UNIVERSIDADE D
COIMBRA

Inês de Sousa Oliveira

Relatórios de Estágio e Monografia intitulada " Exploring new biomarkers of aging " referentes à Unidade Curricular "Estágio", sob a orientação da Dra. Filipa Jesus, do Dr. João Serra e da Professora Doutora Cláudia Cavadas, apresentados à Faculdade de Farmácia da Universidade de Coimbra, para apreciação na prestação de provas públicas de Mestrado Integrado em Ciências Farmacêuticas.

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Dissertation Project in fulfilment of the requirements for a Master's degree in Pharmaceutical Sciences, under the supervision of Professor Cláudia Cavadas, Faculty of Pharmacy of the University of Coimbra and Center for Neuroscience and Cell Biology of the University of Coimbra, and co-supervision of Doctor Célia Alexandra Aveleira and Doctor Marisa Ferreira Marques, Center for Neuroscience and Cell Biology.

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Coimbra, 30 de outubro de 2020.

Inês de Sousa Oliveira

(Inês de Sousa Oliveira)

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PARTE I

Relatório de Estágio em Farmácia Comunitária

Farmácia Godinho Tomaz

Lista de Abreviaturas

ANF – Associação Nacional das Farmácias

API – *Active Pharmaceutical Ingredient* (em português, Ingrediente Farmacêutico Ativo)

ATDPS – *Automatic Tablet Dispensing Packaging System*

DCI – Denominação Comum Internacional

EEE – Espaço Económico Europeu

FFUC – Faculdade de Farmácia da Universidade de Coimbra

FGT – Farmácia Godinho Tomaz

MICF – Mestrado Integrado em Ciências Farmacêuticas

MNSRM – Medicamento não sujeito a receita médica

PIM – Preparação Individualizada da Medicação

SNS – Serviço Nacional de Saúde

SWOT – *Strengths, Weaknesses, Opportunities, Threats* (em português, Forças, Fraquezas, Oportunidades, Ameaças)

1. Introdução

De acordo com a Diretiva 2013/55/UE, do Parlamento Europeu e do Conselho de 20 de novembro de 2013 (Artigo 44º, n.º 2), a formação de um farmacêutico deve incluir quatro anos de formação teórica e prática, a tempo inteiro, lecionada numa instituição de ensino superior. No final destes quatro anos, o estudante deve ser sujeito a um estágio de seis meses numa farmácia aberta ao público ou num hospital sob orientação de um farmacêutico (1).

Face ao descrito na diretiva acima e com o culminar de quatro anos de ensino na Faculdade de Farmácia da Universidade de Coimbra, realizei o estágio curricular em farmácia comunitária na Farmácia Godinho Tomaz.

Como finalista do plano de estudos do Mestrado Integrado em Ciências Farmacêuticas (MICF) da Faculdade de Farmácia da Universidade de Coimbra (FFUC) e após o término do estágio, reitero o papel do farmacêutico na comunidade como agente de saúde pública e especialista do medicamento, enaltecendo o seu papel, quer ao nível de conhecimentos técnicos e científicos, como ao nível do contacto com os utentes e do seu contributo para o melhoramento do estado de saúde da comunidade e promoção na adoção de estilos de vida saudáveis (2).

A experiência de estágio permite aos estagiários o seu crescimento a nível profissional tornando-os farmacêuticos dignos, responsáveis, capazes e detentores de conhecimento (3) tendo sempre como foco principal "o cidadão em geral e o doente em particular" (4).

O presente relatório versa sobre o estágio curricular em Farmácia Comunitária, realizado entre janeiro e junho de 2020 na Farmácia Godinho Tomaz (FGT) em Leiria, sob a orientação da Dra. Filipa Jesus, e encontra-se organizado sob a forma de uma análise SWOT (*Strengths, Weaknesses, Opportunities, Threats*). A FGT inclui-se no grupo Beatriz Godinho que engloba: Laboratório Tomaz (5), Laboratório de Análises Clínicas Beatriz Godinho (6), Polidiagnóstico (7), Polidiagnóstico Empresas (8), Clínica Luís Lourenço (9) e Farmácias (Farmácia Godinho Tomaz, Farmácia Tomaz, Farmácia Beatriz Godinho e Farmácia Praia da Vieira).

2. Análise SOWT

A análise SOWT encontra-se dividida em dois níveis: interno e externo. Externamente devem ser identificadas as oportunidades (*Opportunities*) e as eventuais ameaças (*Threats*). Ao nível interno, são avaliados os pontos fortes (*Strengths*) e os pontos fracos (*Weakness*).

Esta análise permite identificar os eventuais pontos fracos e fortes do desempenho do estagiário, mas também as oportunidades e as ameaças associadas ao ambiente no qual o estágio se desenrola.

2.1 Pontos Fortes

2.1.1 Localização da farmácia

A Farmácia Godinho Tomaz é uma farmácia que apresenta duas vertentes bastante díspares. Para além de ser uma farmácia local com diversos clientes habituais e fidelizados, é também considerada uma farmácia de passagem visto que se encontra numa zona de saída do centro de Leiria. Para além disso, tem uma localização privilegiada uma vez que se encontra perto do Hospital Distrital de Leiria (Hospital Santo André) e de uma clínica dentária.

2.1.2 Perfil demográfico dos utentes

Os utentes que frequentam a farmácia caracterizam-se por pertencerem a diferentes faixas etárias e estratos socioeconómicos. Esta heterogeneidade obrigou-me a ser mais flexível no atendimento ao balcão, tendo de adaptar, rapidamente, a linguagem técnica e a explicação prestada.

2.1.3 Horário alargado de funcionamento

O horário de funcionamento da FGT é das 9h às 22h, sem noites de serviço. A adaptação do horário do estagiário a este horário foi fundamental, uma vez que me permitiu estar presente nas horas com mais afluência e ter a possibilidade de executar mais atendimentos.

2.1.4 Diversidade de tarefas desempenhadas

A FGT é uma farmácia com um número diário de clientes relativamente elevado e, face a isso, as tarefas a executar nunca terminam e são sempre bastante diversas. Esta situação é bastante favorável num estágio uma vez que torna o estagiário uma pessoa mais ativa e dinâmica e com capacidade de desempenhar várias e diversificadas tarefas num só dia.

2.1.5 Realização de rastreios da glicémia e tensão arterial e testes de gravidez

Os rastreios (glicémia e tensão arterial) e testes de gravidez foram realizados diversas vezes o que consistiu num ponto forte e que realizei com à vontade, tendo apenas recorrido à equipa em situações pontuais e mais preocupantes do estado de saúde de alguns utentes, com necessidade de recorrer à assistência médica.

2.1.6 Iniciação do atendimento ao balcão relativamente cedo

Considero que a minha introdução ao atendimento ao balcão logo no primeiro mês de estágio tenha sido uma enorme vantagem visto que me permitiu desenvolver competências técnicas e profissionais mais rapidamente e uma certa destreza no atendimento, de forma a que, no período final do estágio, conseguisse estar mais familiarizada com o sistema e com menos constrangimentos, dispensando mais tempo e atenção no atendimento ao utente e nos seus problemas, alertando para determinadas situações de forma mais espontânea.

2.1.7 Contacto com a equipa técnica

Desde o primeiro dia de estágio, sempre fui bem acolhida e integrada na equipa. A simpatia e o profissionalismo sempre primaram na farmácia o que foi, sem qualquer dúvida, um dos grandes pontos fortes do estágio, não só por estar mais à vontade para colocar qualquer dúvida, mas também por me sentir parte integrante da equipa.

2.1.8 Realização do estágio de verão na FGT

A possibilidade que a FGT me ofereceu para a realização de um estágio de verão contribuiu muito para a minha rápida iniciação no atendimento ao público, visto que me encontrava familiarizada com a maneira de organização e funcionamento da equipa.

2.1.9 Ausência de *robot*

A ausência de *robot* permitiu um melhor conhecimento da embalagem secundária e do nome comercial dos medicamentos, facilitando a compreensão do utente aquando do atendimento ao público.

2.2 Pontos Fracos

2.2.1 Insegurança inicial no atendimento e necessidade de interrupção de atendimentos

Especialmente no início do estágio e devido a alguma insegurança, tive necessidade de interromper alguns atendimentos para esclarecer dúvidas e para pedir ajuda em determinadas situações com as quais ainda não me tinha deparado. Isto tornava os

atendimentos mais extensos, o que constituía uma constante preocupação da minha parte, essencialmente quando a farmácia se encontrava com alguns utentes em espera. Apesar disso, qualquer uma das farmacêuticas se demonstrou sempre disponível para prestar ajuda.

2.2.2 Dificuldade na identificação dos medicamentos pelos nomes comerciais

Inicialmente e face à enorme variedade de marcas de medicamentos, foi difícil estabelecer uma ligação entre os nomes comerciais e a Denominação Comum Internacional (DCI).

É de referir que apesar de ter sido um dos meus pontos fracos, não é da responsabilidade da instituição de ensino referenciar isto. Esta situação deve advir mesmo da aprendizagem inerente ao estágio porque é nessa situação que realmente faz sentido desenvolver esta competência.

2.2.3 Adaptação da linguagem ao utente

Termos como DCI, Ingrediente Farmacêutico Ativo (API) e posologia são pouco conhecidos pela população em geral, logo devem ser evitados ou adaptados às pessoas com mais formação de forma a que os utentes compreendam bem toda a medicação e que as terapêuticas sejam eficazes e eficientes, garantindo a adesão à terapêutica.

2.3 Oportunidades

2.3.1 Oportunidade de frequentar diversas ações de formação

A FGT sempre proporcionou, quer ao estagiário quer às próprias farmacêuticas, uma formação contínua e uma constante renovação de conhecimentos e descoberta de novos produtos de determinadas marcas. Face a isto e, não obstante de ser estagiária, foi-me dada a oportunidade de participar em diversas formações.

2.3.2 Ações de formação na farmácia com os delegados de informação médica

A visita dos delegados de informação médica às farmácias é uma prática comum no dia-a-dia da farmácia. Estas visitas trazem, na sua maioria, novidades e certos apontamentos fulcrais para um aconselhamento farmacêutico mais assertivo e detalhado.

2.3.3 Entrega e leitura de análises clinicas e exames de diagnóstico

A FGT faz parte do grupo Beatriz Godinho e face a isto é um local onde também se entregam os resultados das análises clínicas (realizadas nos Laboratórios Beatriz Godinho) e exames de diagnóstico (realizados na Clínica Luís Lourenço). Diversas pessoas, devido a dificuldades de interpretação de valores ou mesmo da compreensão de determinados

parâmetros, acabam por colocar as questões aquando do seu levantamento. Isto permitiu desenvolver conhecimentos especialmente na área da Hematologia, o que é um ponto extra à formação base do estagiário na Farmácia Comunitária.

2.3.4 Preparação de diversos medicamentos manipulados (fórmulas magistrais)

Ao longo do estágio, realizei a preparação de diversos medicamentos manipulados como: o medicamento manipulado de ácido salicílico, furoato de mometasona (Elocom®), glicerina e vaselina, o medicamento manipulado de ácido salicílico, ureia e vaselina e o medicamento manipulado de enxofre com vaselina sólida (para um utente com sarna) (Anexo 1, 2 e 3).

Para a preparação dos seguintes manipulados, foram seguidas a orientações estabelecidas na Portaria n.º 594/2004, de 2 de junho (10) (que refere as boas práticas a observar na preparação dos medicamentos manipulados), Portaria n.º 769/2004, de 1 de julho (que estabelece que o cálculo do preço de venda ao público dos medicamentos manipulados) (11) e Despacho n.º 18694/2010 (que estabelece os medicamentos manipulados sujeitos a comparticipação por parte do Serviço Nacional de Saúde (SNS)) (12,13).

2.3.5 Observação da Preparação Individualizada da Medicação (PIM) (máquina ATDPS)

A FGT adquiriu, nos últimos tempos, uma máquina ATDPS (*Automatic Tablet Dispensing Packaging System*), adequada para a preparação individualizada da medicação (14).

Considero fundamental este serviço fornecido pela farmácia especialmente no caso de utentes que apresentem dificuldades no processo de uso dos medicamentos, com problemas de adesão à terapêutica de forma não intencional, problemas cognitivos, utentes com terapêuticas complexas, entre outros (15).

2.3.6 Contacto com diversos profissionais de saúde

Devido à necessidade de alguns esclarecimentos durante o atendimento tive que estabelecer contacto telefónico com médicos, enfermeiros e outros farmacêuticos e técnicos de outras farmácias para a prestação de determinados serviços que não se encontravam ao alcance da FGT (como por exemplo, o contacto com Farmácia Luciano e Matos para a preparação de determinados manipulados). Estabeleci também contacto com Associação Nacional das Farmácias (ANF) aquando a necessidade de colocar alguma dúvida (especialmente acerca da comparticipação dos medicamentos manipulados).

2.4 Ameaças

2.4.1 Problemática dos medicamentos esgotados

A situação dos medicamentos esgotados é, infelizmente, um caso recorrente aquando do atendimento. Esta situação nem sempre é bem entendida pelos utentes o que afeta deste logo a relação dos utentes com os profissionais de saúde e, consequentemente, as vendas. A ausência do medicamento por esta situação e a imprevisibilidade do seu reaparecimento no mercado levou ao aconselhamento, em alguns casos, de uma nova visita do médico de forma que este opte por uma nova solução terapêutica.

2.4.2 Automedicação incorreta e desvalorização dos conhecimentos do farmacêutico

Devido ao aumento da literacia da população, ao excesso de informação que circula na internet e à publicidade dos produtos feita, quer na televisão quer nas redes socias, e pelas *influencers* (o que é uma prática cada vez mais comum), leva, muitas vezes, a situações mais complicadas derivadas da automedicação incorreta.

É o papel do farmacêutico alertar para estes casos e tentar explicar os riscos do uso de determinados produtos do *Consumer Health* em combinação com outra medicação crónica ou estado de saúde.

2.4.3 Concorrência dos estabelecimentos de venda de MNSRM's (16)

Por vezes, deparei-me com a dificuldade de venda de alguns medicamentos não sujeitos a receita médica (MNSRM), suplementos alimentares, cosmética,… devido à proximidade de grandes superfícies comerciais, que detêm preços muito mais baixos. Perante esta situação, é imperioso destacar o aconselhamento e a informação que é necessário deter para proceder a uma escolha adequada do medicamento a usar e proporcionar ao utente todas as informações necessárias para assegurar uma correta e segura utilização dos medicamentos e produtos de saúde.

