

JOANA PROENÇA GONÇALVES PITA DE OLIVEIRA

**HISTOLOGICAL CHARACTERISATION OF THE
NLRP3 INFLAMMASOME IN THE OVARY OF
OBESE MICE**

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Co-Orientador: Professor Doutor António Galvão

Lusófona University of Humanities and Technologies

Faculty of Veterinary Medicine

Lisbon

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Presidente: Prof.^a Doutora Laurentina Pedroso
Arguente: Prof. Doutor Alexandre Trindade
Orientador: Prof. Doutor Daniel Murta

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Resumo

A obesidade tem vindo a aumentar durante os últimos anos. Paralelamente, o número de mulheres obesas com problemas reprodutivos tem vindo também a aumentar. Sabe-se que o inflamassoma Nlrp3 está presente no estado inflamatório característico da obesidade e pela produção e secreção de Il-1 β , pelo que é plausível estabelecer uma relação entre a ativação do inflamassoma e as alterações reprodutivas associadas à obesidade. Assim, este trabalho caracteriza histologicamente a expressão do inflamassoma Nlrp3 no ovário de murganhos obesos, tendo como controlo a expressão da Il-1 β . Para tal, levou-se a cabo a técnica de imunohistoquímica de forma a identificar as proteínas Nlrp3 e Il-1 β em ovários dos seguintes quatro diferentes grupos de murganhos: i) murganhos em que a obesidade foi induzida por dieta; ii) murganhos ob/ob; iii) murganhos db/db; iv) murganhos injetados com leptina. No total, foram observados 21 cortes de ovários de murganhos com o anticorpo do inflamassoma Nlrp3 e 22 cortes de ovários de murganhos com o anticorpo Il-1 β . Com este trabalho, foi possível concluir que o inflamassoma Nlrp3 está expresso nos oócitos de todos os folículos de cada fase folicular, nas células da granulosa e no estroma de ovários obesos, o que comprova a associação da inflamação com a obesidade.

Palavras-chave: inflamassoma Nlrp3, obesidade, inflamação, ovário, Il-1 β , imunohistoquímica

Abstract

Obesity has been increasing during the last years. Also, the number of obese women with reproductive disorders has been increasing. It is known that the Nlrp3 inflammasome is present in the inflammatory state characteristic of obesity and for the production and secretion of Il-1 β . Therefore, it is plausible to establish the link between inflammasome activation and obesity related reproductive disorders. Thus, this study histologically characterises the expression of the Nlrp3 inflammasome in the ovary of obese mice, having as a control the expression of Il-1 β . Therefore, it was performed immunohistochemistry staining of Nlrp3 and Il-1 β proteins in ovaries collected from four different groups of mice as follows: i) diet-induced obesity mice; ii) ob/ob mice; iii) db/db mice; iv) leptin-injected mice. A total of 21 mice ovarian cuts with the Nlrp3 inflammasome antibody and 22 mice ovarian cuts with the Il-1 β antibody were processed. With this study, it was possible to conclude that the Nlrp3 inflammasome is expressed in the oocytes of follicles of all stages of the follicle development, in the granulosa cells and in the stroma of obese ovaries, supporting the relation between inflammation and obesity.

Keywords: Nlrp3 inflammasome, obesity, inflammation, ovary, Il-1 β , immunohistochemistry

Resumo das secções em Português

1. Revisão bibliográfica

1.1. Introdução

A obesidade é uma das grandes problemáticas dos países desenvolvidos e tem aumentado a nível mundial nos últimos anos. Em 2016 afetava 13% da população mundial, sendo que 15% das mulheres eram obesas (World Health Organization, 2018). No entanto, já em 2015, em Portugal, 32% das mulheres encontravam-se nesta situação (World Obesity Federation, 2018).

A obesidade está associada a um aumento da incidência de várias afeções, como doenças neurológicas, doenças cardiovasculares, diabetes mellitus do tipo 2, doenças respiratórias, doenças gastrointestinais, doenças autoimunes, doenças do trato urinário, doenças musculoesqueléticas e infertilidade, situação que tem motivado uma preocupação crescente sobre esta temática e levado ao desenvolvimento de estratégias e terapias que previnam ou controlem o excesso de peso.

Os efeitos negativos que a obesidade tem sobre a reprodução são bem conhecidos; contudo, o mecanismo através do qual afeta o sistema reprodutivo ainda não está completamente desvendado (Dağ & Dilbaz, 2015). Contudo, sabe-se que o inflamassoma Nlrp3 está presente no estado inflamatório característico da obesidade e é responsável pela produção e secreção de IL-1 β .

Neste trabalho, irá ser estudada a relação entre a obesidade e a ativação do inflamassoma Nlrp3 em ovários de murganhos obesos. O objetivo geral consiste em caraterizar histologicamente a ativação do inflamassoma Nlrp3. Para além disso, pretende-se comparar a ativação deste inflamassoma em diferentes estadios de obesidade e, também, pretende-se avaliar a influência que a leptina tem na ativação do inflamassoma.

A identificação do mecanismo de regulação e ativação do inflamassoma Nlrp3 no ovário de murganhos obesos permitirá uma melhor compreensão de como a obesidade afeta a capacidade reprodutiva da mulher, o que poderá conduzir para a investigação sobre possíveis terapias para a infertilidade induzida por obesidade.

1.2. O Murganho como modelo experimental

Inicialmente, os murganhos eram vistos como animais de estimação (Guénet & Bonhomme, 2004; Roderick, 2007), mas há séculos que os murganhos têm sido usados para investigações científicas, até mesmo por Gregor Mendel nos anos 1800s (Roderick, 2007).

Há variadas razões para os murganhos serem usados como modelo para estudo da biologia e doenças humanas. Uma dessas razões é porque cerca de 99% dos genes de murganhos têm um homólogo no genoma humano (Mouse Genome Sequencing Consortium [MGSC], 2002). Para além disso, os roedores têm um ciclo éstrico de curta duração, sendo possível estudar mudanças que ocorram durante o ciclo reprodutivo (Caligioni, 2009). De acordo com o Jackson Laboratory (2018), algumas razões – fora as já referidas – para os murganhos serem os animais mais usados para estudar a biologia humana e doenças humanas são as seguintes:

- A capacidade de manipular geneticamente os murganhos para conseguir reproduzir doenças humanas;
- Os murganhos podem ser cruzados consaguinamente para produzirem estirpes geneticamente idênticas;
- Como um ano de vida em murganhos corresponde a cerca de 30 anos de vida em humanos, o seu ciclo de vida poderá ser estudado dentro de dois ou três anos;
- A biologia e fisiologia dos murganhos está muito bem estudada;
- Os murganhos têm uma produção de baixo custo, tendo em conta a sua eficiência para a investigação.

Existem, também, estirpes de murganhos mutantes. Duas delas são os murganhos ob/ob e os murganhos db/db. Os murganhos ob/ob têm uma mutação no gene da leptina, no cromossoma 6 (Smith, 2012; Van der Spek, Kreier, Fliers & Kalsbeek, 2012). Estes murganhos têm deficiência em leptina e, assim, nunca estão saciados, tornando-se obesos. São hiperfágicos, inativos, hiperglicémicos e hiperinsulinémicos; têm níveis elevados de corticoesteroides, têm hipotiroidismo, dislipidemia e temperatura corporal baixa (Smith, 2012; Van der Spek et al., 2012).

Os murganhos db/db têm uma mutação no gene do recetor da leptina (Mohammed-Ali, Carlisle, Nademi & Dickhout, 2017). Apesar destes murganhos terem níveis normais de leptina, a mutação que têm no gene recetor da mesma faz com que tenham um fenótipo similar aos murganhos ob/ob (Smith, 2012).

1.3. Oogénese

A oogénese é um processo que começa com a junção de folículos primordiais, no ovário fetal, e consiste na diferenciação do óvulo (Albertini, 2015).

De acordo com Wang et al. (2017), durante a embriogénese, várias proteínas morfogénicas ósseas (BMPs) induzem os epiblastos a tornarem-se em células germinativas primordiais (PGCs). Depois da proliferação mitótica, as PGCs diferenciam-se em oogónias, entram em meiose e tornam-se oócitos primários. Este processo ocorre uns dias depois do nascimento em murganhos, ratazanas e hamsters, e uns dias antes do nascimento em humanos, vacas e ovelhas (Smitz & Cortvrindt, 2002).

A maioria das células germinativas desaparece por volta dos quatro dias pós-parto e as células sobreviventes são individualmente envolvidas por células da pré-granulosa e, assim, dá-se a formação de um folículo primordial (Wang et al., 2017).

O reservatório de folículos primordiais representa a reserva ovárica de onde os folículos são recrutados para se submeterem ao crescimento e ovulação, durante o ciclo reprodutivo da fêmea.

1.4. Foliculogénese

A foliculogénese é um processo em que os folículos primordiais começam a desenvolver-se e consiste em três processos – proliferação celular, diferenciação e atresia – para promoção do crescimento e maturação dos folículos e acaba com a ovulação (Eppig, 2001; Smitz & Cortvrindt, 2002; Rimon-Dahari, 2016).

Durante a foliculogénese, o folículo primordial desenvolve-se em folículo primário, folículo secundário, folículo pré-antral, folículo antral e folículo atrético (Pedersen & Peters, 1968; Gougeon, 1996; Myers, Britt, Wreford, Ebling & Kerr, 2004). Contudo, pouco se sabe sobre os mecanismos de regulação da formação do folículo, atresia do oócito e desenvolvimento e atresia folicular, apesar de já haver estudos sobre este assunto (Smitz & Cortvrindt, 2002).

A foliculogénese começa quando os folículos primordiais deixam a reserva e entram na fase de crescimento (Gougeon, 1996). Nos murganhos, a duração desta fase de crescimento é de três semanas (Amleh & Dean, 2002).

Os folículos primários formam-se quando há uma só camada de células da granulosa a envolvê-los. Ao haver proliferação das células da granulosa, os folículos primários transformam-se em folículos secundários (Fortune, 2003). Durante esta fase, há a formação de células da teca (Cortvrindt & Smitz, 2001). A teca é composta por duas camadas: a teca interna e a teca externa (Edson, Nagaraja & Matzuk, 2009). O crescimento dos folículos até ao estadio pré-antral e antral, é independente da hormona folículo-estimulante (FSH) e da hormona luteinizante (LH) (Picton, Harris, Muruvi & Chambers, 2008). Após a puberdade, o desenvolvimento dos folículos para o estadio antral é acelerado pela FSH e LH (Kumar, Wang, Lu & Matzuk, 1997; Edson, Nagaraja & Matzuk, 2009). A FSH é, depois, inibida pelo estradiol, que vai aumentar a produção de LH pela pituitária, causando um pico de LH (Edson et al., 2009). Este pico nos níveis de LH faz com que o oócito expulse o primeiro corpo polar, ficando retido na metafase II (Richards, Russell, Ochsner & Espey, 2002). Com o pico de LH, a ovulação ocorre e surge o fim da foliculogénese. A maioria dos folículos sofrem atresia e podem sofrê-la em qualquer estadio de desenvolvimento; contudo, é mais comum sofrerem atresia no estadio pré-antral e antral (Amleh & Dean, 2002).

1.5. Classificação de folículos

Os folículos são classificados de acordo com as suas características morfológicas. De acordo com o aspecto das células da granulosa, os folículos podem ser classificados como primordiais, primários, secundários, pré-antrais e antrais (Gougeon, 1996; Myers et al., 2004).

Pedersen e Peters (1968) propuseram, primeiramente, uma classificação de oócitos e folículos baseada no tamanho do oócito, células da granulosa e morfologia do folículo. Os autores dividiram os folículos em pequenos, médios e grandes e subdividiram-nos em tipo 1, tipo 2, tipo 3a, tipo 3b, tipo 4, tipo 5a, tipo 5b, tipo 6, tipo 7 e tipo 8 (Peters, 1969). O tipo 1, tipo 2 e tipo 3a foram considerados folículos pequenos. O tipo 3b, tipo 4 e tipo 5a foram considerados folículos médios. Os tipos 5b, 6, 7 e 8 foram considerados folículos grandes.

Gougeon (1996) e Myers et al. (2004), categorizaram os folículos como primordiais, primários, secundários, pré-antrais e antrais.

Os folículos primordiais são folículos que consistem num oócito rodeado por uma camada parcial ou completa de células da granulosa (Myers et al., 2004).

Folículos primários são aqueles que contêm apenas uma camada de células da granulosa a envolver o oócito (Myers et al., 2004).

Os folículos secundários consistem num oócito rodeado por múltiplas camadas de células da granulosa e não têm um antro visíveis (Myers et al., 2004).

Os folículos pré-antrais consistem num oócito rodeado por múltiplas camadas de células da granulosa (Amleh & Dean, 2002). Pequenas cavidades com líquido começam por se formar entre as células da granulosa (Priedkalns & Leiser, 2006).

Folículos antrais consistem em folículos cujas células da granulosa se encontra rodeadas por uma camada de células da teca e por uma cavidade com fluido, o antro (Priedkalns & Leiser, 2006).

1.6. Ciclo Éstrico dos Murganhos

O ciclo éstrico nos murganhos dura cerca de quatro a cinco dias e podem ser identificadas diferentes fases durante o ciclo – proestro, estro, metaestro e diestro. Há hormonas específicas responsáveis pelas mudanças que ocorrem durante cada fase do ciclo. Estas hormonas são reguladas pelo eixo hipotálamo-hipófise-gonadal (Bertolin & Murphy, 2014).

A hormona libertadora de gonadotropina (GnRH) vai estimular a secreção de LH e FSH, que, por sua vez, estimulam células específicas no ovário que são responsáveis pela ovulação (Caligioni, 2009).

Quando o estrogénio, secretado pelos folículos ováricos, alcança um pico, no período pré-ovulatório, a GnRH é estimulada (Sarkar, Chiappa, Fink & Sherwood, 1976; Levine, 2015).

O proestro dura menos de 24 horas nos murganhos (Astwood, 1939; Long & Evans, 1922; Mandl, 1951; Allen, 1922; Grasso, Rozhavskaia & Reichert, 1998 citado em Cora, Kooistra & Travlos, 2015). É nesta fase que a FSH faz com que os folículos antrais cresçam rapidamente. Para além disto, neste estágio, o estradiol afeta o hipotálamo através de *feedback* positivo, por isso, quando os níveis de estrogénio aumentam, ocorre um pico de LH e a ovulação ocorre 12 a 14 horas depois do pico de LH (Bertolin & Murphy, 2014).

O estro nos murganhos dura cerca de 24 a 48 horas (Astwood, 1939; Long & Evans, 1922; Mandl, 1951; Allen, 1922; Grasso et al., 1998, citado em Cora et al., 2015). Nesta fase, os níveis de estrogénio continuam elevados durante a manhã, mas baixam para níveis basais à tarde (Walmer, Wrona, Hughes & Nelson, 1992, citado em Caligioni, 2009).

O metaestro pode durar até 24 horas (Allen, 1922; Grasso et al., 1998, citado em Cora et al., 2015). Nesta fase, a progesterona inibe a secreção de LH, e a regressão do corpo lúteo no final do metaestro causa a redução dos níveis de progesterona, o que vai resultar na inibição da secreção de LH (Bertolin & Murphy, 2014).

O diestro tem uma duração de cerca de 48 a 72 horas (Astwood 1939; Long & Evans 1922; Mandl 1951; Allen 1922; Grasso et al., 1998). É nesta fase que os níveis de estrogénio começam a aumentar (Walmer et al., 1992, citado em Caligioni, 2009).

1.7. O inflamassoma Nlrp3 e a obesidade e as suas consequências para o ovário

Os componentes dos inflamassomas são complexos multiproteicos localizados no citoplasma das células. O inflamassoma Nlrp3 é composto por três subunidades - *Nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing 3* (Nlrp3), uma proteína adaptadora, a *apoptosis-associated speck-like protein containing a caspase recruitment domain* (ASC) e caspase-1.

Os macrófagos e outras células do sistema imunitário inato podem induzir reações inflamatórias através da deteção de patogéneos ou *danger-associated molecular patterns* (PAMPs ou DAMPs), através da utilização de *pattern-recognition receptors* (PRRs) (Rheinheimer, de Souza, Cardoso, Bauer & Crispim, 2017). Na presença de PAMPs ou DAMPs, o Nlrp3 vai interagir com o domínio NACHT (Tschopp & Schroder, 2010). Após ativação através de DAMPs, o Nlrp3 recruta pro-caspase-1 via proteína ASC, para formar o inflamassoma. Com a ativação do inflamassoma, há a clivagem da pro-caspase-1 em caspase-1 ativa, que, subsequentemente, cliva a pro-Interleucina-1 β (pro-Il-1 β) na sua forma ativa, a Interleucina-1 β (Il-1 β) (Hernandez, Sirois & Latz, 2011). Este inflamassoma é, assim, responsável por induzir a produção de Il-1 β e também de Il-18, importantes citocinas inflamatórias, tendo, por isso, um papel importante na inflamação. Outros mecanismos que podem induzir a ativação do inflamassoma são espécies reativas de oxigénio, efluxo de potássio e destabilização do lisossoma. Trifosfato de adenosina, ácido úrico, ácidos gordos, glucose, polipéptido amiloide das ilhotas e leptina são substâncias que também podem ativar o inflamassoma.

A obesidade está associada a alterações na função reprodutiva feminina. Honnma et al. (2010), usando fêmeas de murganhos *Zucker Fatty*, modelos de obesidade e hiperinsulinemia, observaram nos ovários de obesas um maior número de folículos em atresia, múltiplos quistos e diminuição de crescimento folicular. Adicionalmente, os autores observaram que o ciclo estrico das fêmeas obesas apresentava anormalidades e que a incidência destas alterações aumentava com a idade.

Jia et al. (2018), usando um grupo de murganhos alimentados com dieta rica em gordura e outro grupo de murganhos alimentados com dieta normal, investigaram os possíveis efeitos que a obesidade tem no oócito. Os autores mostraram que a obesidade pode afetar a meiose do oócito, pode destruir a *spindle assembly*, pode promover stresse oxidativo e distribuição anormal das mitocôndrias e faz com que os óocitos se tornem moles.

Para além de alterações ováricas e no ciclo estrico, Balasubramanian et al. (2011) mostraram que uma dieta rica em gorduras provoca um aumento dos níveis séricos de leptina, levando a uma diminuição dos níveis de estradiol.

Sabe-se que o inflamassoma Nlrp3 está presente no estado inflamatório característico da obesidade e é responsável pela produção e secreção de IL-1 β em vários tecidos e tipos de células, durante o desenvolvimento da obesidade. Tem, também, um papel na fisiopatologia de diabetes mellitus do tipo 2 (Stienstra et al., 2012).

Stienstra et al. (2011) e Vandanmagsar et al. (2011) mostraram que o inflamassoma Nlrp3 é o responsável pelo estado inflamatório associado à obesidade e pela insulinoresistência. Os primeiros autores utilizaram um grupo de murganhos geneticamente manipulados que apresentavam ausência de Nlrp3 e Caspase-1, alimentados com dieta baixa em gordura, e um outro grupo de murganhos, alimentados com dieta rica em gordura. Utilizaram, ainda, dois grupos de murganhos *wild type* com os mesmo parâmetros das referidas dietas. Com este estudo, os autores mostraram que a ausência dos componentes do inflamassoma Nlrp3 protege contra a obesidade induzida por uma dieta rica em gordura e contra a hiperinsulinemia.

Vandanmagsar et al. (2011), utilizando também murganhos transgênicos com ausência do inflamassoma Nlrp3, mostraram igualmente que a remoção deste inflamassoma protege significativamente contra a obesidade induzida pela dieta rica em gordura, bem como contra a insulinoresistência, a intolerância à glucose e a inflamação. Estes autores propuseram ainda que a ativação do inflamassoma Nlrp3 nos macrófagos poderá ser importante para a patofisiologia da inflamação na obesidade. No tecido adiposo de obesos, a acumulação de

ácidos gordos pode levar ao aumento de ceramida. Estes autores também mostraram que, ao adicionarem ceramida ao tecido adiposo, a produção de Il-1 β , dependente de Nlrp3, aumentou, sugerindo, assim, que a ceramida atua como um *danger signal* que estimula a ativação do inflamassoma Nlrp3.

