic syndrome and metabolically healthy obesity in Europe: a collaborative analysis of ten large cohort studies." *BMC endocrine disorders* 14.1 (2014): 1.
69. Vasanthakumar, Aparna, and Lucy A. Godley. "5-hydroxymethylcytosine in cancer: significance in diagnosis and therapy." *Cancer genetics* 208.5 (2015): 167-177.
70. Veenendaal, Marjolein VE, et al. "Transgenerational effects of prenatal exposure to the 1944–45 Dutch famine." *BJOG: An International Journal of Obstetrics & Gynaecology* 120.5 (2013): 548-554.
71. Vieweg, Markus, et al. "Methylation analysis of histone H4K12ac-associated promoters in sperm of healthy donors and subfertile patients." *Clinical epigenetics* 7.1 (2015): 1.
72. Vignera, Sandro, et al. "Negative effect of increased body weight on sperm conventional and nonconventional flow cytometric sperm parameters." *Journal of andrology* 33.1 (2012): 53-58.
73. Volpes, Aldo, et al. "The pellet swim-up is the best technique for sperm preparation during in vitro fertilization procedures." *Journal of assisted reproduction and genetics* (2016): 1-6.
74. Ward, W. Steven. "Function of sperm chromatin structural elements in fertilization and development." *Molecular human reproduction* 16.1 (2010): 30-36.
75. Wei, Yanchang, et al. "Paternally induced transgenerational inheritance of susceptibility to diabetes in mammals." *Proceedings of the National Academy of Sciences* 111.5 (2014): 1873-1878.
76. Wei, Yanchang, HeideSchatten, and Qing-Yuan Sun. "Environmental epigenetic inheritance through gametes and implications for human reproduction." *Human reproduction update* (2014): dmu061.

77. World Health Organization. "WHO laboratory manual for the examination and processing of human semen." (2010).

### **Chapter 3 Intergenerational and transgenerational inheritance of paternal acquired obesity and its associated glomerulopathy and subfertility in a mouse model**

#### **I- Abstract**

The widespread concern about obesity as a medical condition that is exceeding regular dietary measures has stimulated the extension of perspectives of the related research. This is particularly important given that obesity is associated with significant health problem such as development of cardiovascular disease, kidney failure, and infertility. Whereas the impact of epigenetic inheritance of obesity on offspring health is well established, the epigenetic adaptive evolution of HFD has not been investigated. The aim of this study is to determine whether High Fat diet feeding in male mice over several generations impacts the metabolic health of the progeny. For that purpose, *C57BL/6* male mice were fed a high-fat diet (HFD) from weaning for up to 5 months during 5 successive generations. The resulting male offspring were raised either on a control diet (CD6 and CD7) or on a HFD (HFD6 and HFD7).

In effect, there was a gradual increase in the total body weight ( $p < 0.001$ ), in the adipose tissue volume ( $p < 0.001$ ), and in the amount of kilocalories intake ( $p < 0.001$ ) across the HFD generations compared to the controls. In parallel, kidney histological lesions increased progressively from one HFD generation to the other. Thus, when compared to the control group, the seven generations of HFD male mice showed significant increase in kidney's glomerular area/bowman's capsule ratio ( $p < 0.001$ ) and glomerular nuclei ( $p < 0.001$ ), in addition to glomerular extracellular matrix accumulation ( $p < 0.001$ ), and glomerular collagen deposition ( $p < 0.001$ ). Accompanying these histological changes, HFD mice showed a significant increase in water intake ( $p < 0.05$ ), in urine specific gravity ( $p < 0.05$ ), in proteinuria ( $p < 0.01$ ) and ketonuria ( $p < 0.001$ ). In addition, the kidney failure seems to be more severe in the HFD7 compared to the HFD1 group with a significant increase in the proteinuria levels ( $p < 0.05$ ) and in the glomerular fibrosis ( $p < 0.001$ ). Although the CD6 and CD7 mice had never encountered an obesogenic environment, they were heavier ( $p < 0.001$ ), and their kidney glomeruli were smaller ( $p < 0.001$ ) with significant increase in the glomerular collagen deposition ( $p < 0.01$ ) as compared to controls.

The transmission of these health cues through the male line occurred most probably *via* the spermatozoa. At the sperm level, there was a gradual decrease in the percentage of sperm with progressive motility ( $p < 0.001$ ) and with normal morphology ( $p < 0.05$ ) across the HFD generations. At the molecular level, there was a higher histone retention and/or fragmented DNA in the sperm of mice on HFD as compared to controls ( $p < 0.001$ ) and mainly in HFD7 as compared to the HFD1 male mice ( $p < 0.001$ ).

These findings indicated that at each generation the offspring might retain residual effects of the paternal obesity within their cells that could be worsened when they were re-exposed to the same obesogenic environment.

## II- Introduction

The body weight fluctuations may result from an imbalance between the energy intake and the energy expenditure. Obesity is the medical condition related to the increase in the body weight due to excessive accumulation of adipose deposits (Blundell et al. 2000; Proietto et al. 2004). The adipose tissue of obese subjects undergoes pathological remodelling that leads to development of several health problems (e.g., hypertension, type 2 diabetes, kidney disease, and infertility) (Golay et al. 2005; Wozniak et al. 2009; Fulleston et al. 2012). Unfortunately, the prevalence of obesity and its associated health comorbidities are increasing worldwide (Nasreddine et al. et al. 2012; AL-Quwaidhi et al. 2014; Ogden et al. 2014; Vilet-Ostaptchouk et al. 2014) urging the need of innovative medical interventions. Of particular interest, the prevalence of obesity is high among young men of reproductive age. The accumulating epidemiological studies in humans suggest that paternal excessive fatness may influence the health of the subsequent generations (Campbell et al. 2015, Lane et al. 2014).

In order to decipher the mystery behind this kind of inheritance, animal models of diet-induced obesity were developed. It was shown that male rats fed on a high fat diet (HFD), gave birth to female offspring with pancreatic islets dysfunction (Wei et al. 2014) and to male offspring with tubular damage in the kidney (Chowdhury et al. 2016). Moreover, it was demonstrated that paternal acquired obesity in male mice induced metabolic disturbances and reduced the fertility potential in two subsequent generations of mice (Fullston et al. 2013, Fullston et al. 2012). In addition, feeding male mice with a HFD altered the levels of gene expression in the liver of the offspring (Terashima et al. 2015) and potentially increased the susceptibility of malignant transformation in the mammary tissue of the female offspring (Fontelles et al 2016).

The transmission of this paternal acquired pathology occurred most probably *via* the spermatozoa. On one hand, it was demonstrated that the conventional semen parameters and the fertility status could be affected in the rodents on HFD as compared to those on control diet (Mc Pherson, 2014). On the other hand, several authors highlighted the impact of paternal acquired obesity on the molecular composition of the mice spermatozoa. For instance, sperm RNAs content was found to be altered in obese mice as compared to non-obese ones (Fullston et al. 2013, Binder et al. 2015, and Grandjean et al. 2015). Interestingly, microinjection of testis RNAs from the obese mice to normal zygotes induced metabolic alterations in the offspring, indicating a possible role of sperm RNAs in the transmission of this paternal acquired phenotype (Grandjean et al. 2015). Compared to the sperm of normal mice, higher sperm histone 3 (H3) retention was found on the genes involved in cell fate decision and development in obese mice (Terashima et al. 2015). Additionally, differentially methylated regions of sperm DNA were found in obese and diabetic rodents as compared to normal ones (Ng et al. 2010).

Furthermore, eating habits such as feeding a high fat diet (HFD) can also be inherited across generations (Wahlqvist et al. 2014, Fullston et al. 2015). Using animal models, it was demonstrated that kidney failure could develop in the offspring sired by malnourished mothers

and this phenotype could be aggravated when the offspring were also reared on an unhealthy diet (Lloyd, 2012; Cervantes-Rodriguez, 2014). Also, the male offspring derived from an obese father had an exacerbated metabolic and sperm disturbances when they were re-exposed to the same paternal HFD (Fullston, 2015). Our proposal aims to evaluate the adaptive and evolutionary potential of non-genetic heritable mechanisms in experimentally controlled animal models. Using a high fat diet (HFD)-induced obesity mouse model, we have examined how feeding male mice with a high fat diet for multiple generations impacts the phenotype of the resulting mice. Moreover, we examined whether the accumulation of epigenetic marks with an history of multiple induction could be maintained without the inducer (here the High Fat Diet). In this study, we will focus on the evolution of obesity-associated complications such as glomerulopathy and subfertility.

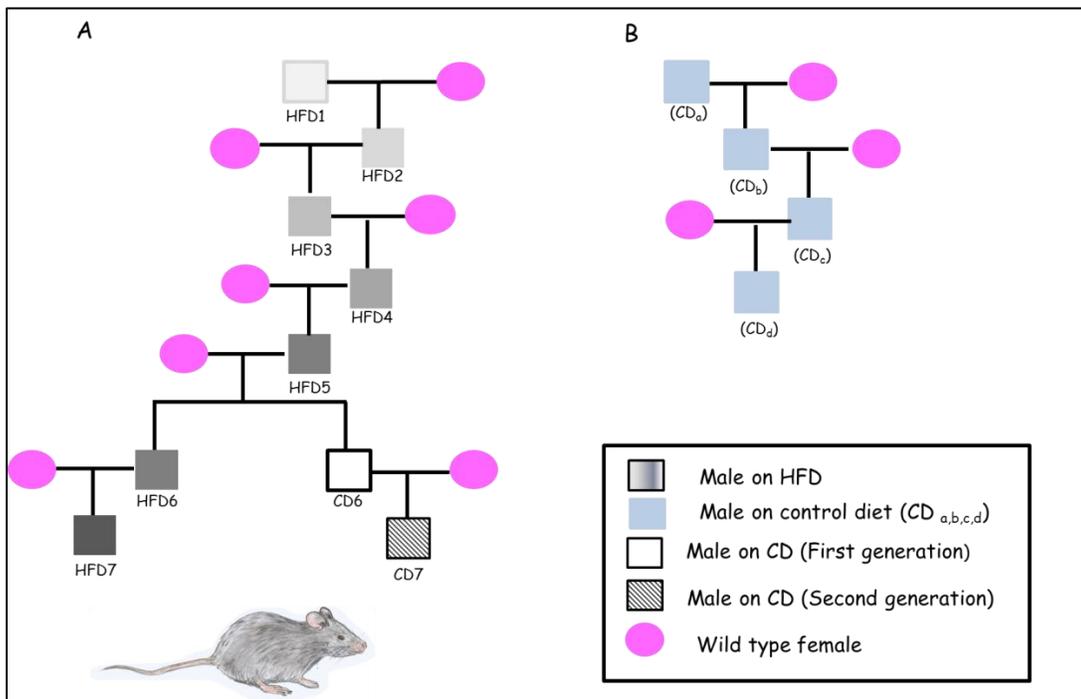
### III- Materials and methods

#### a) Animals and experimental design

All the experiments were conducted in compliance with the relevant institutional and French animal welfare laws, guidelines, and policies. All mice (*C57BL/6* background) had free access to food and water. They were maintained at 24<sup>0</sup>C on a 14-h light, 10-h dark illumination cycle. The male mice were divided into two groups (**Figure 1**):

- The first group (**Figure 1A**): male mice were fed a high-fat diet (HFD)(SAFE-U8954 version 35(4.035kcal/g metabolizable energy))from weaning for up to 5 months during 5 successive generations. The resulting offspring were raised either on a HFD (HFD 6 and HFD 7) or on a control diet (SAFE-A04chow diet(2.791kcal/g metabolizable energy)) (CD6 and CD7). The diets were supplied by SAFE (scientific Animal Food and Engineering, Villmoisson-sur-orge, France).
- The second group (**Figure 1B**): male mice were maintained on a control diet (CD) from 3 weeks old up to 5 months during several successive generations (CD<sub>a, b, c, d</sub>).
- All the males were mated with normal females sired by normal fathers both reared on control diet.

All assessments of sperm function and kidney histological analysis were performed blinded to the diet group. Also, they were performed by the same person throughout the study.



**Figure 46: Two pedigrees showing the high fat diet (HFD) (A) and the control diet (CD a, b, c, d) generations (B).**

### **b) Body measurements**

Body weight for individual males and the amount of remaining food were measured weekly. At the completion of 16-week-period, mice were anesthetised and scanned using a SkyScan- 1178 X-ray micro-CT system. The same scanning parameters were used for all mice: 104  $\mu$ m of voxel size, 49 kV, 0.5 mm thick aluminum filter, 0.9° of rotation step. The analysis of adipose tissue volumes was performed using NRecon and CTAn software (Skyscan)(Beranger et al. 2014). Moreover, the body composition was assessed at euthanasia by measuring all organs weights.

### **c) Metabolic cage experiments**

Each male mouse was placed in a metabolic cage (Tecniplast, Hohenpeissenberg, Germany) with free access to water and food (Control (n=21), HFD1 (n=12), HFD4 (n=7), HFD5 (n=6), HFD6 (n=11), HFD7 (n=9), CD6 (n=11), and CD7 (n=9)). After 12h, the excreted urine was collected for urinalysis. The latter was performed using urine dipstick (Combur 10 test, Roche Diagnostics Corporation) and the standard colorimetric assay was performed according to the manufacturer's instructions (Sison et al. 2010).

### **d) Renal histology and special stains**

Freshly dissected kidneys were washed in PBS 1X, fixed in formalin for 30 min at 4°C, embedded in paraffin, and sectioned at 4 $\mu$ m (Sison et al. 2010). The histological evaluation of kidney glomeruli was performed using the periodic-acid-Schiff (PAS) stain. It is a special stain where the glycol groups of the polysaccharides, mucins, and mucosubstances are oxidized by the periodic acid (1%) to form aldehydes. The oxidized carbohydrates are then combined with Schiff's reagent to form a colored ionic structure termed as magenta. The PAS is recommended for renal biopsies diagnosis due to basement membrane and mesangial matrix staining. Briefly, rehydrated tissue is incubated in periodic acid (1%) solution for 5 min, washed by tap water, and then incubated for 30 min with Schiff's reagent (Kiernan et al. 2010). Moreover, picrosirius red staining was performed to evaluate the amount of collagen deposition in the glomeruli. Sirius red is a strongly acidic dye which strongly reacts with the highly basic collagen molecules. Shortly, rehydrated tissue is stained in weigert's hematoxylin for 8 min, and then stained with the picrosirius red stain for one hour and washed by tap water (Kumar et al. 2010).

### **e) Quantification of renal pathology**

Renal histopathology quantifications were performed using the ImageJ software. From each mouse (5-6 mice/generation), 30 glomeruli cut at their vascular poles were selected for the morphometrical analysis (Deji et al. 2008). The glomerular area was selected using the freehand tool from the ImageJ menu bar and the size of the selected area was obtained by selecting the analyse/measure icons. Similar protocol was used to determine the Bowman's capsule size. Moreover, the number of nuclei inside the glomeruli was obtained *via* an ImageJ plug-in (cell\_counter.jar) which counted nuclei as they were manually tagged on the image. Furthermore, the assessment of the stained areas (PAS or Sirius red staining) was possible *via* the selection of Image/type/8-bit followed by the selection of Image/adjust/threshold and then the calculation of the area percentage (Rangan et al. 2007).

## **Semen analysis**

### **f) Assessment of sperm count and motility**

At 16 weeks of age, the vas deferens and caudal epididymis were collected into 1ml pre-warmed G-MOPS medium (Vitrolife, AB, Gothenburg, Sweden). Spermatozoa were allowed to swim-out from the punctured vas deferens and caudal epididymis for 10 min at 37 °C before any assessment. Sperm concentration was determined according to the world health organization manual for the examination of human semen (WHO 2010). In addition, sperm motility was determined from duplicate measures of 200 spermatozoa under light microscopy using 40x magnification and expressed as a percent of motile sperm for each sample (Fullston et al. 2015).

### **g) Evaluation of sperm morphology**

From the sperm suspended in the G-MOPS medium, 10 µl was used to prepare a smear for the morphological examination of mouse sperm. The slides were air dried and then fixed in a methanol-acetone (3:1) solution for 1 min. Next, the slides were stained with hematoxylin and eosin for few seconds at room temperature. For each mouse, at least 400 spermatozoa were examined under light microscopy using 100x magnification. The cells were classified as normal or abnormal (hook less, banana-like, amorphous, folded, short tail, two tail and two head) morphology (Khan et al. 2015; McPherson et al. 2016).

### **h) Sperm chromatin integrity**

To determine the status of sperm chromatin integrity the toluidine blue test was performed. Toluidine blue is a basic dye that shifts from blue (TB-) to dark blue or violet (TB+) upon binding to sperm histones and/or DNA phosphate residues (Tsarev et al. 2009). Spermatozoa were smeared, air dried, and then fixed in ethanol-acetone (1:1) for half an hour. The smears were then hydrolyzed in HCl (0.1%) for 5 minutes followed by wash in distilled water (3 times: 2 min each). Next, they were stained in toluidine blue 0.05% (prepared with McIlvaine buffer) for 5 min and finally washed with tap water. At least 400 spermatozoa were counted under light microscopy 100x under immersion oil (Pourentezar et al. 2014).