2.4.4 Alteração de preços e a mudança da embalagem secundária

Estas duas situações levam por vezes a uma grande desconfiança por parte do utente.

A constante alteração do preço dos medicamentos pode ser explicada devido ao surgimento dos medicamentos genéricos o que fez com que o valor do medicamento de marca suportado pelo utente aumentasse; isto deve-se ao facto do preço de referência ter diminuído e a comparticipação feita pelo Estado também diminuir visto que está definida por um valor percentual. A alteração de preços pode também dever-se ao *Parallel trade* que decorre das diferenças de preços entre os diferentes mercados nacionais no âmbito do Espaço Económico Europeu (EEE). Os distribuidores paralelos compram produtos

comercializados pelo fabricante original a um preço inferior num país e vendem-nos por um preço mais elevado noutro país.

3. Casos Clínicos

Com o decorrer do estágio, o atendimento é o local onde grande parte dos conhecimentos adquiridos ao longo dos quatro anos de formação são testados e mesmo aprimorados. Face a isso, em seguida, irei mencionar dois casos com os quais me deparei durante a realização do estágio e que demonstram a importância do farmacêutico no aconselhamento farmacoterapêutico.

3.1 Caso I

Utente do sexo feminino, com cerca de 50 anos, apresenta-se na farmácia com sintomas inerentes a uma constipação derivada possivelmente da sazonalidade da época. A senhora refere que tem tosse e que o nariz está congestionado, tendo desenvolvido esta sintomatologia no início do dia anterior. A senhora refere que tem tosse com alguma expetoração (tosse produtiva) e, após algumas questões, refere que não é diabética, não tem nenhuma doença cardíaca, hepática, renal ou respiratória. Também ainda não tinha tomado qualquer tipo de medicação visto que os sintomas apenas se tinham acentuado no dia em que foi à farmácia. Face à tosse com expetoração, aconselhei os comprimidos efervescentes Fluimucil® (17) (acetilcísteina 600 mg) que é um adjuvante mucolítico adequado para situações de hipersecreção brônquica. Referi que deveria ser tomado 1 comprimido efervescente por dia (devia dissolver bem o comprimido na água até a solução ser homogénea), preferencialmente antes de dormir, de forma a facilitar o descanso e que no dia seguinte é que haveria a estimulação para a eliminação das secreções, chamando à atenção para o facto de nos primeiros dias poder dar mais tosse de forma a eliminar as secreções. Aconselhei ainda a senhora a beber bastante água.

Face à congestão nasal relatada, aconselhei Vicks Sinex Aloé® (18)(oximetazolina 0,5 mg/ml), que é um vasoconstritor tópico que atua nos recetores alfa-adrenérgicos dos vasos da mucosa nasal com uma ação rápida e prolongada (12h). Referi que pode ser feita 1 a 2 pulverizações em cada narina 2 a 3 vezes por dia e que não deve ser usado mais de 5 dias devido ao efeito *rebound*. Caso não haja melhorias ao fim de 3 dias, deveria consultar o médico.

Falei ainda na possibilidade de usar uma água do mar isotónica e estéril (Frimar®) de forma a facilitar a libertação das secreções do nariz e a facilitar o assoar, garantindo a higienização da mucosa nasal.

(O caso I ocorreu antes da situação pandémica causada pelo vírus SARS-COV-2).

3.2 Caso II

Uma utente com 70 anos dirige-se à farmácia e refere que tem as unhas dos pés amareladas e solicita ajuda. Face à cor das unhas e à espessura das mesmas, depreende-se que é uma onicomicose (infeção fúngica provocadas por *Tinea unguium*). Visto que a senhora era diabética, desaconselhei o Canespro® *Kit* porque contém uma pomada com 40% ureia, o que aumenta o risco de desenvolvimento do pé diabético. Assim sendo, aconselhei o verniz medicamentoso Locetar EF® (19). Face à idade da senhora e às suas dificuldades locomotoras, aconselhei a aplicação do mesmo 1 vez por semana da seguinte forma: primeiramente limiar as unhas com a lima que se encontra no *kit* (que deve ser descartada após a utilização), de seguida limpar a unha com a compressa fornecida pelo *kit* (que contém álcool de forma a desengordurar a mesma) e, por último, aplicar o verniz com a espátula, que deve ser limpa após aplicação com a compressa. Deixar secar durante 3 minutos. Deve realizar este tratamento durante 9 a 12 meses sendo que após 3 meses do seu início e na ausência de melhorias deve consultar o médico. Referi ainda os cuidados a ter durante a higiene diária (como secar bem os dedos, limpar os pés e unhas como último passo, usar a mesma toalha apenas uma vez e não partilhar a toalha com outros membros da família).

4. Considerações Finais

O estágio é o culminar de 5 anos de aprendizagem técnico-científica nas diversas áreas do saber, sendo que todas as unidades curriculares têm um papel preponderante na nossa formação académica. Como complemento de toda a formação teórica e prática, o estágio em Farmácia Comunitária permite aprimorar grande parte desses conhecimentos e obter outros essenciais para a prática da atividade profissional e conhecer a realidade e o dia-a-dia do farmacêutico enquanto profissional de saúde e agente de saúde pública. É de destacar o papel preponderante do farmacêutico que exerce funções, não só como especialista do medicamento, mas também como promotor de estilos de vida saudáveis e responsável pela educação em saúde, tendo sempre como foco principal o utente.

Enquanto estagiária da FGT, destaco esta experiência de estágio pelo seu carácter enriquecedor, quer a nível de conhecimentos científicos e técnicos como a nível pessoal, graças a nossa intervenção na comunidade em geral e ao contacto direto com os utentes e as suas problemáticas. É de louvar a maneira como foi recebida e integrada na equipa da FGT à qual estarei eternamente grata, não só por todos os conhecimentos que adquiri, pelo seu profissionalismo e competência, mas também pela simpatia e amabilidade com que sempre me receberam dia após dia.

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Anexos

Anexo I

Ficha de preparação do medicamento manipulado de ácido salicílico, furoato de mometasona (Elocom®), glicerina e vaselina.

(Carimbo da Farmácia)

Medicamento: Ácido

Teor em substância ati

Forma farmacêutica:

Nome do Prescritor:

Matéria-Prima	Fabrican Distribui
Ácido salicílico	Labche
Glicerina	GSL-Fa Químicos Farmacê
Vaselina sólida	Labche
Furoato de mometasona	MSD

Preparação: estapulacç sólida e furoato d

Embalagem e Capacida

Prazo de Utilização: 3 n

ambiente (15 - 25

Receita Médica Nº

20110000487888838O6

Utente: CARLA MARIA BARATA MARTINS *295188857*

Telefone: R.C.:

Entidade Responsável: SNS

Nº. de Beneficiário: *295188857*

MM

M34923

HUGO OLIVEIRA

Especialidade: DERMATO-VENEREOLOGIA

Telefone: 244811800

POLIDIAGNÓSTICO - LEIRIA

U970117

Rx DCI / Nome, dosagem, forma farmacêutica, embalagem, posologia	Nº	Extenso	Identificação Ótica
1 Ácido Salicílico 3 Gramas; Glicerina 10 gr; Furoato de Mometasona 30 Mg; Vaselina Q.B.P. 100 Gramas. Fsa e Mande. *Posologia* Aplicar nas palmas à noite até melhoria. Pára >> SOS	1	Uma	
2			
3			
4			

FARMÁCIA GODINHO TOMAZ

Av Francisco Sá Carneiro nº70, R/C
2415-376 Leiria
Telef.: 244 832432
Dir. Téc.. Filipa Jesus

Médico: Hugo Oliveira
Utente: CARLA MARIA BARATA MARTINS
Lote nº 2/20
Data de prep.: 11/02/2020

Ácido salicílico 3g, Glicerina 10g, furoato de mometasona 30g, vaselina q.b.p. 100g

Quantidade: 100g
Uso externo
Manter fora do alcance das crianças

Prazo de utilização: 3 meses
Conservar à temperatura ambiente (15ºC-25ºC) ao abrigo de luz, calor e humidade

Validade: **30 dias**
Data: 2020-02-10

(assinatura do Médico prescritor)

Processado por computador - iReceita-EHR, v1.2 - CimpleCare, Lda

CONTROLO DO PRODUTO ACABADO:

Ensaio	Especificação	Resultado
Caracteres Organolépticos (cor, odor, espeto geral,...)	Branco, inodoro, homogéneo	☒Conforme ☐Não Conforme
Quantidade	100g ± 5%	☒Conforme ☐Não Conforme

Conclusão: Aprovado Nome do Operador: Inês Oliveira Rubrica: Inês Oliveira

VERIFICAÇÃO: Farmacêutico: Data: 11/02/20

(Carimbo da Farmácia)

Ficha de Preparação de Medicamentos Manipulados

Lote nº 2/2020

Data: 11/2/2020

Medicamento: Ácido salicílico 3 g, glicerina 10 g, furoato de mometasona 30g, vaselina q.b.p 100g

Teor em substância ativa: 100 g / ml contêm 3 g / ml de ácido salicílico

Forma farmacêutica: Pomada Quantidade a Preparar: 100 g

Nome do Prescritor: Dr. Hugo Oliveira Nome do Doente: Carla Maria Barata Martins

Matéria-Prima	Fabricante / Distribuidor	Lote nº	Boletim de Análise	Validade	Quantidade pesada / medida	Rubrica do Operador
Ácido salicílico	Labchem	RAS1810400	FPX	14/4/2023	3 g	Inês Oliveira
Glicerina	GSL - Produtos Químicos e Farmacêuticos	19145	FPX	09/2022	10 g	Inês Oliveira
Vaselina sólida	Labchem	12-015719	FPX	01/2024	57g	Inês Oliveira
Furoato de mometasona	MSD	5012799		03/2022	30g	Inês Oliveira

Preparação: Estapulação manual de ácido salicílico, glicerina, vaselina sólida e furoato de mometasona

Embalagem e Capacidade: 1 embalagem de 100 g

Prazo de Utilização: 3 meses Condições de conservação: temperatura ambiente (15 - 25 °C), ao abrigo da luz e calor

CONTROLO DO PRODUTO ACABADO:

Ensaio	Especificação	Resultado
Caracteres Organolépticos (cor, odor, espeto geral,...)	Branco; inodoro; homogéneo	☒ Conforme ☐ Não Conforme
Quantidade	100 g ± 5%	☒ Conforme ☐ Não Conforme

Conclusão: Aprovado Nome do Operador: Inês Oliveira Rubrica: Inês Oliveira

VERIFICAÇÃO: Farmacêutico: _____ Data: 11/02/20

CÁLCULO DO PREÇO DE VENDA:

MATÉRIAS-PRIMAS:

matérias-primas	embalagem existente em armazém		preço de aquisição de uma dada quantidade unitária (s/IVA)		quantidade a usar	factor multiplicativo	valor da matéria-prima utilizada na preparação
	quantidade adquirida	preço de aquisição (s/IVA)	quantidade unitária	preço			
Ácido salicílico	100 g	1,83 €	1 g	0,0183	x 3 g	x 2,2	= 0,1208 €
Glicerina	60 ml	0,48 €	1 g	0,00635	x 10 g	x 1,9	= 0,1207 €
Vaselina Sólida	900 g	4,84 €	1 g	0,00537	x 57 g	x 1,9	= 5,82 €
					x	x	=
					x	x	=
					x	x	=
					x	x	=
						subtotal A	6,07 €

HONORÁRIOS DE MANIPULAÇÃO:

	forma farmacêutica	quantidade	F(€)	factor multiplicativo	valor
valor referente à quantidade base	Pomada	100 g	5,05	x 3	= 15,15 €
valor adicional			x	x	=
				subtotal B	15,15 €

MATERIAL DE EMBALAGEM:

materiais de embalagem	preço de aquisição (s/IVA)	quantidade	factor multiplicativo	valor
Embalagem de plástico	1 €	x 1	x1,2	= 1,2 €
		x	x1,2	=
		x	x1,2	=
		x	x1,2	=
			subtotal C	1,2 €

PREÇO DE VENDA AO PÚBLICO DO MEDICAMENTO MANIPULADO:

(A + B + C) x 1,3	29,15
+ IVA	6%
Preço Final: D	30,90 €

Operador: Inês Alves Supervisor: _____

Rubrica do Diretor Técnico: _____ Data: 11/02/20

Anexo 2

Ficha de preparação do medicamento manipulado de ácido salicílico, ureia e vaselina.