Assim, um melhor entendimento do mecanismo de regulação do inflamassoma Nlrp3 poderá conduzir a estudos sobre terapias de infertilidade induzida por obesidade; aliás, vários autores (Larsen et al, 2007; Rissanen, Howard, Botha & Thuren, 2012; Sloan-Lancaster et al., 2013) mostraram que a utilização de um inibidor da Il-1 β , como Anakinra, um antagonista da Il-1, em murganhos obesos melhorou a homeostase da glucose e reduziu o aumento de peso.

Na área de Reprodução, alguns estudos, como Ortega & Duleba (2015) e Banaszewska, Wrotyńska-Barczyńska, Spaczynski, Pawelczyk & Duleba (2016), mostraram que o resveratrol tem efeitos benéficos para os ovários. Jia et al. (2018) também mostr que o resveratrol pode aliviar os efeitos negativos que a obesidade tem nos oócitos.

2. Objetivos

O presente estudo compreende os seguintes objetivos:

1. Caracterização histológica do inflamassoma Nlrp3 nos diferentes componentes de ovários obesos;
2. Comparação da presença do inflamassoma Nlrp3 entre os diferentes estadios da obesidade;
3. Avaliação da influência da leptina na expressão do inflamassoma Nlrp3.

3. Materiais e Métodos

Neste estudo, foram usados 23 ovários de murganhos. Estes murganhos foram criados e eutanasiados para um estudo maior no *Institute of Animal Reproduction and Food Research of Polish Academy of Sciences*, na Polónia, sendo que no presente trabalho foram só usadas amostras recolhidas previamente nesse Instituto. Após a eutanásia, foi feita excisão cirúrgica dos ovários.

Foram usadas amostras de ovários de murganhos, tendo em conta a dieta e a duração da mesma (dieta normal durante 16 semanas; dieta rica em gordura durante 16 semanas) - obesidade induzida por dieta; também foram usadas amostras de ovários de murganhos

mutantes - murganhos ob/ob e db/db - e foram usadas amostras de ovários de murganhos injetados com leptina durante 16 dias. Os protocolos utilizados para a criação dos murganhos, bem como a fenotipagem, foram realizados pela equipa do Doutor António Galvão no *Institute of Animal Reproduction and Food Research of Polish Academy of Sciences*. Os cortes histológicos e a imunohistoquímica dos mesmos foram realizados pela estudante na Universidade Lusófona, em Lisboa.

Os procedimentos realizados foram aprovados pelo Comité de Ética do Departamento de Imunologia e Patologia Reprodutiva, do *Institute of Animal Reproduction and Food Research of Polish Academy of Sciences*, Olsztyn, Polónia.

No estudo, no grupo de murganhos da obesidade induzida por dieta, não foram incluídos murganhos com menos de 35g que tenham sido alimentados com dieta rica em gordura, e não foram incluídos murganhos com menos de 24g que tenham sido alimentados com comida normal. Todos os animais foram eutanasiados no estro.

As amostras foram observadas num microscópio Olympus (CX21), numa apiação de 400x, por um único observador, a estudante. Em cada lâmina, os folículos foram classificados como primordiais, primários, secundários e antrais. Se os folículos apresentassem uma cavidade visível, seriam classificados como antrais, uma vez que era a classificação usada pelos investigadores do *Institute of Animal Reproduction and Food Research*. Foram também identificadas células da granulosa, células da teca, estroma e oócitos.

As imagens de imunohistoquímica foram tiradas com um microscópio ZEISS Axio Observer Z1, à exceção das imagens do grupo de leptina, que foram tiradas com um microscópio Olympus série CX. As fotos foram editadas com o programa GIMP 2.10.

3.1. Imunohistoquímica

A imunohistoquímica foi realizada em grupos de secções de ovários de murganhos com duas lâminas gémeas – o controlo positivo e o controlo negativo – e tinham 3 μ de espessura.

Todos os passos foram conduzidos à temperatura ambiente, a menos que indicado de outro modo (Anexo I).

Foram usados dois anticorpos primários, separadamente. Um foi usado para identificar a presença de IL-1 β (sc-7884; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) e o outro para identificar a presença do inflamassoma Nlrp3 (ab214185; Abcam, Cambridge, UK).

Antes de começar o protocolo de IHC-P, foi conduzido um protocolo de IHC-P para validação dos anticorpos. Diferentes diluições de ambos os anticorpos foram usadas (1:50; 1:100; 1:200; 1:600). IgG (1:100; 1:200; 1:600) e cortes só com anticorpo secundário foram usados como controlo negativo.

Depois das lâminas terem sido colocadas na estufa, a 37°C, *overnight*, procedeu-se à desparafinização e rehidratação em concentrações decrescentes de álcool. De seguida, as lâminas foram lavadas em água destilada e posteriormente foram incubadas em 3% H₂O₂, a 37°C, por 15 minutos, no escuro. A recuperação antigénica foi feita em 10 mM de tampão citrato pH6 (450 mL of H₂O + 9 mL de citrato 0.1M pH1.8 + 41 mL de citrato de sódio 0.1M pH8.8) em solução aquecida no microondas (700W), três vezes, por cinco minutos. As lâminas foram arrefecidas, por 20-30 minutos. Depois foi distribuída uma solução de bloqueio (PBS com 2% BSA - A7906, Sigma-Aldrich, Inc., St Louis, MO, USA), num volume de 200 µL por lâmina, na câmara húmida, durante 1 hora. Posteriormente as lâminas positivas foram incubadas no anticorpo primário (sc-7884; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA/ ab214185; Abcam, Cambridge, UK) a 4°C, *overnight*. As lâminas de control negative foram incubadas só na solução de bloqueio. O anticorpo para a Il-1β (sc-7884; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) foi usado numa diluição de 1:100 e o anti-inflamassoma Nlrp3 (ab214185; Abcam, Cambridge, UK) foi usado numa diluição de 1:200.

Após terem sido lavadas em PBS, durante 10 minutos, as lâminas foram incubadas no anticorpo secundário, policlonal cabra anti-coelho IgG conjugado com peroxidase (410972, Dako, Carpinteria, CA, USA), por 1 hora, numa diluição de 1:100 1:100. De seguida, as lâminas foram lavadas em PBS, por 10 minutos, 2 vezes. Depois, foram incubadas no cromogénio 3'-*diaminobenzidine* (DAB) (ImmPACT DAB, SK-4105, Vector Laboratories, Burlingame, CA, USA), até haver reação e seguidamente foram lavadas em água destilada e coradas com hematoxilina de Gill, por cerca de 45 segundos.

Procedeu-se à desidratação em concentrações ascendentes de álcool, colocadas em xilol e depois montadas em Entellan®.

4. Resultados

A amostra deste estudo foi constituída por um total de 21 ovários obesos, para o Nlrp3 e 22 para a Il-1β. No grupo dos murganhos com obesidade induzida pela dieta, foram obtidos

5 ovários, para ambos os anticorpos. No grupo ob/ob, foram obtidos 5 ovários, para ambos os anticorpos. No grupo db/db foram obtidos 7 ovários, para o Nlrp3 e 8 ovários para Il-1 β . No grupo do modelo de hiperleptinemia, foram obtidos 4 ovários, para ambos os anticorpos.

Foi feita uma contagem de folículos. Assim, foram observados 213 folículos, em 21 lâminas de ovários com o anticorpo para o inflamassoma Nlrp3. No grupo dos murganhos alimentados com dieta normal, observou-se marcação em um folículo primordial, um primário, dois secundários e um antral. No grupo dos murganhos alimentados com dieta rica em gordura, observou-se marcação em cinco folículos primordiais, quatro primários, cinco secundários e um antral. No grupo dos murganhos ob/ob homozigóticos, observou-se marcação em quatro folículos primordiais, sete primários, 20 secundários e seis antrais. No grupo dos murganhos ob/ob heterozigóticos, observou-se marcação em quatro folículos primordiais, dois primários, 15 secundários e zero antrais. No grupo dos murganhos db/db homozigóticos, observou-se marcação em três folículos primordiais, um primário, um secundário e zero antrais. No grupo dos murganhos db/db heterozigóticos, observou-se marcação em 14 folículos primordiais, cinco primários, 16 secundários e três antrais. No grupo dos murganhos injetados com leptina, observou-se marcação em três folículos primordiais, um primário, zero secundários e dois antrais. No grupo dos murganhos injetados com soro fisiológico, observou-se marcação em três folículos primordiais, quatro primários, sete secundários e 11 antrais.

Em relação às amostras com o anticorpo da Il-1 β , foram observados 267 folículos, em 22 lâminas de ovários. No grupo dos murganhos alimentados com dieta normal, observou-se marcação em um folículo primordial, zero primários, um secundário e um antral. No grupo dos murganhos alimentados com dieta rica em gordura, observou-se marcação em cinco folículos primordiais, zero primários, um secundário e zero antrais. No grupo dos murganhos ob/ob homozigóticos, observou-se marcação em cinco folículos primordiais, 16 primários, 16 secundários e cinco antrais. No grupo dos murganhos ob/ob heterozigóticos, observou-se marcação em 10 folículos primordiais, cinco primários, 11 secundários e 10 antrais. No grupo dos murganhos db/db homozigóticos, observou-se marcação em um folículo primordial, um primário, sete secundários e dois antrais. No grupo dos murganhos db/db heterozigóticos, observou-se marcação em 11 folículos primordiais, três primários, 17 secundários e seis antrais. No grupo dos murganhos injetados com leptina, observou-se marcação em três folículos primordiais, um primário, zero secundários e dois antrais. No grupo dos murganhos injetados

com soro fisiológico, observou-se marcação em cinco folículos primordiais, zero primários, zero secundários e cinco antrais.

Relativamente à imunohistoquímica, observou-se expressão do inflamassoma Nlrp3 nos oócitos de todos os folículos de cada fase folicular, nas células da granulosa e no estroma. As células da teca não apresentaram marcação. A Il-1 β teve os mesmos padrões de expressão.

5. Discussão

O resultado conseguido neste trabalho acerca da expressão do inflamassoma Nlrp3 no ovário confirma outro estudo já descrito (Wang et al., 2017). Para além de confirmar a presença do inflamassoma no ovário, os resultados obtidos no presente estudo vêm acrescentar a caracterização da expressão do mesmo nos componentes do ovário.

Assim, o inflamassoma Nlrp3 é expresso em todos os tipos de folículo de cada fase folicular, nos oócitos, nas células da granulosa e no estroma. Para que se pudesse obter resultados fiáveis, também foi observada a expressão de Il-1 β , uma vez que esta interleucina é produzida devido à ativação do inflamassoma. A Il-1 β também se expressa em todos os tipos de folículo de cada fase folicular, nos oócitos, nas células da granulosa e no estroma.

Que seja do conhecimento da estudante, este é o primeiro estudo que caracteriza histologicamente a expressão do inflamassoma Nlrp3 especificamente no ovário de murganhos obesos. Contudo, o papel inflamatório que este inflamassoma tem na obesidade já foi estudado (Sienstra et al, 2011; Stienstra et al., 2012; Vandanmagsar et al., 2011), e, apesar de o ovário não ser o seu objeto de estudo, estes trabalhos foram usados como referência para a ativação do inflamassoma Nlrp3 em murganhos obesos.

A presença do inflamassoma Nlrp3 e da Il-1 β , observados através da técnica de imunohistoquímica, mostrou que a inflamação está presente no ovário de murganhos obesos. Esta inflamação pode ser uma das razões para os problemas reprodutivos já estudados em fêmeas de murganhos obesas (Honnma et al., 2010; Balasubramanian et al., 2011; Jia et al., 2018).

Stienstra et al. (2011) e Vandanmagsar et al. (2011) mostraram que, ao remover-se o gene do inflamassoma Nlrp3 de murganhos, estes não desenvolvem obesidade, mesmo sendo alimentados com uma dieta rica em gordura. Os resultados do presente estudo, por sua vez, mostraram que murganhos alimentados com uma dieta rica em gordura expressaram o

inflamassoma Nlrp3, e também Il-1 β , nos componentes do ovário, confirmando, de certa maneira, os resultados previamente conseguidos pelos estudos mencionados. Os murganhos alimentados com uma dieta rica em gordura apresentaram um número mais elevado de folículos marcados do que os murganhos alimentados com uma dieta normal, o mesmo se verificando relativamente à Il-1 β . Através da imunohistoquímica, os murganhos alimentados com dieta rica em gordura obtiveram uma maior intensidade de marcação do que os murganhos alimentados com dieta normal.

No que diz respeito às diferenças de expressão do inflamassoma Nlrp3 entre os murganhos ob/ob homozigóticos e os heterozigóticos, esperava-se, de acordo com a literatura (Smith, 2012; Van der Spek et al., 2012), obter uma intensidade de marcação maior nos homozigóticos, porque estes murganhos são os que desenvolvem obesidade e, consequentemente, todas as complicações associadas a esta condição. No presente estudo, os homozigóticos apresentaram um número maior de folículos marcados não só com o anticorpo do inflamassoma Nlrp3, mas também com o da Il-1 β . Na imunohistoquímica, os homozigóticos apresentaram uma intensidade maior de marcação para ambos os anticorpos.

Esperava-se também que os murganhos db/db homozigóticos apresentassem uma expressão mais evidente do inflamassoma Nlrp3 e de Il-1 β do que os heterozigóticos, uma vez que os primeiros são os que desenvolvem obesidade e as complicações a ela associadas (Smith, 2012). Contudo, o número de folículos marcados foi mais elevado no grupo dos heterozigóticos, para ambos os anticorpos, contrariamente ao que se esperava. Com efeito, os murganhos homozigóticos deviam ter tido uma expressão mais evidente do inflamassoma, uma vez que desenvolviam obesidade mais facilmente do que os heterozigóticos. Uma das razões que podem explicar este resultado é o facto de a observação microscópica ter sido feita por apenas um observador, podendo ter ocorrido erros. Relativamente à imunohistoquímica, com o pequeno número de amostras é difícil identificar diferenças.

Quanto ao grupo de murganhos modelos de hiperleptinemia, esperava-se uma expressão mais elevada do inflamassoma Nlrp3 nos murganhos injetados com leptina, uma vez que esta desencadeia a ativação do inflamassoma (Fu et al., 2017). Os resultados da imunohistoquímica apresentam uma maior intensidade de marcação nos murganhos injetados com leptina. O grupo de controlo apresentou um número mais elevado de folículos marcados, o que não era esperado, pois a leptina leva à ativação do inflamassoma e, assim, o grupo de

murganhos injetados com esta hormona é que devia apresentar um maior número de folículos marcados. Uma vez mais, este resultado pode ter sido obtido porque a observação foi feita apenas por uma pessoa. Para além disto, a morfologia dos ovários não era a melhor, pois os mesmos apresentavam muitos quistos.

Durante o desenvolvimento deste trabalho, registaram-se algumas limitações. Em primeiro lugar, foram necessários alguns meses para adequar o protocolo de imunohistoquímica no que diz respeito à melhor diluição a utilizar para os anticorpos primários. Para além disso, em alguns blocos enviados da Polónia, verificaram-se perdas de amostra e problemas com a parafina, o que impossibilitou o corte dos mesmos. Alguns ovários não tinham uma boa morfologia e não puderam ser usados. Assim, o número total de amostras válidas não permitiu uma análise estatística, o que poderia ter possibilitado uma interpretação mais robusta dos resultados. Foi feita apenas uma contagem descritiva de folículos, não se obtendo dados estatisticamente significativos. Os resultados das diferenças entre grupos podem também não ser representativos, pela mesma razão.

Outra limitação está relacionada com a imunofluorescência. O aperfeiçoamento do protocolo respetivo, de forma a obter a melhor diluição para os anticorpos, exigiu algum tempo. Tiveram de ser adquiridos novos anticorpos, porque os disponíveis na altura não eram eficazes. Ocorreram também problemas de auto-fluorescência, que obrigaram a novo aperfeiçoamento do protocolo. Tudo isto fez com que fosse impossível realizar imunofluorescência, uma vez que esta técnica iria ser conduzida na Polónia, onde a estudante apenas esteve dois meses. Se a imunofluorescência tivesse sido realizada, poder-se-iam obter resultados que confirmassem a diferença quantitativa entre grupos e não apenas qualitativa, como acontece com a imunohistoquímica.

Apesar destas limitações, o trabalho levado a cabo neste estudo permitiu caracterizar a expressão do inflamassoma Nlrp3 nos componentes do ovário obeso. Ao identificar a presença deste marcador de inflamação no ovário de murganhos obesos, este estudo poderá levar a outros sobre terapêuticas para as complicações que a obesidade tem na função reprodutiva, como a infertilidade em mulheres obesas.

6. Conclusão

Este estudo caracteriza a expressão do inflamassoma Nlrp3 no ovário obeso. De acordo com os resultados obtidos, é possível afirmar que este inflamassoma está presente nos oócitos de todos os folículos de cada fase folicular, nas células da granulosa e no estroma.

O facto de se ter usado o anticorpo Il-1 β como controlo torna os resultados mais expressivos. Com efeito, dado que esta interleucina é produzida pelo inflamassoma Nlrp3, ambos deverão estar presentes nos mesmos locais, o que se verificou neste trabalho.

Assim, os objetivos do trabalho foram, na generalidade, conseguidos, à exceção do objetivo de avaliar a influência da leptina na expressão do inflamassoma Nlrp3, que não foi completamente cumprido. O número pequeno de amostras e o facto de apenas se ter realizado uma técnica qualitativa, a imunohistoquímica, não permitiu que se obtivessem resultados conclusivos sobre a influência da leptina.

Em estudos futuros, técnicas como imunofluorescência e *western blot* devem ser realizadas, para que se possa obter uma análise quantitativa e resultados mais conclusivos. Algumas destas técnicas já estão a ser usadas num estudo mais amplo sobre o inflamassoma Nlrp3, no Institute of Animal Reproduction and Food Research, na Polónia, ainda em fase de desenvolvimento.

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List of Abbreviations

ASC - Adapter protein apoptosis-associated speck-like protein containing a caspase recruitment domain

BMDMs - Bone marrow-derived macrophages

BMPs - Bone morphogenetic proteins

CD – Chow diet

CD68⁺ - Cluster of differentiation 68

DAMPs - Danger-associated molecular patterns

DMBG - Dymethyldiguanide

E2 - Estrogen

EC - Estrous cycle

FSH - Follicle-Stimulating Hormone

GnRH - Gonadotropin-releasing hormone

HFD - High-Fat Diet

IHC - Immunohistochemistry

IHC-P - Immunohistochemistry-Paraffin

IL- 1 β - Interleukin-1 β

IL- 18 - Interleukin-18

IAPP - Islet amyloid polypeptide

LACH – Laboratory of Clinical Analysis and Histopathology

LH - Luteinizing Hormone

LPS - Lipopolysaccharide

NLRP3 - Nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing- 3

NF- κ B - Nuclear factor kappa-light-chain-enhancer of activated B cells

PAMPs - Pathogen-associated molecular patterns

PGCs - Primordial Germ Cells

PRO-IL-1 β - Pro- interleukin-1 β

PRRs - Pattern-recognition receptors

ROS - Reactive oxygen species

TIR - Toll-IL-1 receptor

TRX - Thioredoxin

TXNIP - Thioredoxin-interacting protein

XOR - Xanthine oxidoreductase

Introduction

The present dissertation was written as part of the Integrated Master in Veterinary Medicine of Lusófona University of Humanities and Technologies (FMV-ULHT). In the last semester of the course, the student was given the opportunity to have an internship and an externship. The first one was at the the Laboratory of Clinical Analysis and Histopathology, of FMV-ULHT, under the supervision of Professor Daniel Murta. The second one was at the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, in Olsztyn, Poland, in the department of Reproductive Immunology and Pathology, under the supervision of Dr. António Galvão. Thus, the study reported in this dissertation represents a result of the research activities developed in these laboratories.

Bearing in mind the general topic of the dissertation, animal reproduction, the histological characterisation of the Nlrp3 inflammasome in the obese ovary was set as the main aim of this study.

This dissertation is divided into three chapters. Chapter One presents a brief description of the developed activities during the internship and externship, as well as a brief description of the externship facilities. Chapter Two presents the literature review for the experimental work. Chapter Three presents the experimental work itself: the aims, materials and methods, results, discussion and conclusion.

The study presented in this dissertation focus on the ovary of obese mice, that were bred, euthanised and that had a surgical removal of its ovaries, at the the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, in Olsztyn, Poland.