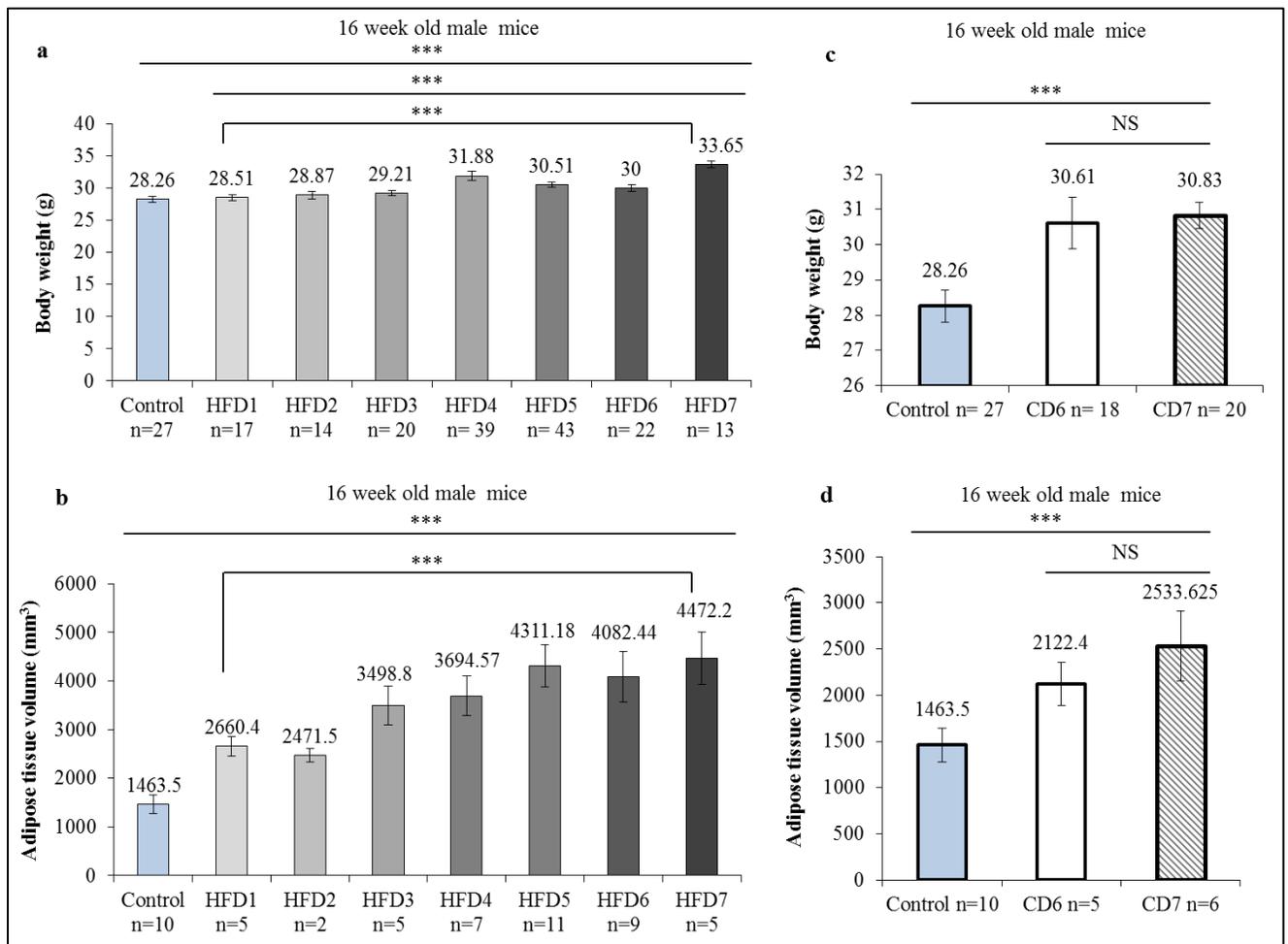
### **i) Statistical analysis**

All the data were statistically analyzed and graphically represented using Microsoft Office Excel software. The error bars represent the standard error of the mean (SEM). Two-tailed Student's t-test was used for the analysis of statistical significance between two groups. Also, one-way ANOVA was used for the analysis of statistical significance between several groups. A p value <0.05 was considered statistically significant.

## IV- Results

### 1- Exacerbation of the overweight phenotype and the adipose tissue volume after seven successive generations of HFD feeding

Since no significant differences was observed in the basic physiological parameters among male mice on control diet (CD<sub>a, b, c, d</sub>) (Figure 1B), they were combined into one control group. All the HFD generations fed the same “western-like diet”; however, the body weight and the adipose tissue volume were gradually increased in the male offspring across generations under continuous HFD stress ( $p < 0.001$ ) (Figure 2a, 2b). In addition, the HFD7 group had a significant heavier body weight and larger adipose tissue when compared to the HFD1 group at 16 weeks of age ( $p < 0.001$ ) (Figure 2a, 2b).



**Figure 47: The body weight and the adipose tissue volume of the different HFD and CD groups.** (a) The body weight of the HFD groups. (b) The volume of the adipose tissue of the seven high fat diet generations. (c) The body weight of the CD6 and CD7 males. (d) The adipose tissue volume of the CD6 and CD7 males. Data is expressed as mean  $\pm$  SEM (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

## 2- Feeding five successive generations of male mice with a HFD negatively impacted the body weight and the adipose tissue volume of the CD6 and the CD7 males

The CD6 and CD7 males were on a control diet and they had never encountered an obesogenic environment (**Figure 1A**). Interestingly, a male line exposed during five successive generations to a HFD significantly increased the body weight and the adipose tissue volume in the CD6 males ( $p<0.001$ ) (**Figure 2c, 2d**). Moreover, the CD7 males generated from unexposed gametes of the CD6 had a heavier body weight and larger adipose tissue when compared to the control group ( $p<0.001$ ) (**Figure 2c, 2d**). There was no statistically significant difference between the CD6 and CD7 males.

## 3- Progressive alterations in the body composition after seven successive generations of HFD feeding

In parallel to the increase in the fatness, there was a significant gradual increase in the ratio of gonadal fat pad/the total body weight across the HFD generations ( $p<0.01$ ) (**Table 1**). In addition, the ratio of the liver weight/total body weight became higher at each generation ( $p<0.001$ ). The latter was significantly elevated in the HFD7 group when compared to the HFD1 one ( $P<0.001$ ) (**Table 1**). There was no statistically significant difference in the ratio of the kidney/body weight and testis/body weight among the HFD generations.

**Table 1: Gross anatomy of the different HFD groups**

	Control (n=27)	HFD1 (n=17)	HFD2 (n=14)	HFD3 (n=30)	HFD4 (n=39)	HFD5 (n=43)	HFD6 (n=22)	HFD7 (n=13)
Ratio gonadal fat pad/ total body weight	0.01 ±0.001	0.03 ±0.004 <sup>c,e</sup>	0.03 ±0.002 <sup>c,e</sup>	0.03 ±0.001 <sup>c,e</sup>	0.04 ±0.002 <sup>c,e</sup>	0.04 ±0.001 <sup>c,e</sup>	0.03 ±0.002 <sup>c,e</sup>	0.04 ±0.001 <sup>c,e</sup>
Ratio Kidney/ total body weight	0.012 ± 0.0002	0.014 ± 0.0005	0.014 ± 0.0004	0.014 ± 0.0004	0.0136 ± 0.0004	0.014 ± 0.0004	0.014 ± 0.0003	0.013 ± 0.0005
Ratio testis/ total body weight	0.0041 ± 0.0003	0.006 ± 0.002	0.004 ± 0.0002	0.0039 ± 0.0002	0.0037 ± 0.0000	0.004 ± 0.0001	0.0038 ± 0.0001	0.0032 ± 0.0001
Ratio liver/body weight	0.050 ± 0.00099	0.052 ± 0.001 <sup>c,f</sup>	0.052 ± 0.001 <sup>c,f</sup>	0.045 ± 0.002 <sup>c,f</sup>	0.057 ± 0.0015 <sup>c,f</sup>	0.056 ± 0.002 <sup>c,f</sup>	0.053 ± 0.0012 <sup>c,f</sup>	0.058 ± 0.0018 <sup>c,f,g</sup>

Data expressed as mean ± SEM. Superscripts denote statistically significant differences: a ( $p<0.05$ ), b ( $p<0.01$ ), c ( $p<0.001$ ) when the HFD groups were compared to the control one; d ( $p<0.05$ ), e ( $p<0.01$ ), f ( $p<0.001$ ) when the HFD groups were compared among each others; g ( $p<0.05$ ), h ( $p<0.01$ ), i ( $p<0.001$ ) when the HFD7 generation was compared to the HFD1 generation.

#### 4- The drawbacks of HFD feeding during 5 generations on the body composition of the CD6 and CD7 males

The control group, the CD6, and the CD7 fed the same control diet but they had different body composition. The ratio of the gonadal fat pad/body weight was significantly higher in the CD6 and CD7 males as compared to the control group ( $p < 0.001$ ) (**Table 2**).

Furthermore, the kidney/body weight ratio was lower in the CD7 when compared to the control group ( $p < 0.01$ ) and to the CD6 ( $p < 0.01$ ) (**Table 2**). However, there was no statistically significant difference in the ratio of testis/total body weight and of the liver/total body weight between groups (**Table 2**).

**Table 2: Gross anatomy of the different CD groups.**

	Control (n=27)	CD6 (n=18)	CD7 (n=20)
Ratio gonadal fat pad/ total body weight	0.01 ± 0.001	0.019 ± 0.002 <sup>c</sup>	0.020 ± 0.001 <sup>c</sup>
Ratio Kidney/ total body weight	0.012 ± 0.0002	0.012 ± 0.0003 <sup>b</sup>	0.011 ± 0.0002 <sup>b,e</sup>
Ratio testis/ total body weight	0.0041 ± 0.0003	0.0039 ± 0.0002	0.0034 ± 0.0001
Ratio liver/body weight	0.050 ± 0.00099	0.050 ± 0.001	0.049 ± 0.001

Data expressed as mean ± SEM. Superscripts denote statistically significant differences: a ( $p < 0.05$ ), b ( $p < 0.01$ ), c ( $p < 0.001$ ) when the CD6 and CD7 groups were compared to the control one; d ( $p < 0.05$ ), e ( $p < 0.01$ ), f ( $p < 0.001$ ) when the CD6 generation was compared to the CD7 one.

#### 5- The amount of food intake in the different HFD and CD groups

The experimental groups (HFD 1 to 7) experienced a significant increase in the daily consumed kilocalories (Kcal) when compared to that of the control group ( $p < 0.001$ ). Also, the amount of food intake was different between the experimental groups: for instance it was significantly higher in the HFD7 than HFD1 (**Table 3**).

Remarkably, the CD7 male mice had a higher mean of daily consumed food than the control group ( $p < 0.001$ ). Compared to the CD6, the CD7 males consumed a higher amount of kilocalories ( $p < 0.001$ )(**Table 4**).

**Table 3: Calories intake, water balance, and kidney physiology of different HFD groups**

		Control	HFD1	HFD4	HFD5	HFD6	HFD7
<b>Food intake</b>	<b>Daily diet consumption ( Kcal)</b>	10.31 ±0.04	11.17 ±0.19 <sup>c,f</sup>	12.57 ±0.17 <sup>c,f</sup>	11.42 ±0.12 <sup>c,f</sup>	13.28 ±0.17 <sup>c,f</sup>	12.19 ±0.19 <sup>c,f,g</sup>
<b>Water balance</b>	<b>Water intake ( ml)</b>	2.54 ± 0.13	2.041 ± 0.13 <sup>c,f</sup>	2.92 ± 0.22 <sup>c,f</sup>	3.41 ± 0.21 <sup>c,f</sup>	4.7 ± 0.24 <sup>c,f</sup>	3.33 ± 0.17 <sup>c,f,g</sup>
	<b>Urine output ( ml)</b>	0.74 ± 0.08	0.71 ± 0.07	0.78 ± 0.11	0.98 ± 0.09	0.86 ± 0.12	0.94 ± 0.13
<b>Urine Biochemistry</b>	<b>specific gravity (g/mL)</b>	1.015 ± 0.0008	1.018 ± 0.0008 <sup>a</sup>	1.017 ±0.001 <sup>a</sup>	1.016 ± 0.001 <sup>a</sup>	1.019 ± 0.0005 <sup>a</sup>	1.017 ± 0.0008 <sup>a</sup>
	<b>pH</b>	5.6 ± 0.102	5 ± 0 <sup>c</sup>	5 ± 0 <sup>c</sup>	5 ± 0 <sup>c</sup>	5 ± 0 <sup>c</sup>	5 ± 0 <sup>c</sup>
	<b>Protein (mg/dl)</b>	0.61 ± 0.105	1 ± 0 <sup>c</sup>	1.28 ± 0.17 <sup>c</sup>	1.16 ± 0.15 <sup>c</sup>	1 ± 0 <sup>c</sup>	1.33 ± 0.15 <sup>c,g</sup>
	<b>Glucose (mg/dl)</b>	0 ± 0	0.08 ± 0.06	0.14 ± 0.08	0.16 ± 0.09	0 ± 0	0.22 ± 0.1
	<b>Ketones (mg/dl)</b>	0.125 ± 0.08	1 ± 0 <sup>c</sup>	1 ± 0 <sup>c</sup>	1 ± 0 <sup>c</sup>	1 ± 0 <sup>c</sup>	1 ± 0 <sup>c</sup>

Data expressed as mean ± SEM. Proteinuria, glucosuria, and Ketonuria were assessed by the urine dipsticks ((protein: 0= negative, 1= 30 mg/dl, 2= 100 mg/dl), (Glucose: 0= normal, 1= 50 mg/dl, 2= 100 mg/dl), and (ketone: 0= negative, 1= 1+, 2= 2+)).

Superscripts denote statistically significant differences: a (p<0.05), b (p<0.01), c (p<0.001) when the HFD groups were compared to the control one; d (p<0.05), e(p<0.01), f (p<0.001) when the HFD groups were compared between them; g (p<0.05), h (p<0.01), i (p<0.001) when the HFD7 generation was compared to the HFD1 generation.

**Table 4: Calories intake, water balance, and kidney physiology of different CD groups**

		Control	CD6	CD7
Food intake	Daily diet consumption ( Kcal)	10.31 ±0.04	10.21 ±0.26	11.55 ±0.19 <sup>c,f</sup>
Water balance	Water intake (ml)	2.54 ± 0.13	2.041 ± 0.32 <sup>b</sup>	2.92 ± 0.28 <sup>b</sup>
	Urine output (ml )	0.74 ± 0.08	0.71 ± 0.07 <sup>b</sup>	0.78 ± 0.12 <sup>b,e</sup>
Urine Biochemistry	Urine osmolality (g/mL)	1.015 ± 0.0008	1.018 ± 0.001	1.017 ±0.0008
	pH	5.6 ± 0.102	5.84 ± 0.18	5.44 ± 0.16
	Protein (mg/dl)	0.61 ± 0.105	1 ± 0 <sup>b</sup>	1 ± 0 <sup>b</sup>
	Glucose (mg/dl)	0 ± 0	0 ± 0	0 ± 0
	Ketones (mg/dl)	0.125 ± 0.34	1 ± 0 <sup>c</sup>	1 ± 0 <sup>c</sup>

Data expressed as mean ± SEM. Proteinuria, glucosuria, and Ketonuria were assessed by the urine dipsticks ((protein: 0= negative, 1= 30 mg/dl, 2= 100 mg/dl), (Glucose: 0= normal, 1= 50 mg/dl, 2= 100 mg/dl), and (ketone: 0= negative, 1= 1+, 2= 2+)). Superscripts denote statistically significant differences: a (p<0.05), b (p<0.01), c (p<0.001) when the CD6 and CD7 groups were compared to the control one; d (p<0.05), e (p<0.01), f (p<0.001) when the CD6 generation was compared to the CD7 one.

#### 6- The effect of the exposure to the same obesogenic environment during seven successive generations on the water balance

The seven groups of HFD had no statistically significant difference in the amount of urine output, but they had a significant increase in the water intake compared to the control group (P<0.001). Additionally, the amount of consumed water was not similar between the HFD generations. The HFD7 group had a significant increase in the water uptake than the HFD1 male mice (p<0.05) (**Table 3**). In contrast, feeding five successive generations of male mice with a HFD increased the water intake and urine output in the CD6 male offspring (p<0.01). Moreover, this phenotype was transmitted to the CD7 male mice. Interestingly, the volume of urine output was significantly higher in CD7 group compared to CD6 one (p<0.01) (**Table 4**).