Farmácia Godinho Tomaz Unip. Lda
Direção Técnica
Dra. Filipa Alexandra Rodrigues de Jesus
Av. Dr. Francisco Sá Carneiro, nº 70, R/C
Marinheiros • 2415-275 Leiria
Contribuinte n.º: 506 541 426
Tel.: 244 832 432

(Carimbo da Farmácia)

Ficha de Preparação de Medicamentos Manipulados

Lote nº 5/2020
Data: 26/2/2020

Medicamento: Ácido Salicílico 5 g, Ureia 10 g, Vaselina q.b.p 50 g

Teor em substância ativa: 100 g / ml contêm 5 g / ml de ácido salicílico

Forma farmacêutica: Pomada Quantidade a Preparar: 50 g

Nome do Prescritor: Dr. Henrique Oliveira Nome do Doente: Francisco Faria Caseiro

Matéria-Prima	Fabricante / Distribuidor	Lote nº	Boletim de Análise	Validade	Quantidade pesada / medida	Rubrica do Operador
Ácido Salicílico	Labchem	RAS1810400	FPX	14/04/2023	5 g	Inês Oliveira
Ureia	José Manuel Gomes dos Santos, Lda	181700	FPX	10/2023	10 g	Inês Oliveira
Vaselina Sólida	Labchem	12-0157 19	FPX	01/2024	35 g	Inês Oliveira

Preparação: espatulação manual de ácido salicílico, ureia e vaselina sólida

Embalagem e Capacidade: 1 embalagem de plástico de 50 g

Prazo de Utilização: 3 meses Condições de conservação: temperatura ambiente (15 - 25 °C) ao abrigo da luz e calor

CONTROLO DO PRODUTO ACABADO:

Ensaio	Especificação	Resultado
Caracteres Organolépticos (cor, odor, espeto geral,...)	Branco; inodoro;	☒ Conforme ☐ Não Conforme
Quantidade	50 g ± 5%	☒ Conforme ☐ Não Conforme

Conclusão: Aprovado Nome do Operador: Inês Oliveira Rubrica: Inês Oliveira

VERIFICAÇÃO: Farmacêutico: _____ Data: _____

CÁLCULO DO PREÇO DE VENDA:

MATÉRIAS-PRIMAS:

matérias-primas	embalagem existente em armazém		preço de aquisição de uma dada quantidade unitária (s/IVA)		quantidade a usar	factor multiplicativo	valor da matéria-prima utilizada na preparação
	quantidade adquirida	preço de aquisição (s/IVA)	quantidade unitária	preço			
Ácido salicílico	100 g	1,83 €	1 g	0,0183	x 5 g	x 2,2	= 0,201
Ureia	250 g	11,74 €	1 g	0,047	x 10 g	x 1,9	= 0,893
Vaselina sólida	900 g	4,84 €	1 g	0,00538	x 35 g	x 1,9	= 0,358
					x	x	=
					x	x	=
					x	x	=
					x	x	=
						subtotal A	1,45 €

HONORÁRIOS DE MANIPULAÇÃO:

	forma farmacêutica	quantidade	F(€)	factor multiplicativo	valor
valor referente à quantidade base	Pomada	50 g	5,05	x 3	= 15,15
valor adicional			x	x	=
				subtotal B	15,15 €

MATERIAL DE EMBALAGEM:

materiais de embalagem	preço de aquisição (s/IVA)	quantidade	factor multiplicativo	valor
Embalagem de plástico	1 €	x 1	x1,2	= 1,2 €
		x	x1,2	=
		x	x1,2	=
		x	x1,2	=
			subtotal C	1,2 €

PREÇO DE VENDA AO PÚBLICO DO MEDICAMENTO MANIPULADO:

(A + B + C) x 1,3 = (17,8 x 1,3) = 23,14 €

+ IVA 6 %

Preço Final: D 24,53 €

Operador: Inês Oliveira Supervisor: _____

Rubrica do Diretor Técnico: _____ Data: _____

CÁLCULO DO PRE

MATÉRIAS-PRIMAS:

matérias-primas	qua ac
Ácido salicílico	1(
Ureia	25
Vaselina sólida	9(

HONORÁRIOS DE MANIPULA

valor referente à quantidade base
valor adicional

MATERIAL DE EMBALAGEM:

materiais de embalage
Embalagem de plás

PREÇO DE VENDA AO PÚBLI

Receita Méc

Local de Pres
Médico prescr
Utente: **FRAN**

Código Acess

(informação a utiliz

DCI / Nome, dos

1 Manipulado
Mande Em

Posologia apl

2

3

4

FARMACIA GODINHO TOMAZ

Av Francisco Sá Carneiro nº1446
2415-436 Leiria
Telef.: 244 832432
Dir. Téc: Drª Filipa Alexandra Rodrigues de Jesus

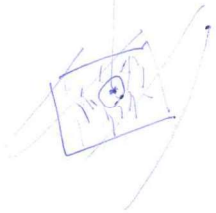
Médico: Dr Henrique Oliveira
Utente: Francisco Faria Caseiro
Lote nº 05/2020
Data de prep.: 26/02/2020

Ac salic 5g + ureia 10g + vaselina qbp 50g

50g de pomada contém 5g ac salicil e 10g de ureia
Quantidade: 50g
Uso externo
Manter fora do alcance das crianças

Prazo de utilização:3 meses

Conservar ao abrigo de luz, calor e humidade



Encargo para o utente de acordo com os medicamentos comercializados que cumprem a prescrição médica

1 É comparticipado. 7750000

2

3

4 24,53€

Para obter mais informações sobre o preço dos medicamentos:
. Consulte «Pesquisa Medicamento», no sítio do INFARMED (www.infarmed.pt);
. Contacte a Linha do Medicamento 800 222 444 (Dias úteis: 09.00-13.00 e 14.00-17.00)
. Fale com o seu médico ou farmacêutico.

Data: 2020-02-25

Processado por computador - iReceita-EHR, v1.2 - CimpleCare, Lda

Operador: Inês Oliveira Supervisor: _____

Rubrica do Diretor Técnico: _____ Data: _____

CÁLCULO DO PRE

MATÉRIAS-PRIMAS:

matérias-primas	em qua adc
Ácido salicílico	10
UREIA	25
Vaselina sólida	90

HONORÁRIOS DE MANIPULAÇ

valor referente à quantidade base
valor adicional

MATERIAL DE EMBALAGEM:

materiais de embalagem
Embalagem de plástic

PREÇO DE VENDA AO PÚBLICO

Guia de tratamento para o utente

Receita Médica Nº *201100004903360690 1*

Local de Prescrição: LPEUPS CENTRO
Médico prescritor: HENRIQUE OLIVEIRA
Utente: **FRANCISCO FARIA CASEIRO**
Telefone: 919462675

Código Acesso: *781903*
Código Direito opção: *4343*

(informação a utilizar para a dispensa de medicamentos na farmácia)

DCI / Nome, dosagem, forma farmacêutica, embalagem, posologia — Nº

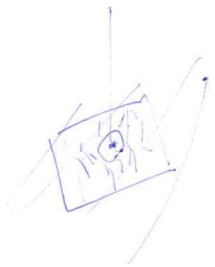
1 Manipulado : Ácido Salicilico: 5 Grama, Ureia:10 Grama, Vaselina:Q.B.P. 50 Grama. F.S.A. e Mande Em Boião — 1

Posologia aplica ao deitar após isolar e depois película aderente por cima e adesivo

2

3

4



Encargo para o utente de acordo com os medicamentos comercializados que cumprem a prescrição médica

1 É comparticipado. 7,50 0000

2

3 24,53€

4

Para obter mais informações sobre o preço dos medicamentos:
. Consulte «Pesquisa Medicamento», no sítio do INFARMED (www.infarmed.pt);
. Contacte a Linha do Medicamento 800 222 444 (Dias úteis: 09.00-13.00 e 14.00-17.00)
. Fale com o seu médico ou farmacêutico.

Data: 2020-02-25

Processado por computador - iReceita-EHR, v1.2 - CimpleCare, Lda

Operador: Inês Oliveira Supervisor: _____

Rubrica do Diretor Técnico: _____ Data: _____

Anexo 3

Ficha de preparação do medicamento manipulado de enxofre e vaselina.

Farmácia Godinho Tom
Direção Técnica
Dra. Filipa Alexandra Rodrigues de J
Av. Dr. Francisco Sá Carneiro n.º 70, R/C
Marinheiros • 2415-376 Leiria
Contribuinte n.º: 506 541 436
Tel: 244 832 432
(Carimbo da Farmácia)

Medicamento: Enxo
Teor em substância at
Forma farmacêutica:
Nome do Prescritor:

Matéria-Prima	Fabrica Distribu
Enxofre	Acofar
Vaselina sólida	Labch

Preparação: espalha

Embalagem e Capaci

Prazo de Utilização:

(15-25 °C), ao ab

Receita Médica Nº

REPÚBLICA PORTUGUESA 40 SNS SERVIÇO NACIONAL DE SAÚDE

201100004857350920O

Utente: EVANDRO PESCADINHA EUSÉBIO
Telefone:
R.C.:
285640860
Entidade Responsável: SNS
Nº. de Beneficiário:
285640860

MM

M34475
HENRIQUE OLIVEIRA
Especialidade: DERMATO-VENEREOLOGIA
Telefone:
LPEUPS CENTRO
U989892

℞ DCI / Nome, dosagem, forma farmacêutica, embalagem, posologia	Nº	Extenso	Identificação Ótica
1 Manipulado:Enxofre-24 Gr, Vaselina-Q.B.P. 300 Gr.F.S.A. e Mde Em Boião	2	Duas	

Posologia aplicar ao deitar -10 noites e banho de manhã

2

3

4

FARMACIA GODINHO TOMAZ
Av Francisco Sá Carneiro nº1446
2415-436 Leiria
Telef.: 244 832432
Dir. Téc Drª Filipa Alexandra Rodrigues de Jesus
Médico: Dr. Henrique Oliveira
Utente: Evandro Eusébio
Lote nº 01/20
Data de prep.: 31/01/2020
Enxofre 48g + Vaselina 600g
100g de pomada propriamente dita contém 8g de enxofre
Quantidade:600g
Uso externo
Prazo de utilização 3 meses
Conservar ao abrigo da luz, calor e humidade
Manter fora do alcance das crianças

Processado por computador - iReceita-EHR, v1.2 - CimpleCare, Lda

Validade: **30 dias**
Data: 2020-01-28
(assinatura do Médico prescritor)

CONTROLO DO PRODUTO ACABADO:

Ensaio	Especificação	Resultado
Caracteres Organolépticos (cor, odor, espeto geral,...)	Amarelo e homogéneo	☒Conforme ☐Não Conforme
Quantidade	600 g ± 5%	☒Conforme ☐Não Conforme

Conclusão: Aprovado Nome do Operador: Inês Oliveira Rubrica: Inês Oliveira

VERIFICAÇÃO: Farmacêutico: Rita Laga Data: 3/11/2020

Farmácia Godinho Tomaz
Direção Técnica
Dra. Filipa Alexandra Rodrigues de Jesus
Av. Dr. Francisco Sá Carneiro nº 70, R/C
Marinheiros • 2415-376 Leiria
Contribuinte n.º: 506 541 436
Tel: 244 832 432

(Carimbo da Farmácia)

Ficha de Preparação de Medicamentos Manipulados

Lote nº 01/2020

Data: 31/01/2020

Medicamento: Enxofre 48 g + vaselina sólida 600 g

Teor em substância ativa: 100 g / ml contêm 8 g / ml de enxofre

Forma farmacêutica: pomada Quantidade a Preparar: 600 g

Nome do Prescritor: Drª Henrique Oliveira Nome do Doente: Evandro Eusébio

Matéria-Prima	Fabricante / Distribuidor	Lote nº	Boletim de Análise	Validade	Quantidade pesada / medida	Rubrica do Operador
Enxofre	Acofarma	170918	FP X	01/07/2020	48 g	Inês Oliveira
Vaselina sólida	Labchem	12-086519	FP X	31/08/2024	552 g	Inês Oliveira

Preparação: espatulação manual de vaselina sólida com enxofre

Embalagem e Capacidade: 6 embalagens de 100 g

Prazo de Utilização: 3 meses Condições de conservação: temperatura ambiente (15-25 °C), ao abrigo da luz, calor e humidade

CONTROLO DO PRODUTO ACABADO:

Ensaio	Especificação	Resultado
Caracteres Organolépticos (cor, odor, espeto geral,...)	Amarelo e homogéneo	☒ Conforme ☐ Não Conforme
Quantidade	600 g ± 5%	☒ Conforme ☐ Não Conforme

Conclusão: Aprovado Nome do Operador: Inês Oliveira Rubrica: Inês Oliveira

VERIFICAÇÃO: Farmacêutico: Rita Lago Data: 31/1/2020

CÁLCULO DO PREÇO DE VENDA:

MATÉRIAS-PRIMAS:

matérias-primas	embalagem existente em armazém		preço de aquisição de uma dada quantidade unitária (s/IVA)		quantidade a usar	factor multiplicativo	valor da matéria-prima utilizada na preparação
	quantidade adquirida	preço de aquisição (s/IVA)	quantidade unitária	preço			
Enxofre	250 g	8,15	1 g	0,033	x 48	x 1,9	= 3,03
Vaselina sólida	900 g	4,09	1 g	0,0045	x 552	x 1,6	= 3,99
					x	x	=
					x	x	=
					x	x	=
					x	x	=
					x	x	=
						subtotal A	7,02

HONORÁRIOS DE MANIPULAÇÃO:

	forma farmacêutica	quantidade	F(€)	factor multiplicativo	valor
valor referente à quantidade base	pomada	100	5,05	x 3	= 15,15
valor adicional		500	x 5,05	x 0,01	= 25,25
				subtotal B	40,4

MATERIAL DE EMBALAGEM:

materiais de embalagem	preço de aquisição (s/IVA)	quantidade	factor multiplicativo	valor
Caixas de plástico	1 €	x 6	x1,2	= 7,2
		x	x1,2	=
		x	x1,2	=
		x	x1.2	=
			subtotal C	7,2

PREÇO DE VENDA AO PÚBLICO DO MEDICAMENTO MANIPULADO:

(A + B + C) x 1,3	71,01 €
+ IVA	6 %
Preço Final: D	75,27 €

Operador: Inês Oliveira Supervisor: B.K

Rubrica do Diretor Técnico: _____ Data: 31/01/20

PARTE II

Relatório de Estágio em Indústria Farmacêutica

Grupo Tecnimede

Lista de Abreviaturas

FFUC – Faculdade de Farmácia da Universidade de Coimbra

HPLC – *High Performance Liquid Chromatography*

I&D – Investigação e Desenvolvimento

IET – Instruções Específicas de Trabalho

IGQ – Instruções Gerais da Qualidade

LQ – LaborQualitas

MICF – Mestrado Integrado em Ciências Farmacêuticas

OEP – Operações, Equipamentos e Processos

QUA – Qualidade

SOWT – *Strengths, Weaknesses, Opportunities,Threats*

TEC – Técnicas

1. Introdução

A Indústria Farmacêutica constitui uma área de grande interesse para os futuros farmacêuticos, nomeadamente no desenvolvimento da sua carreira profissional. Dotados de diversos conhecimentos técnicos e científicos na área do medicamento, os farmacêuticos têm a capacidade de integrar inúmeras áreas na Indústria Farmacêutica, nomeadamente as áreas de Investigação e Desenvolvimento, Produção, Registos e Assuntos Regulamentares do Medicamento, Marketing, Departamento Médico, Garantia de Qualidade, entre outras.

Face à oportunidade única proporcionada pela Faculdade de Farmácia da Universidade de Coimbra (FFUC) aos alunos finalistas do Mestrado Integrado em Ciências Farmacêuticas (MICF), foi-me possível realizar o estágio curricular no Grupo Tecnimede. Dado o meu especial interesse pela área de investigação e desenvolvimento e pela vertente laboratorial inerente a esta área, integrei uma das equipas no Grupo Tecnimede, mais concretamente, no LaborQualitas (LQ) de setembro a novembro de 2020, sob orientação do Dr. João Serra e da Engª. Joana Ramos.

O presente relatório apresenta-se sob a forma de uma análise SOWT (*Strengths, Weaknesses, Opportunities,Threats*) e faz uma breve sumula do estágio curricular em Indústria Farmacêutica, identificando, internamente, os pontos fortes e fracos e, externamente, as oportunidades e ameaças.

2. Grupo Tecnimede

O Grupo Tecnimede é um grupo farmacêutico privado que iniciou a sua atividade em 1980. O seu principal foco centra-se no desenvolvimento e comercialização de medicamentos para uso humano, tendo por missão contribuir para a melhoria dos cuidados de saúde e da qualidade da vida, a nível mundial.