Chapter I – Internship and Externship Report

1. Lusófona University of Humanities and Technologies – Faculty of Veterinary Medicine

The internship at the faculty of Veterinary Medicine took part between 13th of November 2018 and 28th of March 2019, at the Laboratory of Clinical Analysis and Histopathology (LACH), under the supervision of Professor Daniel Murta.

1.1. Developed Activities

In this intership, the intern was able to perform IHC-P on mice ovaries.

The first two weeks of the internship were a period of adaptation: it was possible to get to know where the materials were and the intern checked if there were any reagents missing; if there were, the intern had to prepare them. During this period, it was also possible to learn the Immunohistochemistry-Paraffin (IHC-P) protocol and to practice this technique, before working with the intern's own samples.

The intern also provided some support to Cellular Biology classes from the 1st year of the Veterinary Medicine course, by preparing the reagents and materials to be used in class, doing certain steps of the IHC-P protocol and helping the Professors during the classes. Furthermore, the intern helped a colleague with her IHC-P work.

Additionally, the intern learnt how to prepare slides for histological analysis, including performing histological cuts.

For the intern's IHC-P, it was used 23 samples of mouse ovaries. Four ovaries were from ovaries of mice that were fed with a high-fat diet (HFD) for 16 weeks. Two ovaries were from ovaries of mice that were fed with chow diet (CD) for 16 weeks. Five ovaries were from ob/ob mice, eight ovaries were from db/db mice. Two ovaries were from mice injected with saline and two ovaries were from mice injected with leptin for 16 days.

2. Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn, Poland

The second part of the curricular internship took place at the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, in Olsztyn, Poland, in the department of Reproductive Immunology and Pathology. This Institute was established in 1988; it holds the status of The National Leading Science Centre (KNOW) and it is a member of the European Institute for Innovation & Technology (EIT) – Food Knowledge and Innovation Community (KIC). The Institute is divided into two facilities: the division of food sciences and the division of reproductive biology, the latter being the one where the intern was working, which was located at Bydgoska Street. The externship had the duration of two months, from the 29th of March 2019 to the 31th of May 2019, under the supervision of Dr. António Galvão.

2.1. Developed Activities

In this externship, the intern was able to continue the work from the first internship and to contribute to a wider study on the same area of the student's master's dissertation.

The intern started by learning microscope image processing, including image acquisition and then 2D image techniques, for image enhancement. After the intern learnt how to process images by herself, she started to process the images from her IHC-P slides she performed in Lisbon, at the LACH, FMV-ULHT.

The student also performed immunohistochemistry in validation slides, both in ovary as in liver of obese mice, to validate the concentration of the antibodies used in her IHC-P slides. Validation on the ovaries included rabbit IgG (as a negative control), besides Nlrp3 and Il-1 β . Validation on the liver was done to be included on a PhD thesis of an Institute's PhD student and not on the student's master's dissertation. This validation included antibody for CD68⁺, besides rabbit IgG, Nlrp3 and Il-1 β . Figures 1 to 4 illustrate some of the results of the validation process. The immunohistochemistry protocol used will be discussed in Chapter III- section 2.6.

Another activity developed in the externship, was the validation of antibodies for immunofluorescence. One of the aims of this externship was to be able to perform immunofluorescence on the intern's slides, however, validation of antibodies had to be repeated several times and new antibodies (from different companies) had to be bought, so the intern was only able to perform validations and not the final immunofluorescence in her slides.

The immunofluorescence protocol used is going to be in Annex I. Conclusions of the immunofluorescence were not achieved during the externship, because of the need of new validations. In spite of the student not being able to perform new validations and achieve a final concentration of antibodies and the correct brand of the antibodies, a researcher from the Institute carried on with this process of validations.

Besides performing immunohistochemistry, immunofluorescence and microscope image processing, the intern also observed RNA extraction techniques and cell culture techniques being performed.

The intern also attended lectures about scientific articles and/or PhD thesis from researchers working at the student's department, every Monday. Plus, the intern had to give a short presentation about her master's dissertation for the department's researchers.

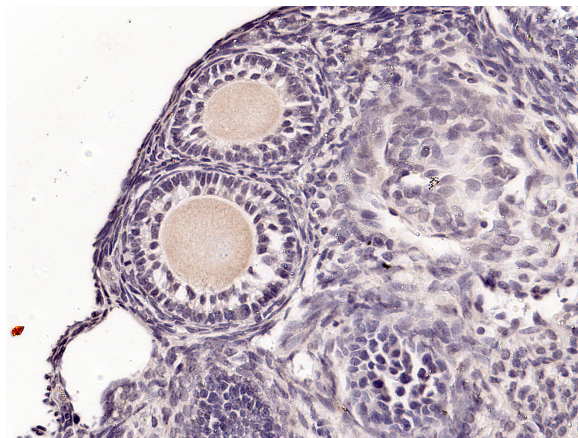


Figure 1 - Nlrp3 1:200. 400x. Ovarian follicles.
Author's original.

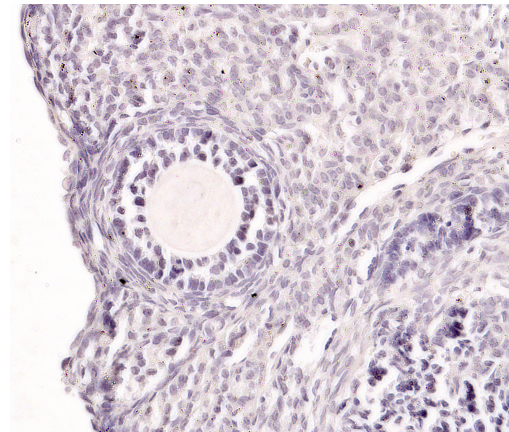


Figure 2- IgG 1:50. 400x. Ovarian follicle.
Author's original.

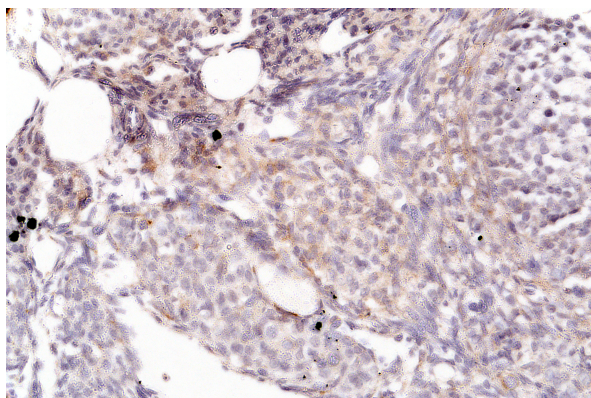


Figure 3- IL-1 β 1:50. 630x. Ovarian stroma.

Author's original.

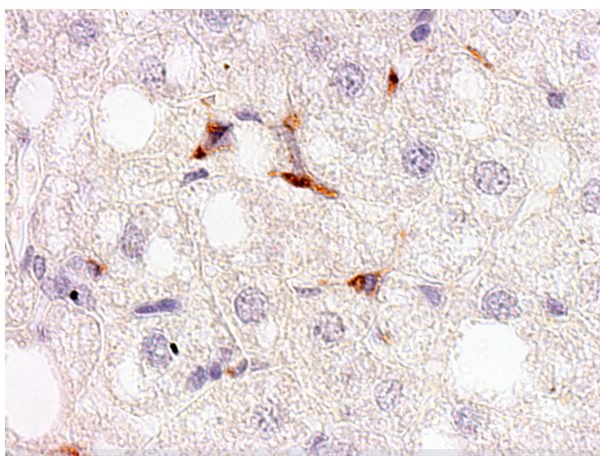


Figure 4- CD68⁺ 1:100. 630x. Liver. Author's original.

Chapter II- Introduction to the experimental work: the role of the Nlrp3 inflammasome in the obese ovary

1. Introduction

Obesity is one of the main problems in developed countries and it has been increasing globally over the past few years. In 2016, it affected 13% of the world's population and 15% of the women were obese (World Health Organization, 2018). In Portugal, in 2015, 32% of the women were affected by this condition (World Obesity Federation, 2018).

Obesity is associated with an increase of the occurrence of certain diseases (table1). According to Kinlen, Cody and O'Shea (2017), table 1 shows the most common complications of obesity. Thus, rising concern about this subject, it has led to the development of strategies and therapies that prevent or control the overweight.

The negative effects that obesity has on reproduction are well known; however, the mechanism in which it affects the reproductive system is not completely unveiled (Dağ & Dilbaz, 2015).

It is known that one of the mechanisms behind these alterations on fertility is the inflammasome. The main aim of this study is to do a histological characterisation of the Nlrp3 inflammasome activation in the obese ovaries. Furthermore, it is intended to make a comparison of the Nlrp3 inflammasome's presence between different stages of obesity and also an evaluation of the influence of leptin in the Nlrp3 inflammasome's expression.

The identification of the regulation and action of the Nlrp3 inflammasome in the ovary of obese mice will allow a better comprehension of how obesity affects women's reproductive capacity and it may lead to scientific investigation on possible treatment for obesity-induced infertility.

Table 1- Complications of obesity

System	Disease
Nervous	Increased risk of dementia and Alzheimer's disease.
Cardiovascular	Hypertension; Dislipidaemia; Coronary Heart Disease; Stroke; Heart Failure.
Endocrine	Type 2 Diabetes Mellitus.
Respiratory	Obstructive sleep apnoea; Asthma.
Immune	Increased risk of getting auto-immune diseases
Gastrointestinal	Gallbladder disease; Pancreatitis.
Urinary	Renal failure; Increased risk of kidney stones.
Musculoskeletal	Osteoarthritis.

2. The mouse as a model for human biology and diseases

The relationship between humans and mice has been thought to have started centuries ago. Initially, mice were probably just seen as pets (Guénet & Bonhomme, 2004; Roderick, 2007), but soon started being seen as an interest in research purposes, even by Gregor Mendel in the 1800s (Roderick, 2007).

According to Berry (1987), the earliest record of the use of mice for research purposes was probably in England, in 1664: Robert Hooke used mice to study the consequences the increase in air pressure had in biological terms.

But it was only in 1909, when Clarence Cook Little started working on mice genetics, at Harvard University - eventually creating a mouse inbred strain - that mice began being meaningful for the study of the human organism (Rader, 1999). The strain that was created, DBA/2, formerly dba, then DBA, is the most ancient of all the inbred strains. Ten years later, Abbie Lathrop established strain C57BL/6, in Granby, Massachusetts, USA (Guénet &

Bonhomme, 2004), while strains C3H, CBA and A were established by Leonell C. Strong, a cancer geneticist working at Cold Spring Harbor Laboratory (Strong, 1978).

Regarding mice breeding, the Jackson Laboratory (the JAX-Lab), founded in 1929, in Maine, USA, was, at that time, the main research institution promoting the mouse as a laboratory model. Nowadays, it still works on mammalian genetics research to improve human health (Guénet & Bonhomme, 2004).

There are many reasons for mice being a good model to study human biology and diseases. One of them is because approximately 99% of mouse genes have a homologue in the human genome (Mouse Genome Sequencing Consortium [MGSC], 2002). Furthermore, rodents have a short estrous cycle which makes them a good model for investigating changes during the reproductive cycle (Caligioni, 2009). According to the Jackson Laboratory (2018), some of the reasons - besides the ones referred previously - why mice are the most common animals used for studying human biology and diseases are the following:

- The ability to genetically manipulate mice to mimic virtually any human disease;
- Mice can be inbred to produce genetically identical strains. This allows more accurate and repeatable experiments;
- Since one mouse year equals about 30 human years, their life cycle can be studied within only two or three years;
- The biology and physiology of mice are very well understood, because they have been used in biomedical research for centuries;
- Mice have a low-cost production, concerning their research efficiency.

The analysis of the mouse genome and the use of this animal as a model organism seems to be unlimited. With the ability to genetically modify almost every mouse gene, it is likely that mice will continue to be the main animal used for human biology and diseases studies (Davisson & Linder, 2004).

2.1. ob/ob and db/db mice

The ob/ob mice are used to model diabetes mellitus type II and obesity. The main characteristic of ob/ob mice is a mutation in the leptin gene on chromosome 6 (Smith, 2012; Van der Spek, Kreier, Fliers & Kalsbeek, 2012). Leptin is a hormone responsible for the inhibition of hunger, secreted by white adipose tissue.

These mice are leptin deficient, thus, they do not have the feeling of satiety, becoming severely obese. The ob/ob mice are hyperphagic, inactive, hyperglycemic and hyperinsulinemic; they have high levels of corticosteroids, hypothyroidism, dyslipidemia and decreased body temperature (Smith, 2012; Van der Spek et al., 2012).

The mutation in the leptin gene in these mice causes sleep disorders and it lessens diurnal and overall locomotor activity (Van der Spek et al., 2012)

The db/db mouse is also used for modelling diabetes mellitus type II. The main characteristic of these mice is a mutation in the leptin receptor gene (Mohammed-Ali, Carlisle, Nademi & Dickhout, 2017). Although these mice have normal levels of leptin, the mutation they have in its receptor gene makes them have a similar phenotype as the ob/ob mice (Smith, 2012).

3. Anatomy and histology of the mouse ovary

3.1. Anatomy of the ovary

The mouse's ovaries "are located at the caudal poles of the kidneys, attached to the dorsal body wall by the mesovarium and enclosed in a transparent elastic bursa" (Boyd, Muchlenbachs, Rendi, Garcia & Gibson-Corley, 2018). As in Carretero, Ruberte and Navarro (2017), "they are suspended from the roof of the abdominal cavity by the mesovarium, a portion of the broad ligament of the uterus. The mesovarium joins the mesosalpinx, forming a membranous bag, the ovarian bursa, which surrounds the ovary". The mouse ovaries have a yellowish red colour. The vessels that are responsible for the arterial supply and for the venous drainage are the ovarian artery and vein, which are, respectively, branches from the dorsal aorta and inferior vena cava (Kaufman, Nikitin & Sundberg, 2010). Figure 5 represents the reproductive tract of a female mouse.

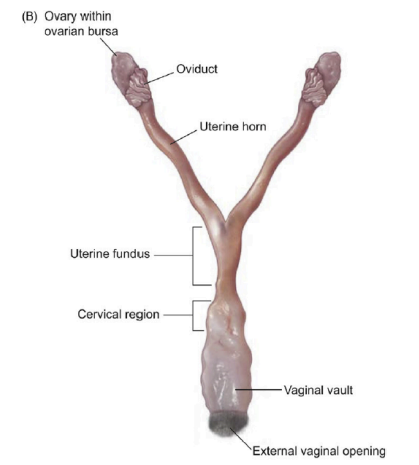


Figure 5- Reproductive tract of a female mouse (Boyd, Muchlenbachs, Rendi, Garcia & Gibson-Corley, 2018).

3.2. Histology of the ovary

The ovary consists of two parts: the cortex and the medulla.

The cortex is covered by a low cuboidal surface epithelium and it contains follicles in different stages of development. These follicles (the ovarian follicles) are round structures constituted by an oocyte with epithelial cells surrounding it (Priedkalns & Leiser, 2006). The cortex also encloses *corpora lutea* inserted in a loose connective tissue stroma. Cords of polyhedral interstitial endocrine cells are present in the cortical stroma of rodents, bitches and queens. (Priedkalns & Leiser, 2006).

The medulla, containing blood, lymph vessels and nerves, is the inner part of the ovary. It is made up of loose connective tissue and strands of smooth muscle (Scudamore, 2014).

A histological section of the mouse's ovary is represented in figure 6.



Figure 6- Histological section of a mouse ovary showing the bursa, cortex, medulla and bursal fat (Scudamore, 2014).

4. Oogenesis

Oogenesis is a process starting with the assembly of primordial follicles, in the fetal ovary, and consists of the differentiation of the *ovum*. (Albertini, 2015).

According to Wang et al. (2017), during mouse's embryogenesis, various bone morphogenetic proteins (BMPs) induce some epiblast cells to become the primordial germ cells (PGCs). After mitotic proliferation, PGCs differentiate into mitotic oogonia, enter into meiosis and then become primary oocytes. This process occurs a few days after birth in mice, rats and hamsters, and before birth in humans, cows and sheep (Smitz & Cortvrindt, 2002).

When the primary oocytes are formed, oocytes are arrested at the diplotene stage of prophase I. Wang et al. (2017) report that about one third of the oocytes degenerate by apoptosis during this phase. The oocytes will remain arrested in the cell cycle until luteinizing hormone (LH) induces ovulation and meiosis starts again (Albertini, 2015).

The majority of germ cells have disappeared, at around 4 days *post-partum*, and the surviving cells are individually surrounded by pre-granulosa cells, and a primordial follicle is formed (Wang et al., 2017).

The primordial follicle pool represents the ovarian reserve from which follicles are recruited to undergo growth and ovulation during the female's reproductive life.

Four to twelve fertilizable oocytes are produced every four to five days, for about seven to eight months, by an adult female mouse, depending on its strain (Wang et al., 2017).

5. Folliculogenesis

Folliculogenesis is a process in which the primordial follicles start developing and it consists of specific processes - cell proliferation, differentiation and atresia - to promote the growth and maturation of follicles and it concludes with ovulation (Eppig, 2001; Smitz & Cortvrindt, 2002; Rimon-Dahari, 2016).

During folliculogenesis, the primordial follicle goes through the following different stages: primary follicle, secondary follicle, pre-antral follicle, antral follicle and atretic follicle (Pedersen & Peters, 1968; Gougeon, 1996; Myers, Britt, Wreford, Ebling & Kerr, 2004). However, not all is known about the mechanisms of regulation of follicle formation, oocyte attrition and follicle development and atresia, though there are already studies that began working on this subject (Smitz & Cortvrindt, 2002).

As in Smitz and Cortvrindt (2002), the primordial follicle pool formed during fetal life and the rate of depletion of the same after birth will determine the duration of the female's reproductive life. Folliculogenesis starts when follicles leave this pool and enter the growth phase (Gougeon, 1996). In the mouse, the duration of the growth phase is three weeks (Amleh & Dean, 2002).

Primary follicles are formed when there is a single layer of cuboidal granulosa cells involving the primary oocyte. In rodents, contrary to what happens in domestic animals and primates, follicle formation occurs synchronously during the first few days after birth (Fortune, 2003). The transition of primary follicles to secondary follicles happens because of the continuous proliferation of granulosa cells (Fortune, 2003). During this phase, theca cells are formed (Cortvrindt & Smitz, 2001). The theca is composed by two layers: the theca interna and the theca externa (Edson, Nagaraja & Matzuk, 2009). The growth of follicles until the pre-antral and antral stages is follicle-stimulating hormone (FSH) and luteinizing hormone (LH) independent (Picton, Harris, Muruvi & Chambers, 2008). After puberty, the development of

follicles to the antral stage is accelerated by FSH and LH (Kumar, Wang, Lu & Matzuk, 1997; Edson, Nagaraja & Matzuk, 2009). FSH is then suppressed by estradiol, which enhances pituitary production of LH, causing a LH surge (Edson et al., 2009). This surge in levels of LH makes grown oocytes to extrude the first polar body, becoming arrested at metaphase II (Richards, Russell, Ochsner & Espey, 2002). With the surge of LH, ovulation occurs and this marks the end of folliculogenesis. The majority of follicles undergo atresia; they can become atretic at any stage of development; however, this is more common at pre-antral and early antral stages (Amleh & Dean, 2002). Figure 7 represents the regulators involved in the development of follicles.