## **7- Physiological adaptation of the kidney to the continuous HFD stress during several generations**

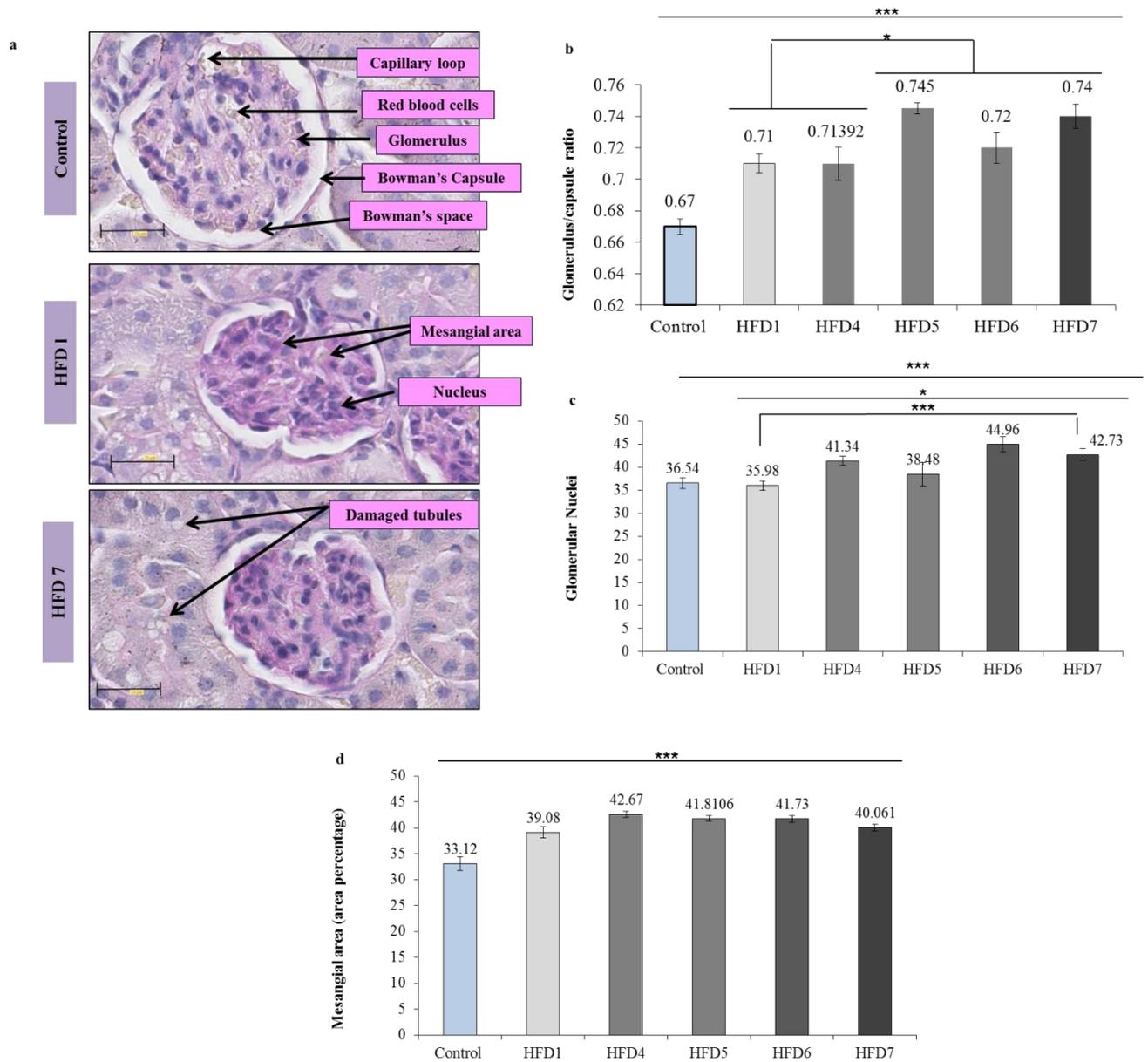
The urinalysis of the HFD groups showed a statistically significant increase in the specific gravity ( $p < 0.05$ ), in the ketonuria ( $p < 0.001$ ), in the proteinuria ( $p < 0.001$ ), and in the urine acidity ( $p < 0.001$ ) when compared to the control group. Also, the HFD7 generation exhibited significant increase in the proteinuria than the HFD1 male offspring ( $p < 0.05$ ). However, no statistically significant difference was detected in the glucosuria among the generations of male mice (**Table 3**).

Although the CD6 and CD7 generations were maintained on a control diet, they had a significant higher level of proteinuria ( $p < 0.01$ ) and ketonuria ( $p < 0.001$ ) than the control group. The urine specific gravity and the glucosuria were not different between the CD6, CD7, and the control mice (**Table 4**).

## **8- The effect of exposure to HFD during successive generations on glomerular histology**

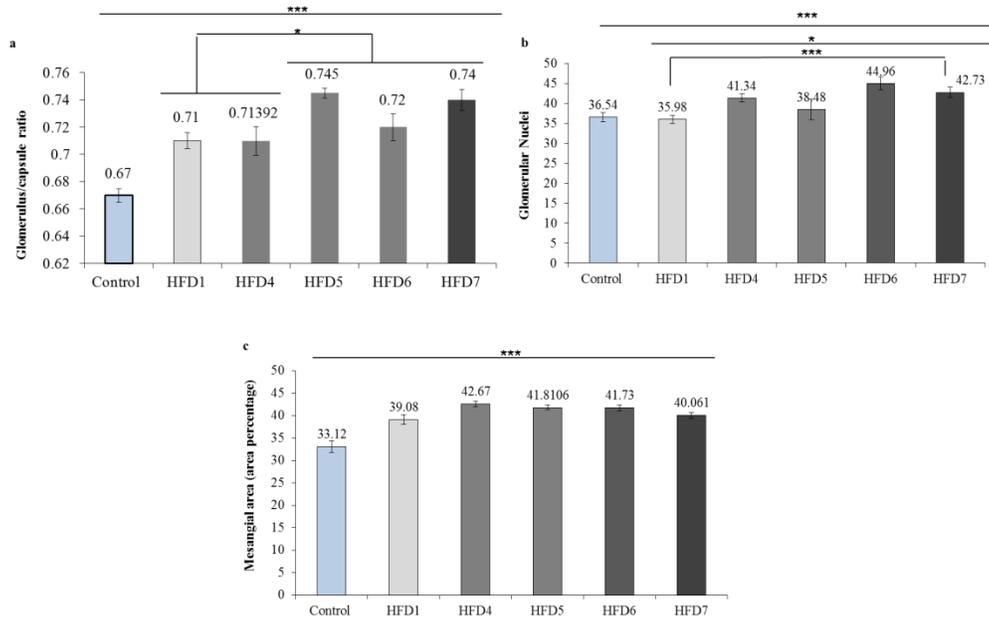
Nephron is the functional autonomous unit of the kidney which is made up of a glomerulus containing capillaries, podocytes, mesangial cells, and extracellular matrix. Moreover, the glomerulus is surrounded by the capsule of Bowman and it is connected to the nephron tubules which serve in urine filtration (**Figure 3a**) (**Berridge et al. 2012**). Our results showed that at week 16 the HFD groups demonstrated an overall increase in the ratio of the glomerulus over the Bowman's capsule as compared to the control group ( $p < 0.001$ ) (**Figure 3b**). In addition, the HFD5, HFD6, and HFD7 had a statistically significant increase in the glomerulus/capsule ratio in comparison to HFD1 and HFD4 ( $p < 0.05$ ) (**Figure 3b**). The changes in this ratio were contributed by an accumulation of Periodic-Acid-Schiff (PAS)-positive matrix in the mesangium ( $p < 0.001$ ) and an increase of nuclei number ( $p < 0.001$ ) in the HFD generations as compared to the control group (**Figure 3b, 3c**). Interestingly, the nuclei number was also different between the HFD groups ( $p < 0.05$ ) and was significantly increased in HFD 7 when compared to that of the HFD 1 ( $p < 0.001$ ) (**Figure 3b**). Furthermore, there was an important increase in the glomerular matrix because the collagen fibres were significantly increased in the glomeruli of the HFD generations compared to the control group ( $p < 0.001$ ) and in the HFD 7 and HFD 6 compared to the HFD 1, HFD4, and HFD5 ( $p < 0.001$ ) (**Figure 5a, 5b**).

Fascinatingly, the glomerulus/capsule ratio in the CD6 and CD7 male mice was significantly higher rather than that in the mice of the control group ( $p < 0.001$ ) (**Figure 4a**). Also, the mesangial matrix area was significantly larger in the CD6 and CD7 males compared to the controls ( $p < 0.001$ ) (**Figure 4c**). The mesangial expansion was accompanied by an increase in collagen deposition in the extracellular matrix of the CD6 and CD7 glomeruli compared to the control group ( $p < 0.01$ ) (**Figure 5c**). However, the number of glomerular nuclei was not statistically different between the controls, CD6, and CD7 ( $p > 0.05$ ) (**Figure 4b**).

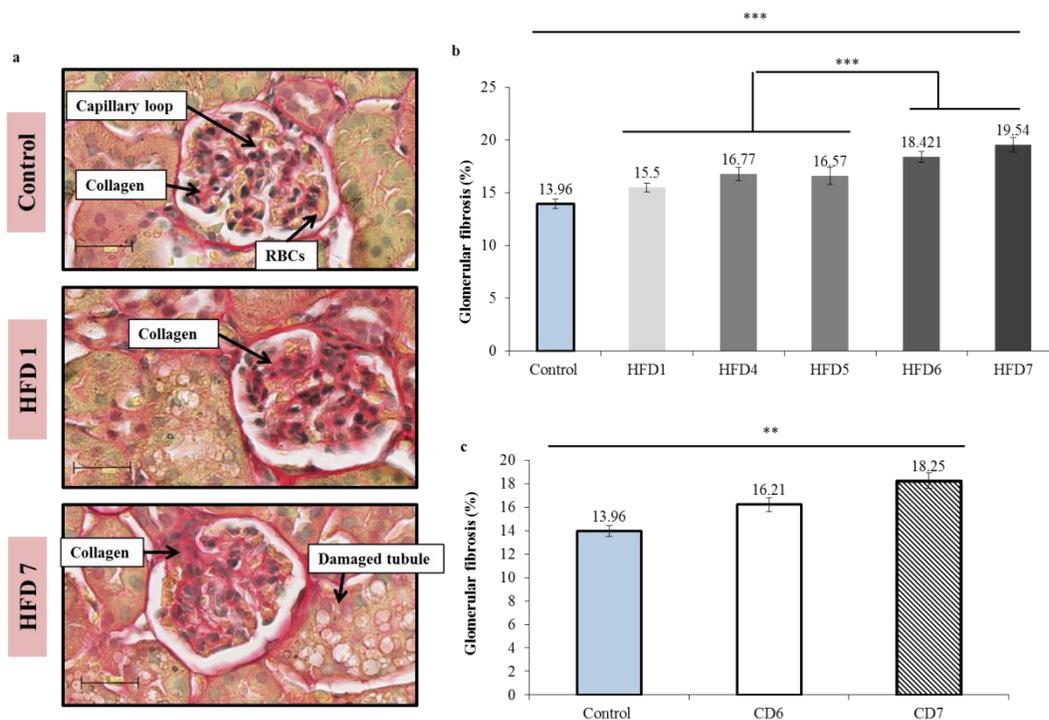


**Figure 48: Feeding male mice with a HFD during seven generations alters the glomerulus/capsule ratio and increases the matrix accumulation.**

(a) Periodic-acid-Schiff staining on kidney sections from mice on control diet, HFD1, and HFD7 at 16 weeks. The arrows indicate the different histological structure of the glomerulus. Scale bar = 25  $\mu$ m, original magnification x80. Quantitative analysis of the glomeruli (n=30 glomeruli/mice and 5 mice/group) demonstrates an increase in the glomerulus/corpuscle ratio (b), in the glomerular nuclei (c), and in the mesangial area expansion (d). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.



**Figure 4: Quantitative analysis of the glomeruli in CD6 and CD7 males compared to the controls at 16 weeks.** These graphs demonstrate an increase in the glomerulus/capsule ratio (a) and in the mesangial expansion of CD6 and CD7 compared to controls (c). However, the number of glomerular nuclei is not statistically different between groups (b). \*\*\* $p < 0.01$ , NS= no significance.



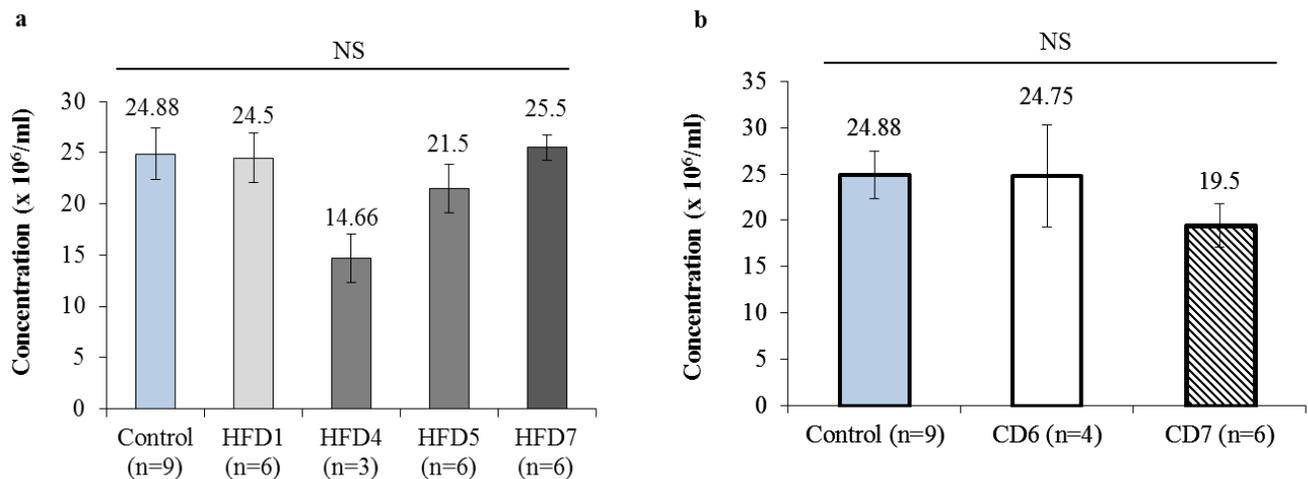
**Figure 49: Pathological alterations in the kidney of mice on HFD (1-7) and control diet (CD6 and CD7) at 16 weeks.**

(a) Picrosirius red staining on kidney sections from control, HFD1, and HFD7 mice. Scale bar = 25  $\mu$ m, original magnification x80. Quantitative analysis for glomerular fibrosis in the HFD generations (b) and the CD generations (CD6, CD7) (c). \* $p < 0.05$ , \*\*\* $p < 0.01$ , \*\*\*\* $p < 0.001$ .