O departamento de Investigação e Desenvolvimento (I&D) é um dos pilares do Grupo Tecnimede, tendo iniciado a sua atividade em 1998, no concelho de Torres Vedras. As suas principais áreas de trabalho são o desenvolvimento farmacêutico de medicamentos genéricos, medicamentos de valor acrescentado e medicamentos inovadores. O departamento de I&D, denominado de LaborQualitas (LQ), tem como objetivo o desenvolvimento de produtos farmacêuticos em diversas áreas terapêuticas, nomeadamente doenças cardiovasculares e metabólicas, doenças degenerativas do sistema nervoso e doenças infeciosas (1,2).

Face à diversidade de atividades desenvolvidas no LQ, este encontra-se dividido em diversos departamentos que se articulam entre si: o Departamento de Química Fina

(encarregue da investigação na área da síntese química), Laboratório de Ensaios (que se subdivide no laboratório de Química Analítica e no Laboratório de Microbiologia, tendo como principal objetivo a execução de análises físico-químicas, ensaios de estabilidade e a execução de análises microbiológicas, respetivamente), Desenvolvimento Farmacêutico (desenvolvimento analítico e galénico de medicamentos genéricos e/ou medicamentos inovadores) e o Departamento de Cultura de Células (realiza estudos *in vitro* em base celular).

O meu estágio foi realizado no departamento do Desenvolvimento Farmacêutico, onde participei maioritariamente na área da analítica, no desenvolvimento de um medicamento genérico, sob a forma farmacêutica de comprimidos revestidos por película.

3. Análise SWOT

3.1 Pontos Fortes

3.1.1 Receção e integração na empresa

O acolhimento de um novo membro de uma empresa é, sem dúvida, um ponto fulcral que reflete a imagem da empresa.

Realço positivamente, a receção que me foi feita pelo Dr. João Serra, que me acolheu com total disponibilidade, tendo esclarecido todas as minhas dúvidas.

A primeira semana foi bastante enriquecedora, uma vez que foi absorvente em termos de aquisição de conhecimento a vários níveis. Tive a possibilidade de visitar os diversos laboratórios que constituem o LaborQualitas, tendo-me sido dada uma explicação detalhada do trabalho desenvolvido em cada um deles. Adicionalmente, fui apresentada às diversas equipas que desempenham funções no LQ, tendo sido bem acolhida por todos.

Sempre que o LQ recebe alguém pela primeira vez, é disponibilizado um manual de acolhimento constituído por um conjunto de procedimentos que definem o modo *operandis* do trabalho desenvolvido em I&D. Saliento igualmente, a disponibilidade demonstrada por todas as pessoas que me acompanharam e que se mostraram recetivas em clarificar todas minhas dúvidas. Adicionalmente, tive a oportunidade de assistir a uma ação de formação interna sobre o funcionamento de um equipamento frequentemente utilizado em análises físicas (durómetro).

Posteriormente, fui integrada na equipa de trabalho da Engª. Joana Ramos que me recebeu com toda a amabilidade e se mostrou sempre disponível para responder a todas as minhas questões e dúvidas. O mesmo se verificou com a restante equipa e com todos os elementos integrantes do LQ, que graças à sua pluridisciplinaridade permitiram um aprofundamento e interligação de conhecimentos.

3.1.2 Diversidade de tarefas desempenhadas

A Indústria Farmacêutica é mundialmente conhecida pelo elevado rigor e pelo cumprimento de requisitos técnicos e regulamentares que a si está associada. Para tal, todo o desenvolvimento farmacêutico é conduzido segundo documentos oficiais, tendo sempre como objetivo a eficácia, qualidade e segurança do fármaco. Não obstante, o foco no cumprimento de prazos é fulcral, devido à elevada competitividade associada a este tipo de indústria. Assim sendo, tive o privilégio de poder participar em inúmeras tarefas bastante diversificadas e que implicaram rapidez de execução associado simultaneamente a uma elevada capacidade de análise.

Acompanhei o estudo do impacto que determinadas variáveis de processo (por exemplo, força de compressão aplicada e a quantidade de água adicionada aquando da granulação) poderiam ter no perfil de dissolução da forma farmacêutica em estudo. Para tal, foram realizados perfis de dissolução na gama de pH 1,2 a 6,5, utilizando condições de dissolução discriminativas que possam prever pequenas alterações no medicamento, garantindo a consistência de lote para lote e sinalizando potenciais problemas com a biodisponibilidade in vivo.

Também foi avaliado a etapa de revestimento, e em que medida afetaria a curva de dissolução. Para tal, foram realizados perfis de dissolução comparativos dos núcleos e dos comprimidos revestidos. Resumindo, o objetivo da realização dos perfis de dissolução é simular as condições fisiológicas e, deste modo, estudar o comportamento do fármaco *in vitro*, uma vez que o passo limitante para a dissolução num medicamento é, em geral, o fármaco. Face a isto, e como referido anteriormente, é preponderante que o ensaio de dissolução seja preditivo e discriminatório (4).

3.1.3 Promoção do sentido crítico e ganho de autonomia

O espírito crítico é uma característica fundamental em qualquer profissão, especialmente na de um farmacêutico a desempenhar funções na área de I&D. Ao longo do estágio, deparei-me com diversos desvios nos resultados obtidos e com a necessidade de reflexão sobre os mesmos de forma a identificar a sua causa e proceder à sua resolução.

É essencial e indispensável desenvolver e aumentar a capacidade de análise no trabalho realizado na área da analítica. Só assim se poderá obter os resultados desejados para a concretização de uma determinada tarefa associada ao correto desenvolvimento de um projeto. Relativamente a este ponto, tive a oportunidade de desenvolver esta competência, analisando de forma crítica os resultados obtidos por análise cromatográfica (resultados obtidos em HPLC - *High Performance Liquid Chromatography*).

A autonomia na realização das tarefas atribuídas torna também o estagiário mais proactivo e dinâmico no que toca, quer à organização das suas tarefas, mas também à procura da melhor maneira de as executar.

A liberdade e confiança que me foram concedidas culminou num acréscimo de responsabilidade fomentando a minha autonomia e confiança nas minhas capacidades.

3.1.4 Rigor no cumprimento das regras laboratoriais

Face ao rigor e à elevada regulamentação da Indústria Farmacêutica, o Grupo Tecnimede prima pelo cumprimento de inúmeras regras e rege-se por diversos protocolos.

Saliento o uso do *audit trail* e de várias bases de dados referentes à utilização de equipamentos e consumíveis (reagentes e substâncias) onde é possível ter uma evidência do que foi utilizado e facilitar a rastreabilidade de algum dado, quando necessário. Esta prática facilita uma maior organização e agilização das tarefas diárias.

Adicionalmente, o uso destas ferramentas informáticas, permite uma gestão mais rigorosa dos consumíveis e dos equipamentos disponíveis no laboratório. Acrescento que todos os equipamentos obedecem a um plano de verificação e calibração (quando aplicável), por forma a garantir que todos os equipamentos se encontram em perfeitas condições de utilização.

A elaboração dos cadernos de laboratório é também uma prática diária e sujeita a rigor técnico e científico, visto que é onde se efetuam todas as anotações detalhadas dos ensaios realizados diariamente.

Acrescenta-se ainda as regras básicas de segurança inerentes ao trabalho laboratorial, tais como uso de bata de proteção, óculos, luvas e máscara de proteção.

3.2 Pontos Fracos

3.2.1 Duração do estágio

A complexidade e a diversidade de tarefas que podem ser desempenhadas no LQ e, particularmente, no departamento de Desenvolvimento Farmacêutico aliadas ao tempo inerente ao processo de desenvolvimento, *per se*, requem um processo de aprendizagem mais extenso de forma a permitir o acompanhamento na integra de todo o processo de desenvolvimento farmacêutico.

Desta forma, considero que, apesar da realização do estágio no Grupo Tecnimede ter sido uma ótima experiência para desenvolver novas competências e conhecer melhor o contexto de indústria farmacêutica, em particular do I&D, a duração de 3 meses foi restrita, dada à imensidão de conhecimentos que ficaram por aprimorar ou mesmo por desenvolver. Deste modo, concluo que este foi um dos aspetos menos positivos do estágio.

3.3 Oportunidades

3.3.1 Contacto com a Indústria Farmacêutica

É, sem dúvida, de ressalvar a oportunidade dada, pelo Grupo Tecnimede, aos alunos finalistas de MICF da FFUC mesmo em tempos pandémicos mais conturbados. No meu caso, em particular, foi graças a esta oportunidade que pude contactar pela primeira vez com a Indústria Farmacêutica e perceber quão vasta e polivalente é esta indústria.

A possibilidade de integração na área de I&D foi muito gratificante e permitiu-me ter uma visão mais real e aproximada do funcionamento e desenrolar do processo de desenvolvimento farmacêutico e do papel do farmacêutico como parte integrante do mesmo.

3.3.2 Contacto com diferentes equipamentos laboratoriais

Graças à diversidade de tarefas a realizar pude contactar e aprender as técnicas inerentes ao uso de diversos equipamentos, quer na área do desenvolvimento analítico (equipamentos de dissolução, cromatógrafos líquidos (HPLC), balanças analíticas, potenciómetros, entre outros), quer a nível de desenvolvimento galénico (máquina de compressão, máquina de revestimento, durómetro, equipamento de desagregação, entre outros).

Durante o meu período de estágio, aprofundei conhecimentos na técnica de cromatografia líquida, nomeadamente na utilização de HPLC. Constatei que a cromatografia líquida é uma ferramenta essencial no desenvolvimento farmacêutico analítico.

3.3.3 Formação contínua interna

A primeira semana de estágio, especialmente os primeiros dias, dedicaram-se a uma formação mais geral e teórica, no que toca aos procedimentos instituídos no LQ, como Instruções Específicas de Trabalho (IET) (entre as quais destaco, Técnicas (TEC), Operações, Equipamentos e Processos (OEP) e Qualidade (QUA)) e Instruções Gerais da Qualidade (IGQ).

Posteriormente, e com a necessidade de usar diferentes equipamentos para realizar os ensaios em curso, foi-me disponibilizado o procedimento específico para cada equipamento e fornecida uma explicação para o seu uso de forma autónoma e responsável.

Como referi anteriormente, tive também a possibilidade de frequentar uma ação de formação interna relativa ao funcionamento do durómetro, que se mostrou bastante vantajosa no desenrolar do estágio.

Neste tópico, gostaria de ressalvar e enaltecer a simpatia e disponibilidade de todos os membros do LQ que sempre se disponibilizaram para prestar qualquer esclarecimento e ajuda.

3.4 Ameaças

3.4.1 Custo elevado da inovação

A área de I&D é uma das áreas que requer investimentos avultados, quer ao nível da indústria farmacêutica quer ao nível de outras indústrias e empresas. Contudo, é também graças a esta área que as empresas se conseguem diferenciar e expandir. Deste modo e apesar dos processos de investigação e desenvolvimento serem bastante demorados e dispendiosos, é uma área bastante aliciante e promissora.

Dado ao elevado investimento financeiro e à enorme concorrência ao nível do setor farmacêutico, cabe à área de I&D desenvolver produtos e/ou formulações que comercialmente sejam competitivos, não descurando as bases fundamentais de um produto farmacêutico: a qualidade, eficácia e segurança.

A competitividade inerente à Indústria Farmacêutica ficou bem vincada ao longo do estágio e permitiram ter uma noção mais real do ambiente competitivo que decorre de um processo de investigação.

4. Considerações Finais

Face ao papel do farmacêutico como especialista do medicamento, a possibilidade do mesmo realizar um estágio curricular na Indústria Farmacêutica é de elevada importância uma vez que, num futuro próximo, poderá vir a integrar qualquer uma das áreas inerentes às etapas do ciclo do medicamento.

O estágio em Indústria Farmacêutica no Grupo Tecnimede foi fundamental para aprimorar e desenvolver novas competências técnicas e profissionais, mas também para ter uma noção mais real e aprofundada do funcionamento desta indústria.

A enorme aposta do Grupo Tecnimede em I&D e a disponibilidade para aceitar estagiários é de louvar, uma vez que permite aos novos formandos não só integrar e consolidar novos conhecimentos teóricos e práticos, como também fomentar o seu espírito crítico e autonomia, proporcionando um enriquecimento pessoal e profissional.

O estágio superou as minhas expetativas e constituiu para uma oportunidade única no meu percurso académico. Aproveito, desse já, para agradecer ao Grupo Tecnimede esta oportunidade de estágio bem como a todos os profissionais que tão bem me acolheram durante estes 3 meses.