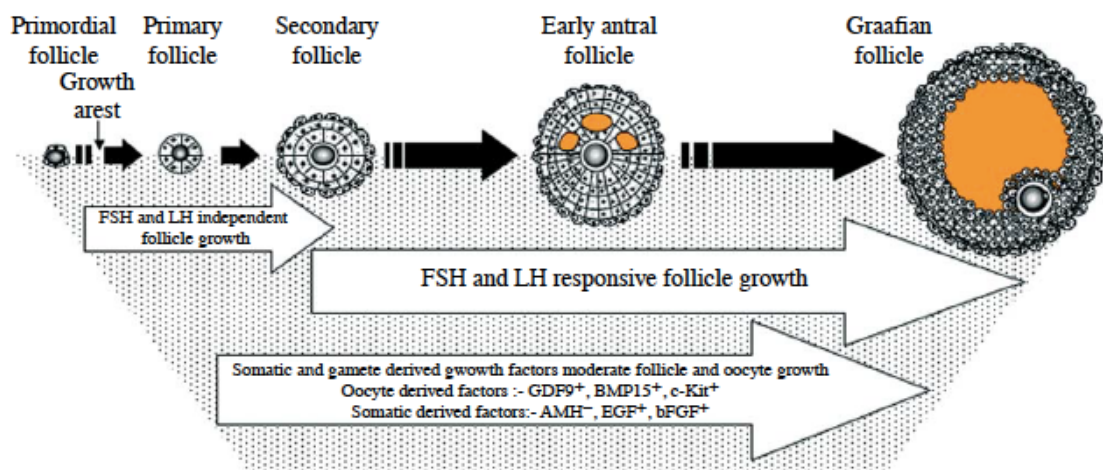


Figure 7 – Representation of the regulators of the development of follicles (Picto et al., 2008)

5.1. Follicle Classification

Follicles are categorised according to their morphological characteristics. According to the aspect of the granulosa cell layers, the follicles can be, thus, categorised into primordial, primary, secondary, pre-antral and antral follicles (Gougeon, 1996; Myers et al., 2004).

Pedersen and Peters (1968) first proposed a classification of oocytes and follicles that were based on the size of the oocyte, granulosa cell numbers and morphology of the follicle. They divided the follicles into small, medium and large and subdivided them into type 1, type 2, type 3a, type 3b, type 4, type 5a, type 5b, type 6, type 7 and type 8 (Peters, 1969). Type 1,

type 2 and type 3a were considered small follicles. Type 3b, type 4 and type 5a were considered medium-sized follicles. Type 5b, type 6, type 7 and type 8 were considered large follicles.

The description that Pedersen and Peters (1968) gave to each type of follicles was the following (Figure 8):

- Type 1: consists of a small oocyte that has no follicle cells surrounding it;
 - Type 2: consists of a small oocyte that has a few cells surrounding it;
 - Type 3a: consists of a small or growing oocyte that has a complete layer of follicle cells surrounding it;
 - Type 3b: consists of a growing oocyte that is surrounded by a complete layer of follicle cells;
 - Type 4: consists of a growing oocyte that is surrounded by two layers of follicle cells;
 - Type 5a: consists of an oocyte that may either be less than 70 μ or may have reached its full size; this oocyte is surrounded by three layers of follicle cells;
 - Type 5b: consists of a fully-grown oocyte that is surrounded by many layers of follicle cells;
 - Type 6: consists of a large oocyte surrounded by many layers of cells; the follicle cells are separated by areas of fluid;
 - Type 7: consists of a follicle with a single cavity that contains fluid; the *cumulus oophorus* has formed;
- Type 8: consists of a large follicle with a single cavity containing fluid and a well-formed *cumulus* stalk; it represents the pre-ovulatory follicle.

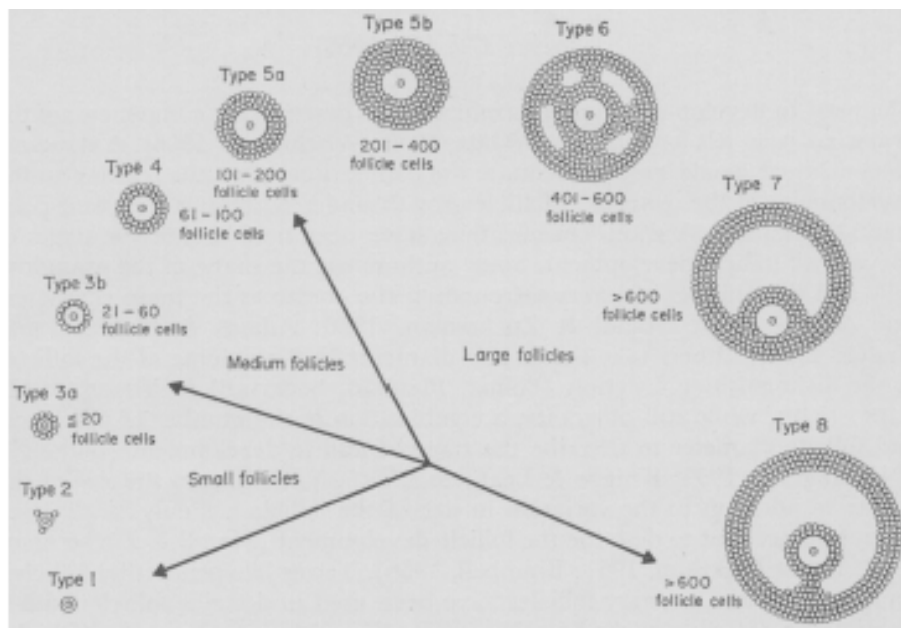


Figure 8 – Classification of follicles according to Pedersen and Peters (Pedersen & Peters, 1968)

In the classification proposed by Gougeon (1996) and Myers et al. (2004), follicles can be categorised in primordial, primary, secondary, pre-antral and antral follicles.

Primordial follicles are follicles that consist of an oocyte surrounded by a partial or complete layer of granulosa cells. (Myers et al., 2004). Since the early 1990's, there have been studies that suggest that there are two different types of primordial follicles in mice: the first primordial follicles that are activated immediately after they are formed and the adult primordial follicles that are activated gradually in later life (Zheng, Zhang & Liu, 2014 and studies cited there).

Primary follicles are follicles that have just one layer of cuboidal granulosa cells surrounding an oocyte (Myers et al., 2004).

Secondary follicles are follicles that consist of an oocyte surrounded by multiple layers of granulosa cells and have no visible antrum (Myers et al, 2004). Secondary follicles have a layer, the *zona pellucida*, around the plasma membrane of the oocyte and theca cells begin to form around the granulosa cells (Priedkalns & Leiser, 2006).

Pre-antral follicles are follicles in which the oocyte is surrounded by multilayers of granulosa cells, within the follicular basement membrane (Amleh & Dean, 2002). Small fluid-filled cavities start to form among the granulosa cells (Priedkalns & Leiser, 2006).

Antral follicles or Graafian follicles are follicles in which the granulosa cells are surrounded by a layer of theca cells and cavity with fluid, the antrum (Priedkalns & Leiser, 2006).

Figure 9 represents the aspect of the different stages of follicles.

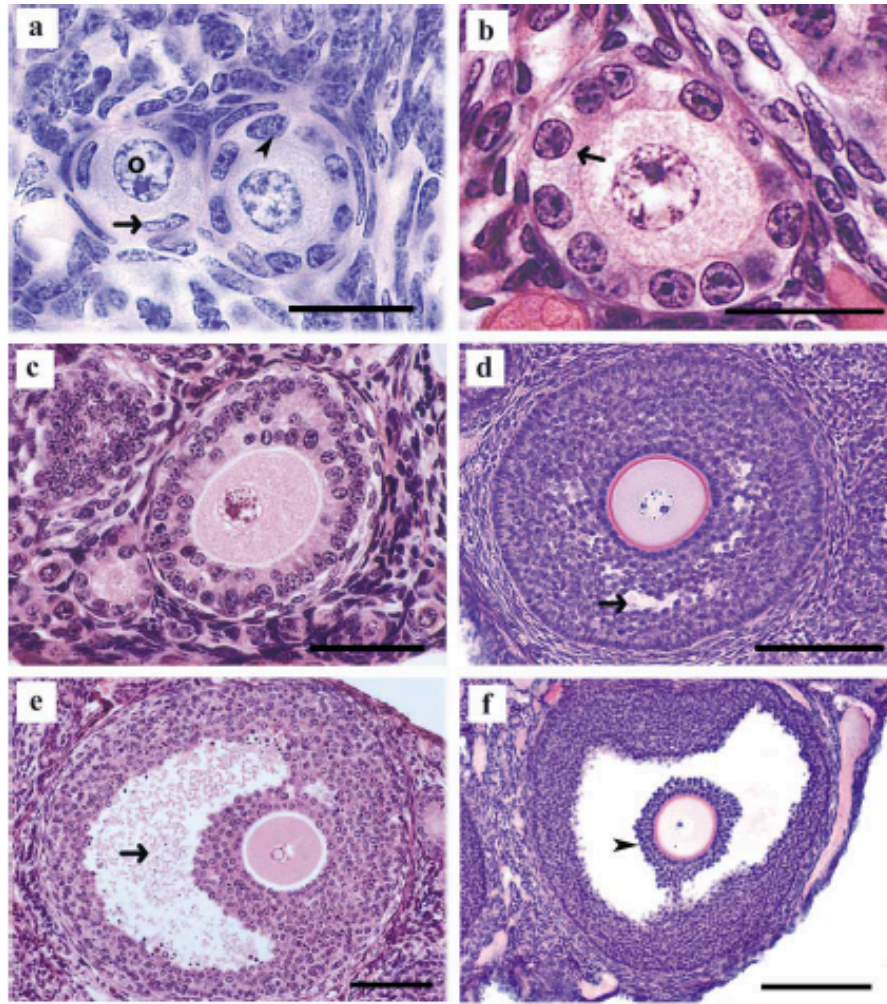


Figure 9 - Classification of follicles. A: primordial follicle; B: primary follicle; C: secondary follicle; D: pre-antral follicle; E: antral follicle; F: pre-ovulatory follicle (Adapted from Myers et al., 2004).

6. Mouse Estrous Cycle

Estrous cycle (EC) in the mouse has a duration of about four to five days and different phases can be identified during the cycle - proestrus, estrus, metestrus and diestrus. Specific hormones are responsible for the changes that occur during each phase of the cycle. These

hormones are regulated by the hypothalamus-pituitary-gonadal axis (Bertolin & Murphy, 2014).

The median eminence, in the hypothalamus, will receive axons from neurons of the gonadotropin-releasing hormone (GnRH); when reaching the anterior pituitary, GnRH stimulates the gonadotrophs to secrete LH and FSH, which are responsible for stimulating specific cells in the ovary that will be responsible for ovulation (Caligioni, 2009).

Estrogen (E2) is secreted by ovarian follicles and its concentration, normally, is low. Nonetheless, E2 reaches a peak, in the pre-ovulatory period, and GnRH neurons are stimulated (Sarkar, Chiappa, Fink & Sherwood, 1976; Levine, 2015).

Proestrus lasts for less than 24 hours in mice (Astwood, 1939; Long & Evans, 1922; Mandl, 1951; Allen, 1922; Grasso, Rozhavskaya & Reichert, 1998 as cited in Cora, Kooistra & Travlos, 2015). It is the stage of the cycle in which FSH causes the antral follicles to have an accelerated growth. Also, in this phase, estradiol affects the hypothalamus in a positive feedback, so when E2 increases, LH peaks and ovulation occurs 12-14 hours after a surge of LH (Bertolin & Murphy, 2014). The vaginal opening of mice during proestrus is wide and it is swollen, moist and pink tissue; there can be wrinkles or striations along the dorsal and ventral edges (Byers, Wiles, Dunn & Taft, 2012). Concerning the vaginal smear, in proestrus, there is a predominance of small nucleated epithelial cells and these can appear in clusters or individually; sometimes cornified cells may appear, which corresponds to the pre-ovulatory day (Caligioni, 2009).

Estrus in mice lasts between 24 and 48 hours (Astwood, 1939; Long & Evans, 1922; Mandl, 1951; Allen, 1922; Grasso et al, 1998 as cited in Cora et al., 2015). In this stage, levels of E2 remain elevated during morning but lower to basal levels in the afternoon (Walmer, Wrona, Hughes & Nelson, 1992, as cited in Caligioni, 2009). The vaginal opening of mice during estrus starts to be less swollen, less moist and less pink (Byers et al., 2012). Regarding vaginal smear, estrus is characterised by the presence of predominately anucleated keratinized epithelial cells, bacteria can be observed, nucleated epithelial cells may be observed and neutrophils are absent or can be sometimes observed in late estrus (Cora et al, 2015).

Metestrus duration can last up to 24 hours (Allen, 1922; Grasso et al., 1998 as cited in Cora et al., 2015). In this stage, progesterone inhibits the secretion of LH and the regression of *corpus luteum* (CL) towards the end of metestrus causes the reduction of progesterone levels, which will result in inhibition of LH secretion (Bertolin & Murphy, 2014). The vaginal opening in metestrus is not open wide, not swollen and white cellular debris may be visible (Byers et al.,

2012). Respecting vaginal smear, there is the presence of different cell types. There is a predominance of leukocytes and a few nucleated epithelial and/or cornified squamous epithelial cells can be observed (Caligioni, 2009).

Diestrus has a duration of 48-72 hours (Astwood 1939; Long & Evans 1922; Mandl 1951; Allen 1922; Grasso et al., 1998). In the unbred mouse the regression of CL occurs in late diestrus (Bertolin & Murphy, 2014). The levels of E2 start increasing (Walmer et al., 1992, as cited in Caligioni, 2009). The vaginal opening is small, closed and not swollen (Byers et al., 2012). In regard to vaginal smear, in diestrus there is predominantly leukocytes (Caligioni, 2009). Table 2 represents the classification of mice estrous cycle based on the type of cells.

Table 2- Classification of mice estrous cycle based on the type of cells and its numbers in vaginal smears (Adapted from Cora et al., 2015).

Stage	Neutrophils	Small nucleated epithelial cells	Large nucleated epithelial cells	Anucleated keratinized epithelial cells	Relative cell density
Proestrus	0 to +	++ to +++	0 to +	0 to +	Low to moderate
Estrus	0 to +	0 to +	0 to +	++ to +++	Moderate to high
Metestrus	+ to +++	0 to +	0 to +	++ to +++	Moderate to high
Diestrus	++ to +++	+ to ++	+ to ++	0 to +	Low to moderate

Note: 0 = none; + = few; ++ = moderate; +++ = high

7. Interleukin-1 β

Interleukin-1 β (IL-1 β) is an important pro-inflammatory cytokine. Cytokines are small proteins released by cells of the immune system and are important in cell signalling. They have a very important role between the communication of the immune system and host tissue cells (McInnes, 2017).

It belongs to the IL-1 family. This family represents a group of cytokines whose functions are mainly the formation and maintenance of inflammatory processes (Dembic, 2015).

According to Dinarello (2011), after binding to IL-1 receptor type 1, IL-1 β recruits the coreceptor chain accessory protein IL-1RAcP and thus, it is formed a complex between the receptor and the coreceptor. After the complex is assembled, the signal starts with recruitment of the adaptor protein MyD88 to the toll-IL-1 receptor (TIR) domain. Therewith, several kinases are phosphorylated, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) moves to the nucleus and then the induction of the expression of a wide range of inflammatory genes happens. This interleukin is synthesized as a precursor and it is processed to its mature form through specific cellular proteases, for example, cleavage by caspase-1.

The activation and production of IL-1 β will be described in the following section.

8. Nlrp3 inflamassome and obesity and its consequences for the ovary

The components of the inflammasomes are multiprotein complexes located in the cytoplasm of cells. The Nlrp3 inflammasome is formed by Nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing 3 (Nlrp3), an adapter protein, the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and caspase-1. The Nlrp3 is part of the nucleotide-binding domain and leucine-rich repeat (LRR)-containing (NLR) family and the pyrin and HIN domain (PYHIN) (He, Hara & Núñez, 2016). The Nlrp3 inflammasome is one of four confirmed inflammasomes forming pattern recognition receptors for activation of caspase-1, the other ones being Nlrp1, Nlrc4 and Aim2 (Elliot & Sutterwala, 2015).

Macrophages and other cells from the innate immune system can induce inflammatory reactions through detection of pathogens or danger-associated molecular patterns (PAMPs or DAMPs), using pattern-recognition receptors (PRRs) (Rheinheimer, de Souza, Cardoso, Bauer & Crispim, 2017). Therefore, the activation of the Nlrp3 inflammasome consists of two steps. The first one is invading pathogens driving IL-1 β mRNA transcription through PRRs, such as toll-like receptors (TLRs); the other step consists of the activation of Nlrp3 inflammasome by

reactive oxygen species (ROS) signaling, potassium efflux and lysosomal destabilization (Jo, Kim, Shin & Sasakawa, 2016). Lysosomal destabilization can happen because of microbial components such as microbial DNA, cell-wall-components and toxins, and endogenous ligands like adenosine triphosphate (ATP) or uric acid (Lamkanfi et al., 2008; Muruve et al., 2008; Ali et al., 2011; Mariathasan et al., 2006 as cited in Stienstra et al., 2012). Plus, arrangement of intracellular organelles, fatty acids, glucose and islet amyloid polypeptide are other molecular mechanisms that can activate the inflammasome (Jo, Kim, Shin & Sasakawa, 2016).

Under the presence of PAMPs or DAMPs, the Nlrp3 will interact with the NACHT domain (Tschopp & Schroder, 2010). Upon this activation by DAMPs or PAMPs, Nlrp3 recruits pro-caspase-1 through ASC protein, to form the inflammasome, then self-cleavage of pro-caspase-1 into the active form caspase-1 happens, which consequently cleaves pro-interleukin-1 β (pro-IL-1 β) into the active form IL-1 β (Hernandez, Sirois & Latz, 2011). This inflammasome is, as a result, responsible for inducing the production of IL-1 β and also of Interleukin-18 (IL-18) that are important inflammatory cytokines, hence having an important role on inflammation. Figure 10 illustrates how the formation of the Nlrp3 inflammasome happens.

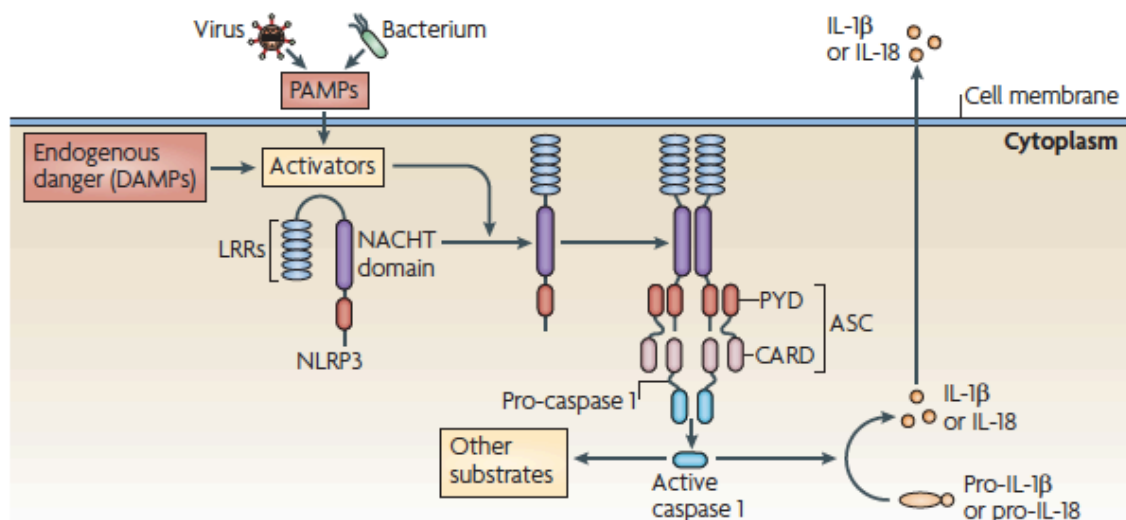


Figure 10 - Formation of the Nlrp3 inflammasome (Tschopp & Schroder, 2010).

About the activation by ROS Signaling, the mitochondria contribute the most to cellular ROS. Some examples that make ROS signalling one of the molecular mechanisms of the Nlrp3

activation are the following: palmitate activates the Nlrp3 inflammasome and, thus, IL-1 β is released in a mitochondrial ROS-dependent manner; multiple Nlrp3 triggering agents lead to mitochondrial dysfunction and cell death which increases oxidized mitochondrial DNA that binds to Nlrp3, activating the inflammasome (Jo, Kim, Shin & Sasakawa, 2016).

Concerning potassium efflux, a decrease in the intracellular concentration of K⁺ is very important in the Nlrp3 inflammasome activation; also, the known triggers that activate the inflammasome use the K⁺ efflux to activate it (Jo, Kim, Shin & Sasakawa, 2016).

Regarding lysosomal destabilization, the rupture of the lysosome releases cathepsin B, lysosomal hydrolases, prior to death cell; these factors all contribute to the activation of the Nlrp3 inflammasome (Jo, Kim, Shin & Sasakawa, 2016).