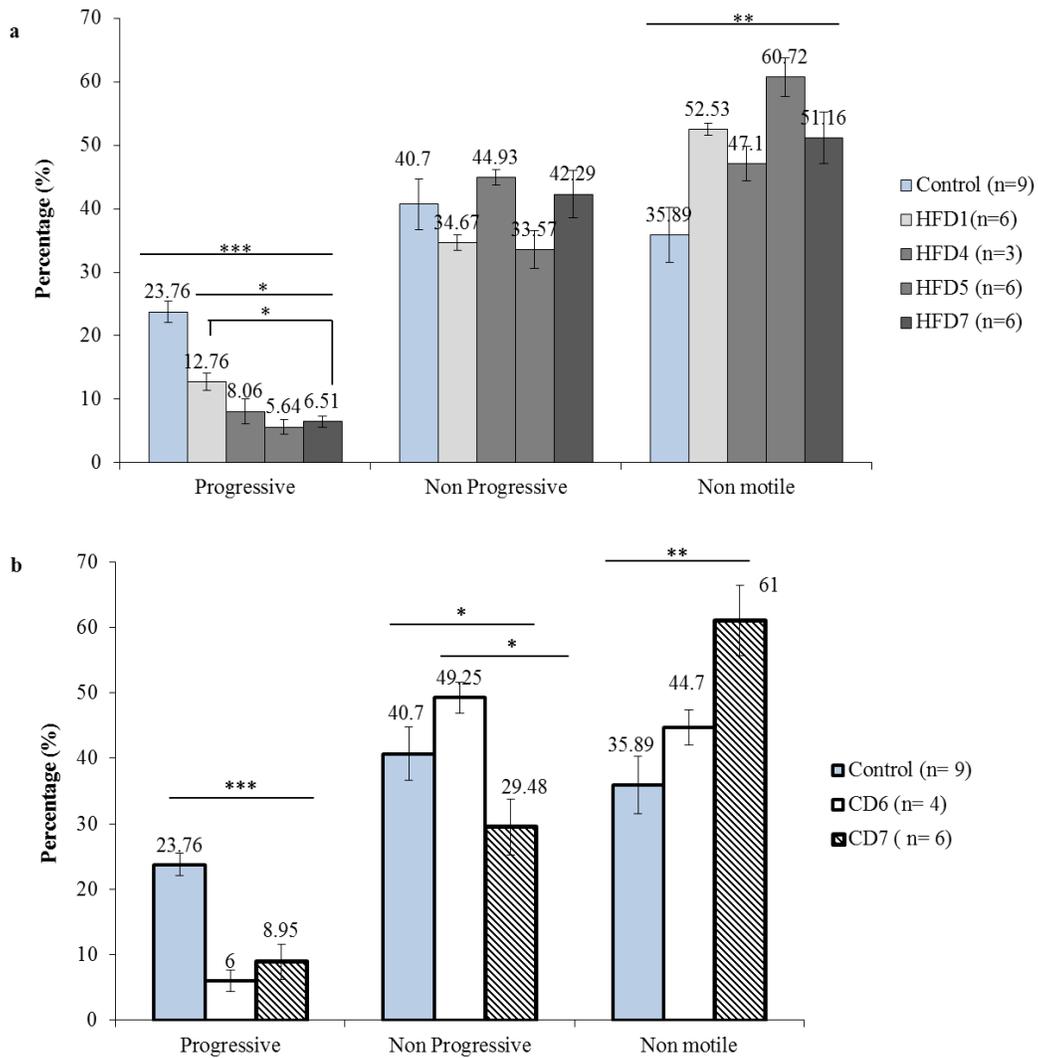
**9- The impact of the continuous exposure to a HFD stress during several generations on the conventional semen parameters**

No significant difference was found in relation to sperm concentration between the different HFD and control diet groups (control, CD6, CD7) (**Figure 6a, 6b**). In contrast, the percentage of progressive motile spermatozoa was significantly decreased in the HFD groups compared with controls ( $p < 0.001$ ), and in the HFD7 compared with HFD1 ( $p < 0.01$ ) (**Figure 7a**). Moreover, HFD feeding during successive generations had significantly reduced the percentage of progressive motile spermatozoa in the CD6 and CD7 male offspring ( $p < 0.001$ ). In addition, it had significantly increased the percentage of non-motile spermatozoa in the CD6 and CD7 generations compared with offspring sired by control diet founder males ( $p < 0.01$ ) (**Figure 7b**).

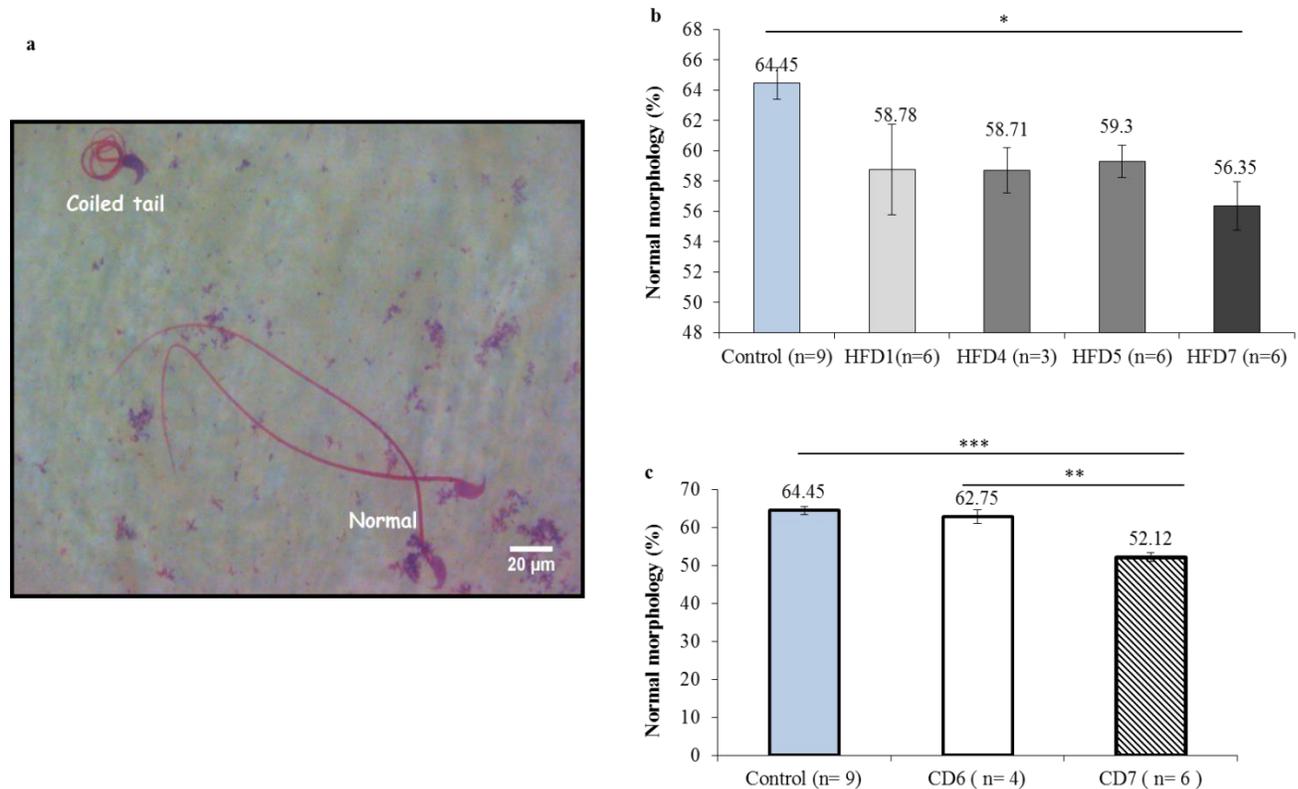
Furthermore, the sperm morphology was assessed using haematoxylin and eosin staining. Normal mice sperm is made up of a head with a definite shape characterized by a marked hook, a rectangular mid-piece, and single unfolded tail (Mosuro et al. 2007)(**Figure 8a**). The percentage of normal sperm morphology was significantly lower in the HFD groups when compared to controls ( $p < 0.05$ ) (**Figure 8b**). Plus, the CD7 male mice generated from unexposed gametes had a significant lower percentage of normal morphology compared with CD6 ( $p < 0.01$ ) and with control group ( $p < 0.001$ ) (**figure 8c**).



**Figure 50: Graphs showing the sperm concentrations of the different HFD (a) and CD (b) generations. Data expressed as mean  $\pm$  SEM. NS= No statistically significant difference was detected between groups.**



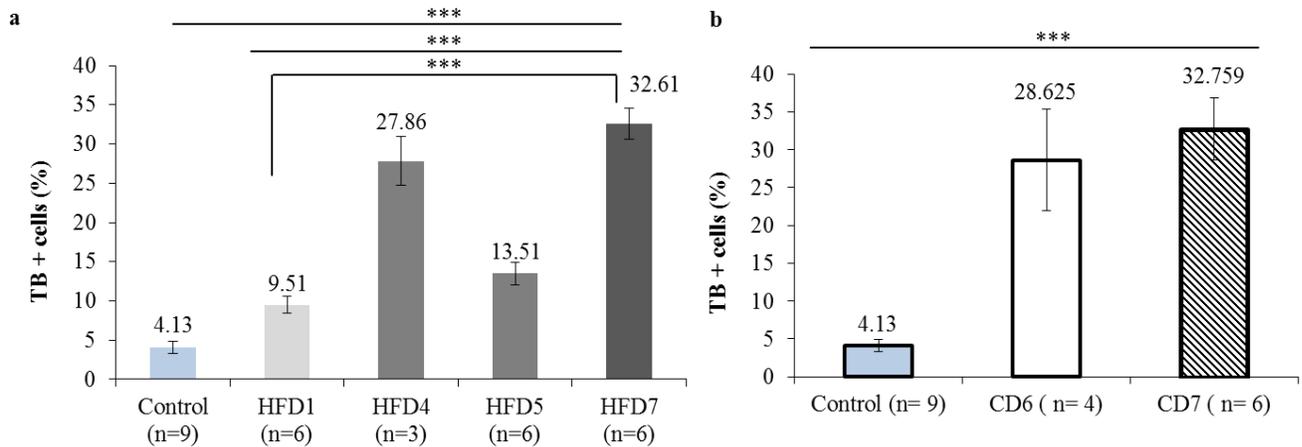
**Figure 51: Graphs showing the percentage of spermatozoa with progressive motility, non-progressive motility, and the non-motile ones in the different HFD (a) and CD (b) generations. Data expressed as mean  $\pm$  SEM (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001).**



**Figure 52: The normal sperm morphology percentage in the different HFD and CD groups.** Figure illustrating the normal and abnormal mice sperm morphology (a). Two graphs showing the percentage of spermatozoa with normal morphology in the different experimental groups (b, c). Data expressed as mean  $\pm$  SEM (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001). Scale bar= 20 $\mu$ m. Magnification x100.

### 10- The changes in the sperm chromatin integrity across HFD and CD generations

Toluidine blue (TB) is a basic dye that stains phosphate residues of sperm DNA with fragmented ends and attaches with Lysine-rich histones, so it determines both DNA fragmentation and chromatin packaging at the same time. When it binds to the substrate, the TB shifts from blue (TB-) to dark blue or violet (TB+) (Tsarev et al. 2009). On one hand, the percentage of TB+ cells was significantly increased in the HFD generations compared with the controls ( $p$ <0.001). On the other hand, the level of TB+ cells had gradually increased across the HFD generations ( $p$ <0.001) with an exception in the HFD4. The result of the HFD4 could be caused by the small sample size in this group (Figure 9a). Moreover, the CD6 and CD7 male mice had statistically significant increased levels of TB+ cells ( $p$ <0.001) compared to the control group (Figure 9b).



**Figure 53: The mean percentage of TB positive cells in the different HFD (a) and CD groups (b). Data expressed as mean  $\pm$  SEM (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001).**

## V- Discussion

Food choices and eating habits could be shared between elders and children. Therefore, exposure to an obesogenic environment could be intergenerationally inherited (Wahlqvist et al. 2014). In animal models, it was demonstrated that maternal malnutrition may increase the risk of obesity and kidney failure in the next generation. Additionally, these health problems were worsened when the offspring were also exposed to an unhealthy diet later in life (Lloyd et al. 2012; Cervantes-Rodriguez et al. 2014). This finding was termed as “second-hit phenomenon”. In parallel, Fullston and colleagues showed that metabolic and sperm disturbances of the male mice sired by an obese father were exacerbated when the offspring also fed a HFD (Fullston et al. 2015). Here we aimed to assess the impact of continuous exposure of male mice during seven generations to a HFD stress on the phenotype of the resulting mice. We focused mainly on the evolution of the glomerulopathy and the fertility status.

Despite their genetic similarity, the seven generations of male mice which were reared on a HFD and sired by fathers that were also on a HFD had different phenotypes at each generation. Our results showed that there was a gradual increase in the total body weight ( $p$ <0.001), in the ratio of gonadal fat pad/total body weight ( $p$ <0.01), and in the ratio of the liver weight/total body weight ( $p$ <0.001) across the HFD generations. Compared to the HFD1, the HFD7 male mice had the poorest outcomes in the total body weight ( $p$ <0.001), in the adipose tissue volume ( $p$ <0.001), and in the liver weight/total body weight ( $p$ <0.05). These findings indicated that at each generation the offspring might retain residual effects of the paternal nutritional imbalance within their cells that could be worsened when they were re-exposed to the same obesogenic environment.

In parallel, the analysis of the feeding behavior showed a significant increase in the daily diet consumption in the HFD generations when compared to the control group ( $p < 0.001$ ). Also, there was a statistically significant increase in the amount of food intake among the different HFD groups ( $p < 0.001$ ) and specifically in the HFD7 compared to the HFD1 males ( $p < 0.05$ ). It was demonstrated in mice that offspring derived from an obese mother were hyperphagic compared to those sired by a normal weight mother (Samuelsson et al. 2008). However, the impact of paternal obesity on the control of food intake in the offspring was underestimated in the literature. Our findings were in line with the results of a recent study performed on *Drosophila melanogaster* where the authors showed that paternal obesity increased the food intake in the offspring (Ost et al. 2014).

Furthermore, it was confirmed in several studies that feeding *C57BL/6* mice with a HFD induced obesity and helped in the development of its associated complications such as hypertension and type 2 diabetes (Harris et al. 2014). The kidneys of these mice presented an abnormal lipid accumulation, glomerular lesions and fibrosis, impaired sodium handling, and increased urinary albumin excretion. Moreover, the mRNA expression levels of the renin, the angiotensin converting-enzyme (ACE), and the angiotensinogen were up regulated in the kidney of mice on HFD (Kume et al. 2007; Deji et al. 2008). These molecules may be implicated in the regulation of blood pressure (Berridge et al. 2012). The obesity-related hypertension may induce glomerular hemodynamic alterations resulting in an increase in the renal plasma flow and in the glomerular filtration rate (Hall et al. 2003). The consecutive activation of the renin-angiotensin system (RAS) helps in the sodium reabsorption by the tubules to reduce its excessive loss, induces vasoconstriction of the renal afferent arteriole to regulate the filtration rate, acts on the brain to increase water uptake, and stimulates water retention (Berridge et al. 2012). Prolonged obesity may lead to a severe hypertension and consequently progressive loss of the renal function (Hall et al. 2003). In order to assess the kidney physiology of the male mice that were exposed during seven generations to a HFD, we analysed the hydration status and the urine biochemistry of these animals. Compared to the controls, the HFD generations had an increase in the amount of water uptake ( $p < 0.001$ ), in the urine specific gravity (ions concentration in the urine) ( $p < 0.05$ ), in the urine acidity ( $p < 0.001$ ), in the proteinuria levels ( $p < 0.001$ ), and in the ketonuria levels (results from fat degradation) ( $p < 0.001$ ). However, the HFD groups had no statistically significant increase in the amount of urine output ( $p > 0.05$ ) and in the glucosuria levels ( $p > 0.05$ ) when compared to the controls. In addition, the level of proteinuria was significantly higher in the HFD7 than the HFD1 mice indicating a more severe glomerular lesion. At the histological level, continuous exposure to a HFD stress significantly increased the glomerulus/capsule ratio ( $p < 0.001$ ), the glomerular nuclei number ( $p < 0.001$ ), the extracellular matrix ( $p < 0.001$ ), and the glomerular collagen deposition ( $p < 0.001$ ) across the HFD generations. The histological lesions were exacerbated in the HFD7 mice when compared to the HFD1 group. Interestingly, the presence of small and condensed glomeruli, narrowed Bowman's space, and occluded capillary lumen on histological sections of the kidney was also found in mice with severe hypertension (Siddiqui et al. 2011). Altogether, these findings indicated that, probably, the increased fatness

across the HFD generations had gradually increased the severity of the hypertension and subsequently accentuated the kidney lesions.

Curiously, feeding five generations of male mice with a HFD had negative impacts on the two subsequent generations of male mice (CD6 and CD7) which were reared on a control diet. Although the CD6 and CD7 mice had never encountered an obesogenic environment, they had an increase in the total body weight ( $p < 0.001$ ), in the adipose tissue volume ( $p < 0.001$ ), in the ratio of gonadal fat pad/total body weight ( $p < 0.001$ ), and in the amount of calories intake ( $p < 0.001$ ) when compared to controls. In addition, we aimed to assess the renal physiology in the CD6 and CD7 generations. For this reason, we collected the urine and then the kidneys from these animals. Compared to controls, the CD6 and CD7 male mice had an increase in the amount of water intake ( $p < 0.01$ ), in the urine output ( $p < 0.01$ ), in the proteinuria ( $p < 0.01$ ), in the ketonuria ( $p < 0.001$ ), in the glomerular/corpuscle ratio ( $p < 0.001$ ), in the mesangial area ( $p < 0.001$ ), and in glomerular fibrosis ( $p < 0.01$ ). This was the first observation of paternal transmission of kidney disease to the future generations and could explain the transgenerational amplification of obesity and its associated complications worldwide in humans.