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PARTE III

Monografia

"Exploring new biomarkers of aging"

List of Abbreviations

AGEs – Advanced Glycation End-products

AgRP – Agouti-Related Protein

ANS – Autonomic Nervous System

ARC – Arcuate Nucleus

B2M – β-2-microglobulin

BBB – Blood–Brain Barrier

BDNF – Brain-Derived Neurotrophic Factor

BFCS – Basal Forebrain Cholinergic System

BMI – Body Mass Index

bp – base pair

Ca^{2+} – Calcium Ions

cAMP – Cyclic Adenosine Monophosphate

CART – Cocaine- and Amphetamine-Regulated Transcript

cDNA – complementary Deoxyribonucleic Acid

CNS – Central Nervous System

CRH – Corticotropin-releasing Hormone

CRP – C-Reactive Protein

CSF – Cerebrospinal Fluid

DCV – Dense Core Vesicles

DMH – Dorsomedial Hypothalamic Nucleus

DNA – Deoxyribonucleic Acid

ELISA – Enzyme-linked immunoassay

ER – Endoplamatic Reticulum

FDA – US Food and Drug Administration

fMRI – Functional Magnetic Resonance Imaging

FSH – Follicle-stimulating Hormone

GABA – Gamma-Aminobutyric Acid

GADD34 – DNA-Damage-Inducible 34

GH – Growth Hormone

GHRH – Growth Hormone-releasing Hormone

$G_{i/o}$ protein – Inhibitory Regulative G-protein

GIRK – G-Protein-Coupled Inwardly Rectifying Potassium ions

GnRH – Gonadotropin-Releasing Hormone

GPCRs – G protein-coupled receptors

$G_{q/11}$ protein – G_q protein alpha subunit

G_s protein – Stimulative Regulative G-protein

HCRT – Hypocretin (humans)

Hcrt – Hypocretin (rodents)

HGPS – Hutchison Gilford progeria syndrome

HLA – Human Leukocyte Antigen

HPA – Hypothalamus-Pituitary-Adrenal

HPRT – Hypoxanthine-guanine Phosphoribosyltransferase

IL-6 – Interleukin-6

IP_3 – Inositol Triphosphate

iPSCs – Induced Pluripotent Stem Cells

K^+ – Potassium Ions

LC – Locus Coeruleus

LH – Luteinizing Hormone

LH/PFA – Lateral Hypothalamus and Perifornical Area

LHA – Lateral Hypothalamus Area

LMNA – Lamin A/C

MBH – Mediobasal Hypothalamic region

MCH – Melanin-Concentrating Hormone

MHC I – Major Histocompatibility Complex Class I

miR-9 – microRNA-9

miRNA – micro Ribonucleic Acid

MR – Magnetic Resonance Imaging

mRNA – messenger Ribonucleic Acid

mtDNA – Mitochondrial DNA

MTs – Metallothioneins

N/OFQ – Nociceptin/Orphanin FQ

NADPH – Nicotinamide-adenine dinucleotide phosphate

NF-kB – Factor Nuclear kappa B

NIH – National Institutes of Health

NOP – Nociceptin Opioid

NPY – Neuropeptide Y

NSC – Neural Stem/Progenitor Cells

OCT – Optimum Cutting Temperature

OD – Optical Density

ORF – Open Reading Frame

OX – Orexin

OX1R – Orexin Receptor Type 1

OX2R – Orexin Receptor Type 2

OX-A – Orexin A

OX-B – Orexin B

PCR – Polymerase Chain Reaction

PeF or PFA – Perifornical Nucleus

PET – Positron Emission Tomography

PKA – Protein Kinase A

PKC – Protein Kinase C

POMC – Proopiomelanocortin

PPO – Preprohypocretin

PVN – Paraventricular Nucleus

qRT-PCR – Quantitative Real-time Polymerase Chain Reaction

REM – Rapid Eye Movement

RER – Rough Endoplasmic Reticulum

ROS – Reactive Oxygen Species

SPA – Spontaneous Physical Activity

SPECT – Single Photon Emission Computed Tomography

TNF-α – Tumor Necrosis Factor-alpha

TRH – Thyrotropin-Releasing Hormone

TSH – Thyroid-Stimulating Hormone

UTR – Untranslated Region

VMH – Ventromedial Hypothalamic Nucleus

WHO – World Health Organization

Resumo

Ao longo dos anos, a esperança média de vida tem sofrido um aumento gradual, contudo a qualidade de vida tem vindo a decrescer e as terapêuticas farmacológicas para as novas patologias derivadas do envelhecimento são ainda escassas ou desconhecidas.

Atualmente, o processo de envelhecimento tem-se tornado cada vez mais uma preocupação para a sociedade moderna, visto que se encontra estreitamente relacionado com o declínio das funções fisiológicas e da homeostase do organismo, resultando em alterações das funções endócrina, comportamental e cognitiva.

A desregulação, observada durante o envelhecimento, dos sistemas centro-periféricos e os mecanismos celulares e moleculares subjacentes a este estado culminaram na descoberta dos biomarcadores de envelhecimento. Apesar da pesquisa de novos biomarcadores ser altamente complexa e extensa, a sua descoberta pode estar na origem de novas e bem-sucedidas terapêuticas e, portanto, o interesse nos biomarcadores tem vindo a aumentar, especialmente na área do envelhecimento.

Tendo em consideração os mecanismos celulares e moleculares associados ao envelhecimento, o hipotálamo e as funções desempenhadas pelo mesmo (especialmente o controlo do sistema nervosa central e do sistema endócrino, a regulação do ritmo circadiano e a capacidade de resposta a estímulos internos e externos e de mediar a transmissão de informação através da transmissão sináptica) revelaram-no como um órgão de especial importância no processo de envelhecimento. Esta estrutura anatómica altamente funcional é considerada um regulador primário do processo de envelhecimento do organismo, apesar dos seus núcleos e grupos neuronais sofrerem especial impacto durante este processo.

Em suma, esta revisão bibliográfica explora um neuropeptídeo como biomarcador do envelhecimento e reflete sobre a possibilidade de terapêuticas farmacológicas com base nesse neuropeptídeo.

Palavras-chave: Envelhecimento, Progeria, Biomarcadores, Hipotálamo.

Abstract

The lifespan expectancy has been increasing over the years, however the quality of life is slightly decreasing and the pharmacological treatments for the new age-related diseases are scarce or even unknown.

Recently, the aging process has increasingly been a concern for the modern society since it is related to a decline in physiological functions and in the homeostasis of all body. Such changes might lead to alterations in endocrine, behavioural and cognitive functions, observed in several elderly people.

The impaired function of centre-peripherical systems observed during the aging process and the cellular and molecular mechanisms underlying this process, culminated in the discovery of biomarkers. The research of biomarkers is highly complex and extensive. Nevertheless, their discovery could be in the origin of new and successful treatments and, thus, the interest for them has been growing, especially in aging research.

Taking into account the cellular and molecular mechanisms related with aging, the hypothalamus and its functions- namely, the control of the CNS and the endocrine system, the regulation of circadian rhythm and also the capacity to respond to internal (temperature and glucose levels) and external (visual or auditory stimuli) stimuli and to mediate the transmission of information, from and to brain, through synaptic transmission- might have a crucial role in aging. This highly functional anatomic structure is considered as a primary regulator of the aging process in the whole body, despite of their nuclei and neuronal groups suffer a special impact during this process.

To sum up, this review analyses the interest that a neuropeptide might have in the context of aging biomarkers and, furthermore, explores the possibility of a future pharmacological treatment based on this neuropeptide.

Keywords: Aging, Progeria, Biomarkers, Hypothalamus.

I. Introduction

1. Aging

According to the World Health Organization (WHO), the world's population is getting older and it is expected that, by 2050, the number of people with 65 years old or older is going to be about 1.5 billion, which represents 16 percent of the world's population.

Consequently, the improvements in life expectancy were related to a shift in the leading causes of disease and death. The infectious and acute diseases, associated to younger populations, were decreasing and the chronic and degenerative diseases are emerging. Over the next 10 or 15 years, people will suffer more from non-communicable diseases, as cancer, diabetes and neurological diseases. In face of this, one of the biggest concerns about the massive aging is the increase of life expectancy followed by an increase in morbidity.

In order to promote an improvement in quality of life of the elderly population, it is of great importance to understand the aging process and their cellular and molecular mechanisms and how it links disease (1).

1.1. Cellular and molecular mechanisms involved in the aging process

Aging is defined as a breakdown of self-organizing systems and is characterized by a decrease in the ability to cope with environment alterations. It results in a complex biological process that is still not completely understood (2).

Aging is associated with many alterations in central and peripheral systems (3) including alterations in regulatory, endocrine, behavioural, physiological and neurological functions (2) as food intake, energetic balance and sleep patterns (3-7). Therefore, aging is characterized by a progressive loss of physiological functions and by the increase susceptibility to several diseases related with age (2,8).

Several theories were emerged in order to explain the causes of aging and, by 1990, Medvedev had classified more than 300 theories of aging. Due to the high number of theories and the continuous research, these theories were divided into 3 categories: program theories, damage theories and combined theories.

The program aging theories refer that there is a deliberate deterioration with age and the limited lifespan results in evolutionary benefits. Some researchers suggested that aging is an altruistic plan in which the oldest individuals were eliminated in order to avoid overpopulation and to promote the adaptation of the newest generations. This plan is a result of "aging genes" and mutations.

The neuroendocrine theory of aging is another program theory and is a hormone-based theory. This theory emerged due to the fact that the brain regulates the endocrine system and the production of growth hormone (GH) is decreased with aging.

Apart from the program theories, the damage theories defend that the aging process is not due to natural selection and it is not programmed. They suggest that the accumulation of damage is a spontaneous process and its kinetics can be modified genetically and environmentally, which explain the wide range of lifespans. The most accepted theory is based on oxidative damage. Reactive oxygen species (ROS) were generated during metabolism and, when they are accumulated, they might lead to deoxyribonucleic acid (DNA), protein and lipid damage, which affects translation and protein turnover due to the oxidative stress. The mitochondria are the major producer of ROS in mammalian cells, then the mitochondrial DNA (mtDNA) is the most affected. This results in impaired mitochondrial maintenance and altered cellular homeostasis.

ROS induces DNA damage and lead to the hypomethylation of the DNA and histone modifications and, consequently, to alterations in several organs (such as heart, brain and skeletal muscle) and in the epigenetic process. Besides DNA, ROS also might affect lipids and proteins and their damage are irreversible. Therefore, they may be degraded by the proteasome. However, with age, this proteolytic machinery is impaired, resulting in the accumulation of oxidizing proteins and aggresomes (currently observed in neurodegenerative diseases).

The cellular oxidative damage might also be induced by advanced glycation end-products (AGEs), that results from the Maillard reaction. The interaction between AGEs and their receptors lead to an upregulation of nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase and, consequently, to an increase of intracellular oxidative stress. Thereby, AGEs are associated to several chronic diseases, as diabetes and cardiovascular pathologies.

Another damage theory is based on genome instability and telomere shortening. The integrity and the stability of DNA is challenged by endogenous and exogenous factors, as DNA replications errors and biological and chemical agents. The highly complex DNA repair mechanisms and the DNA helicases are essential to the integrity of the DNA, however they might defective. The telomere shortening is also observed in aging, besides it is not considered the major driver of the aging process, and is caused by the presence of shelterin, that acts as a barrier against DNA repair proteins.

As a result from the program theories and the damage theories, the combined theories emerged, in 1976, by Strehler, in order to unify these theories of aging. He considered that aging is a universal process that affects all species and must be intrinsic (do not depend on extrinsic factors), progressive and deleterious. Several hypotheses were included in this theory, such as the membrane hypothesis of aging (based on the fact that cell's membranes are becoming more rigid which might lead to a decrease of the intracellular potassium content), the dysdifferentiative hypothesis (based on the impaired differentiation that result in a cascade of changes) and, more recently, the fading electricity hypothesis (based on the gradual loss of the cells to produce their own electricity, which might lead to senescence) (2,9).

In these theories, Medvedev attributed some molecular and cellular changes to aging, that lately, were identified and categorized as cellular and molecular hallmarks of aging by Lopéz-Otin and colleagues (2,10). The cellular and molecular hallmarks of aging are: cellular senescence, stem cell exhaustion, altered intercellular communication, genomic instability, telomere shorting, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing and mitochondrial dysfunction (11). The hallmarks explain the time-dependent functional decline during aging (11) which involves molecular changes (alterations in gene expression and genetic variations), physiological changes and pathological changes (cognitive deficits and alterations in sleep patterns) (2).

The "integrative" hallmarks (stem cell aging and dysfunction of intercellular communication) are of particular interest as they could provide insights into the aging process and, especially the intercellular communication could help to identify biomarkers of aging because they interact with the secretion of proteins, lipids and metabolites that might change due to alterations in the network of communication factors (12). Therefore, proteomic and metabolomic studies of aging have sought potential biomarkers of age-related diseases and neurodegenerative diseases (12,13). The protein most correlated with aging is chordin-like protein 1, which might be involved in neural stem-cell fate and angiogenesis. The immune factor eotaxin (also known as CCL11), a chemokine involved in allergies, and β-2-microglobulin (B2M), a component of major histocompatibility complex class I (MHC I) molecules, that was found to be a pro-aging factor, are also linked to aging. The inflammation process linked to aging is known as inflammageing and might lead to senescence and a senescence-associated secretory phenotype, which may result in epigenetic changes (12).

The molecular hallmarks of aging, the cell-intrinsic changes (such as telomere shortening, mitochondrial dysfunction or DNA damage), might be considered biomarkers of aging.

Most biomarkers of aging measured in blood samples are related to cardiovascular function, glucose metabolism, inflammation, nutritional status, endocrinology and hematology (14). Studies of immunosenescence revealed the increase of inflammatory peptide biomarkers (interleukin (IL)-6, IL-1, tumor necrosis factor-α (TNF-α) and C-reactive protein (CRP)) during aging, that are collectively named as inflammaging (14,15).

Recently, markers related to red blood cells, specifically hematocrit, hemoglobin and the red blood cell count are associated with chances of adverse health-status measures such as morbidity, cognitive impairment and mortality. And more recently, adipokines, such as adiponectin, ghrelin and leptin, the key regulators of inflammation and appetite, were linked with age-related health outcomes, however further research on this association is required.

Bilirubin is also an emerging biomarker of aging but also a biomarker for reduced chronic disease prevalence and for the prediction of all-cause mortality.

AGEs, that are a result of a posttranslational modification of proteins known as non-enzymatic protein glycation or "Maillard reaction", might be future biomarkers of aging as they accumulate during normal aging. Therefore, AGEs might have some potential to monitor healthy aging as well as the pathophysiology of numerous age-related diseases, including cardiovascular diseases, renal disease and neurodegeneration.

Metallothioneins (MTs) have an essential role in the transcriptional regulation of genes involved in growth, proliferation, differentiation, and development of neuronal cells as well as in pathways of importance in neural function and also contributes to the neuroprotection of the brain, avoiding the oxidative stress. However, MTs are down-regulated in older groups.

Very recently, another interesting biomarker has been discovered based on epigenetic changes. The novel molecular marker, p16INK4a, has the capacity to inhibit cyclin-dependent kinase activity and, consequently, promote cellular senescence, a process of irreversible cell-cycle arrest and loss of regenerative capacity. Therefore, the precise regulation of p16INK4a is essential to homeostasis.

Aging microRNAs (miRNAs), that regulate a broad spectrum of biological activities, have also been proposed as biomarkers of aging. Of the many miRNAs expressed in the human genome, potential candidate analysis is focused on miR-146, miR-155, miR-21 and miR-126. Furthermore, miRNAs might also be considered as circulating biomarkers for

cardiovascular aging or aging-associated diseases. However, further research needs to be conducted to evaluate their sensitivity, selectivity and potential as predictive biomarkers (14).

Biomarkers were defined, for the first time, in 1988, as the biological parameters that might predict the changes in physiological functions in the absence of disease (15,16). The National Institutes of Health (NIH) defines them as a "characteristic that is considered as an indicator of normal biological processes, diseases, or pharmacological responses to a therapeutic intervention". Biomarkers of aging are used to monitor the health of the population and to know the susceptibility to health problems (9,17).

Biomarkers emerged due to the fact that chronological age is not a sufficient marker of the functional status and the susceptibility to age-related diseases and, then, biomarkers show promise in capturing specificity of biological aging (18). Therefore, these biological parameters should be better measures of rate of aging than chronological age (19).