According to Stienstra et al. (2012), palmitate, a saturated fatty acid, may be the first signal for the production of IL-1 β . Also, palmitate may induce ceramide biosynthesis, which acts as a danger signal that stimulates activation of the Nlrp3 inflammasome, as suggested by Vandanmagsar et al. (2011). Thus, palmitate can supply danger signals for the inflammasome.

The study by Zhou et al. (2010) has suggested that high levels of glucose activate the Nlrp3 inflammasome. According to the authors, glucose induces the expression of thioredoxin-interacting protein (TXNIP) which interacts with thioredoxin (TRX). After an increase in ROS, TXNIP is released from oxidized TRX and interacts with Nlrp3, activating caspase-1 and stimulating the production of IL-1 β .

It is not well known whether acid uric can activate the inflammasome during obesity. However, there is a hypothesis that uric acid can cause the activation of the Nlrp3 inflammasome and, thus, the production of IL-1 β . Cheung et al. (2007) showed that xanthine oxidoreductase (XOR) is highly expressed in adipose tissue and that its expression, enzymatic activity and its product urate are all increase in obesity. The authors also showed that adipocyte-derived XOR activity may play a role in the hyperuricemia associated with obesity. The uric acid levels were elevated in obese mice.

Another substance that is thought to activate the Nlrp3 inflammasome and, thus, stimulate the production of IL-1 β by the pancreas is islet amyloid polypeptide (IAPP). A study by Masters et al. (2010) compared the capacity of IAPP to trigger production of mature IL-1 β from lipopolysaccharide (LPS)-primed bone marrow-derived macrophages (BMDMs) with the capacity of agents that are known to activate the inflammasome. With this study, the authors

showed that human IAPP produced amounts of IL-1 β that were similar with the ones produced by the known agents that can activate de inflammasome. Also, they showed that active caspase-1 was much higher after stimulation for one hours with IAPP. Oleat and palmitate increased the expression of IAPP in MIN6 cells (Westermarck, Andersson & Westermarck, 2011). Hence, this may suggest that fatty acids can lead to pancreatic production of IL-1 β in type 2 diabetes.

Other substance that can activate the inflammasome is leptin. A study by Fu, Liu, Han and Yu (2017) showed that leptin enhanced the mRNA and protein expression levels of Il-18 in RAW 264.7 cells via the Nlrp3 inflammasome. The authors cultured murine RAW 264.7 cells and incubated them with increasing doses of leptin, to evaluate the expression levels of Il-1 β and Il-18 through ELISA and reverse transcription-quantitative polymerase chain reaction. They also used an inhibitor of caspase-1. They also showed that the activation of the inflammasome happened because of an increase in ROS and K⁺ efflux, in response to leptin.

The Nlrp3 inflammasome is expressed in macrophages. Studies by Zhang, Dai, Li, Chen and Chai (2017) and by Stouch et al. (2018), showed, by immunofluorescent staining, that Nlrp3 inflammasome is expressed in CD68⁺ macrophages. Also, it is known that the Nlrp3 inflammasome is expressed in the ovary (Wang et al., 2017). The study by Wang et al. (2017) examined whether or not redox-dependent activation of Nlrp3 inflammasome contributes to the development of polycystic ovary syndrome. The authors also examined if dymethyldiguanide (DMBG), an insulin sensitiser, improves the clynical symptoms of polycystic ovary syndrome, by targeting the Nlrp3 inflammasome. For this study, Wang et al. used six-week old female rats with two consecutive 4-day estrous cycle divided into 3 groups: control group, polycystic ovary syndrome group and DMBG group. They showed that Nlrp3 and ASC mRNA levels were increased in polycystic ovary syndrome, indicating that the Nlrp3 inflammasome is expressed in the ovary and may be involved in polycystic ovary syndrome. The authors also showed that caspase-1 and Il1 β activity was increased in polycystic ovary syndrome.

Obesity has been associated with alterations in the female reproductive function. Honnma et al. (2010) and Jia et al. (2018) have shown that obesity causes significantly alterations in the ovaries.

Honnma et al. (2010), using female Zucker Fatty rats, a model for obesity and hyperinsulinemia, observed in the ovaries of obese rats a higher number of atretic follicles, multiple cysts and a decrease in the growth of follicles. Additionally, the authors observed that

obese rats estrous cycle presented some abnormalities such as prolonged diestrus and that the occurrence of these in the estrous cycle would increase with age.

Jia et al. (2018), using a group of mice fed with an HFD and another one fed with a normal diet, investigated the possible effects of obesity on oocyte quality, including metabolism, lipid accumulation, ROS levels, meiosis and the change of spindle structure in metaphase II. With this study, the authors showed that obesity can impair oocyte meiosis, can destroy spindle assembly, can promote oxidative stress and abnormal mitochondrial distributions and it makes mice oocytes soften.

Apart from alterations in the ovaries and in the estrous cycle, Balasubramanian et al. (2011) showed that a high-fat diet causes an increase of leptin levels which leads to a decrease of estradiol levels.

It is known that the Nlrp3 inflammasome is present in the inflammatory state characteristic of obesity and for the production and release of IL-1 β in various tissues and cell types during the development of obesity and has a role in the pathogenesis of type 2 diabetes mellitus (Stienstra et al., 2012). Thus, studies by Stienstra et al. (2011) and Vandanmagsar et al. (2011) showed that Nlrp3 inflammasome is the responsible for the inflammation associated to obesity and insulin resistance. The first authors used two groups of genetically modified mice in which Nlrp3 and caspase-1 were absent: one group was fed with a low-fat diet and the other one was fed with a high-fat diet. They also used two groups of wild type mice with the same diet parameters of the referred other two groups. With this study, the authors showed that the absence of the Nlrp3 inflammasome's components protects against HFD-induced obesity and against hyperinsulinemia. The ablation of these components also resulted in a decrease of IL-1 β production in animals fed with an HFD, revealing, just like that, the role of the inflammasome in obesity.

Vandanmagsar et al. (2011), using as well genetically modified mice with ablation of Nlrp3 inflammasome, showed equally that the absence of this inflammasome protects significantly against HFD-induced obesity, along with hyperinsulinemia, glucose intolerance and inflammation. These authors also proposed that the activation of Nlrp3 inflammasome in the macrophages has an important for the pathophysiology of inflammation in obesity, a term called meta-inflammation. In obese adipose tissue, accumulation of fatty acids can lead to an increase of ceramide. These authors also showed that, by adding ceramide to adipose tissue, the Nlrp3-

dependent IL-1 β production increased, suggesting, thus, that ceramide acts as a danger signal that stimulates activation of the Nlrp3 inflammasome.

Therefore, a better knowledge of the Nlrp3 inflammasome's mechanism of regulation may lead to studies about obesity-induced infertility therapies.

9. Therapies already studied for the inhibition of the Nlrp3 inflammasome

The Nlrp3 has an important role in the production of IL-1 β , caspase-1 and in the pathogenesis of obesity-induced insulin resistance. Therefore, there have been some studies about therapies of Type 2 diabetes mellitus that aimed at the inhibition of IL-1 β .

Larsen et al. (2007), used anakinra, a recombinant human interleukin-1-receptor antagonist, and administered it subcutaneously to 34 patients with type 2 diabetes mellitus; other 36 patients received a placebo. With this study, the authors showed that the glycated haemoglobin level was lower in the anakinra group, C-peptide secretion was enhanced, there was a reduction in the ratio of proinsulin to insulin and there was a decrease in levels of interleukin-6 and in levels of C-reactive protein. Thus, the blockage of interleukin-1 with anakinra improved glycemia and beta-cell secretory function and reduced markers of systemic inflammation.

Nonetheless, a study by van Asseldonk (2011), administered anakinra to non-diabetic, obese with metabolic syndrome patients and showed that anakinra did not improve insulin sensitivity in these subjects.

Other study by Rissanen, Howard, Botha and Thuren (2012) also supported the fact that using an IL-1 β inhibitor would improve glycemic control. The authors ran a placebo-controlled study in which they administered canakinumab, an anti- IL-1 β antibody, to type 2 diabetes mellitus patients and observed that insulin secretion improved in patients given the anti- IL-1 β antibody.

Along with Rissanen, Howard, Botha and Thuren (2012), Sloan-Lancaster et al. (2013) also used a neutralizing IL-1 β antibody to study the effects of IL-1 β inhibition. The authors conducted a placebo-controlled study in which they administered LY2189102, a neutralizing

IL-1 β antibody, to type 2 diabetes mellitus patients. They showed that there was a decrease in haemoglobin A_{1C} and fasting glucose; plus, there was a decrease in postprandial glycemia and in inflammatory biomarkers.

Apart from the aforementioned studies, Lamkanfi et al. (2009) and Masters et al. (2010) showed that Glyburide, a drug used for the treatment of type 2 diabetes mellitus, prevents LPS+ATP-induced secretion of IL-1 β and the activation of the Nlrp3 inflammasome.

Regarding the area of Reproduction, some studies by Ortega & Duleba (2015) and Banaszewska, Wrotyńska-Barczyńska, Spaczynski, Pawelczyk & Duleba (2016) have shown that resveratrol, a polyphenolic phytoalexin that is produced by grapes, berries and some plants, has beneficial effects on ovaries.

Regarding the ovaries, there are not many studies about specific therapies for the consequences of the Nlrp3 inflammasome on this organ, nor for the impact of obesity on the ovaries. However, the study by Wang et al. (2017), already referred, showed that the use of DMBG suppressed the activation of the Nlrp3 inflammasome.

Also, as described before, the study by Jia et al. (2018), not only studied the effects that an HFD-induced obesity has on the quality of oocytes, but also studied whether resveratrol could alleviate the negative effects of obesity in the oocyte's quality. The authors showed that resveratrol has a certain inhibitory effect on the negative impact of obesity, such as the increase of the hardness of the *zona pellucida* of the fat mouse.

Chapter III – Study of the Nlrp3 inflammasome in the ovary

1. Aims

The present study comprehended the following goals:

1. Histological characterisation of the Nlrp3 inflammasome in the ovary components of obese mice;
2. Qualitative comparison of the Nlrp3 inflammasome's presence between different stages of obesity;
3. Evaluation of the influence of leptin in the Nlrp3 inflammasome's expression.

2. Materials and Methods

2.1. Sample's characterisation

For this study, samples of 23 mice ovaries were used. These mice were bred and euthanised for a wider study in Poland, being that in this study it was only used samples collected previously.

It was used samples of mice ovaries bearing in mind the diet and the duration of the same; it was also used samples of ovaries from mutant mice - ob/ob mice, which have a mutation in the leptin gene and db/db mice, which have a mutation in the leptin receptor gene and it was used samples of ovaries from mice who were injected with leptin for 16 days (table 3). It was used both homozygous and heterozygous ob/ob and db/db mice, in order to establish a link between leptin signalling and Nlrp3 activation. Animal protocols and phenotyping were performed by Dr. António Galvão's team at the Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, in Poland. After that, histological cuts were performed by the student at University Lusófona, in Lisbon, as well as immunohistochemistry staining of that same cuts.

The carried procedures were approved by the Ethics Committee of the Department of Reproductive Immunology and Pathology, Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, Olsztyn, Poland.

Table 3 – Mice groups used in this study

Group 1 – Diet Induced Obesity Mice	Group 2 – ob/ob mice	Group 3 – db/db mice	Group 4 – Hyperleptinemia model
Fed with CD 16 weeks	ob/ob homozygous	db/db homozygous	Administration of 100 µg of leptin for 16 days
Fed with HFD 16 weeks	ob/ob heterozygous	db/db heterozygous	Administration of 100 µg of saline for 16 days

2.2. Inclusion criteria

All mice were euthanised during estrous. Plus, in the diet induced obesity group, mice over 35 g of weight, fed with a high fat diet, and mice over 24g, fed with a chow diet, were included in this study. The rest of the groups did not have anymore inclusion criteria.

2.3. Exclusion criteria

All mice that were not in estrous, were not used. Plus, mice under 35 g of weight, fed with a high fat diet, and mice under 24g of weight, fed with a chow diet, were excluded from this study. The rest of the group did not have exclusion criteria.

2.4. Collection of samples

The collection of samples was made by surgical excision of the ovaries. The samples were fixated with 10% Formalin and then embebed in paraffin, so that histological sections could be done. 3 μ histological sections were cut in the microtome (Thermo Scientific Microm HM325) at the LACH of Lusófona University of Humanities and Technologies. The sections were positioned into SuperFrost and SuperFrost Ultra microscope slides (ThermoFisher Scientific) and put in the incubator overnight.

2.5. Immunohistochemistry- Paraffin

Immunohistochemistry-Paraffin was the method used on sections groups. Each groups's samples were divided into two slides - one was the positive control and the other one was the negative control - with 3 μ of thickness. The samples were from ovaries of mice.

All the steps of immunohistochemistry were conducted at room temperature, unless when it is indicated otherwise (Annex II).

Two primary antibodies were used, separately, in the IHC-P protocol. One was used to identify the presence of IL-1 β (sc-7884; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) and the other one was used to identify the presence of the Nlrp3 inflammasome (ab214185; Abcam, Cambridge, UK).

Before starting the IHC-P protocol with the study's slides, an IHC-P for validation of antibodies was conducted. It was used different dilutions of both primary antibodies. IgG and slide only with secondary antibody were used as negative controls.

After the slides were put in the incubator, overnight, the deparaffinization in xylene and rehydration in descending concentrations of ethanol proceeded. Afterwards, the slides were washed for 5 minutes in distilled water. Thereafter, the slides were incubated with 3% H₂O₂, at 37°C, for 15 minutes, in a dark place. The antigen retrieval in 10 mM sodium citrate buffer pH6 (450 mL of H₂O + 9 mL of citrate 0.1M pH1.8 + 41 mL of sodium citrate 0.1M pH8.8) was the following - the slides were incubated with the buffer solution, at the microwave (700W), 3

times, during 5 minutes. The slides were cooled down for 20-30 minutes. Then, the slides were incubated with a blocking solution (PBS with 2% BSA - A7906, Sigma-Aldrich, Inc., St Louis, MO, USA), in a volume of 200 μ L per slide, at a humidity chamber, for an hour. Posteriorly, the positive control slides were incubated with the primary antibody (sc-7884; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA/ ab214185; Abcam, Cambridge, UK) at 4°C, overnight. The negative control slides were incubated with blocking solution instead of the primary antibody. The Il-1 β antibody (sc-7884; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) was used at a dilution of 1:100 and the anti-Nlrp3 inflammasome antibody (ab214185; Abcam, Cambridge, UK) was used at a dilution of 1:200 (Table 2).

After washing the slides 4 times, for 10 minutes, with PBS, the slides were incubated with the secondary antibody, polyclonal goat anti-rabbit IgG conjugated with peroxidase (410972, Dako, Carpinteria, CA, USA), for an hour, at a dilution of 1:100 (Table 4). Afterwards, the slides were washed with PBS, twice, for 10 minutes. After, the slides were incubated with 3'-diaminobenzidine (DAB) (ImmPACT DAB, SK-4105, Vector Laboratories, Burlingame, CA, USA), until reaction occurred and then the slides were washed with distilled water, twice, during 5 minutes. The slides were, then, stained with Gill's hematoxylin, for about 45 seconds.

Afterwards, the slides were dehydrated in ascending concentrations of ethanol, they were put in xylene, twice, for 5 minutes and then they were mounted in Entellan®.

Table 4 – Dilutions used for each antibody

Antibody	Dilution
Primary Antibody Il-1 β	1:100
Primary Antibody Anti-Nlrp3 inflammasome	1:200
Secondary Antibody polyclonal goat anti-rabbit IgG conjugated with peroxidase	1:100

2.6. Samples' Analysis

The samples were observed in an Olympus microscope (CX21) with 40x objective lens, by a single observer, the student. In each slide, the follicles were classified as primordial, primary, secondary and antral. If the follicle had a visible cavity, it would be classified as antral, as it was the classification used by researchers at the Institute of Animal Reproduction and Food Research, in Olsztyn, Poland. It was also identified granulosa cells, theca cells, stroma and oocytes.

The immunohistochemistry pictures were taken with a ZEISS Axio Observer Z1 microscope, with the exception of the hyperleptinemia model group whose photos were taken with an Olympus microscope. The images were edited with GIMP 2.10.

3. Results

3.1. Characterisation of the sample

The sample for this study was composed of a total of 21 obese ovaries, for Nlrp3, and 22 for Il-1 β . In the diet-induced obesity, it was obtained 5 ovaries, for both antibodies. In the ob/ob group, 5 ovaries were obtained, for both antibodies. In the db/db group, it was obtained 7 ovaries for Nlrp3 and 8 ovaries for Il-1 β . In the hyperleptinemia group, 4 ovaries were obtained, for both antibodies. The number of ovaries' samples observer per group will be described in table 5.

Table 5 – Number of mice ovaries' samples observed per group

Mice Groups	Number of samples	
	Nlrp3	Il-1 β
HFD for 16 weeks	3	3
CD for 16 weeks	2	2
ob/ob homozygous	3	3
ob/ob heterozygous	2	2
db/db homozygous	2	3
db/db heterozygous	5	5
Leptin for 16 days	2	2
Leptin control (saline)	2	2
Total	21	22

A follicle count was also performed, both in Nlrp3 and in Il-1 β antibodies, as described in tables 6 and 7. Afterwards, follicles were counted as stained or not stained, which will be presented in sections 3.2 and 3.3.

Regarding the Nlrp3 inflammasome antibody's samples, a total of 213 follicles were counted. It was observed 58 primordial follicles, 40 primary follicles, 85 secondary follicles and 30 antral follicles. On the other hand, a total of 267 follicles were counted in the samples with the Il-1 β antibody. It was observed 76 primordial follicles, 52 primary follicles, 90 secondary follicles and 49 antral follicles.

Table 6 – Total number of follicles per group (N= 213). Nlrp3 inflammasome antibody.

NLRP3	Primordial Follicles	Primary Follicles	Secondary Follicles	Antral Follicles	Total
DIO CD	1	1	2	1	5
DIO HFD	8	7	6	1	22
Ob/ob homozygous	11	11	25	6	53
Ob/ob heterozygous	8	5	21	0	34
Db/db homozygous	5	2	1	1	9
Db/db heterozygous	16	8	23	6	53
Leptin +	4	1	0	3	8
Leptin control	5	5	7	12	29
Total	58	40	85	30	213

Table 7 – Total number of follicles per group (N= 267). Il-1 β antibody.

Il-1 β	Primordial Follicles	Primary Follicles	Secondary Follicles	Antral Follicles	Total
DIO CD	6	2	3	5	16
DIO HFD	8	6	7	0	21
Ob/ob homozygous	11	16	16	7	50
Ob/ob heterozygous	12	6	13	10	41
Db/db homozygous	9	6	19	8	42
Db/db heterozygous	18	8	28	11	65
Leptin +	4	4	1	3	12
Leptin control	8	4	3	5	20
Total	76	52	90	49	267

3.2. Follicle Count

Follicles were counted through observation on the optical microscope. Stained cells presented a brownish colouration, meaning that there was a positive reaction to the presence of the proteins.

Regarding the Nlrp3 inflammasome antibody's samples, 213 follicles were observed in 21 slides of mice ovaries. In the DIO group, 27 follicles were observed; in the ob/ob group, 87 follicles were observed; in the db/db group, 62 follicles were observed and in the leptin group, 37 follicles were observed.

Concerning the Il-1 β antibody's samples, it was observed 267 follicles in 22 slides of mice ovaries. In the DIO group, 37 follicles were observed; in the ob/ob group, 91 follicles were observed; in the db/db group, 107 follicles were observed and in the leptin group, 32 follicles were observed.

Respecting the Nlrp3 inflammasome's samples the results are described following.

In the DIO CD group (n=2), it was observed one stained primordial follicle and zero not stained primordial follicles, it was observed one stained primary follicle and zero not stained primary follicles, it was observed two stained secondary follicles and zero not stained secondary follicles, and it was observed one stained antral follicle and zero not stained antral follicles.

In the DIO HFD group (n=3), it was observed five stained primordial follicles and three not stained primordial follicles, it was observed four stained primary follicles and three not stained primary follicles, it was observed five stained secondary follicles and one not stained secondary follicle, it was observed zero stained antral follicles and one not stained antral follicle.

In the ob/ob homozygous group (n=3), it was observed four stained primordial follicles and seven not stained primordial follicles, it was observed seven stained primary follicles and four not stained primary follicles, it was observed 20 stained secondary follicles and five not stained secondary follicles, and it was observed six stained antral follicles and zero not stained antral follicles.