The transmission of these health cues through the male line occurred most probably *via* the spermatozoa. Several studies showed that male mice with acquired obesity had a lower sperm count, impaired sperm motility, lower percentage of spermatozoa with normal morphology, and higher percentage of sperm with fragmented DNA (Bakos et al. 2011; Chen et al. 2013; Duale et al. 2013; Fullston et al. 2013; Vendramini et al. 2014; Zhao et al. 2014; McPherson et al. 2015). By itself, sperm DNA fragmentation could modulate the phenotype of the next generation since microinjection of mice oocytes using DNA-fragmented mice sperm reduced the testicular weight and the sperm count of the male offspring (Ramos-Ibeas et al. 2014). Here we demonstrated that continuous feeding of male mice with a HFD did not significantly affect the sperm concentration. However, the percentage of sperm with progressive motility was progressively reduced in the HFD groups when compared to the controls ( $p < 0.05$ ). Moreover, the percentage of sperm with higher histone retention and/or fragmented DNA (TB+ cells) was significantly higher in the HFD groups rather than the controls ( $p < 0.001$ ). In general, *mice sperm* retain approximately 2% of their histones and they are paternally inherited (Carone et al. 2014). In contrast, Terashima et al. demonstrated that compared to normal weight mice the sperm of obese mice had a higher H3 (histone 3) and H3K4me1 (monomethyl Histone H3 Lysine 4) retention at specific genes implicated in development and cell fate decision. Of particular interest, the generated offspring had alterations in the liver gene expression (Terashima et al. 2015). Also, Palmer and colleagues showed that male obesity inhibited the metabolic sensing protein sirtuin 6 (SIRT6) in the spermatids. The latter may be involved in the chromatin remodelling and its decreased levels resulted in a higher H3K9ac (acetylated histone 3 Lysine 4) retention in the spermatids of obese males compared to non-obese ones. In summary, our findings were in line with the results of several studies that highlighted the possible role of sperm chromatin in the non-genetic inheritance of paternal acquired pathologies (Stuppia et al. 2015).

Fascinatingly, the percentages of sperm with abnormal morphology, reduced motility, and altered chromatin integrity were also higher in the CD6 and CD7 groups compared to controls. Our results were similar to previously published reports that demonstrated that paternal obesity might reduce the reproductive health of the offspring (Fullston et al. 2012; Fullston et al. 2015).

## **VI- Conclusion and perspectives**

In conclusion, this is the first observation of paternal transmission of kidney disease and infertility after successive exposure during 7 generations to an obesogenic environment. It will be interesting to compare the health status of males sired by obese fathers and those sired by fathers that were exposed during five generations to an obesogenic environment.

Our study suggests a role for the male germ line to capture messages from our continuous changing environmental exposures and to transfer this information to subsequent generations. This could have significant implications for the transgenerational amplification of obesity and related comorbidities worldwide in humans.

## VII- Bibliography

1. Al-Quwaidhi, A. J., et al. "Trends and future projections of the prevalence of adult obesity in Saudi Arabia, 1992-2022/Tendances et projections de la prévalence de l'obésité chez l'adulte en Arabie saoudite, 1992-2022." *Eastern Mediterranean Health Journal* 20.10 (2014): 589.
2. Bakos, H. W., et al. "The effect of paternal diet-induced obesity on sperm function and fertilization in a mouse model." *International journal of andrology* 34.5pt1 (2011): 402-410.
3. Beranger, Guillaume E., et al. "Oxytocin reverses ovariectomy-induced osteopenia and body fat gain." *Endocrinology* 155.4 (2014): 1340-1352.
4. Berridge, Michael J. "Cellular processes." *Cell signalling biology* 10 (2014): csb0001011.
5. Binder, Natalie K., et al. "Male obesity is associated with changed spermatozoa Cox4i1 mRNA level and altered seminal vesicle fluid composition in a mouse model." *Molecular human reproduction* (2015): gav010.
6. Blundell, John E., and John Cooling. "Routes to obesity: phenotypes, food choices and activity." *British journal of nutrition* 83.S1 (2000): S33-S38.
7. Carone, Benjamin R., et al. "High-resolution mapping of chromatin packaging in mouse embryonic stem cells and sperm." *Developmental cell* 30.1 (2014): 11-22.
8. Cervantes-Rodriguez, M., et al. "Sugared water consumption by adult offspring of mothers fed a protein-restricted diet during pregnancy results in increased offspring adiposity: the second hit effect." *British Journal of Nutrition* 111.04 (2014): 616-624.
9. Chen, X. L., L. Z. Gong, and J. X. Xu. "Antioxidative activity and protective effect of probiotics against high-fat diet-induced sperm damage in rats." *animal* 7.02 (2013): 287-292.
10. Chowdhury, Sabiha S., et al. "Paternal High Fat Diet in Rats Leads to Renal Accumulation of Lipid and Tubular Changes in Adult Offspring." *Nutrients* 8.9 (2016): 521.
11. Deji, Naoko, et al. "Structural and functional changes in the kidneys of high-fat diet-induced obese mice." *American Journal of Physiology-Renal Physiology* 296.1 (2009): F118-F126.

12. Deji, Naoko, et al. "Structural and functional changes in the kidneys of high-fat diet-induced obese mice." *American Journal of Physiology-Renal Physiology* 296.1 (2009): F118-F126.
13. Duale, N., et al. "Impaired sperm chromatin integrity in obese mice." *Andrology* 2.2 (2014): 234-243.
14. Fontelles, Camile Castilho, et al. "Paternal overweight is associated with increased breast cancer risk in daughters in a mouse model." *Scientific Reports* 6 (2016).
15. Fullston, T., et al. "Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice." *Human reproduction* 27.5 (2012): 1391-1400.
16. Fullston, T., et al. "Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice." *Human reproduction* 27.5 (2012): 1391-1400.
17. Fullston, Tod, et al. "Paternal obesity induces metabolic and sperm disturbances in male offspring that are exacerbated by their exposure to an "obesogenic" diet." *Physiological reports* 3.3 (2015): e12336.
18. Fullston, Tod, et al. "Paternal obesity induces metabolic and sperm disturbances in male offspring that are exacerbated by their exposure to an "obesogenic" diet." *Physiological reports* 3.3 (2015): e12336.
19. Fullston, Tod, et al. "Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete penetrance to the F2 generation and alters the transcriptional profile of testis and sperm microRNA content." *The FASEB Journal* 27.10 (2013): 4226-4243.
20. Golay, A., and J. Ybarra. "Link between obesity and type 2 diabetes." *Best Practice & Research Clinical Endocrinology & Metabolism* 19.4 (2005): 649-663.
21. Grandjean, Valérie, et al. "RNA-mediated paternal heredity of diet-induced obesity and metabolic disorders." *Scientific reports* 5 (2015).
22. Griffin, Karen A., Holly Kramer, and Anil K. Bidani. "Adverse renal consequences of obesity." *American Journal of Physiology-Renal Physiology* 294.4 (2008): F685-F696.

23. Hall, John E. "The kidney, hypertension, and obesity." *Hypertension* 41.3 (2003): 625-633.
24. Harris, Louise E., David G. Morgan, and Nina Balthasar. "Growth hormone secretagogue receptor deficiency in mice protects against obesity-induced hypertension." *Physiological reports* 2.3 (2014).
25. Khan, Shahanshah, et al. "Radioprotective potential of melatonin against 60 Co  $\gamma$ -ray-induced testicular injury in male C57BL/6 mice." *Journal of biomedical science* 22.1 (2015): 1.
26. Kiernan, John A. "On chemical reactions and staining mechanisms." *Connection* 19.3 (2010): 127.
27. Kumar, George L., and John A. Kiernan, eds. *Education Guide-Special Stains and H & E: Pathology*. Dako North America, 2010.
28. Kume, Shinji, et al. "Role of altered renal lipid metabolism in the development of renal injury induced by a high-fat diet." *Journal of the American Society of Nephrology* 18.10 (2007): 2715-2723.
29. Lane, Michelle, Rebecca L. Robker, and Sarah A. Robertson. "Parenting from before conception." *Science* 345.6198 (2014): 756-760.
30. Lloyd, Louise J., et al. "Protein-energy malnutrition during early gestation in sheep blunts fetal renal vascular and nephron development and compromises adult renal function." *The Journal of physiology* 590.2 (2012): 377-393.
31. McPherson, Nicole O., and Michelle Lane. "Male obesity and subfertility, is it really about increased adiposity?." *Asian journal of andrology* 17.3 (2014): 450-458.
32. McPherson, Nicole O., and Michelle Lane. "Male obesity and subfertility, is it really about increased adiposity?." *Asian journal of andrology* 17.3 (2014): 450-458.
33. McPherson, Nicole O., et al. "Paternal under-nutrition programs metabolic syndrome in offspring which can be reversed by antioxidant/vitamin food fortification in fathers." *Scientific reports* 6 (2016): 27010.
34. Nasreddine, Lara, et al. "Trends in overweight and obesity in Lebanon: evidence from two national cross-sectional surveys (1997 and 2009)." *BMC public health* 12.1 (2012): 1.

35. Ng, Sheau-Fang, et al. "Chronic high-fat diet in fathers programs [bgr]-cell dysfunction in female rat offspring." *Nature* 467.7318 (2010): 963-966.
36. Ogden, Cynthia L., et al. "Prevalence of childhood and adult obesity in the United States, 2011-2012." *Jama* 311.8 (2014): 806-814.
37. Öst, Anita, et al. "Paternal diet defines offspring chromatin state and intergenerational obesity." *Cell* 159.6 (2014): 1352-1364.
38. Pouretezari, Majid, et al. "Effects of acrylamide on sperm parameters, chromatin quality, and the level of blood testosterone in mice." *International Journal of Reproductive BioMedicine* 12.5 (2014): 335-342.
39. Proietto, Joseph, and Louise A. Baur. "10: Management of obesity." *Medical journal of Australia* 180.9 (2004): 474-481.
40. Ramos-Ibeas, Priscila, et al. "Intracytoplasmic sperm injection using DNA-fragmented sperm in mice negatively affects embryo-derived embryonic stem cells, reduces the fertility of male offspring and induces heritable changes in epialleles." *PloS one* 9.4 (2014): e95625.
41. Rangan, Gopala K., and Greg H. Tesch. "Quantification of renal pathology by image analysis (Methods in Renal Research)." *Nephrology* 12.6 (2007): 553-558.
42. Samuelsson, Anne-Maj, et al. "Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance A novel murine model of developmental programming." *Hypertension* 51.2 (2008): 383-392.
43. Siddiqui, Athar H., et al. "Recombinant vascular endothelial growth factor 121 attenuates autoantibody-induced features of pre-eclampsia in pregnant mice." *American journal of hypertension* 24.5 (2011): 606-612.
44. Sison, Karen, et al. "Glomerular structure and function require paracrine, not autocrine, VEGF-VEGFR-2 signaling." *Journal of the American Society of Nephrology* 21.10 (2010): 1691-1701.
45. Stuppia, Liborio, et al. "Epigenetics and male reproduction: the consequences of paternal lifestyle on fertility, embryo development, and children lifetime health." *Clinical epigenetics* 7.1 (2015): 1.

46. Terashima, Minoru, et al. "Effect of high fat diet on paternal sperm histone distribution and male offspring liver gene expression." *Epigenetics* 10.9 (2015): 861-871.
47. Tsarev, I., et al. "Evaluation of male fertility potential by Toluidine Blue test for sperm chromatin structure assessment." *Human reproduction* 24.7 (2009): 1569-1574.
48. van Vliet-Ostaptchouk, Jana V., et al. "The prevalence of metabolic syndrome and metabolically healthy obesity in Europe: a collaborative analysis of ten large cohort studies." *BMC endocrine disorders* 14.1 (2014): 1.
49. Vendramini, V., et al. "Reproductive Function of the Male Obese Zucker Rats Alteration in Sperm Production and Sperm DNA Damage." *Reproductive Sciences* (2013): 1933719113493511.
50. Wahlqvist, Mark L., et al. "Dietary quality of elders and children is interdependent in Taiwanese communities: a NAHSIT mapping study." *Ecology of food and nutrition* 53.1 (2014): 81-97.
51. World Health Organization. "WHO laboratory manual for the examination and processing of human semen." (2010).
52. Wozniak, Susan E., et al. "Adipose tissue: the new endocrine organ? A review article." *Digestive diseases and sciences* 54.9 (2009): 1847-1856.
53. Zhao, Jian, et al. "Leptin level and oxidative stress contribute to obesity-induced low testosterone in murine testicular tissue." *Oxidative medicine and cellular longevity* 2014 (2014).

The spermatozoon was considered to be simply the carrier of the paternal genome to the oocyte. However, emerging data indicate that the sperm also deliver different molecules to the oocyte such as, soluble factors that can orchestrate the fertilization events (e.g., phospholipase C zeta), paternally inherited histones, covalent modifications of the DNA, and sperm-borne RNAs which play an essential role in the regulation of embryo development (Nomikos et al. 2011; Casas et al. 2014).

Impressively, several studies showed that the lifestyle and the exposure to some environmental factors like smoke, alcohol, and ionizing radiations might affect the male reproduction by several ways such as impairing the sperm production, modulating the sperm epigenome (non-coding RNAs, histones modifications, and DNA modifications), altering the embryo development, and predisposing the offspring to several pathologies later in life (Stuppia et al. 2015).

In particular, paternal obesity has recently attracted more attention given the prevalence of obesity among men of reproductive age and its long-term consequences on the health of the offspring (Schagdarsurengin et al. 2016). To-date, the impact of excessive weight on male reproduction is neither fully investigated nor well understood. Within that context, the design of this dissertation was planned to study the drawbacks of male excessive fatness on the sperm molecular composition to advance a step in the comprehension of this medical condition.

The first aim of this thesis was to assess the chromatin composition in the motile sperm of obese men and its effects on pre-implantation embryo morphokinetics. The semen samples were obtained from 96 men attending the A-clinic fertility center, Lebanon. Patients were categorized into three groups according to their body mass index (BMI) and waist circumference (WC): normal weight ( $18 < \text{BMI} < 24 \text{ kg/m}^2$  and  $\text{WC} < 90 \text{ cm}$ ), overweight ( $25 < \text{BMI} < 29.9 \text{ kg/m}^2$  and  $\text{WC} < 102 \text{ cm}$ ), and obese ( $\text{BMI} > 30 \text{ kg/m}^2$  and  $\text{WC} > 103 \text{ cm}$ ).

Firstly, the impact of obesity on the conventional semen parameters was assessed. The raw semen of obese men was more viscous and presented a delayed liquefaction when compared to that of normal weight men. Abnormal viscosity and delayed liquefaction time were associated with impaired sperm motility and quality (Stephanus du Plessis et al. 2013). Moreover, sperm concentration, the percentage of spermatozoa with progressive motility, and the percentage of sperm with normal morphology were reduced in the obese and overweight groups as compared to the normal weight one. After the semen processing using the swim-up technique, the motile sperm-enriched fraction of obese men presented a lower sperm concentration, a decreased percentage of sperm with progressive motility, and an increased percentage of vacuolated spermatozoa when compared to the normal weight group.

Since the morphology and motility do not exactly reflect the molecular composition of the spermatozoa, we set out to evaluate their nuclear components. Interestingly, we found that the motile sperm of obese men had higher levels of retained histones, elevated percentage of decompacted chromatin, hypomethylated DNA and hypohydroxymethylated DNA as compared to the motile spermatozoa of normal weight men. On one hand, these results indicated that the sperm of obese men would deliver a higher load of histones to the zygotes, probably altering the gene expression profile in the embryos and/or in the offspring later in life (Terashima et al. 2015). In order to understand the molecular mechanisms lying behind this finding, it will be interesting to assess in the near future which types of histones modifications are present and if they are randomly distributed in the sperm of obese men. On the other hand, the sperm of obese men would deliver a hypomethylated and hypohydroxymethylated DNA to the new forming zygote. It was demonstrated that altered metabolism in cancer cells (Vasanthakumar et al. 2015) and oxidative stress (Delatte et al. 2015) could generate a hypohydroxymethylated DNA, that is why studying the molecular mechanisms by which the testis environment in obese patients modifies the sperm global DNA hydroxymethylation pattern is of paramount importance. Moreover, in depth epigenomic analysis of sperm DNA methylation/hydroxymethylation in obese men (e.g. whole genome bisulfite sequencing (WGBS), and hydroxymethylated DNA immunoprecipitation) may help in the identification of the genes involved in the transmission of this paternal acquired pathology.

Recently, time-lapse technology has emerged in the reproductive medicine field resulting in a better understanding of early embryo development (Kirkegaard et al. 2013). Our study showed for the first time that human zygotes derived from obese fathers had earlier pronuclei appearance (PNa) and fading (PNf) as compared to those derived from fathers with a normal weight. Subsequently, the zygotes from the obese fathers underwent slower cleavage divisions and presented lower blastulating potential as compared to embryos derived from fathers with normal weight. Moreover, the trophectoderm layer quality of the blastocyst was impaired in the obese group. Remarkably, it was demonstrated that an early PNf was associated with a decreased live birth (Azzarello et al. 2012) and a slow embryo cleavage pattern was associated with lower embryos developing to expanded blastocysts, and lower ability to implant in the endometrium (Dal Canto et al. 2012). Altogether, these data indicated that male obesity could affect the molecular composition of the motile sperm nucleus and negatively impacted the preimplantation embryo morphokinetics.

In parallel, food choices and eating habits could be inherited across generations. Interestingly, Fullston *et al.*, showed that metabolic and sperm disorders in the male mice sired by fathers reared on high fat diet (HFD) were aggravated when the offspring also fed the same diet (Fullston et al. 2015). The second aim of this work was to assess how feeding male mice with a HFD during 7 successive generations can impact the phenotype of the resulting mice.