However, one single biomarker correlates with numerous and complex mechanisms underlying aging is limited by poor specificity. Thereby, it is recommended an integrative and simultaneous analysis of multiple biological markers in order to provide an opportunity to identify the biomarkers of aging (18,19).

The criteria for biomarkers of aging have some little differences depending upon the writer and their primary area of interest (19). The American Federation for Aging Research defined the following criteria for a biomarker of aging (9,15,16):

- It must predict the rate of aging. In other words, it would tell exactly where a person is in their total lifespan and must be a better predictor of lifespan than chronological age.
- It must monitor a basic process that underlies the aging process, not the effects of disease.
- It must be able to be tested repeatedly without harming the person, for example, a blood test or an imaging technique (for example the magnetic resonance imaging, MR (13)).
- It must be something that works in humans and in laboratory animals, such as mice. This is so that it can be tested in lab animals before being validated in humans.

Unfortunately, some biomarkers of aging differ between different species. One example is telomere shortening that is detected in humans, but, in wild-type laboratory mouse, it is not observed (16).

Hence, currently, there are no standardized biomarkers ("gold standard") of aging process or healthy aging because biomarkers described in literature do not meet all criteria of an ideal aging biomarker (9).

In order to understand the difficult to find an ideal biomarker of aging, it is important to link the theories of aging with the high complexity of the human brain.

The aging process is related to alterations in several tissues throughout the body, in particular, the central nervous system (CNS). CNS is especially vulnerable to the effects of aging and responds to changes from external environment. The interaction of CNS, mainly the hypothalamus, have been implicated in regulating organism lifespan (20).

Autopsy studies of brains of elderly people, without any diagnose of neurological disease, reported synaptic dystrophy, loss of neurons and loss of brain volume in most of the brains. The causes for these lesions are unknown, however they might be linked to the accumulation of proteins and lipids in aging brains, either in the extracellular space or in neuronal cells. Environmental factors may also have profound effects on healthspan and longevity (12). Despite the loss of neurons in the normal aging process are not large, the number, diameter, length and branching of the dendrites and the density of the dendritic spines decrease with age. These changes are very heterogeneous in different parts of the brain and the decrease of neurons is local (13).

Due to these all alterations during the aging process and considering that hypothalamus represents a vulnerable site to aging effects, but also is a potential master regulator of systemic aging itself (21), the new chapter is focus on the hypothalamus and its relation with the normal process of aging.

2. Hypothalamus

The human hypothalamus is a very heterogeneous brain area that contains distinct neuronal populations (4) and is located at the medio-basal region of the brain, ventral to the thalamus and dorsal to the pituitary gland (22,23). The adult hypothalamus is divided in three distinct longitudinal zones: the periventricular, medial and lateral zones. The periventricular zone contains a few distinct nuclei and the more prominent ones are the arcuate nucleus and the paraventricular nucleus (PVN), which are involved in neuroendocrine and autonomic regulation. Adjacent to periventricular zone is the medial zone that is responsible for regulating the autonomic nervous system (ANS) and the neuroendocrine system. The lateral zone has few nuclei; however, it has important fiber pathways, as median forebrain bundle. Its role is related to the regulation of the ANS (24). These three zones are divided into four rostrocaudal regions: preoptic, anterior, tuberal and mammillary (25). Each zone and region

has different nuclei that result from the segregated agglomeration of cell bodies and they have the possibility to interconnect with several cell populations (24).

The interaction between different nuclei and the neuropeptides produced by them determine the hypothalamic functions (Table 1), that include the regulation of energy and fluid balance (26), the triggering of the stress response (27) and the regulation of reproductive, emotional and social behaviours through stimulation of GH and gonadotropin-releasing hormone (GnRH) release (25). Hence, the hypothalamus is considered the major regulator center of autonomic and endocrine homeostasis (28-30).

Table 1 – The hypothalamus' structure, nuclei and their functions. *The data presented is from:* (24).

Nucleus	Zone	Region	Functions
Paraventricular	Periventricular	Anterior Tuberal	Integration center for ANS (31). Expression of regulating peptides (CRH and TRH), which lead to the production of hormones in the anterior pituitary (25). Secretion of vasopressin to the systemic circulation (32).
Arcuate	Medial	Tuberal	Regulation of food intake (33,34) and GHRH (35).
Anterior	Medial	Anterior	Thermoregulatory control (36).
Suprachiasmatic		Anterior	Regulation of circadian rhythms (37,38).
Dorsomedial		Tuberal	Regulation of several behaviours influenced by circadian rhythm as sleep-wake behaviour, locomotor activity and heart rate (39-41).
Ventromedial		Tuberal	Regulation of homeostatic and behavioural functions as reproduction, obesity (42) and cardiovascular function (43,44).
Posterior		Posterior	Emotional behaviour (45). Control of sleep-wake cycles (46). Cardiovascular regulation (47). Defensive-aggressive behaviours (48,49).
Mammillary		Posterior	Emotion and reward behaviours (50). Spatial and episodic memory consolidation (50).
Preoptic	Medial	Anterior	Thermoregulatory control (51). Cardiovascular and body fluid regulation (51).
Supraoptic	Lateral	Anterior	Production and secretion of (52): - Vasopressin (osmotic balance and regulation of blood pressure); - Oxytocin (lactation and parturition).
Lateral	Lateral	Tuberal	Control of energy balance (53). Regulation of feeding behaviours (53).

CRH - Corticotropin-releasing Hormone; GHRH - Growth Hormone-releasing Hormone; TRH - Thyrotropin-releasing Hormone.

The wide range of hypothalamic functions (Table 1) might be explained by the production and secretion of different neuropeptides/neurotransmitters. The orexigenic agouti-related peptide (AgRP), neuropeptide Y (NPY), orexin, melanin-concentrating hormone (MCH) and the anorexigenic peptide, proopiomelanocortin (POMC), produced by the arcuate nucleus, are neuropeptides involved in promoting food intake (54).

The periventricular part of the hypothalamus is responsible for the secretion of NPY, leptin and gastrin, that are involved in feeding and energy balance, but also, lead to the secretion of somatostatin (which acts directly on the acid-producing parietal cells and reduce acid secretion) and hormones, as TRH (that stimulates the release of thyroid-stimulating hormone (TSH) and prolactin from the anterior pituitary) and GnRH (that is responsible for the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary). PVN and supraoptic nuclei of the hypothalamus also contain neurons producing CRH (which is involved in stress response), TRH, oxytocin, and vasopressin (54).

Some data suggest that the hypothalamus might have other functions related to the control of lifespan and aging (55-57).

On the other hand, the age, *per se*, has a specific impact on hypothalamic neuronal cells (3) and its majority of physiological functions, (8) such as impaired neurogenesis, microglia activation, activation of factor nuclear kappa B (NF-kB) and up-regulation of inflammatory cytokines (20).

NF-kB has a role in controlling gene expression during aging, mediating immune cell communication and inflammatory responses (58). During aging, hypothalamic NF-kB, microglia-induced tumor necrosis factor-alpha (TNF-α), neuronal NF-kB and many cytokines and immune regulators are increased which lead to epigenetic changes in neuroendocrine genes and, consequently, to dysregulation of hypothalamic function. GnRH is one of the gene affected by the aging-related inflammatory process and contribute to decline in muscle and bone fitness, skin atrophy, reduced neurogenesis and memory impairment (20).

Another connection between hypothalamic dysregulation and aging is based on adult neural stem/progenitor cells (NSC), which are presented in mediobasal hypothalamic region (MBH) and mediate neurogenesis and brain function. During the aging process, the hypothalamic NSC suffers a decline which might be the cause for the whole-body aging. Despite of this, the hypothalamic NSC also have an anti-aging effect mediated by the secretion of exossomal miRNA by them and an implantation of hypothalamic NSC in the brain leads to an increase of GnRH-expressing cells, which allow the control of the aging process (59).

In order to shed light on how the hypothalamus and their neurotransmitters contribute to hypothalamic functions, in 1998, it was created a complementary deoxyribonucleic acid's library (cDNA´s library) with the most prevalent messenger ribonucleic acids (mRNAs), in hypothalamus, in rats, using polymerase chain reaction (PCR) (28,60-62). This list contains 43 different sequences which proves that hypothalamus is a "specialist" in the production of intracellular signalization molecules and demonstrates that 40% of hypothalamus sequences encode for neurotransmitters. One of the most frequent cDNA that encodes for a neuropeptide precursor is known as preprohypocretin (PPO) (61).

As the orexin neuropeptide consists in one of the multiple signalling systems and considering that the latter interaction with the hypothalamus is crucial for the regulation of several hypothalamic functions, the following chapter will shed light on how this neuropeptide impacts in hypothalamus and their functions (7).

3. Hypocretin/Orexin

PPO mRNA is produced by a few number of neurons (about 5000 in rodents and 20-80000 in humans), which are exclusively localized bilaterally in posterior lateral hypothalamus (28,29,63,64), more specifically in the lateral hypothalamus and contiguous perifornical area (3). These cells produce only one neuropeptide, the PPO (61) and are named as orexin neurons (65).

Orexin A (OX-A) and orexin B (OX-B) are neuropeptides derived from a common precursor peptide, PPO (Figure 1) (28,66).

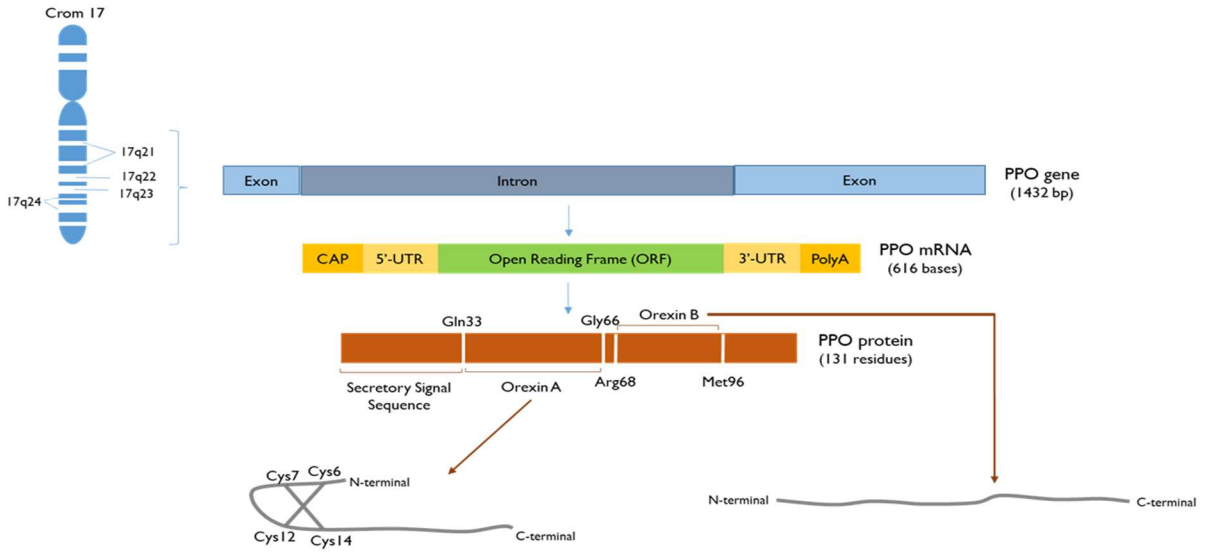

Figure 1 – Schematic representation of OX-A and OX-B synthesis. *Hypocretin (Hcrt) gene is present in chromosome 11, in mice and rats* (62), *which is similar to 17q21-q24 in humans* (66-69). *Hypocretin (HCRT) gene (PPO gene) consists in 2 exons (143 and 473 base pairs (bp)) and 1 intron (816 bp) distributed in 1432 bases. This gene is transcripted in mRNA (with 616 bases). The first exon includes the 5'-untranslated region (UTR) and the first seven amino acids of the peptide. The second exon contains the remainder of the open reading frame (ORF) and 3'-UTR. The protein (PPO) has 130 residues in rodents* (29) *and 131 residues in Humans* (66,67,70). *The first amino acid of OX-A is Gln33 that undergoes enzymatic cyclization and originates pyroglutamyl residue by transamidation of N-terminal* (69,71). *OX-A*

sequence is composed by 33 amino acids (67,69) *and ends with Gly66 (which is a potential donor of* NH_2 *that allows the amidation of C-terminal). Lys67-Arg68 is the recognition site to prohormone convertase (which is an endonuclease) and OX-B starts with Arg68 and ends with Met96, followed by Gly-Arg-Arg that allow the amidation of C-terminal* (69). *Then, OX-B has 28 aminoacids* (7,28,66,67,69).

In eukaryotes, translation occurs not only in the cytosol (Figure 1) but also across the membrane of the endoplasmatic reticulum (ER) (called vectorial synthesis). The entire ribosome/mRNA binds to the outer membrane of the rough endoplasmic reticulum (RER) and the newly polypepetide is stored inside the ER. After the posttranslational modifications, it is incorporated in synaptic secretory vesicles (dense core vesicles (DCV)) (28,62,72). Glutamate, dynorphine and nociceptin/orphanin FQ (N/OFQ) are also in DCV and they are neurotransmitters of orexin neurons (73,74).

Orexin (OX) is a neuropeptide, produced by hypothalamus, that guarantees the homeostatic state of human body due to the diversity of functions performed (explained in detail in *4. Orexin in physiology and pathologies*) (4). It has an excitatory effect (due to its agonist effect of OX receptors (75)) and affect pre and post synaptic mechanisms (7,76).

N/OFQ is an endogenous agonist of nociceptin opioid (NOP) receptor and performs functions at behavioural level, pain (such as hyperalgesia and analgesia), anxiety, learning and memory. It is coupled with G-proteins and their activation leads to the inhibition of adenylyl cyclase and, consequently, to the inhibition of OX neurons (77).

Dynorphine is a neuropeptide that hyperpolarizes orexin neurons by a k-opioid receptor-mediated activation of G protein-dependent rectifying potassium channels and suppresses calcium ion (Ca^{2+}) currents (71,78). Therefore, it has a pre and post-synaptic inhibitory effect (79). Besides that, it is also co-localized with gamma-aminobutyric acid (GABA) forming a GABAergic pathway ("direct" striatal output pathway) (80).

Glutamate is an excitatory neurotransmitter and, together with orexin, it could keep the activate state and/or recruit a great number of OX neurons (71). However, it decreases OX and MCH neurons immunoreactivity, due to the release of some neuropeptides which cause a transitory depletion of them (81).