In the ob/ob heterozygous group (n=2), it was observed four stained primordial follicles and four not stained primordial follicles, it was observed two stained primary follicles and three not stained primary follicles, it was observed 15 stained secondary follicles and six not stained secondary follicles, and it was observed zero stained antral follicles and zero not stained antral follicles.

In the db/db homozygous group (n=2), it was observed three stained primordial follicles and two not stained primordial follicles, it was observed one stained primary follicle and one not stained primary follicle, it was observed one stained secondary follicle and zero not stained secondary follicles, and it was observed zero stained antral follicles and one not stained antral follicle.

In the db/db heterozygous group (n=5), it was observed 14 stained primordial follicles and four not stained primordial follicles, it was observed five stained primary follicles and three not stained primary follicles, it was observed 16 stained secondary follicles and seven not stained secondary follicles, and it was observed three stained antral follicles and three not stained antral follicles.

In the leptin group (n=2), it was observed three stained primordial follicles and one not stained primordial follicle, it was observed one stained primary follicle and zero not stained primary follicles, it was observed zero stained secondary follicles and zero not stained secondary follicles, and it was observed two stained antral follicles and one not stained antral follicle.

In the leptin control group (n=2), it was observed three stained primordial follicles and two not stained primordial follicles, it was observed four stained primary follicles and one not stained primary follicle, it was observed seven stained secondary follicles and zero not stained secondary follicles, and it was observed 11 stained antral follicles and one not stained antral follicle.

Table 8 illustrates the number of stained follicles observed per group with the Nlrp3 inflammasome antibody.

Table 8 – Total number of stained follicles per group. Nlrp3 inflammasome antibody.

NLRP3	Primordial Follicles	Primary Follicles	Secondary Follicles	Antral Follicles	Total
DIO CD	1	1	2	1	5
DIO HFD	5	4	5	0	14
Ob/ob homozygous	4	7	20	6	37
Ob/ob heterozygous	4	2	15	0	21
Db/db homozygous	3	1	1	0	5
Db/db heterozygous	14	5	16	3	38
Leptin +	3	1	0	2	6
Leptin control	3	4	7	11	25
Total	37	25	66	23	151

Respecting the Il-1 β 's samples, the results are described following.

In the DIO CD group (n=2), it was observed one stained primordial follicle and five not stained primordial follicles, it was observed zero stained primary follicles and two not stained primary follicles, it was observed one stained secondary follicle and two not stained secondary follicles, and it was observed one stained antral follicle and four not stained antral follicles.

In the DIO HFD group (n=3), it was observed five stained primordial follicles and three not stained primordial follicles, it was observed zero stained primary follicles and two not stained primary follicles, it was observed one stained secondary follicle and two not stained secondary follicles, and it was observed zero stained antral follicles and zero not stained antral follicle.

In the ob/ob homozygous group (n=3), it was observed five stained primordial follicles and six not stained primordial follicles, it was observed 16 stained primary follicles and zero not stained primary follicles, it was observed 16 stained secondary follicles and zero not stained

secondary follicles, and it was observed five stained antral follicles and two not stained antral follicles.

In the ob/ob heterozygous group (n=2), it was observed 10 stained primordial follicles and two not stained primordial follicles, it was observed five stained primary follicles and one not stained primary follicle, it was observed 11 stained secondary follicles and two not stained secondary follicles, and it was observed 10 stained antral follicles and zero not stained antral follicles.

In the db/db homozygous group (n=3), it was observed one stained primordial follicle and eight not stained primordial follicles, it was observed one stained primary follicle and five not stained primary follicle, it was observed seven stained secondary follicles and 12 not stained secondary follicles, and it was observed two stained antral follicles and six not stained antral follicles.

In the db/db heterozygous group (n=5), it was observed 11 stained primordial follicle and seven not stained primordial follicles, it was observed three stained primary follicles and five not stained primary follicles, it was observed 17 stained secondary follicles and 11 not stained secondary follicles, and it was observed six stained antral follicles and five not stained antral follicles.

In the leptin group (n=2), it was observed three stained primordial follicles and one not stained primordial follicle, it was observed one stained primary follicle and three not stained primary follicles, it was observed zero stained secondary follicles and one not stained secondary follicle, and it was observed two stained antral follicles and one not stained antral follicle.

In the leptin control group (n=2), it was observed five stained primordial follicles and three not stained primordial follicles, it was observed zero stained primary follicles and four not stained primary follicle, it was observed zero stained secondary follicles and three not stained secondary follicles, and it was observed five stained antral follicles and zero not stained antral follicles.

Table 9 illustrates the number of stained follicles observed per group with the Il-1 β antibody.

Table 9 – Total number of stained follicles per group. Il-1 β antibody.

Il-1 β	Primordial Follicles	Primary Follicles	Secondary Follicles	Antral Follicles	Total
DIO CD	1	0	1	1	3
DIO HFD	5	5	3	0	13
Ob/ob homozygous	5	16	16	5	42
Ob/ob heterozygous	10	5	11	10	36
Db/db homozygous	1	1	7	2	11
Db/db heterozygous	11	3	17	6	37
Leptin +	3	1	0	2	6
Leptin control	5	0	0	5	10
Total	41	31	55	31	158

3.3. Expression of the Nlrp3 inflammasome and Il-1 β

Through immunohistochemistry it was possible to identify the expression of the Nlrp3 in the obese ovary. All the stained cells showed a brownish colour, in contrast to the negative controls, that only showed a blue colour. It was possible to identify staining in several ovarian structures from samples of all groups. The Il-1 β 's expression was also detected as a control, since this interleukin is produced as a consequence of the Nlrp3 inflammasome's activation.

Nlrp3 inflammasome is expressed in stroma in every sample. It is also expressed in oocytes of all groups. Expression in granulosa cells also occurred, though with variable intensities. Thus, this protein is expressed in all types of follicles, in the granulosa cells, stroma cells and oocytes (figures 11, 11a, 11b and 11c). This protein is not expressed in theca cells (figure 12).

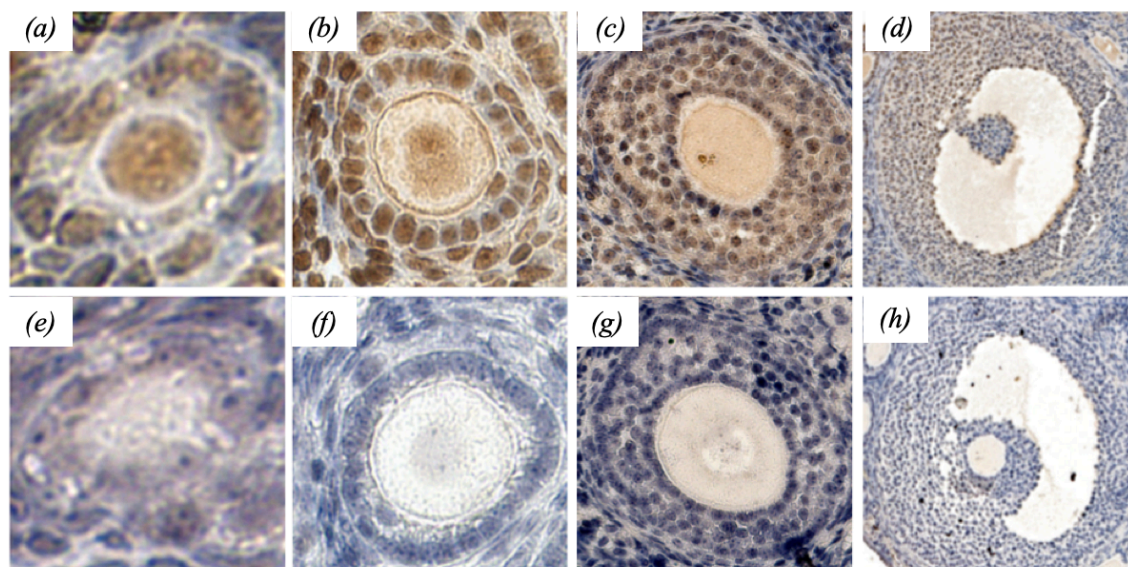


Figure 11- Detection of Nlrp3 inflammasome during follicular development. Positive staining in brown; counterstaining with hematoxylin. Primordial follicles 630x (a, e); Primary follicles 630x (b, f); Secondary follicles 630x (c, g); Antral follicles 200x (d, h). Negative controls (e-h). Author's original.

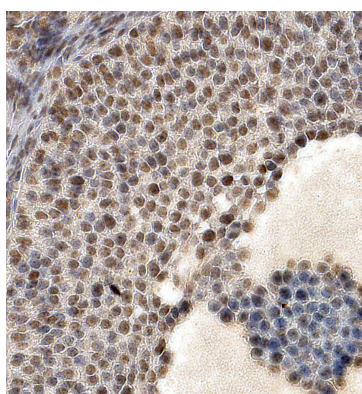


Figure 11a- Stained granulosa cells (400x). Author's original.

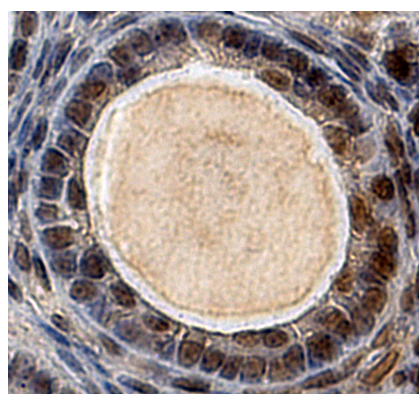


Figure 11b- Stained oocyte (630x). Author's original.

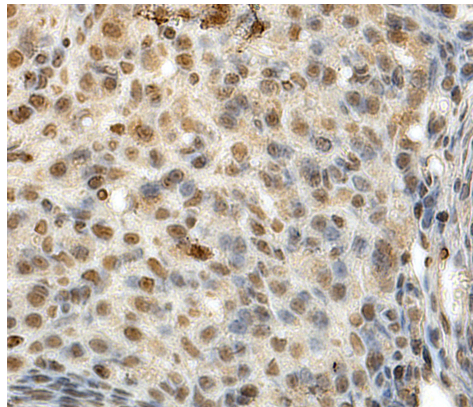


Figure 11c- Stained stroma cells (630x).
Author's original.

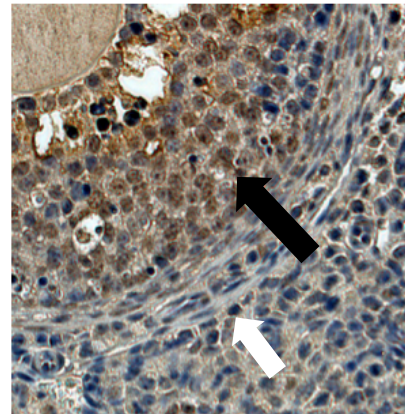


Figure 12- Secondary follicle (400x).
Positive staining in brown; counterstaining with hematoxylin. White arrow – not stained theca cells. Black arrow – stained granulosa cells. Author's original.

As a control, it was also identified the expression of Il-1 β in the obese ovary. This interleukin was expressed on the same sites as the Nlrp3 inflammasome.

Il-1 β is expressed in all types of follicles, in the oocytes, in granulosa cells and in the stroma. The only difference between Il-1 β and Nlrp3 inflammasome's expression is that the first is expressed in the oocyte's nucleus, while Nlrp3 inflammasome is located in the cytoplasm of the oocyte (figures 13 and 14). These results, along with the ones from Nlrp3, are schematically illustrated in figure 15.

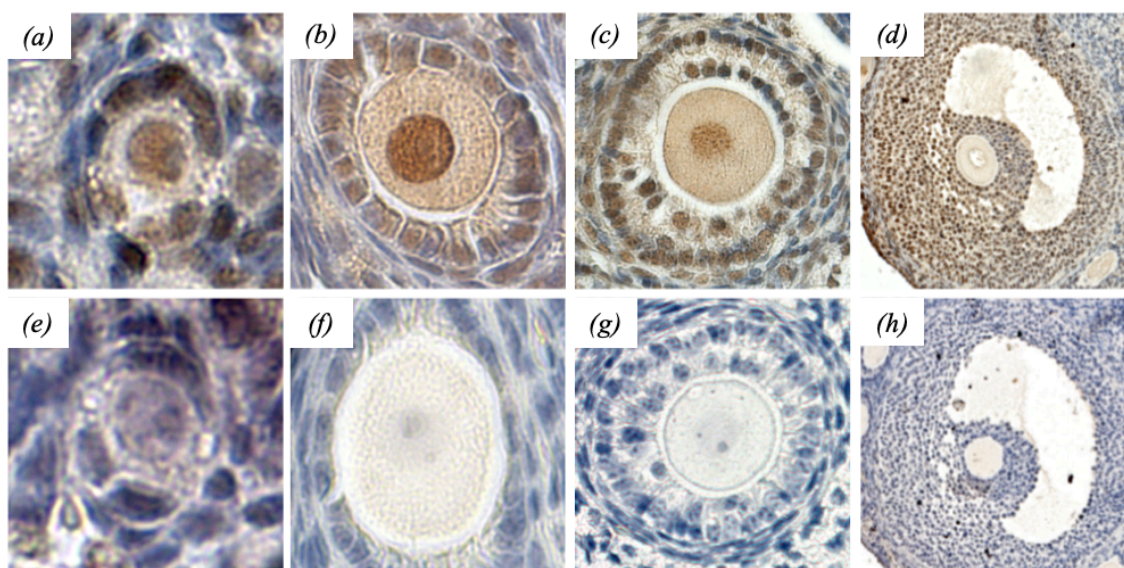


Figure 13- Detection of IL-1 β during follicular development. Positive staining in brown; counterstaining with hematoxylin. Primordial follicles 630x (a, e); Primary follicles 630x (b, f); Secondary follicles 630x (c, g); Antral follicles 200x (d, h). Negative controls (e-h). Author's original.

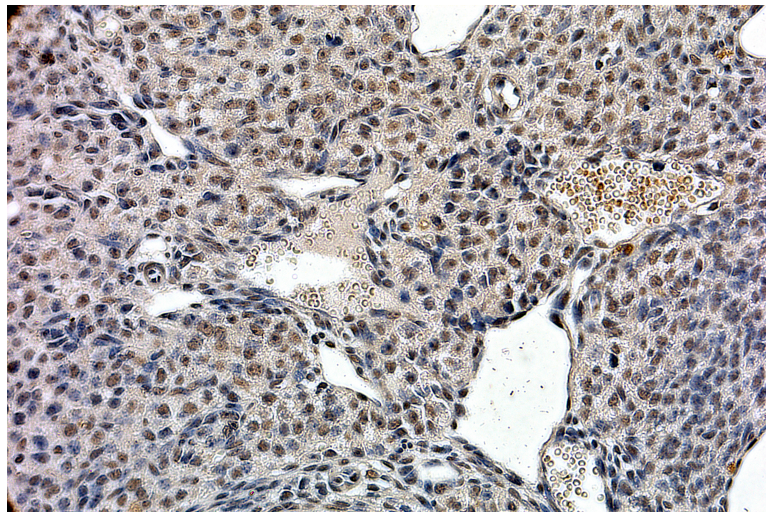


Figure 14- Stained stroma cells (630x). Author's original.

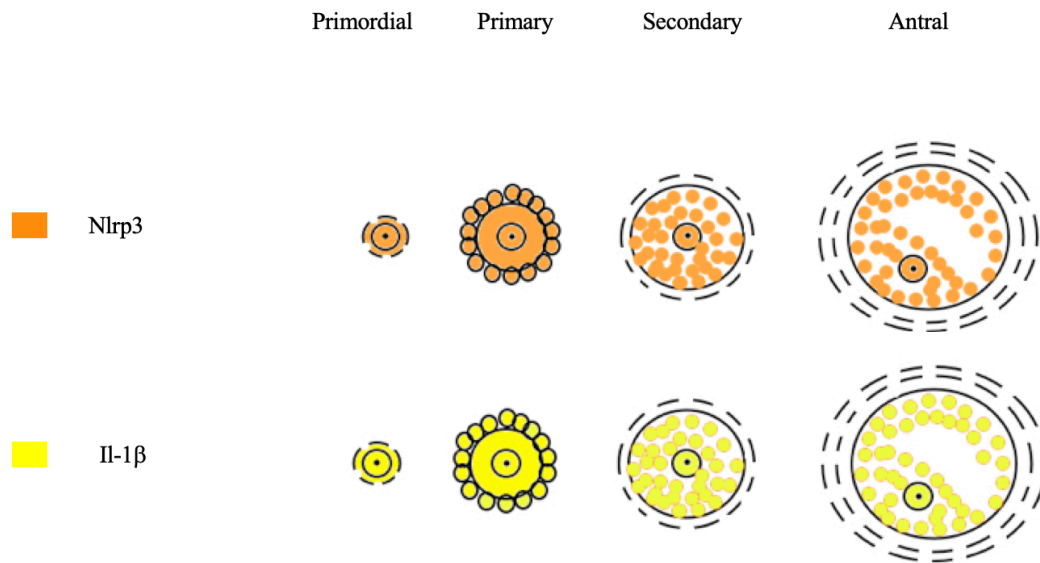


Figure 15- Schematic illustration of Nlrp3 inflammasome and Il-1β's expression patterns (Representation adapted from Murta et al., 2014).

3.4. Differences between groups

Qualitative comparison between groups was done by analysing the pictures, in order to observe if there were any differences between groups. Pictures from the full ovary, follicles and stroma cells were chosen as a way of comparison.

Figures 16 and 17 show the differences of Nlrp3 and Il-1β expression, respectively, between mice fed with CD and mice fed with HFD. It is possible to observe that in the HFD group, the staining intensity is higher.

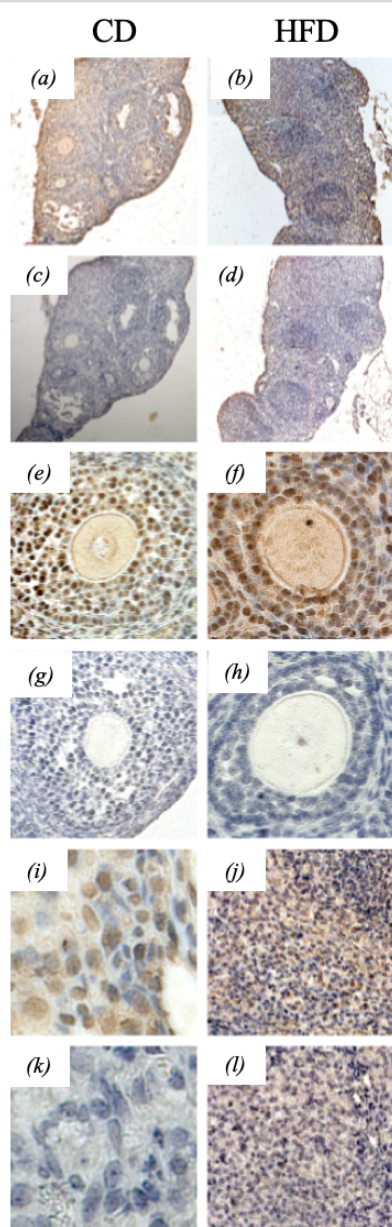


Figure 16- Comparison of the Nlrp3 inflammasome's expression in mice fed with CD and HFD. Positive staining in brown; counterstaining with hematoxylin. Ovary 100x (a-d); Secondary Follicles 630x (e-h); Stroma 630x (i-l). Negative controls (c, d, g, h, k, l). Author's original.