All the mice had the same genetic background (*C57BL/6*), whereas the phenotype was different between the HFD generations. Compared to the mice on control diet, there was a gradual

increase in the total body weight, in the adipose tissue volume, and in the amount of kilocalories intake across the HFD generations. Moreover, histological lesions in the glomeruli were accentuated from one generation to the other with the glomeruli getting smaller, more condensed, and more fibrotic. This phenotype was also found in the kidney of mice with severe hypertension (Siddiqui et al. 2011). Additionally, Deji and colleagues showed that mRNA expression levels of the renin, angiotensin-converting enzyme, and angiotensinogen were up-regulated in the kidneys of HFD mice as compared to controls (Deji et al. 2008); these molecules are implicated in the blood pressure regulation (Berridge et al. 2012). Thus, studying the expression levels of these molecules in the kidneys of the different HFD generations will likely give insight into the mechanism underlying the increase in the severity of kidney failure in these animals.

Furthermore, feeding male mice with a HFD during 5 successive generations increased the total body weight and the volume of adipose tissue in two subsequent generations of mice (CD6 and CD7) maintained on a control diet. Although they had never fed a HFD, obesity-related glomerulopathy was developed in the kidney of the CD6 and CD7 male mice. Altogether, these findings indicated that at each generation, the offspring might retain residual effects of the paternal obesity within their cells that could be worsened when they were re-exposed to the same obesogenic environment. In order to determine whether the accumulation of epigenetic marks with a history of multiple induction could be maintained without the inducer (here the High Fat Diet), we are planning to compare the fertility status, adiposity, and kidney physiology in CD2 (the grandsons of an exposed grandfather), and CD5 (the grandsons of grandfathers exposed during three generations to a HFD), and CD7 progenies.

At the sperm level, the concentration of spermatozoa was not significantly different between the HFD groups. However, the sperm motility and morphology were negatively affected by the paternal obesity. Interestingly, the sperm progressive motility was gradually decreased across the HFD generations and in the CD6 and CD7 male mice when compared to controls. At the molecular level, the sperm of obese mice had higher levels of retained histones and/or fragmented DNA as compared to the controls. Also, these molecular aberrations were significantly higher in the HFD7 as compared to the HFD1 group, and in the CD6 and CD7 mice as compared to the controls. In addition, deep sequencing analysis of testis and sperm RNAs from obese mice allowed the identification of set of differentially expressed non-coding RNAs. Moreover, Grandjean and colleagues has showed that offspring derived from microinjection of testis or sperm RNAs from obese male mice into a naïve zygote had metabolic disturbances. These results indicated that the testicular RNAs could be a vector of transmission of paternal acquired obesity (Grandjean et al. 2015). Transcriptomic analysis of spermatozoa from different HFD generations will help in identifying the level of expression of the sperm RNAs at each generation and in understanding how paternal obesity can affect embryo development and offsprings' health later in life.

In conclusion, we demonstrated that the motile sperm of obese men may transfer specific epigenetic signatures to the oocytes during fertilization and may affect the early embryo development before and after the embryonic genome activation. Moreover, using a mice model of diet-induced obesity, we showed that paternal obesity might affect the body composition, the kidney physiology, and the fertility status of the offspring and these phenotypes might be worsened when the offspring were re-exposed to the same high fat diet. Future studies focusing on the prevention of father-to-child transmission of the acquired obesity are a cardinal need.

## Bibliography

1. Azzarello, A., T. Hoest, and A. L. Mikkelsen. "The impact of pronuclei morphology and dynamicity on live birth outcome after time-lapse culture." *Human reproduction* (2012): des210.
2. Berridge, Michael J. *Cell signalling biology*. Portland Press (2007): 100.
3. Binder, Natalie K., et al. "Paternal obesity in a rodent model affects placental gene expression in a sex-specific manner." *Reproduction* 149.5 (2015): 435-444.
4. Casas, Eduard, and Tanya Vavouri. "Sperm epigenomics: challenges and opportunities." *Frontiers in genetics* 5 (2014): 330.
5. Dal Canto, Mariabeatrice, et al. "Cleavage kinetics analysis of human embryos predicts development to blastocyst and implantation." *Reproductive biomedicine online* 25.5 (2012): 474-480.
6. Deji, Naoko, et al. "Structural and functional changes in the kidneys of high-fat diet-induced obese mice." *American Journal of Physiology-Renal Physiology* 296.1 (2009): F118-F126.
7. Delatte, Benjamin, et al. "Genome-wide hydroxymethylcytosine pattern changes in response to oxidative stress." *Scientific reports* 5 (2015).
8. Fullston, Tod, et al. "Paternal obesity induces metabolic and sperm disturbances in male offspring that are exacerbated by their exposure to an "obesogenic" diet." *Physiological reports* 3.3 (2015): e12336.
9. Gheorghe, Ciprian P., et al. "Gene expression in the placenta: maternal stress and epigenetic responses." *The International journal of developmental biology* 54.2-3 (2010): 507.

10. Grandjean, Valérie, et al. "RNA-mediated paternal heredity of diet-induced obesity and metabolic disorders." *Scientific reports* 5 (2015).
11. Kirkegaard, Kirstine, et al. "Time-lapse parameters as predictors of blastocyst development and pregnancy outcome in embryos from good prognosis patients: a prospective cohort study." *Human Reproduction* 28.10 (2013): 2643-2651.
12. Kume, Shinji, et al. "Role of altered renal lipid metabolism in the development of renal injury induced by a high-fat diet." *Journal of the American Society of Nephrology* 18.10 (2007): 2715-2723.
13. Nasreddine, Lara, et al. "Trends in overweight and obesity in Lebanon: evidence from two national cross-sectional surveys (1997 and 2009)." *BMC public health* 12.1 (2012): 1.
14. Nomikos, Michail, Karl Swann, and F. Anthony Lai. "Starting a new life: Sperm PLC-zeta mobilizes the Ca<sup>2+</sup> signal that induces egg activation and embryo development." *Bioessays* 34.2 (2012): 126-134.
15. Schagdarsurengin, Undraga, et al. "Developmental origins of male subfertility: role of infection, inflammation, and environmental factors." *Seminars in Immunopathology*. Vol. 38. No. 6. Springer Berlin Heidelberg, 2016.
16. Siddiqui, Athar H., et al. "Recombinant vascular endothelial growth factor 121 attenuates autoantibody-induced features of pre-eclampsia in pregnant mice." *American journal of hypertension* 24.5 (2011): 606-612.
17. Du Plessis, Stefan Stephanus, Sheila Gokul, and Ashok Agarwal. "Semen hyperviscosity: causes, consequences, and cures." *Front Biosci (Elite Ed)* 5 (2013): 224-231.
18. Stuppia, Liborio, et al. "Epigenetics and male reproduction: the consequences of paternal lifestyle on fertility, embryo development, and children lifetime health." *Clinical epigenetics* 7.1 (2015): 1.
19. Terashima, Minoru, et al. "Effect of high fat diet on paternal sperm histone distribution and male offspring liver gene expression." *Epigenetics* 10.9 (2015): 861-871.
20. Vasanthakumar, Aparna, and Lucy A. Godley. "5-hydroxymethylcytosine in cancer: significance in diagnosis and therapy." *Cancer genetics* 208.5 (2015): 167-177.

## **APPENDIX**



Middle East Fertility Society  
Middle East Fertility Society Journal

www.mefsjournal.org  
www.sciencedirect.com



## ORIGINAL ARTICLE

# High level of DNA fragmentation in sperm of Lebanese infertile men using Sperm Chromatin Dispersion test

Fadi B. Choucair<sup>a</sup>, Eliane G. Rachkidi<sup>a</sup>, Georges C. Raad<sup>a</sup>, Elias M. Saliba<sup>a</sup>,  
Nina S. Zeidan<sup>b</sup>, Rania A. Jounblat<sup>a</sup>, Imad F. Abou Jaoude<sup>c</sup>, Mira M. Hazzouri<sup>a,\*</sup>

<sup>a</sup> Lebanese University, Faculty of Sciences 2, Fanar, Lebanon

<sup>b</sup> Lebanese University, Faculty of Public Health, Fanar, Lebanon

<sup>c</sup> Abou Jaoude Hospital, Department of Obstetrics and Gynecology, Lebanon

Received 4 June 2016; accepted 15 June 2016

## KEYWORDS

Sperm DNA fragmentation;  
Sperm chromatin dispersion test;  
Male infertility;  
Nuclear quality

**Abstract** Assessment of male fertility has been based on routine semen analysis established by the World Health Organization (WHO), evaluating sperm concentration, motility and morphology. Actually, these parameters become less reliable markers to evaluate male fertility potential. Therefore, a search for better markers has led to an increased focus on sperm chromatin integrity testing in fertility work-up and assisted reproductive technologies (ART). In this study, we evaluate sperm DNA fragmentation in 185 Lebanese infertile patients attending fertility clinics all over the country, in comparison with 30 control men of proven fertility, using the sperm chromatin dispersion test (SCD).

Our results showed a significantly higher sperm DNA fragmentation in infertile group compared to control group (20.62% vs 12.96%;  $p < 0.001$ ). In addition, we found that sperm DNA fragmentation is correlated with alterations in sperm parameters: count, motility and morphology. Moreover, we showed that 28% of normozoospermic patients have high sperm DNA fragmentation. Also, a positive correlation was found between sperm DNA fragmentation and ART failure in infertile group patients. Finally, sperm DNA fragmentation is suggested to be associated with tobacco and environmental conditions.

© 2016 Middle East Fertility Society. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

\* Corresponding author.

E-mail address: [hazzourim@yahoo.com](mailto:hazzourim@yahoo.com) (M.M. Hazzouri).

Peer review under responsibility of Middle East Fertility Society.



Production and hosting by Elsevier

## 1. Introduction

Infertility is recognized today as a public health issue. Approximately 9% of couples worldwide suffer from fertility problems, and 45% of them are due to the male partner (1). Male fertility evaluation is based primarily on routine semen analysis, assessing sperm count, motility and morphology

<http://dx.doi.org/10.1016/j.mefs.2016.06.005>

1110-5690 © 2016 Middle East Fertility Society. Production and hosting by Elsevier B.V.

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Please cite this article in press as: Choucair FB et al. High level of DNA fragmentation in sperm of Lebanese infertile men using Sperm Chromatin Dispersion test, Middle East Fertil Soc J (2016), <http://dx.doi.org/10.1016/j.mefs.2016.06.005>

(2). However, 15% of patients with male factor infertility showed a normal semen analysis (3), thus making it insufficient to predict male fertility potential. Consequently, science is shifting toward analyzing the molecular aspects of spermatozoa.

The status of sperm chromatin has a great importance, specifically in intracytoplasmic sperm injection (ICSI), where natural sperm selection barriers are overridden and a single sperm cell is selected based on its morphology, regardless of its nuclear quality (4). Several studies have shown that male fertility could be affected by sperm DNA damage; therefore, an assessment of sperm DNA content may be a useful adjunct to the routine semen analysis for a better male fertility prediction (5–7). Given the evidence supporting the presence of highly sperm DNA damage in infertile men compared to fertile ones, it is consequently postulated that fertility potential of infertile men is negatively affected (8–16).

While high levels of sperm DNA damage often correlate with poor semen parameters such as reduced count and motility or abnormal morphology (14,17–20), it is also found in infertile men with normal semen parameters (21–23).

In addition to the association between sperm DNA damage and male infertility, sperm DNA fragmentation was also correlated with high sexual chromosomes aneuploidy risk in spermatozoa of infertile men (24–27). High sperm DNA fragmentation was also linked to a low fertilization rate and an altered embryo quality (13,20,28–36). Moreover, previous studies have shown that a paternal effect can be responsible for repeated failure of ART attempts (37–39) and some evidences suggest that abnormal integrity of sperm DNA possibly increases miscarriages (40–44).

Lebanon has been recognized for his contribution to infertility treatments within the Middle East region but the nuclear sperm quality is still ignored by most of the Lebanese clinicians. The present study aims to explore, for the first time in Lebanon, the nuclear quality of spermatozoa in Lebanese infertile men, using the sperm chromatin dispersion test (SCD). A correlative relation between sperm DNA fragmentation, male infertility, semen parameters, lifestyle factors and ART failures will also be investigated. Moreover, the study will match the introduction of a new technique into routine semen analysis for male infertility diagnosis and ART outcome prediction.

## 2. Materials and methods

### 2.1. Patients

This study included 30 fertile men (control group) and 185 men presenting to different infertility clinics (infertile group) from different regions in Lebanon between 2010 and 2013. A detailed medical history was obtained from the participants, including reproductive history and fertility evaluation, mainly semen analysis, ART history and lifestyle factors (smoking, professional risk of exposure to heat stress or toxicants, antioxidant treatment). We included in the study only males whose partners are clinically considered fertile, which have normal reproductive history after gynecological exam and endocrinological testings. It is interesting to mention that the

two groups were matched at the same age range; infertile group: 30–45 years old, control group: 30–50 years old.

The ethics committee of the Lebanese University approved the study protocol.

### 2.2. Semen analysis

The semen samples of the two groups were collected by masturbation into a sterile container after 2–5 days of sexual abstinence. After complete liquefaction of the sample, semen analysis was performed according to World Health Organization guidelines (2).

### 2.3. Sperm Chromatin dispersion (SCD) test

The SCD test is based on the principle that when sperm immersed in an agarose matrix on a slide, treated with an acid solution to denaturate DNA, and then lysed with a buffer to remove membranes and proteins, the result is the formation of nucleoids with a central core and a peripheral halo of dispersed DNA loops. The dispersion halos that are observed in sperm nuclei with non-fragmented DNA after the removal of nuclear proteins are either minimally present or not produced at all in sperm nuclei with fragmented DNA. The agarose matrix allows working with unfixed sperm on a slide in a suspension-like environment. Nucleoids are visualized either with fluorescent microscopy after staining with a DNA specific fluorochrome (DAPI), or with bright-field microscopy with Wright's staining solution.

SCD test protocol:

In this study, we performed the SCD test as proposed and improved by Fernandez et al., in 2005 (45), using the Halosperm® kit (INDAS laboratories, Madrid, Spain).

In brief, an aliquot of a semen sample was diluted to 10 million/mL in phosphate-buffered saline (PBS). Gelled aliquots of low-melting point agarose in eppendorf tubes were provided in the kit, each one to process a semen sample. Eppendorf tubes were placed in a water bath at 90–100 °C for 5 min to fuse the agarose, and then in a water bath at 37 °C. After 5 min of incubation for temperature equilibration at 37 °C, 60 mL of the diluted semen sample was added to the eppendorf tube and mixed with the fused agarose. Of the semen-agarose mix, 20 µL was pipetted onto slides pre-coated with agarose, provided in the kit, and covered with a 22 × 22 mm coverslip. The slides were placed on a cold plate in the refrigerator (4 °C) for 5 min to allow the agarose to produce a microgel with the sperm cells embedded within. The coverslips were gently removed and the slides immediately immersed horizontally in an acid solution, previously prepared by mixing 80 µL of HCl from an eppendorf tube in the kit with 10 mL of distilled water, and incubated for 7 min. The slides were horizontally immersed in 10 mL of the lysing solution for 25 min. After washing 5 min in a tray with abundant distilled water, the slides were dehydrated in increasing concentrations of ethanol (70%, 90%, and 100%) for 2 min each and then air-dried. For bright field microscopy slides were horizontally covered with a mix of Wright's staining solution (Merck, Darmstadt, Germany) and PBS (Merck) (1:1) for 5–10 min with continuous airflow. Slides were briefly washed in tap water and allowed to dry.

Four dispersion patterns were defined:

1. Sperm with large halo: the halo has a 2 times larger width than that of the sperm core, with a darker spot (sperm head) in the middle.
2. Sperm with moderate halo: having a halo size between large and small halos.
3. Sperm with small halo: a very small, clear film, that has a halo appearance, surrounds the sperm head.
4. Sperm with no halo.

Sperm DNA fragmentation percentage (SCD percentage) was calculated as the proportion of sperm with small and no halos, to the total sperm count per slide. We assessed two slides for every patient, and a total of 1000 sperm were counted per slide.

#### 2.4. Statistical analysis

We used a Wilcoxon Rank Sum test to determine the significance of differences in sperm DNA fragmentation rates obtained from the infertile group and sub-groups compared to the control. The extent and significance of correlations between semen analysis parameters, ART outcomes, lifestyle factors and sperm DNA fragmentation rates were determined using Spearman Rank Correlation test. The statistical tests were performed using Sigma Stat Version 3.5 software (Systat Software, Erkrath, Germany). Comparisons were considered to be statistically significant at  $p < 0.05$ .

### 3. Results

DNA fragmentation was evaluated by SCD test in sperm of 185 Lebanese infertile men in comparison with a control fertile group.

#### 3.1. Sperm DNA fragmentation in Lebanese infertile men

Our results have shown a DNA fragmentation rate in sperm of infertile group significantly higher compared to the control group. The rates were distributed between 3% and 78.80% with a mean value of 20.62% ( $p < 0.001$ ) (Fig. 1).