After the incorporation into DCV, they are transported to the synaptic terminal (82) and accumulated in axonal terminals. Therefore, the OX immunoreactivity is mainly associated to Golgi network vesicles, myelinated axons and post-synaptic terminals (68) and also axons and terminals are more immunoreactivity than cell bodies (80). This suggests their action in intercellular signalization (68). Afterwards, the OX contained in DCV is released and is dispersed over the OX receptors present in somato-dendritic membrane and excites or modulates transduction (83). The duration and intensity of firing activity depends

on the release of OX and the post-synaptic response of OX neurons to the 4 coexpressed neurotransmitters (71).

OX-A and OX-B bind exclusively to two G protein-coupled receptors (GPCRs) (orexin receptor type 1 - OX1R - and orexin receptor type 2 - OX2R) (7,65,66). These receptors have a wide and partially overlapped distribution but distinct along the hypothalamus (61). Their expression pattern differs greatly between the different brain zones (7).

OX binds to OX receptors and, then activates at least 3 subtypes of G proteins (stimulative regulative G-protein (G_s) (Figure 2), inhibitory regulative G-protein ($G_{i/o}$) or G_q protein alpha subunit ($G_{q/11}$)) or other proteins, as β-arrestin, and could regulate phospholipases, ionic channels and kinase proteins (67).

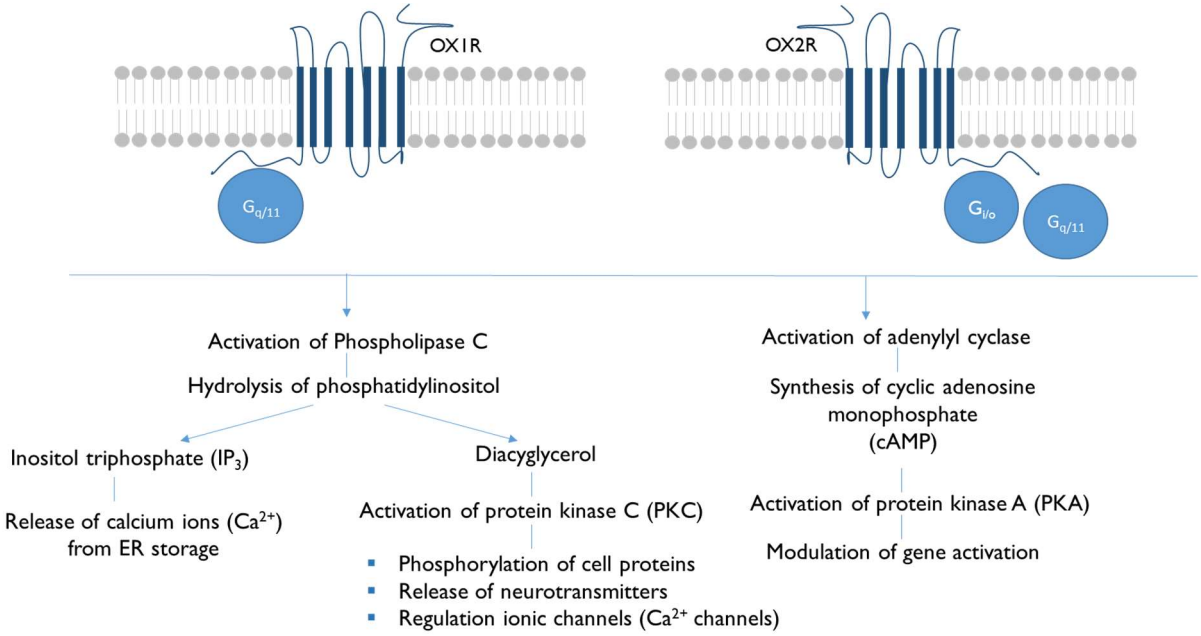

Figure 2 – The activation of Gq-protein by orexin. *Orexin binds their receptors, activates Gq-protein and leads to the depolarization of neurons and to the increase of synaptic activity and, consequently, to excitotoxicity (7,30,71,84).*

OX-A has more affinity to OX1R than OX-B (65). OX1R is 30 to 100 times more responsive to OX-A than OX-B (63) whereas OX-B is 100-1000 times less affinity to OX1R (68). OX-B binds preferentially to OX2R (7). OX-A and OX-B have equal affinity to OX2R (65,66).

After the mechanism reported, in Figure 2, and to decrease the excitability and the synaptic potentials, G-protein, when is activated, also induce the activation and opening of G-protein coupled inwardly rectifying potassium ions (K^+) channels (GIRK channels) that lead to efflux of K^+, hyperpolarization and, consequently, a decrease in neuronal activity (85).

OX2R also binds to $G_{i/o}$ – α subunit sensible to pertussis toxin and activates it, when OX is overexpressed, in cell cultures. This results in a decrease of cyclic adenosine monophosphate (cAMP) production (7,68).

Due to a constitutively active nonselective cation current mediated by transient receptor potential channels, the depolarization resting membrane potential is higher in OX neurons comparatively with the normal potential membrane resting (-54.6 ± 1.5 mV) (62). This could promote long firing activity by releasing the neuropeptides from DCV (71). Therefore, OX neurons are intrinsically in a depolarized state and are spontaneously active (61,81). During prolonged firing activity, the excitatory effect of OX is predominate (71) and OX neurons stay reactive to stimuli (61,71).

OX receptors are expressed by OX neurons, which corresponds to 1/3 of all hypothalamic neurons (5,6). This suggests that OX has activity in the majority of hypothalamic systems (6).

OX neurons are localized exclusively in lateral hypothalamus area (LHA) (more specifically in perifornical nucleus, PeF) and dorsomedial hypothalamic nucleus (DMH) in all ages (3,7,29,30,65,66) and their density is higher in DMH and PeF than in LHA (65) and much higher in hypothalamus than in other areas of brain (62). Despite the low quantity of OX-A and OX-B (due to the restricted localization of their neurons in LHA (61)), their axons are widely projected, not only in hypothalamus, but also in other brain areas, such as basal forebrain cholinergic system (BFCS), preoptic area, PVN, central gray, locus coeruleus (LC) (28), pre-frontal medial cortex (86), diencephalic, brain stem, substantia nigra, ventral tegmental area, nucleus raphe magnus, insular cortex and spinal cord regions, with the expection of cerebellum (3,4,7,29,30,63,65,87).

The dispersion of orexin projections throughout all brain might explain their ability to modulate different humoral signs and neuronal inputs (Figure 3) (7). As a result of this, orexin neurons are descripted as "physiological integrators" (3,88) because they act endogenously as homeostatic regulators in CNS (28).

Besides the fact that OX is only synthetized in the brain, it has not only a central action. The presence of OX-A, in plasma, in humans, explains its production and action in peripheral tissues (89).

In rats, plasma OX-A concentration is 11.5 ± 1.0 pg/mL, in male, and 13.6 ± 1.1 pg/mL, in female (90). In young healthy male volunteers, without narcolepsy (aged 20–24

years), the range of OX plasmatic concentrations are between 0.5 and 16 pg/mL. OX-A plasmatic levels are low but stable e they do not correlate with gender (90-94).

PPO mRNA levels are extremely high in brain but they are also present in testes and, in very low levels, in heart. These levels are undetectable in the pituitary, adrenal kidney, stomach, duodenum, jejunum, pancreas, liver, spleen, aorta, lung, thyroid, adipose tissue, muscle and ovaries (90). However, lately, Nakabayashi and colleagues referred that OX-A was found in gut, pancreas, in the cytoplasm of ganglion cells and in ganglion cells of myenteric plexus of gastrointestinal tract (stomach, duodenum, jejunum, ileum, colon and rectum) (89).

Although OX1R and OX2R are presented in the brain, they are also in peripheral organs and tissues. OX1R mRNA was found in rat brain, pituitary, adrenal, thyroid, kidney, jejunum, testis, and ovaries. These mRNA levels were highest in the brain, followed by lower levels in the pituitary and very low levels in the kidney, thyroid gland, testes, ovaries, and adrenals. OX2R mRNA was found in rat brain, lung, adrenal, and pituitary. The OX2R mRNA levels were 4 times higher in adrenal than brain. Both OX1 and OX2 receptor mRNA were detected in stomach, duodenum, pancreas, liver, spleen, heart, aorta, adipose tissue, or muscle, however their levels are no significance (90).

The expression of OX1R and OX2R in pituitary and adrenal, respectively, indicates the OX action in the regulation of endocrine systems, in particular, the hypothalamic-pituitary-adrenal (HPA) axis. OX2R might even coordinate the synthesis and release of corticosterone in rats (90).

Currently, there is still a lack of information in this area, therefore more investigation needs to be conduct to fill out some information gaps.

4. Orexin in physiology and pathologies

Initially, OX was only associated to the regulation of food intake and the meaning of OX is appetite (60,69,80). Later, it was discovered its role on narcolepsy and sleep regulation (80). Currently, it is known that OX has a role in sleep/excitation, sleep-wake cycle, behaviour stabilization and food intake and energy homeostasis (3,95). This neuropeptide has also an action in reward (78,86), spontaneous physical activity (SPA) (7), arousal (95), sensorial, locomotor, cognitive (such as attention, learning and memory (88)), endocrine and visceral systems (5,61,63), cardiovascular and gastrointestinal systems, water regulation, pain modulation (87), reproduction, motivation, stress, anxiety (95), addiction to alcohol and drugs of abuse (78), hormone release and autonomic control (93). (Figure 3)

OX functions are greatly diverse and, thereby, orexin is denominated by "physiological regulator" because it guarantees the homeostatic state of human body (4).

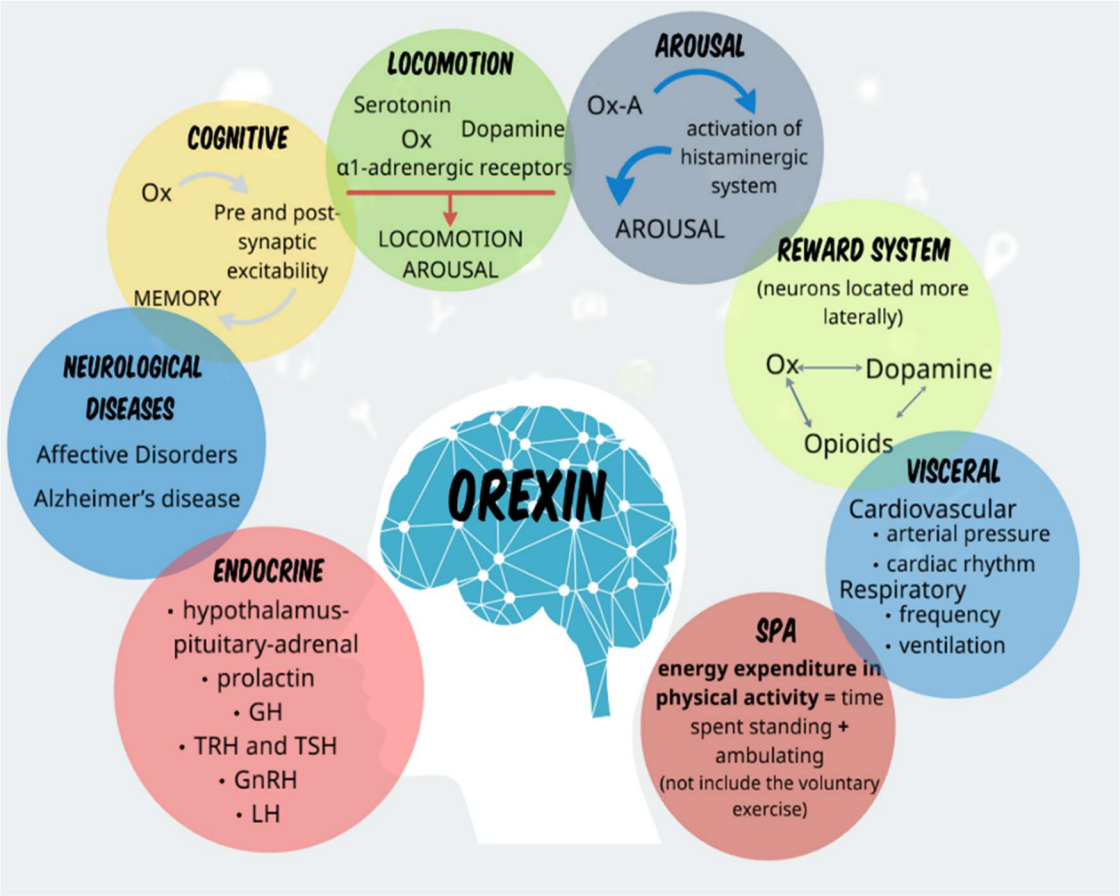

Figure 3 – Interaction between orexin and physiological processes and pathologies. *OX as a role in different systems and pathologies as endocrine system* (5,29,30,61,63,68,87,90)*, neurological diseases* (67,87,96,97)*, cognitive functions* (7,61,86,88,98,99)*, locomotion* (61,70,100)*, arousal* (62,68,99)*, reward system (3,4*,61)*, visceral systems (*29,61,68,80,90) *and SPA* (7,101)*. The interaction between orexin and sleep and metabolism will be explained bellow in detail. GH - Growth Hormone; GnRH - Gonadotropin-Releasing Hormone; LH - Luteinizing Hormone; SPA - Spontaneous Physical Activity; TRH - Thyrotropin-Releasing Hormone; TSH - Thyroid Stimulating Hormone.*

4.1. Sleep

The OX central system is crucial for regulating sleep/wake state (63) because OX is wake-active and wake-promoting (79). It has not known yet if OX action is instructive or permissive to awake (61), however it is known that the time that OX neurons take to reach the activity peak precedes wakefulness and this suggests a relation between OX and wake bout. OX has only effect on maintenance of wake bout and not in brief wake bouts because the initial release of glutamate is not enough to recruit and maintain the activity of a huge number of OX neurons (71). Then, the phasic activation of these cells is involved on transitions to awake state but not on maintenance of awake state (61).

It is thought that OX effects in wakefulness are due to primary downstream activation in tuberomammillary system (which is a histaminergic nucleus) (93). The

histaminergic and orexin neurons perform a synergistic and complementary action in awake control. The histamine is responsible for cortical activation (the posterior hypothalamus is considered to be the "wakefulness center" (102)) and regulates the cognitive function, whereas orexin is involved in behavioural arousal during wakefulness (such as muscle tone, locomotion, food intake and emotions) (103).