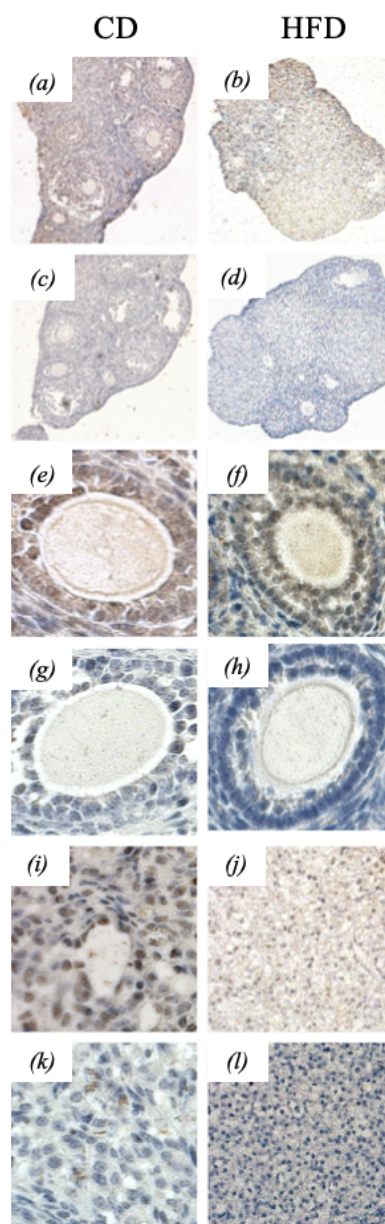


Figure 17- Comparison of the IL-1 β 's expression in mice fed with CF and HFD. Positive staining in brown; counterstaining with hematoxylin. Ovary 100x (a-d); Secondary Follicles 630x (e-h); Stroma 630x (i-l). Negative controls (c, d, g, h, k, l). Author's original.

In figures 18 and 19, pictures from ob/ob heterozygous mice and ob/ob homozygous mice are compared. The Nlrp3 expression is showed in picture 18 and the Il-1 β expression is showed in picture 19. In this case, the staining intensity is higher in the homozygous group.

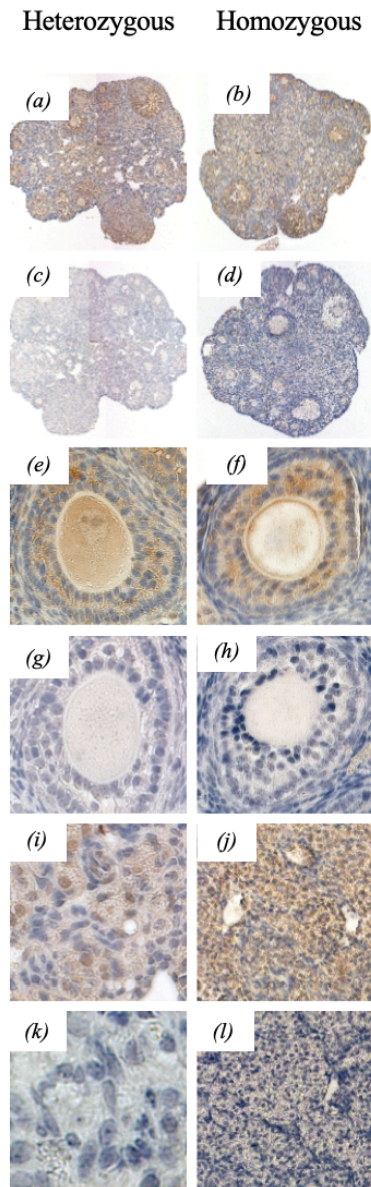


Figure 18- Comparison of the Nlrp3 inflammasome's expression in heterozygous and homozygous ob/ob mice. Positive staining in brown; counterstaining with hematoxylin. Ovary 100x (a-d); Secondary Follicles 630x (e-h); Stroma 630x (i-l). Negative controls (c, d, g, h, k, l). Author's original.

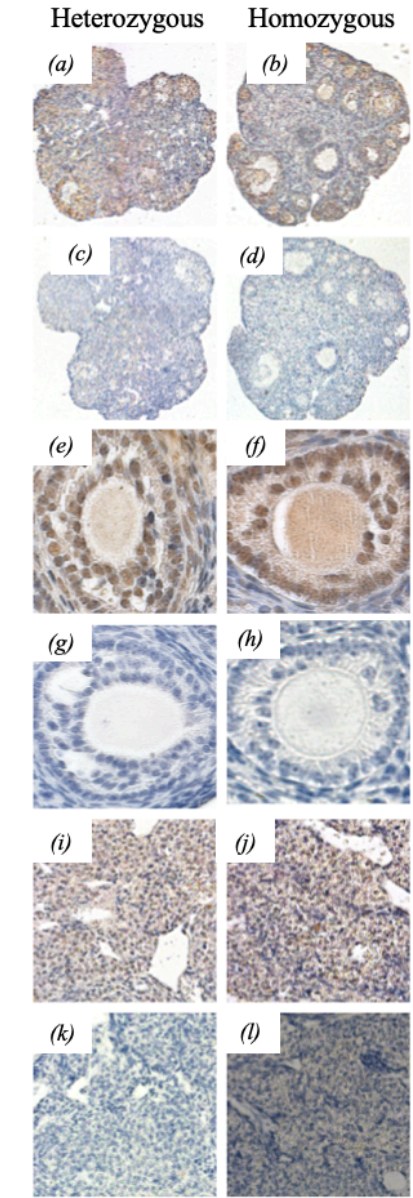


Figure 19- Comparison of the Il-1 β 's expression in heterozygous and homozygous ob/ob mice. Positive staining in brown; counterstaining with hematoxylin. Ovary 100x (a-d); Secondary Follicles 630x (e-h); Stroma 630x (i-l). Negative controls (c, d, g, h, k, l). Author's original.

Regarding the db/db group, it was not possible to obtain pictures from stroma. However, on the microscope it was possible to observe stained stroma cells. Figure 20 shows the Nlrp3 inflammasome's expression in the ovary components and figure 21 shows the Il-1 β 's expression.

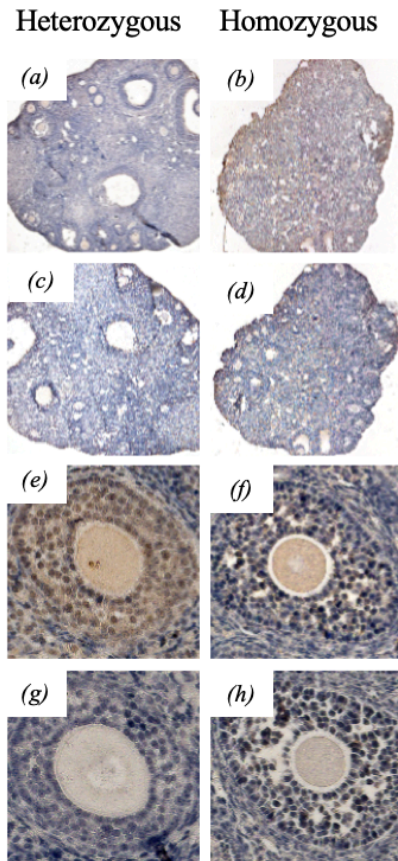


Figure 20- Comparison of the Nlrp3 inflammasome's expression in heterozygous and homozygous db/db mice. Positive staining in brown; counterstaining with hematoxylin. Ovary 100x (a-d); Secondary Follicles 630x (e-h). Negative controls (c, d, g, h). Author's original.

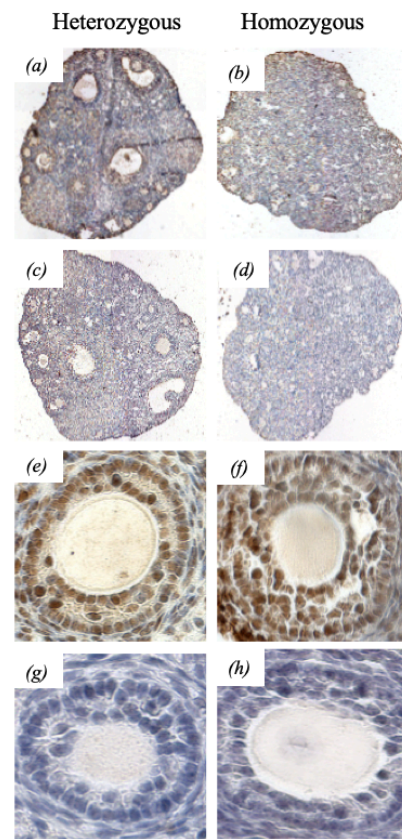


Figure 21- Comparison of the Il-1 β 's expression in heterozygous and homozygous db/db mice. Positive staining in brown; counterstaining with hematoxylin. Ovary 100x (a-d); Secondary Follicles 630x (e-h). Negative controls (c, d, g, h). Author's original.

In pictures 22 and 23 it is possible to observe the expression of Nlrp3 inflamassome and Il-1 β , respectively, in mice injected with saline and in mice injected with leptin for 16 days. In the samples showing the Nlrp3 inflamassome expression, it was not possible to get pictures from the whole ovary and in the samples with the Il-1 β expression, it was not possible to get pictures from follicles. The pictures from the leptin/saline group were taken with an Olympus CX series microscope, having a much inferior quality than the pictures from the other mice groups. Nonetheless, it was possible to identify staining on the microscope, so this group was not discarded. In this case, the saining intensity is higher in the leptin-injected group.

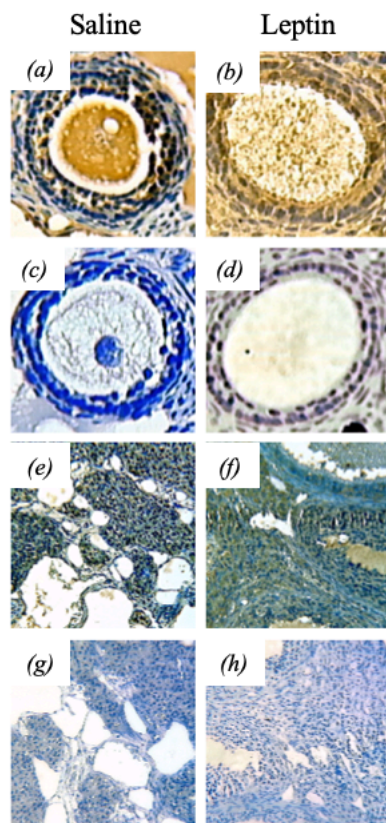


Figure 22- Comparison of the Nlrp3 inflammasome's expression in saline-injected and leptin-injected mice. Positive staining in brown; counterstaining with hematoxylin. Secondary Follicles 600x (a-d); Stroma 600x (c-f). Negative controls (c, d, g, h). Author's original.

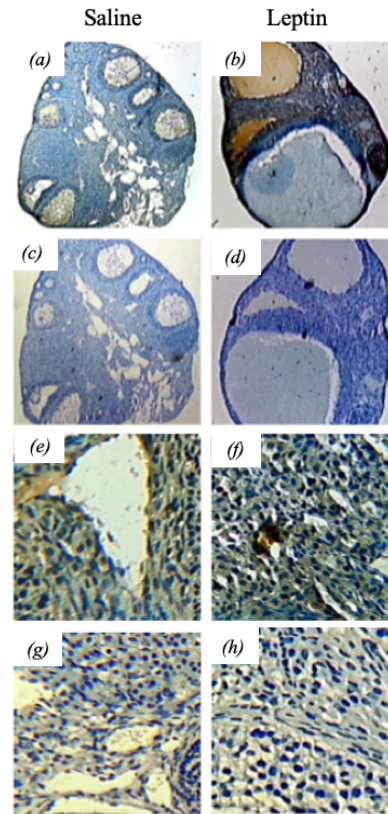


Figure 23- Comparison of the Il-1 β 's expression in saline-injected and leptin-injected mice. Positive staining in brown; counterstaining with hematoxylin. Ovary 100x (a-d); Stroma 600x (c-f). Negative controls (c, d, g, h). Author's original.

4. Discussion

The result achieved in this study regarding the expression of the Nlrp3 inflammasome in the ovary supports the other previously described (Wang et al., 2017). Besides confirming the presence of the inflammasome in the ovary, the results obtained in the present study characterise its expression within the ovary.

Thus, the Nlrp3 inflammasome is expressed in all types of follicles during follicle development, in the oocytes, granulosa cells and stroma (see figures 11, 11a, 11b, 11c and 12). In order to get more reliable results, Il-1 β was also used as a control, since this interleukin is produced due of the Nlrp3 activation. Il-1 β was also expressed in all types of follicles during follicle development, in the oocytes, granulosa cells and in the stroma, and occurs in the same sites where the Nlrp3 inflammasome is expressed (see figures 13 and 14).

As far as the student knows, this is the first study providing the histological characterisation of the Nlrp3 inflammasome's expression specifically in the ovary of obese mice. However, the inflammatory role that the Nlrp3 inflammasome has in obesity has already been studied before (Sienstra et al, 2011; Stienstra et al., 2012; Vandanmagsar et al., 2011). Even though the ovary is not their focus, these studies were used as a reference for the activation of the Nlrp3 inflammasome in obese mice.

The presence of the Nlrp3 inflammasome and of the Il-1 β in this study, observed through immunohistochemistry in obese mice, shows that inflammation is indeed present in the ovary of obese mice. This inflammation can be one of the reasons for the reproductive disorders that were already studied in obese female mice (Honnma et al., 2010; Balasubramanian et al., 2011; Jia et al., 2018).

Stienstra et al. (2011) and Vandanmagsar et al. (2011) already showed that, by removing the Nlrp3 inflammasome gene from mice, the latter will not develop obesity, if fed with an HFD. In turn, the results of the present study showed that mice fed with an HFD expressed the Nlrp3 inflammasome and also Il-1 β in the ovary components (see figures 16 and 17), confirming, in a certain way, the results achieved by the mentioned studies. Mice fed with an HFD had a higher number of stained follicles than mice fed with a CD, as also happened with

Il-1 β (see tables 8 and 9). Through immunohistochemistry, HFD-fed mice seemed to have a higher intensity of staining than CD-fed mice.

Regarding the differences of the Nlrp3 inflammasome's expression between ob/ob homozygous and ob/ob heterozygous mice, it was expected to obtain a higher intensity of expression in ob/ob homozygous, since these mice are the ones that develop obesity and, thus, all the complications associated with this condition (Smith, 2012; Van der Spek et al., 2012). In the present study, the ob/ob homozygous mice showed a higher number of stained follicles not only with the Nlrp3 inflammasome antibody, but also with the Il-1 β antibody (see tables 8 and 9). Concerning immunohistochemistry, ob/ob homozygous mice presented a higher intensity of staining for both Nlrp3 and Il-1 β antibodies (see figures 18 and 19).

Db/db homozygous were also expected to show a more evident expression of the Nlrp3 inflammasome and Il-1 β than db/db heterozygous mice, since the first are also more prone to develop obesity and all of its complications (Smith, 2012). However, the number of stained follicles was higher in the heterozygous group, for both antibodies. This is not in line with what the literature says about db/db homozygous developing obesity (Smith, 2012). In fact, db/db homozygous mice should have had a more evident expression of the inflammasome, since they easily develop obesity. Because the microscope observation was made by only one person, errors may have occurred. Regarding immunohistochemistry, it is hard to say if there were any differences due to the small number of samples (see figures 20 and 21).

As for the hyperleptinemia model group, it was expected to have a higher expression of the Nlrp3 inflammasome in the leptin-injected mice, since leptin can lead to the activation of this inflammasome (Fu et al., 2017). The results from immunohistochemistry showed a more evident staining in the leptin-injected mice (see figures 22 and 23). Unexpectedly, the control group presented a higher number of stained follicles (see tables 8 and 9), not being concordant with the fact that leptin-injected mice should have a higher number of stained follicles, since leptin activates the inflammasome. Once more, this could be due to the fact that only one observer did the follicle count. Plus, the ovaries' morphology was not the best one, since they presented some cysts.

During the development of this study, some limitations occurred.

First of all, the improvement of the immunohistochemistry's protocol took some months, in order to find the best dilution of the primary antibodies.

Plus, there were sample losses in some blocks sent from Poland and also problems with the paraffin, inhibiting their cut. Some ovaries did not have a good morphology and could not be used. Therefore, the total number of valid samples did not allow a statistical analysis, which could give a more accurate interpretation of the results. Only a descriptive count of follicles was performed and significant data could not be attained. For the same reason, the results concerning differences between groups, in immunohistochemistry, may also not be representative.

Other limitation concerns immunofluorescence. The improvement of the respective protocol, in order to get the best dilution of antibodies took some months. New antibodies had to be acquired, because the ones used were not efficient. Some problems of autofluorescence also occurred, which led to a new improvement of the protocol. All of this made immunofluorescence impossible to be performed, since this technique was going to be done in Poland, where the student stayed for only two months. If immunofluorescence was carried out, one could get results confirming the quantitative, and not only qualitative, difference between groups unlike immunohistochemistry.

In spite of these limitations, this study characterises the expression of the Nlrp3 inflammasome within the obese ovary. By identifying the presence of this inflammation marker in the obese ovary, this study can lead to others about therapeutics for the disorders that obesity causes in the reproductive function, such as infertility in obese women.

5. Conclusion

This study characterised the expression of the Nlrp3 inflammasome within the obese ovary. According to the results obtained, it is possible to say that this inflammasome is present in the oocytes of follicles of all stages of the follicle development, in the granulosa cells and in the stroma.

Using the Il-1 β antibody as a control makes the results more expressive. Indeed, since this interleukin is produced by the Nlrp3 inflammasome, they should be both present in the same sites, a fact that the results confirmed.

Thus, the aims of this study were generally achieved, except for the evaluation of the influence of leptin in the Nlrp3 inflammasome's expression, a goal that was not completely achieved. The reduced number of samples and the fact that only immunohistochemistry was done, a qualitative technique, did not allow conclusive results about the influence of leptin.

In future work, staining techniques such as immunofluorescence and western blot should be done, so that a quantitative analysis can be performed, and more conclusive results can be obtained. Some of these techniques are already being conducted in a wider study about the Nlrp3 inflammasome, at the Institute of Animal Reproduction and Food Research, in Poland, still in progress.