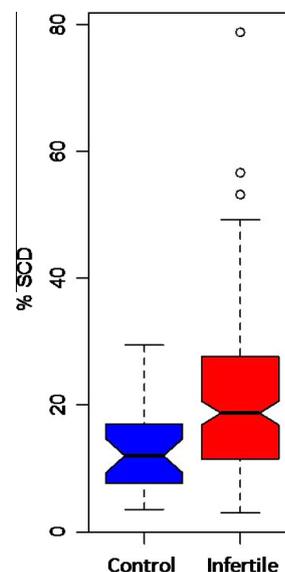
#### 3.2. Sperm DNA fragmentation and semen analysis abnormalities

After finding a significant difference between fertile and infertile men, we suggested to divide the infertile group into sub-groups, based on semen analysis characteristics, notably sperm count, morphology and motility.

Seven sub-groups were established, and compared with the control group.

The results showed a significantly higher SCD percentage, and therefore a high sperm DNA fragmentation in the sub-groups shows altered semen analysis.

A very strong significant difference was established between the control group and the OAT (oligoasthenoteratozoospermic), OA (oligoasthenozoospermic) and O (oligozoospermic) infertile sub-groups ( $p \leq 0.001$ ). A lower significant difference was also found in A (asthenozoospermic) and AT (asthenoter-



**Figure 1** Comparison of sperm DNA fragmentation between the infertile group and the control group, measured by SCD test.

atozoospermic) infertile subgroups ( $p = 0.025$ ,  $p = 0.002$ ). No significant difference was noted with T (teratozoospermic) and N (normozoospermic) sub-groups ( $p = 0.103$ ,  $p = 0.269$ ) (Table 1).

#### 3.3. DNA fragmentation and sperm count, motility and morphology

Sperm DNA fragmentation rate was also plotted with sperm count, motility and morphology.

A clear negative correlation was found between SCD percentage and sperm count, with a very significant  $p$  value ( $< 0.001$ ) and a negative correlation coefficient ( $\rho = -0.349$ ) (Fig. 2A). A similar negative correlation was also shown between SCD percentage and sperm motility ( $p < 0.001$ ;  $\rho = -0.331$ ) (Fig. 2B). The sperm motility and count decrease following a sharp slope when the DNA fragmentation rate increases. Concerning abnormal sperm morphology, a positive correlation was found with sperm DNA fragmentation and teratozoospermia ( $p = 0.003$ ,  $\rho = +0.222$ ) (Fig. 2C).

#### 3.4. Sperm DNA fragmentation and ART failure

In our infertile group, a positive correlation was noted between the number of ART failures and sperm DNA fragmentation rate ( $\rho = +0.352$ ,  $p < 0.001$ ) (Fig. 3).

#### 3.5. Sperm DNA fragmentation and lifestyle

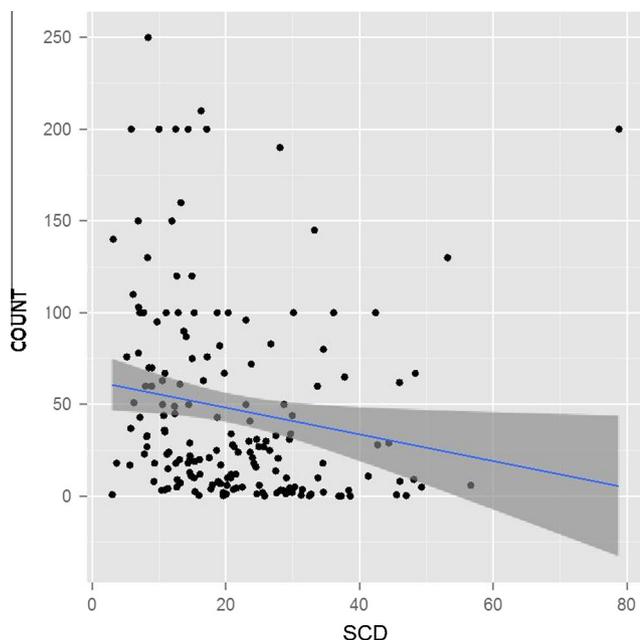
An association between sperm DNA fragmentation and lifestyle was then tested.

Within the infertile group, patients were identified regarding their exposure to heat stress or toxicants in their work as well as if they smoked or took antioxidant treatment. In every category two groups were obtained, and compared to each other according to their SCD percentages.

**Table 1** Sperm DNA fragmentation comparison between control group and infertile sub-groups.

Groups comparison with control	A <i>n</i> = 43	AT <i>n</i> = 19	N <i>n</i> = 43	O <i>n</i> = 22	OA <i>n</i> = 27	OAT <i>n</i> = 26	T <i>n</i> = 5	Control group (C) <i>n</i> = 30
Mean ± SD	18.76% ± 12.95	25.23% ± 12.93	15.97% ± 10.01	23.31% ± 10.67	24.54% ± 11.22	24.64% ± 11.91	20.62% ± 10.37	12.96% ± 6.82
Median	14.65	23.60	12.86	20.44	21.58	24.58	23.80	12.00
Low/high values	6.88 78.80	6.94 48.34	3.16 53.17	3.00 49.27	9.24 48.11	3.67 56.64	5.78 29.69	3.54 29.45
<i>p</i> values	0.025	0.0022	0.269	< 0.001	< 0.001	< 0.001	0.103	

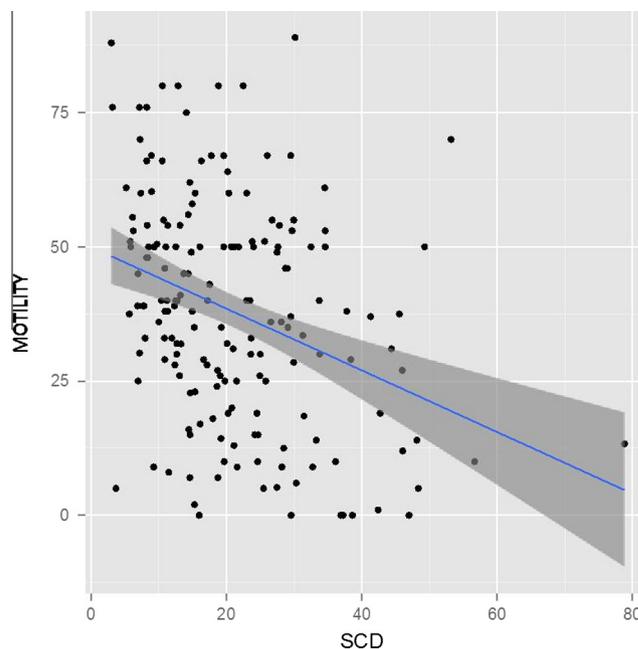
C = control group; O = oligozoospermic; A = asthenozoospermic; T = teratozoospermic; N = normozoospermic.

**Figure 2A** Correlation between sperm DNA fragmentation (SCD percentage) and Sperm Count.

The analysis has shown a trend of positive correlation between smoking and sperm DNA fragmentation ( $\rho = +0.050$ ,  $p = 0.606$ ). The same trend was also found between risky profession and sperm DNA fragmentation ( $\rho = +0.059$ ,  $p = 0.704$ ). In contrast, a trend of negative correlation was noted between antioxidant treatment and sperm DNA fragmentation ( $\rho = -0.079$ ,  $p = 0.531$ ).

#### 4. Discussion

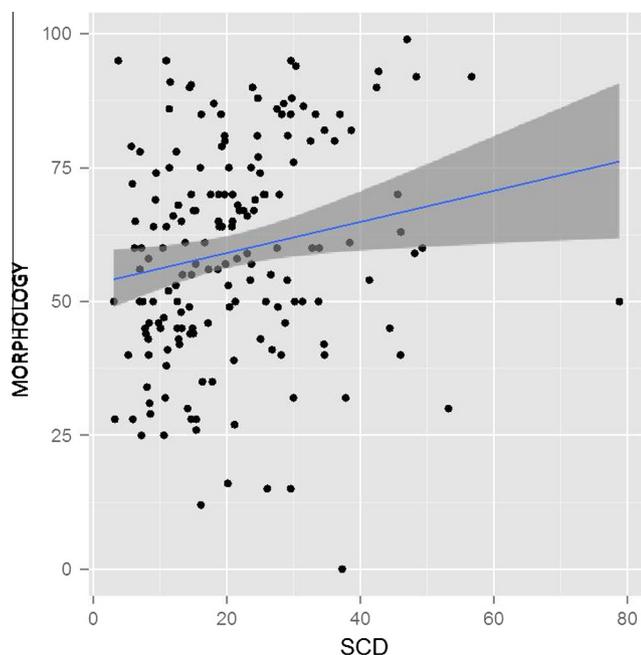
In the present study, we investigated the incidence of sperm DNA damage among 185 infertile men, attending Lebanese infertility clinics for infertility evaluation and treatment. We aimed to understand the association between sperm DNA damage, sperm characteristics, medical history and lifestyle factors. Although several studies have reported that sperm DNA damage could be associated with male infertility. The present study attempts to clarify the association between sperm DNA damage and specific categories of defects in the Lebanese infertile population.

**Figure 2B** Correlation between sperm DNA fragmentation (SCD percentage) and Sperm Motility.

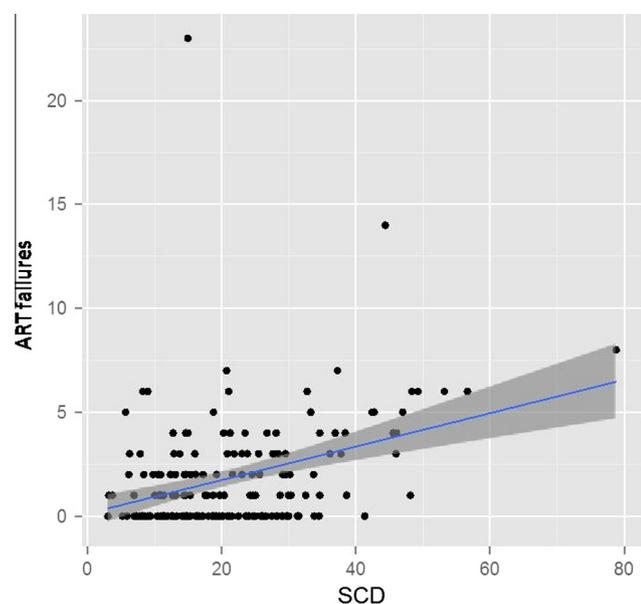
Sperm DNA fragmentation levels measured by Sperm Chromatin Dispersion test (SCD), were compared between two groups of Lebanese fertile and infertile men. The infertile men group showed a significantly higher SCD percentage; thus, higher DNA fragmentation is compared to control. Indeed, these findings reveal that the average incidence of sperm DNA damage in fertile patients is 12.96% whereas patients from the infertile group show significantly higher level of sperm DNA damage (20.78%). Given that, this could provide strong evidence for a negative effect of sperm DNA fragmentation on male fertility potential.

Our result perfectly complies with previous studies that have reported the association between defects in sperm chromatin and male infertility (23,25,45,46).

The group of infertile men is divided into seven sub-groups based on semen analysis phenotypes (O, A, T, OA, AT, OAT, and N). After comparing the sperm DNA fragmentation percentages between infertile sub-groups and control, we found high rates of sperm DNA damage associated with asthenozoospermia, and higher rates associated with oligozoospermia,



**Figure 2C** Correlation between sperm DNA fragmentation (SCD percentage) and Sperm Morphology.



**Figure 3** Relation between SCD percentage and ART failure.

oligoasthenozoospermia, asthenoteratozoospermia and oligoasthenoteratozoospermia. This finding was also reported by previous studies (21,47) and would suggest that sperm DNA fragmentation could be a promising predictive index of sperm alteration gravity, and therefore it is an important diagnostic tool for male fertility assessment.

When comparing the control group with the group of teratozoospermic infertile patients, there was no significant difference (C/T:  $p = 0.103$ ). It should be mentioned that this specific group of infertile men (T) only comprises five patients with moderated teratozoospermia, so a larger sample size will be needed in order to rule out significance.

In our study, the normozoospermic group does not appear to be significantly different from the control group (C/N:  $p = 0.269$ ). Within this group, the mean value of SCD percentage (15.97%) was higher than that of the control group (12.96%). Moreover, 28% of the normozoospermic infertile patients present a higher SCD percentage. Previous studies mentioned that around 10% of infertile patients with normal semen analysis showed a high degree of sperm DNA fragmentation (22,48), which could perfectly explain cases of male infertility, classified as idiopathic.

Besides the inter-group DNA fragmentation comparison, it was necessary to evaluate the association between DNA fragmentation and semen analysis parameters. The SCD percentage was negatively associated with sperm count and sperm motility but positively associated with abnormal sperm morphology (6,13,14,16,20,21,46,47,49–53). This finding suggests that an abnormal semen analysis reflects not only an altered spermatogenesis, but a deep negative effect on sperm nuclear quality.

As for teratozoospermia, its association with sperm DNA damage is much debated and has been the focus of many studies (54–57). Our study showed high SCD percentage in infertile men with teratozoospermia. This finding is extremely important, because abnormal sperm shape anomalies are easily noticed during clinical sperm processing and help to identify spermatozoa possibly carrying fragmented DNA. This result emphasizes the importance of sperm selection during ICSI as the damage carried by morphologically abnormal spermatozoa, could entail nuclear chromatin defect, and thus may affect ART outcomes such as fertilization rate, embryo cleavage, development, implantation and ongoing pregnancy (33).

Furthermore, the SCD percentage shows a positive correlation with failed ART attempts. ART cycles appear to fail more often with high sperm DNA fragmentation. Our finding is consistent with results from previous studies (9,35,43,58–60). Moreover, normal females whose partner had high sperm DNA fragmentation, suffered from recurrent pregnancy loss (RPL) (61,62).

The present study expanded into exploring the possible causes of sperm DNA fragmentation. While a number of studies have been conducted regarding the association between lifestyle factors and sperm quality (56,63–65), studies on the Lebanese population are lacking.

Different lifestyle factors were studied. A trend of a positive correlation was observed between smoking and sperm DNA fragmentation in the Lebanese population. Smoking seems to be associated with increased sperm DNA fragmentation. Consequently, patients aiming to conceive should be encouraged to reduce or even stop smoking which has already shown a deleterious effect on the motility of spermatozoa and its DNA integrity (66,67).

The impact of heat stress and toxicants on male fertility can be elucidated by the trend of a positive correlation between sperm DNA fragmentation and risky professions. The occupations were classified as risky when the patient is exposed in his work, to toxicants, or heat. Many studies confirmed the negative effect of heat stress (68–70), and exposure to toxicants (64,71,72) on sperm DNA quality.

It was denoted that high sperm DNA fragmentation after tobacco, heat and toxicants exposures, will cause testicular oxidative stress which is detrimental to sperm function

(73,74). This will be considered as a significant contributing factor to male infertility because it leads to germ cell apoptosis and dysfunction (75–77).

On the other hand, the antioxidant treatment was found to be negatively correlated with SCD percentage. Thus, it is considered useful in preventing damage to spermatozoa and reducing DNA fragmentation levels (78–82). This could be a promising strategy in treating male infertility linked to nuclear sperm damage.

## 5. Conclusion

Our study is conducted in the Lebanese population to explore sperm DNA fragmentation. The SCD percentage is a potential discriminator between fertile and infertile patients. Moreover, sperm DNA fragmentation appears to be a powerful indicator of semen quality and ART outcomes because normal semen analysis is not sufficient to assess male fertility. We also suggested that tobacco, heat stress and toxicants exposure can affect sperm DNA integrity and we proposed the use of antioxidants as a treatment. The present study may serve as a platform for future meta-analysis studies, with a larger sample size and more variables to consider, in order to present a more definitive clarification about the role of sperm DNA fragmentation in male infertility.

## Declaration of interests

The authors report no declarations of interest.

## Funding

This work was funded by the National Council for Scientific Research – Lebanon (CNRS).

## References

- Boivin J, Bunting L, Collins J, Nygren K. International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Hum Reprod* 2007;22:1506–12.
- Organization W. WHO laboratory manual for the examination and processing of human semen. Geneva: World Health Organization; 2010.
- Agarwal A, Allamaneni S. Sperm DNA damage assessment: a test whose time has come. *Fertil Steril* 2005;84:850–3.
- Seli E. Spermatozoal nuclear determinants of reproductive outcome: implications for ART. *Human Reprod Update* 2005;11:337–49.
- Tarozzi N, Bizzaro D, Flamigni C, Borini A. Clinical relevance of sperm DNA damage in assisted reproduction. *Reprod BioMed Online* 2007;14:746–57.
- Evgeni E, Charalabopoulos K, Asimakopoulos B. Human sperm DNA fragmentation and its correlation with conventional semen parameters. *J Reprod Infert* 2014;15:2.
- Evgeni E, Lymberopoulos G, Gazouli M, Asimakopoulos B. Conventional semen parameters and DNA fragmentation in relation to fertility status in a Greek population. *Euro J Obst Gynecol Reprod Biol* 2015;188:17–23.
- Kodama H, Yamaguchi R, Fukuda J, Kasai H, Tanaka T. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril* 1997;68:519–24.
- Evenson D. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum Reprod* 1999;14:1039–49.
- Spanò M, Bonde JP, Hjøllund HI, Kolstad HA, Cordelli E, Leter G. Danish first pregnancy planner study team. Sperm chromatin damage impairs human fertility. *Fertil Steril* 2000;73:43–50.
- Zini A. Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. *Fertil Steril* 2001;75:674–7.
- Saleh R, Agarwal A, Nada E, El-Tonsy M, Sharma R, Meyer A, et al. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil Steril* 2003;79:1597–605.
- Velez de la Calle J, Muller A, Walschaerts M, Clavere J, Jimenez C, Wittemer C, et al. Sperm deoxyribonucleic acid fragmentation as assessed by the sperm chromatin dispersion test in assisted reproductive technology programs: results of a large prospective multicenter study. *Fertil Steril* 2008;90:1792–9.
- Zhang L, Qiu Y, Wang K, Wang Q, Tao G, Wang L. Measurement of sperm DNA fragmentation using bright-field microscopy: comparison between sperm chromatin dispersion test and terminal uridine nick-end labeling assay. *Fertil Steril* 2010;94:1027–32.
- Chi H, Chung D, Choi S, Kim J, Kim G, Lee J, et al. Integrity of human sperm DNA assessed by the neutral comet assay and its relationship to semen parameters and clinical outcomes for the IVF-ET program. *Clin Exp Reprod Med* 2011;38:10.
- Venkatesh S, Kumar R, Deka D, Deccaraman M, Dada R. Analysis of sperm nuclear protein gene polymorphisms and DNA integrity in infertile men. *Syst Biol Reprod Med* 2011:1–9.
- Irvine S, Twigg P, Gordon L, Fulton N, Milne A, Aitken R. DNA integrity in human spermatozoa: relationships with semen quality. *J Androl* 2000;21:33–44.
- Muratori M, Piomboni P, Baldi E, Filimberti E, Pecchioli P, Moretti E, Gambera L, Baccetti B, Biagiotti R, Forti G, Maggi M. Functional and ultrastructural features of DNA-fragmented human sperm. *J Androl* 2000;21:903–12.
- Caglar GS, Koester F, Schoepper B, Asimakopoulos B, Nehls B, Nikolettos N, Diedrich K, Al-Hasani S. Semen DNA fragmentation index, evaluated with both TUNEL and Comet assay, and the ICSI outcome. *In Vivo* 2007;21:1075–80.
- Fang L, Lou LJ, Ye YH, Jin F, Zhou J. A study on correlation between sperm DNA fragmentation index and age of male, various parameters of sperm and in vitro fertilization outcome. *Zhonghua yi xue yi chuan xue za zhi = Zhonghua yixue yichuanxue zazhi = Chinese. J Med Genet* 2011;28:432–5.
- Varshini J, Srinag B, Kalthur G, Krishnamurthy H, Kumar P, Rao S, et al. Poor sperm quality and advancing age are associated with increased sperm DNA damage in infertile men. *Andrologia* 2011;44:642–9.
- Mayorga-Torres B, Cardona-Maya W, Cadavid Á, Camargo M. Evaluation of sperm functional parameters in normozoospermic infertile individuals. *Actas Urológicas Españolas (English Edition)* 2013;37:221–7.
- Oleszczuk K, Augustinsson L, Bayat N, Giwercman A, Bungum M. Prevalence of high DNA fragmentation index in male partners of unexplained infertile couples. *Andrology* 2012;1:357–60.
- Muriel J, Goyanes V, Segrelles E, Gosalvez J, Alvarez J, Fernandez J. Increased aneuploidy rate in sperm with fragmented dna as determined by the sperm chromatin dispersion (SCD) test and FISH analysis. *J Androl* 2006;28:38–49.
- Qiu Y, Wang L, Zhang L, Yang D, Zhang A, Yu J. Analysis of sperm chromosomal abnormalities and sperm DNA fragmentation in infertile males. *Zhonghua yi xue yi chuan xue za zhi = Zhonghua yixue yichuanxue zazhi = Chinese. J Med Genet* 2008;25:681–5.
- Perrin A, Basinko A, Douet-Guilbert N, Gueganic N, Le Bris M, Amice V, et al. Aneuploidy and DNA fragmentation in sperm of

- carriers of a constitutional chromosomal abnormality. *Cytogenet Geno Res* 2011;133:100–6.
- (27) Younes B, Hazzouri K, Chaaban M, Karam W, Jaoude I, Attieh J, et al. High frequency of sex chromosomal disomy in spermatozoa of Lebanese infertile men. *J Androl* 2010;32:518–23.
- (28) Tesarik J. Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. *Hum Reprod* 2004;19(3):611–5.
- (29) Meseguer M, Santiso R, Garrido N, Gil-Salom M, Remohí J, Fernandez J. Sperm DNA fragmentation levels in testicular sperm samples from azoospermic males as assessed by the sperm chromatin dispersion (SCD) test. *Fertil Steril* 2009;92:1638–45.
- (30) Simon L, Castillo J, Oliva R, Lewis S. Relationships between human sperm protamines, DNA damage and assisted reproduction outcomes. *Reprod BioMed Online* 2011;23:724–34.
- (31) Avendano C, Oehninger S. DNA fragmentation in morphologically normal spermatozoa: how much should we be concerned in the ICSI Era? *J Androl* 2010;32:356–63.
- (32) Zini A, Jamal W, Cowan L, Al-Hathal N. Is sperm dna damage associated with IVF embryo quality? A systematic review. *J Assist Reprod Genet* 2011;28:391–7.
- (33) Simon L, Murphy K, Shamsi M, Liu L, Emery B, Aston K, et al. Paternal influence of sperm DNA integrity on early embryonic development. *Hum Reprod* 2014;29:2402–12.
- (34) Tandara M, Bajić A, Tandara L, Bilić-Zulle L, Šunjić M, Kozina V, et al. Sperm DNA integrity testing: big halo is a good predictor of embryo quality and pregnancy after conventional IVF. *Andrology* 2014;2:678–86.
- (35) Sivanarayana T, Ravi Krishna C, Jaya Prakash G, Krishna K, Madan K, Sudhakar G, et al. Sperm DNA fragmentation assay by sperm chromatin dispersion (SCD): correlation between DNA fragmentation and outcome of intracytoplasmic sperm injection. *Reprod Med Biol* 2013;13:87–94.
- (36) Lazaros L, Vartholomatos G, Pamporaki C, Kosmas I, Takenaka A, Makrydimas G, Sofikitis N, Stefanos T, Zikopoulos K, Hatzi E, Georgiou I. Sperm flow cytometric parameters are associated with ICSI outcome. *Reprod Biomed online* 2013;26:611–8.
- (37) Parinaud J, Mieusset R, Vieitez G, Labal B, Richoille G. Influence of sperm parameters on embryo quality. *Fertil Steril* 1993;60:888–92.
- (38) Janny L, Menezes Y. Evidence for a strong paternal effect on human preimplantation embryo development and blastocyst formation. *Mol Reprod Dev* 1994;38:36–42.
- (39) Shoukir Y, Chardonens D, Campana A, Sakkas D. Blastocyst development from supernumerary embryos after intracytoplasmic sperm injection: a paternal influence? *Hum Reprod* 1998;13:1632–7.
- (40) Carrell DT, Liu L, Peterson CM, Jones KP, Hatasaka HH, Erickson L, Campbell B. Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. *Arch Androl* 2003;49:49–55.
- (41) Borini A, Tarozzi N, Bizzaro D, Bonu M, Fava L, Flamigni C, et al. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. *Hum Reprod* 2006;21:2876–81.
- (42) Gil-Villa A, Cardona-Maya W, Agarwal A, Sharma R, Cadavid Á. Assessment of sperm factors possibly involved in early recurrent pregnancy loss. *Fertil Steril* 2010;94:1465–72.
- (43) Absalan F, Ghannadi A, Kazerooni M, Parifar R, Jamalzadeh F, Amiri S. Value of sperm chromatin dispersion test in couples with unexplained recurrent abortion. *J Assist Reprod Genet* 2011;29:11–4.
- (44) AlKusayer G, Amily N, Abou-Setta A, Bedaiwy M. Live birth rate following IVF/ICSI in patients with sperm DNA fragmentation: a systematic review and meta-analysis. *Fertil Steril* 2014;102:e305.
- (45) Fernández JL, Muriel L, Goyanes V, Segrelles E, Gosálvez J, Enciso M, LaFromboise M, De Jonge C. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. *Fertil Steril* 2005;84:833–42.
- (46) Shamsi M, Imam S, Dada R. Sperm DNA integrity assays: diagnostic and prognostic challenges and implications in management of infertility. *J Assist Reprod Genet* 2011;28:1073–85.
- (47) Belloc S, Benkhalifa M, Cohen-Bacrie M, Dalleac A, Chahine H, Amar E, et al. Which isolated sperm abnormality is most related to sperm DNA damage in men presenting for infertility evaluation. *J Assist Reprod Genet* 2014;31:527–32.
- (48) Khodair H, Omran T. Evaluation of reactive oxygen species (ROS) and DNA integrity assessment in cases of idiopathic male infertility. *Egypt J Dermatol Venerol* 2013;33:51.
- (49) Trisini A, Singh N, Duty S, Hauser R. Relationship between human semen parameters and deoxyribonucleic acid damage assessed by the neutral comet assay. *Fertil Steril* 2004;82:1623–32.
- (50) Cohen-Bacrie P, Belloc S, Ménéz Y, Clement P, Hamidi J, Benkhalifa M. Correlation between DNA damage and sperm parameters: a prospective study of 1633 patients. *Fertil Steril* 2009;91:1801–5.
- (51) Varghese A, Bragais F, Mukhopadhyay D, Kundu S, Pal M, Bhattacharyya A, et al. Human sperm DNA integrity in normal and abnormal semen samples and its correlation with sperm characteristics. *Andrologia* 2009;41:207–15.
- (52) Matsuura R, Takeuchi T, Yoshida A. Preparation and incubation conditions affect the DNA integrity of ejaculated human spermatozoa. *Asian J Androl* 2010;12:753–9.
- (53) Curti G, Cánepa M, Cantú L, Montes J. Diagnosis of male infertility: a need of functional and chromatin evaluation. *Actas Urológicas Españolas (English Edition)* 2013;37:100–5.
- (54) Avendaño C, Franchi A, Taylor S, Morshedi M, Bocca S, Oehninger S. Fragmentation of DNA in morphologically normal human spermatozoa. *Fertil Steril* 2009;91:1077–84.
- (55) Avendaño C, Franchi A, Duran H, Oehninger S. DNA fragmentation of normal spermatozoa negatively impacts embryo quality and intracytoplasmic sperm injection outcome. *Fertil Steril* 2010;94:549–57.
- (56) Elshal M, El-Sayed I, Elsaied M, El-Masry S, Kumosani T. Sperm head defects and disturbances in spermatozoal chromatin and DNA integrities in idiopathic infertile subjects: association with cigarette smoking. *Clin Biochem* 2009;42:589–94.
- (57) Mehdi M, Khantouche L, Ajina M, Saad A. Detection of DNA fragmentation in human spermatozoa: correlation with semen parameters. *Andrologia* 2009;41:383–6.
- (58) Duran E. Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. *Hum Reprod* 2002;17:3122–8.
- (59) Speyer B, Pizzey A, Ranieri M, Joshi R, Delhanty J, Serhal P. Fall in implantation rates following ICSI with sperm with high DNA fragmentation. *Hum Reprod* 2010;25:1609–18.
- (60) Jiang H, He R, Wang C, Zhu J. The relationship of sperm DNA fragmentation index with the outcomes of in-vitro fertilisation-embryo transfer and intracytoplasmic sperm injection. *J Obstet Gynaecol* 2011;31:636–9.
- (61) Ribas-Maynou J, Garcia-Peiró A, Fernandez-Encinas A, Amengual M, Prada E, Cortés P, et al. Double stranded sperm DNA breaks, measured by Comet assay, are associated with unexplained recurrent miscarriage in couples without a female factor. *PLoS ONE* 2012;7:e44679.
- (62) Robinson L, Gallos I, Conner S, Rajkhowa M, Miller D, Lewis S, et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. *Hum Reprod* 2012;27:2908–17.
- (63) Soares S, Melo M. Cigarette smoking and reproductive function. *Curr Opin Obstet Gynecol* 2008;20:281–91.
- (64) Calogero A, La Vignera S, Condorelli R, Perdichizzi A, Valenti D, Asero P, et al. Environmental car exhaust pollution damages human sperm chromatin and DNA. *J Endocrinol Invest* 2010;34:e139–43.

- (65) Vilorio T, Meseguer M, Martínez-Conejero J, O'Connor J, Remohí J, Pellicer A, et al. Cigarette smoking affects specific sperm oxidative defenses but does not cause oxidative DNA damage in infertile men. *Fertil Steril* 2010;94:631–7.
- (66) Sepaniak S, Forges T, Gerard H, Foliguet B, Bene M, Monnier-Barbarino P. The influence of cigarette smoking on human sperm quality and DNA fragmentation. *Toxicology* 2006;223:54–60.
- (67) Anifandis G, Bounartzi T, Messini C, Dafopoulos K, Sotiriou S, Messinis I. The impact of cigarette smoking and alcohol consumption on sperm parameters and sperm DNA fragmentation (SDF) measured by Halosperm®. *Arch Gynecol Obstet* 2014;290:777–82.
- (68) Paul C, Murray A, Spears N, Saunders P. A single, mild, transient scrotal heat stress causes DNA damage, subfertility and impairs formation of blastocysts in mice. *Reproduction* 2008;136(1):73–84.
- (69) Kanter M, Aktas C, Erboğa M. Heat stress decreases testicular germ cell proliferation and increases apoptosis in short term: an immunohistochemical and ultrastructural study. *Toxicol Ind Health* 2011;29(2):99–113.
- (70) Santiso R, Tamayo M, Gosálvez J, Johnston S, Mariño A, Fernández C, et al. DNA fragmentation dynamics allows the assessment of cryptic sperm damage in human: evaluation of exposure to ionizing radiation, hyperthermia, acidic pH and nitric oxide. *Mutat Res/Funda Mol Mech Mutag* 2012;734:41–9.
- (71) Gomes M, Gonçalves A, Rocha E, Sá R, Alves A, Silva J, et al. Effect of in vitro exposure to lead chloride on semen quality and sperm DNA fragmentation. *Zygote* 2014;23:384–93.
- (72) Hosseinpour E, Shahverdi A, Parivar K, Sedighi Gilani M, Nasr-Esfahani M, Salman Yazdi R, et al. Sperm ubiquitination and DNA fragmentation in men with occupational exposure and varicocele. *Andrologia* 2013;46:423–9.
- (73) Cocuzza M, Sikka S, Athayde K, Agarwal A. Clinical relevance of oxidative stress and sperm chromatin damage in male infertility: an evidence based analysis. *Int Braz J Urol* 2007;33:603–21.
- (74) Tremellen K. Oxidative stress and male infertility—a clinical perspective. *Human Reprod Update* 2008;14:243–58.
- (75) Sakkas D, Mariethoz E, St. John J. Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the fas-mediated pathway. *Exp Cell Res* 1999;251:350–5.
- (76) Sakkas D. Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biol Reprod* 2002;66:1061–7.
- (77) Novotny J, Aziz N, Rybar R, Brezinova J, Kopecka V, Filipcikova R, Reruchova M, Oborna I. Relationship between reactive oxygen species production in human semen and sperm DNA damage assessed by Sperm Chromatin Structure Assay. *Biomed Papers* 2013;157:383–6.
- (78) Greco E. Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. *J Androl* 2005;26:349–53.
- (79) Greco E. ICSI in cases of sperm DNA damage: beneficial effect of oral antioxidant treatment. *Hum Reprod* 2005;20:2590–4.
- (80) Ménéz Y, Hazout A, Panteix G, Robert F, Rollet J, Cohen-Bacrie P, et al. Antioxidants to reduce sperm DNA fragmentation: an unexpected adverse effect. *Reprod BioMed Online* 2007;14:418–21.
- (81) Abad C, Amengual M, Gosálvez J, Coward K, Hannaoui N, Benet J, et al. Effects of oral antioxidant treatment upon the dynamics of human sperm DNA fragmentation and subpopulations of sperm with highly degraded DNA. *Andrologia* 2012;45:211–6.
- (82) Talevi R, Barbato V, Fiorentino I, Braun S, Longobardi S, Gualtieri R. Protective effects of in vitro treatment with zinc, d-aspartate and coenzyme q10 on human sperm motility, lipid peroxidation and DNA fragmentation. *Reprod Biol Endocrinol* 2013;11:81.