6. References

- Abcam (2019). Introduction to immunohistochemistry (IHC). Accessed on 19th February 2019 at <https://www.abcam.com/kits/introduction-to-immunohistochemistry-ihc>
- Albertini, D. F. (2015). The Mammalian Oocyte. In T. M. Plant & A. J. Zeleznik (Eds.), *Knobil and Neill's Physiology of Reproduction* (4th Ed., pp. 59-98). Oxford: Elsevier.
- Amleh, A. & Dean, J. (2002). Mouse genetics provides insight into folliculogenesis, fertilization and early embryonic development. *Human Reproduction Update*, 8(5), 395-403. doi:10.1093/humupd/8.5.395
- Balasubramanian, P., Jagannathan, L., Mahaley, R. E., Subramanian, M., Gilbreath, E. T., Mohankumar, P. S. & Mohankumar, S. M. (2012). High Fat Diet Affects Reproductive Functions in Female Diet-Induced Obese and Dietary Resistant Rats. *J Neuroendocrinol.*, 24(5), 748-755. doi: 10.1111/j.1365-2826.2011.02276
- Banaszewska, B., Wrotyńska-Barczyńska, J., Spaczynski, R. Z., Pawelczyk, L. & Duleba, A. J. (2016). Effects of Resveratrol on Polycystic Ovary Syndrome: A Double-blind, Randomized, Placebo-controlled Trial. *J Clin Endocrinol Metab.*, 101(11), 4322-4328. doi: 10.1210/jc.2016-1858
- Barnett, K. R., Schilling, C., Greenfeld, C. R., Tomic, D. & Flaws, J. A. (2006). Ovarian follicle development and transgenic mouse models. *Human Reproduction Update*, 12(5), 537-555. doi: 10.1093/humupd/dml022
- Berry, R. J. (1987). House mouse. *Biologist*, 34, 177-186.
- Bertolin, K. & Murphy, B. D. (2014). Mouse Estrous Cycle. In B. A. Croy, A. T. Yamada, F. J. DeMayo & S. L. Adamson (Eds.), *The Guide to Investigation of Mouse Pregnancy* (pp. 85-94). London: Academic Print.
- Boyd, K. L., Muehlenbachs, A., Rendi, M. H., Garcia, R. L. & Gibson-Corley, K. N. (2018). Female Reproductive System. In P. M. Treuting, S. M. Dintzis & K. S. Montine (Eds.),

- Comparative Anatomy and Histology: A Mouse, Rat, and Human Atlas* (2nd Ed., pp. 303-334). London: Academic Press.
- Byers, S. L., Wiles, M. V., Dunn, S. L. & Taft, R. A. (2012). Mouse Estrous Cycle Identification Tool and Images. *PLoS One*, 7(4). doi:10.1371/journal.pone.0035538
- Caligioni, C. S. (2009). Assessing Reproductive Status/Stages in Mice. *Curr Protoc Neurosci.*, A4 I.1-A4 I.8. doi:10.1002/0471142301.nsa04is48
- Carretero, A., Ruberte, J. & Navarro, M. (2017). Female Genital Organs. In A. Carretero, J. Ruberte & M. Navarro (Eds.), *Morphological Mouse Phenotyping: Anatomy, Histology and Imaging* (pp.227-251). Madrid: Editorial Medica Panamericana, S.A.
- Cheung, K. J., Tzamelis, I., Pissios, P., Rovira, I., Gavrilova, O., Ohtsubo, T., ... Friedman, J. M. (2007). Xanthine Oxidoreductase Is a Regulator of Adipogenesis and PPAR γ Activity, *Cell Metab.*, 5(2), 115–128. doi: 10.1016/j.cmet.2007.01.005
- Cora, M. C., Kooistra, L. & Travlos, G. (2015). Vaginal Cytology of the Laboratory Rat and Mouse: Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears. *Toxicol Pathol.*, 43(6), 776-793. doi:10.1177/0192623315570339
- Cortvrindt, R. & Smitz, J. (2001). *In vitro* Follicle Growth: Achievements in Mammalian Species, *Reprod Domest Anim.*, 36(1), 3-9. doi: 10.1046/j.1439-0531.2001.00261.x
- Davisson, M. T. & Linder, C. C. (2004). Historical Foundations. In H. Hedrich (Ed.), *The Laboratory Mouse* (pp. 15-24). Oxford: Elsevier.
- Dembic, Z. (2015). Cytokines of the Immune System: Interleukins. In Z. Dembic (Ed.), *The Cytokines of the Immune System* (pp. 143-239). London: Academic Press.
- Dinarello, C. A. (2011). Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood*, 117(4), 3720-3732. doi:10.1182/blood-2010-07-273417

- Duraiyan, J., Govindarajan, R., Kaliyappan, K. & Palanisamy, M. (2012). Application of immunohistochemistry. *J Pharm Bioallied Sci.*, 4(Suppl 2), S307-S309. doi: 10.4103/0975-7406.100281
- Edson, M. A., Nagaraja, A. K. & Matzuk, M. M. (2009). The Mammalian Ovary from Genesis to Revelation, *Endocr Rev.*, 30(6), 624-712. doi: 10.1210/er.2009-0012
- Elliot, E. I. & Sutterwala, F. S. (2015). Initiation and perpetuation of NLRP3 inflammasome activation and assembly. *Immunol Rev.*, 265(1), 35-52. doi: 10.1111/imr.12286
- Eppig, J. J. (2001). Oocyte control of ovarian follicular development and function in mammals. *Reproduction*, 122(6), 829-838.
- Fu, S., Liu, L., Han, L. & Yu, Y. (2017). Leptin promotes IL-18 secretion by activating the NLRP3 inflammasome in RAW 264.7 cells. *Mol Med Rep.*, 16(6), 9770-9776. doi: 10.3892/mmr.2017.7797
- Gougeon, A. (1996). Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocr. Rev.*, 17(2), 121-155. doi: 10.1210/edrv-17-2-121
- Guénet, J. L. & Bonhomme, F. (2004). Origin of the Laboratory Mouse and Related Subspecies. In H. Hedrich (Ed.), *The Laboratory Mouse* (pp. 3-14). Oxford: Elsevier.
- He, Y., Hara, H. & Núñez, G. (2016). Mechanism and Regulation of NLRP3 Inflammasome Activation. *Trends Biochem Sci.*, 41(12), 1012-1021. doi:10.1016/j.tibs.2016.09.002
- Hernandez, J. C., Sirois, C. M. & Latz, E. (2011). Activation and Regulation of the NLRP3 Inflammasome. In I. Couillin, V. Pétrilli & F. Martinon (Eds.), *The Inflammasomes* (pp. 197-208). Basel: Springer.
- Honnma, H., Endo, T., Kiya, T., Shimizu, A., Nagasawa, K., Baba, T., ... Saito, T. (2010). Remarkable features of ovarian morphology and reproductive hormones in insulin-resistant Zucker fatty (fa/fa) rats. *Reprod Biol Endocrinol.*, 73(8). doi: 10.1186/1477-7827-8-73

- Jia, Z., Feng, Z., Wang, L., Li, H., Wang, H., Xu, D., ... Feng, X. (2018). Resveratrol reverses the adverse effects of diet-induced obese murine model oocyte quality and zona pellucida softening. *Food Funct*, 9(5), 2623-2633. doi: 10.1039/C8FO00149A
- Jo, E. K., Kim, J. K., Shin, D.M. & Sasakawa, C. (2016). Molecular mechanisms regulating NLRP3 inflammasome activation. *Cellular & Molecular Immunology*, 13(2), 148-159. doi: 10.1038/cmi.2015.95
- Kalyuzhny, A. E. (2016). *Immunohistochemistry: Essential Elements and Beyond*. Switzerland: Springer.
- Kaufman, M., Nikitin, A. Y. & Sundberg, J. P. (2010). *Histologic Basis of Mouse Endocrine System Development: A Comparative Analysis*. Boca Raton: CRC Press.
- Kumar, T. R., Wang, Y., Lu, N. & Matzuk, M. M. (1997). Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat. Genet.*, 15(2), 201-204. doi:10.1038/ng0297-201
- Larsen, C. M., Faulenbach, M., Vaag, A., Vølund, A., Ehres, J. A., Seifert, B., ... Donath, M. Y. (2007). Interleukin-1–Receptor Antagonist in Type 2 Diabetes Mellitus. *N engl j med*, 356, 1517-1526. doi: 10.1056/NEJMoa065213
- Levine, J. E. (2015). Neuroendocrine Control of the Ovarian Cycle of the Rat. In T. M. Plant & A. J. Zeleznik (Eds.), *Knobil and Neill's Physiology of Reproduction* (4th Ed., pp. 1199-1258). Oxford: Elsevier.
- McInnes, I. B. (2017). Cytokines. In: G. S. Firestein, R. C. Budd, S. E. Gabriel, I. B. McInnes & J. R. O'Dell (Eds.), *Kelley and Firestein's Textbook of Rheumatology, Volume 1* (10th Ed., pp. 396-407). Philadelphia: Elsevier.
- Mohammed-Ali, Z., Carlisle, R. E., Nademi, S. & Dickhout, J. G. (2017). Animal Models of Kidney Disease. In: P. M. Conn (Ed.), *Animal Models for the Study of Human Disease* (2nd Ed., pp. 379-417). London: Academic Press.

Mouse Genome Sequencing Consortium. (2002). Initial sequencing and comparative analysis

- of the mouse genome. *Nature*, 420(6915), 520-562. doi:10.1038/nature01262
- Murta, D., Batista, M., Silva, E., Trindade A., Mateus, L., Duarte, A. & Lopes-da-Costa, L. (2014). Differential expression of Notch component and effector genes during ovarian follicle and corpus luteum development during the oestrous cycle. *Reprod Fertil Dev.*, 27(7), 1038-1048. doi: 10.1071/RD13399
- Myers, M., Britt, K. L., Wreford, N. G. M., Ebling, F. J. P. & Kerr, J. B. (2004). Methods for quantifying follicular numbers within the mouse ovary. *Reproduction*, 127(5), 569-580. doi: 10.1530/rep.1.00095
- Ortega, I., & Duleba, A. J. (2015). Ovarian actions of resveratrol, 1348, 86–96. <https://doi.org/10.1111/nyas.12875>
- Pedersen, T. & Peters, H. (1968). Proposal for a classification of oocytes and follicles in the mouse ovary. *J Reprod. Fert.*, 17(3), 555-557. doi:<https://doi.org/10.1530/jrf.0.0170555>
- Peters, H. (1969). The development of the mouse ovary from birth to maturity. *Acta Endocrinologica*, 62(1), 98-116. doi:10.1530/acta.0.0620098
- Picton, H. M., Harris, S. E., Muruvi, W. & Chambers, E. L. (2008). The *in vitro* growth and maturation of follicles, *Reproduction*, 136(6), 703-715. doi: 10.1530/REP-08-0290
- Priedkalns, J. & Leiser, R. (2006). Female Reproductive System. In J. A. Eurell & B. L. Frappier (Eds.), *Dellmann's Textbook of Veterinary Histology* (6th Ed., pp. 256-278). Oxford: Blackwell Publishing, Ltd.
- Rader, K. A. (1999). Of Mice, Medicine, and Genetics: C. C. Little's Creation of the Inbred Laboratory Mouse, 1909–1918. *Stud. Hist. Phil. Biol. & Biomed. Sci.*, 30(3), 319–343. doi: 10.1016/S1369-8486(99)00015-1
- Rheinheimer, J., de Souza, B. M., Cardoso, N. S., Bauer, A. C & Crispim, D. (2017). Current role of the NLRP3 inflammasome on obesity and insulin resistance: A systematic review. *Metabolism: clinical and experimental*, 74, 1-9. doi: 10.1016/j.metabol.2017.06.002

- Richards, J. S., Russell, D. L., Ochsner, S. & Espey, L. L. (2002) Ovulation: new dimension and new regulators of the inflammatory-like response. *Annu. Rev. Physiol.*, 64, 69-92. doi:10.1146/annurev.physiol.64.081501.131029
- Rimon-Dahari, N., Yerushalmi-Heinemann, L., Alyagor, L. & Dekel, N. (2016). Ovarian Folliculogenesis. In R. P. Piprek (Ed.), *Molecular Mechanisms of Cell Differentiation in Gonad Development* (pp. 167-190). Switzerland: Springer.
- Roderick, T. H. (2007). Foreword For Volume I. In J. G Fox, S. W. Barthold, M. T. Davisson, C. E. Newcomer, F. W. Quimby & A. L. Smith (Eds.), *The Mouse in Biomedical Research – Volume I: History, Wild Mice, and Genetics* (2nd Ed., pp. Xiii). Oxford: Elsevier.
- Sarkar, D. K., Chiappa, S. A., Fink, G. & Sherwood, N. M. (1976). Gonadotropin-releasing hormone surge in pro-oestrous rats. *Nature*, 264(5585), 461-463. doi:https://doi.org/10.1038/264461a0
- Scudamore, C. L. (2014). Reproductive system. In C. L. Scudamore (Ed.), *A Practical Guide to the Histology of the Mouse* (pp. 87-108). Oxford: John Wiley & Sons, Ltd.
- Smith, G. P. (2012). Animal Models of Cutaneous and Hepatic Fibrosis. In: P. M. Conn (Ed.), *Progress in Molecular Biology and Translational Science, Volume 105* (pp. 371-409). London: Academic Press.
- Smitz, J. E. J. & Cortvrindt, R. G. (2002). The earliest stages of folliculogenesis *in vitro*. *Reproduction*, 123(2), 185-202.
- Sloan-Lancaster, J., Abu-Raddad, E., Polzer, J., Miller, J. W., Scherer, J. C., De Gaetano, A., ... Landschulz, W. H. (2013). Double-Blind, Randomized Study Evaluating the Glycemic and Anti-inflammatory Effects of Subcutaneous LY2189102, a Neutralizing IL-1 β Antibody, in Patients with Type 2 Diabetes. *Diabetes Care*, 36(8), 2239-2246. doi: 10.2337/dc12-1835

- Stienstra, R., Tack, C. J., Kanneganti, T. D., Joosten, L. A. B. & Netea, M. G. (2012). The Inflammasome Puts Obesity in the Danger Zone. *Cell Metab.*, 15(1), 10-18. doi: 10.1016/j.cmet.2011.10.011
- Stienstra, R., van Diepen, J. A., Tack, C. J., Zaki, M. H., van de Veerdonk, F. L., Perera, D., Kanneganti, T. D. (2011). Inflammasome is a central player in the induction of obesity and insulin resistance. *Proc Natl Acad Sci USA*, 108(37), 15324-15329. doi:10.1073/pnas.1100255108.
- Stouch, A. N., McCoy, A. M., Greer, R. M., Lakhdari, O., Yull, F. E., Blackwell, T. S., Hoffman, M. & Prince, L. S. (2017). Il-1 β and Inflammasome Activity Link Inflammation to Abnormal Fetal Airway Development. *J. Immunol.*, 196(8), 3411-3420. doi: 10.4049/jimmunol.1500906
- Strong, L. C. (1978). Inbred Mice in Science. In H. C. Morse (Ed.), *Origins of Inbred Mice* (3rd Ed., pp. 45-68). New York: Academic Press.
- The Human Protein Atlas (2019). Immunohistochemistry. Accessed on 14th March 2019 at <https://www.proteinatlas.org/learn/method/immunohistochemistry>.
- The Jackson Laboratory (2018). The mouse as a model for human disease. Accessed on 6th December 2018 at <https://www.jax.org/personalized-medicine/why-mouse-genetics>.
- ThermoFisher Scientific (2019). Overview of Immunohistochemistry (IHC). Accessed on 19th February 2019 at <https://www.thermofisher.com/pt/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-immunohistochemistry.html>
- Tschopp, J. & Schroder, K. (2010). NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? *Nat Rev Immunol.*, 10(3), 210-215. doi: 10.1038/nri2725
- Van Asseldonk, E. J. P., Stienstra, R., Koenen, T. B., Joosten, L. A. B., Netea, M. G. & Tack, C. J. (2011). Treatment with Anakinra Improves Disposition Index But Not Insulin

- Sensitivity in Nondiabetic Subjects with the Metabolic Syndrome: A Randomized, Double-Blind, Placebo-Controlled Study. *J Clin Endocrinol Metab.*, 96(7), 2119-2126. doi: 10.1210/jc.2010-2992
- Van der Spek, R., Kreier, F., Fliers, E. & Kalsbeek, A. (2012). Circadian rhythms in white adipose tissue. In: A. Kalsbeek, M. Meroow, T. Roenneberg and R. G. Foster (Eds.), *Progress in Brain Research, Volume 199* (pp. 183-201). Oxford: Elsevier.
- Vandanmagsar, B., Youm, Y., Ravussin, A., Galgani, J. E., Stadler, K., Mynatt, R., ... Dixit, V. D. (2011). The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nat Med*, 17(2), 179-188. doi: 10.1038/nm.2279
- Cheung, K. J., Tzamelis, I., Pissios, P., Rovira, I., Gavrilova, O., Ohtsubo, T., ... Friedman, J. M. (2007). Article Xanthine Oxidoreductase Is a Regulator of Adipogenesis and PPAR γ Activity, (February), 115–128. <https://doi.org/10.1016/j.cmet.2007.01.005>
- Wang, J., Ge, W., Liu, J., Klinger, F. G., Dyce, P. W., De Felici, M. & Shen, W. (2017). Complete *in vitro* oogenesis: retrospects and prospects. *Cell Death and Differentiation*, 24(11), 1845-1852. doi:10.1038/cdd.2017.134
- Zhang, X., Dai, J., Li, L., Chen, H. & Chai, Y. (2017). NLRP3 Inflammasome Expression and Signaling in Human Diabetic Wounds and in High Glucose Induced Macrophages. *J Diabetes Res.*, 2017, 1-7. doi: 10.1155/2017/5281358
- Zheng, W., Zhang, H. & Liu, K. (2014). The two classes of primordial follicles in the mouse ovary: their development, physiological functions and implications for future research. *Molecular Human Reproduction*, 20(4), 286-292. doi:10.1093/molehr/gau007
- Zhou, R., Tardivel, A., Thorens, B., Choi, I. & Tschopp, J. (2010). Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol*, 11(2), 136-140. doi: 10.1038/ni.1831

ANNEXES

ANNEX I – Immunofluorescence Protocol

Deparaffinization:

- 2 hours at 59°C (incubator)
- Xylene 15 minutes
- Xylene 10 minutes (change basket)
- 2x3 minutes EtOH 99,8%
- 2x3 minutes EtOH 90%
- 2x3 minutes EtOH 70%

10 minutes in ultrapure water

Antigen retrieval:

- Warm solution for 3 minutes, at 700W, in the microwaves
- Incubate slides for 3x5 minutes, at 700W
- Let the slides cool down for 20-30 minutes

Wash slides 1x5 minutes with PBS

Wash slides 2x5 minutes with PBS-Triton 0,3%

Wash slides 1x5 minutes with PBS

Blocking in 2%BSA+0,3M Glycine, for 2 hours

Sudan Black Treatment for 10 minutes

Wash slides 8x5 minutes with PBS

Incubate sections with primary antibodies (20 ul/slice) in 5% blocking solution at 4°C, overnight

Wash slides 6x10 minutes with PBST 0,1%

Incubate sections with secondary antibody (20 ul/slice) in 5% blocking solution at 37°C, for 2 hours

Wash slides 6x10 minutes with PBST 0,1%

Apply DAPI to the slides and incubate in the dark for 30 minutes at room temperatures

Wash slides 3x5 minutes with PBST 0,1%

Apply mounting medium to the coverslip and apply slip to slide

Store at 4°C

ANNEX II – Immunohistochemistry Protocol

Day 1

1. The slides must be put in the incubator at 57°C, 15 minutes before deparaffinization starts.
2. Proceed to the slide's deparaffinization in xylene and rehydration in descending concentrations of ethanol:
 - a. Xylene _____ 10'
 - b. Xylene _____ 5'
 - c. Ethanol 100% _____ 2x2'
 - d. Ethanol 95% _____ 2x2'
 - e. Ethanol 75% _____ 2x2'
3. Wash the slides with distilled water for 5 minutes.
4. Incubate the slides in 3% H₂O₂, in the dark, for 15 minutes at 37°
5. Proceed to the antigen retrieval in 10 mM of citrate buffer pH6
 - a. Heat the solution for 3 minutes at 700W without the slides
 - b. Incubate for 5' x 3 at 700W
 - c. The slides must be cooled down at room temperature, for about 20-30 minutes.
6. Incubate the slides in the blocking solution - 2% BSA
 - put 200µl of the blocking solution per slide.
 - let it incubate for **1 hour** at a humidity chamber.
7. Incubate with the primary antibody
 - put 100µl/slide and cover with a coverslip
 - Incubate at 4 °C, in humidity chamber, **overnight**.
 - Il-1β antibody (sc-7884; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA): 1:100
 - Anti-Nlrp3 inflammasome antibody (ab214185; Abcam, Cambridge, UK): 1:200

Day 2

1. Wash the slides with PBS (**4 × 10 min**)
2. Incubate with the secondary antibody
 - put 100µl/slide and cover with a coverslip.
 - incubate at room temperature, in a humidity chamber, for **1 hour**.
 - polyclonal goat anti-rabbit IgG conjugated with peroxidase (410972, Dako, Carpinteria, CA, USA): 1:100
3. Wash the slides with PBS (**2× 10 min**)
4. Incubate the slides in DAB until reaction occurs
5. Wash the slides with distilled water (**2× 5 min**)
6. Proceed to Gill's Hematoxylin stain for 45 seconds (3 mins)
7. Wash the slides with tap water to remove the excess of hematoxylin
8. Dehydrate in ascending concentrations of ethanol:
 - a. Ethanol 95% _____ 2'
 - b. Ethanol 100% _____ 2'
 - c. Xylene _____ 5'
 - d. Xylene _____ 5'
9. Mount the slides with Entellan.

ANNEX III – Immunofluorescence Solutions

1. Sodium Citrate Buffer

- 900ml distilled water
- 2,94g Tri-sodium citrate, set pH 6.0
- Add 0,5 ml Tween 20

2. Phosphate Buffered Saline (PBS)

- 8g NaCl
- 0,2g KCl
- 1,44g Na₂HPO₄
- 0,24g KH₂PO₄
- 1L H₂O
- pH 7.4

3. PBS TRITON 0,3%

- 3 ml Triton-X
- 1000 ml PBS

4. Sudan Black

- 0,3% Sudan Black
- 70% EtOH
- Stirred in the dark, for 2 hours

5. PBS-Tween (PBST) 0,1%

- 1 ml Tween 20
- 1000 ml PBS

ANNEX IV – Immunohistochemistry Solutions

1. Hydrogen Peroxide 3% (H₂O₂)

- 30% H₂O₂, FV 60ml = 54 ml H₂O + 6 ml H₂O₂

2. Sodium Citrate Buffer 10 mM

- Sodium Citrate 0,1 M
- Citric Acid 0,1 M
- 450mL H₂O + 9mL acid citric 0.1M pH 1.8+ 41mL sodium citrate 0.1M pH8.8

3. Phosphate Buffered Saline (PBS) 10%

- 100 ml PBS 1%
- 1000 ml H₂O