4.1.1. Narcolepsy

Narcolepsy is a sleep disorder that is defined as a primary disease and affects 1:2000 of the population (68,81) (then, its prevalence is 0.03-0.05%). It appears at any age, however it is more prevalent between adolescence and the fourth decade (mostly between 15 and 30 years old)(87) and is an autonomic recessive disease (68). The symptoms are: excess of sleepiness and irresistible sleep attacks during daytime (68,87). Other symptoms are: cataplexy (that is related to a population of neurons present in amygdala (104)), sleep paralysis, hypnogogic hallucinations and dream-like images. The sleep paralysis is due to a persistence of rapid eye movement (REM) sleep atonia on waking (68,87). One predisposing factor for this disease is specific class II human leukocyte antigen (HLA) haplotype (81).

In humans, narcolepsy is apparently related to loss of OX immunoreactivity (4,65) or loss of their receptors due to an autoimmune attack that cause a selective degeneration of OX cells (60,68,83). The normal number of OX neurons are reduced approximately by 90% (85-95%) (64,87,104,105) and this decrease is accompanied by gliosis and signs of inflammation (81,87). The destruction of OX neurons (by genetic factors or by toxins) results in dysfunction of OX system which is identified as the first cause of clinic manifestations of narcolepsy in humans. This dysregulation leads to multiple cognitive and behaviour deficits (88) as well as severe and chronic sleepiness (64,104).

Several pharmacological treatments have been arising such as amphetamines and modafinil (68) (that increase monoaminergic transmission by stimulating the monoamine release and blocking the monoamine reuptake (106)) or hypnotic drugs (that activate orexin receptors) (107).

4.1.2. Sleep and aging

Sleep disorders, like excessive daytime sleep or fragmentation of sleep (8), are common situations in elderly people (3,6). Decreasing amount of sleep and increasing number and duration of intrasleep arousal are also observed during aging (94,108).

Alterations in sleep/wake behaviour arise, initially, in adolescence and continuous with age. In adolescence, it occurs a delayed onset of the sleep phase. This might be due to

physiological, environmental and social factors or to alterations in melatonin and orexin. In fact, there is a decrease of 10% in OX expression between early and later adulthood (65).

Between 50 and 85 years, the sleep quality decreases about 50%, due to alterations in melatonin secretion and circadian rhythm associated to aging (65). The melatonin is released earlier and the peak is low comparatively to young people (109). These alterations suggest alterations in OX (65,70) or a dysfunction in OX system (94) and could lead to an increase of sleep latency and the number of times waking up throughout the night, short periods of sleep during daytime, insomnia at night and sleepiness during daytime (65,70,94,108). The duration of sleep bouts are also decreased with age (6).

The number of noradrenergic neurons in LC (region associated to arousal) is decreased with age and this decrease explains the decrease of vigilance and disruptions in sleep-wake behaviour (108).

4.2. Metabolism

In 1998, it was discovered that OX-A and OX-B stimulated food intake and PPO mRNA were accumulated during feeding (29) which suggested that the expression of Hcrt gene is upregulated in fasting (63,66,68). Later, in 2001, it was demonstrated that OX signalling was also involved in appetite, satiety and energy balance and, thus, OX neurons were involved in short-term feeding and energy homeostasis (63,110,111).

As OX is an orexigenic peptide and LHA is considered to be the "feeding center" (87,100,112), the electrical stimulation of LHA by OX increases feeding (30) and hyperphagic (110), whereas lesions in LHA (named as "LHA syndrome") decreases body weight and food intake (30) and provokes hypophagia (96,113,114). The ventromedial hypothalamic nucleus (VMH) is considered to be the "satiety center" (30), then, lesions in this area produce hyperphagic and obesity and it is known as "VMH syndrome" (96,115,116).

The plasma OX-A levels are correlated negatively with body mass index (BMI) (110). Then, the lack of OX due or not to progressive loss of OX neurons also provokes obesity and low caloric intake (7).

OX neurons act as adaptive glucosensors and are able to modify glucose levels in blood (63). 1/3 of OX neurons are activated by hypoglycaemia induced by insulin (87) or intermittent periods of fasting and caloric restriction (7,91). The activation of OX neurons raises the sensibility to insulin and promotes glucose mobilization (whereas high levels of glucose inhibit them (7)), which suggests an increase in the accessibility to energy store (7,61).

In 2014, OX was demonstrated to modulate energy homeostasis by coordination hormonal factors, like leptin and ghrelin (61).

Leptin is an adipocyte hormone produced by adipose tissue. Plasma leptin levels are higher in people with a higher BMI and body fat and, thereby, leptin decreases food intake and increases energy expenditure (117). Leptin seems to have a role in short-term regulation of food intake and body weight (118).

The interaction between leptin and their receptors influences the activity of some hypothalamic neurons receptors and the expression of orexigenic and anorexigenic neuropeptides. The orexigenic peptides are: orexin (61), NPY (117), MCH, AgRP, galanin and galanin-like peptide (96). They have a role in stimulating appetite (96). The anorexigenic peptides are appetite-supressing peptides and include POMC, cocaine- and amphetamine-regulated transcript (CART), neurotensin, CRH and brain-derived neurotrophic factor (BDNF) (118).

Nevertheless, leptin inhibits orexigenic peptides, supresses the strength of excitatory pre-synaptic synapses (78) and stimulates anorexingenic neuropeptides (119), whereas ghrelin has the opposite effect (61) because it is an appetite-stimulatory peptide (118).

Ghrelin is a hormone produced and secreted by stomach and its release depends on the nutritional state. It increases in preprandial periods and decreases in postprandial periods. This hormone might stimulate food intake in the presence of gastric emptying. Then, its secretion is inhibited in the presence of leptin. Therefore, ghrelin has a role in short-term regulation of food intake and also in long-term regulation of energy balance (118).

Metabolism might suffer alterations during human development due to the interaction with OX and considering the fact that energy homeostasis is one of the hallmarks of physiological alterations in aging (8).

Aging leads to a decrease of 50% in hunger and appetite (65) which results in an imbalance between nutrition and energy metabolism (8) and in alterations in corporal composition and glucose homeostasis (3). These suggest alterations in OX such as decrease of activity, expression or number of OX neurons (3,65).

Even though it is verified a decrease in food intake in elderly people without degenerative diseases, there are variations in metabolic activity and in corporal composition that results on a sarcopenic obesity, which is characterized by an increase of fat due to a loss of muscles. This is why the prevalence of metabolic syndromes increases with age (8). It may

be explained by the decrease of OX signalling in CNS that could contribute to insulin resistance, hyperglycaemias and obesity (7,111).

5. Orexin and Aging

OX signalling suffer a decline in aging (3), more specifically a decrease of OX expression or OX levels, decrease orexin innervations of target regions (3), reduction of OX neurons (98), depletion of OX receptors (7) or decrease of OX receptors mRNA (111) (65). However, authors showed controversial results.

The response of OX neurons in aging is different and specific for each specie (108).

The decrease of OX levels might be due to several situations such as:

a) Decline of OX synthesis without neuronal loss (65,109) showing a slowdown of the activity of OX neurons (96):

Initially, in 2002, it was thought that the expression of PPO and prodynorphine remained unchanged with age in hypothalamus (94). However, in 2004, it was contradicted and cells expressing PPO gene and the levels of expression are effectively reduced (6). The decrease of PPO gene expression and, consequently, the reduction of PPO mRNA production (7) lead to desynchronization and coordination defect of autonomic, metabolic and endocrine functions (6).

b) Decrease of OX neurons synthesis (65);

c) Phenotype silencing:

OX neurons are preserved during aging; however, they undergo a mute function through phenotype (neurons remain intact however they fail to express this neuropeptide in detectable levels) (3).

d) Loss of OX cells (65,109):

Despite the loss of neuronal cells in aging have been reported to non-hypothalamic systems involved in arousal or energy balance, hypothalamic cells are also affected and orexin cells are the most affected (3,7). Their loss is higher than 40 % both in medial and lateral sectors of lateral hypothalamus and perifornical area (LH/PFA) (3,98). The loss of OX cells with age is reflected in a decrease of optical density (OD) and density of OX neurons *per* mm^2, which is more pronounced in caudal sections. Then, the functions associated to caudal cells are more affected (6).

Although the evaluation of neural loss is difficult, it is observed by several authors and may be due to (111):

1. Excitotoxicity mediated by glutamate: Glutamate depolarizes the membrane and, consequently, activates neurons (3). The excessive activation of glutamate induces excitotoxicity and leads to damage and cellular death, which results in loss of OX neurons (65).

2. ER stress: It is due to a Ca^{2+} low store capacity and an increase of oxidative stress exposition and it could result in neuronal injury and neurological diseases (65).

The ER dysfunction leads to an increased expression of the transcription factor C/EBP homologous protein, that rises growth arrest and DNA-damage-inducible 34 (GADD34) expression, resulting in stress-induced cell death (apoptosis). This dysfunction, consequently, limit OX synthesis and reduce orexin immunoreativity (65).

In order to make up for the loss and/or decrease of central OX production, the peripheral OX levels increase; despite, in some situations, the alterations in PPO production and/or the efficacy of activation of OX receptors in brain are undetectable (7).

During the aging process, the decrease (due to degeneration) or dysregulation in the production of OX receptors or a loss of their capacity to bind OX might be also observed. This is proved by the decrease of the mRNA that encodes for OX1R and OX2R and a decrease of OX1R and OX2R in rats (108). The decrease of both OX mRNA receptors is detected in LHA and hippocampus and could be due to a reduction in the number of mRNA copies *per* cell or a decrease of the number of cells producing these mRNA (94,108).

Numerous studies were made in order to understand the relation between orexin and the human development and, especially, aging.

In humans, OX and their receptors are detectable in earlier development stages even at low levels and they are activated at birth (76). OX and their receptors and OX neurons are high in infants and then they start to decrease. In comparison with infants (0 to 1-year age), children (4 to 10 years old) show a decrease of 5% of the OX neurons, young adults (22 to 32 years old) show a decrease of 13% and older adults (48 to 60 years old) a decrease of 23% (in a post-mortem study). Then, the proportion and density of OX neurons decrease 23-26% from infants to elderly (0-60 years) in humans' hypothalamus (65).

With age, the expression of OX is in gradual decline, which is more evident in maturation than in aging (65). However, there is an exception; during adolescence, the OX levels increases because of sexual maturation (110).

In face of this, OX levels, in cerebrospinal fluid (CSF) and plasma, are also affected by aging and vary during the human development, as seen below in Figure 4.

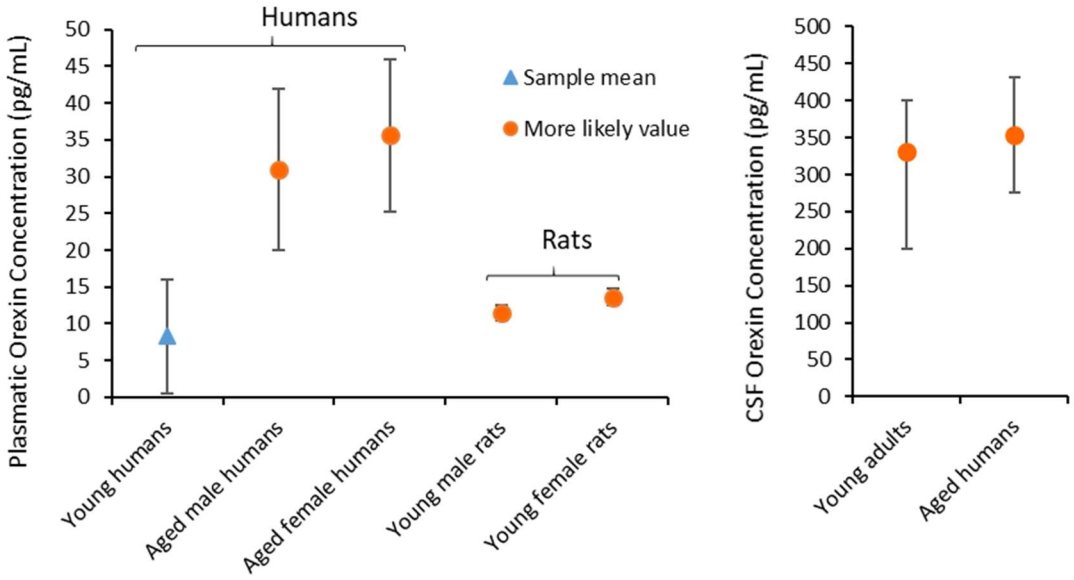

Figure 4 – Comparison between orexin concentration (in plasma and in CSF) in young and aged humans and rats. *The data presented is from:* (90,91,120,121).

OX-A levels, in plasma and in CSF, are higher during infants and decrease after adolescence. However, in adults, these differences are variable (65).

OX-A levels, in CSF, increase linearly during the 3rd embryonic trimester and continue to growth after 40 weeks of gestation and reaches the peak after 2-4 months postnatal (OX-A concentration is 476 ± 72 pg/mL). At this age, the consolidation of wakefulness and sleep occurs (65,76). Then, this level decreases in childhood and puberty (65).

OX-A, in CSF, remains unchanged from 22 to 80 years while OX-A, in plasma, increases from 23 to 79 years (65).

Relatively to OX levels in plasma, preterm infants already have high OX-A levels. The high levels of OX in infants (2-4 months) have a protector effect against ischemic neuronal injury. However, these levels decrease gradually and, 35-36 weeks after birth, OX levels are lower comparatively to infants between 2 and 4 months (76).

The plasmatic levels of OX-A and OX-B are higher in neonates and children during puberty (Table 2) because the increase of OX concentration is associated to periods of fast growing and high metabolism (110).

Table 2 – Plasma OX concentration in humans between 0 and 18 years old.

Age	OX-B (ng/mL)	OX-A (ng/mL)			
Newborns (0-1month)	0.67 ± 0.18	1.02 ± 0.17			
Infants (1-12 months)	0.61 ± 0.11	0.97 ± 0.14			
Children before puberty (2-9 years)	0.60 ± 0.11	0.85 ± 0.16		1.03 ± 0.36	Female 0.66 ± 0.35 Male 0.57 ± 0.36
Children during puberty (10-15 years)	0.65 ± 0.09	1.01 ± 0.12	0.014 ± 0.001		
Children after puberty (16-18 years)	0.48 ± 0.07	0.80 ± 0.07			
	(a)		*(b)*	*(c)*	*(d)*

(a) *Newborns (0-1 month, n = 7), infants (1-12 months, n = 15), prepubertal children (2–9 years, n = 12), pubertal children (10–15 years, n = 8) and postpubertal adolescents (16–18 years, n = 8). Plasma orexin concentrations were determined by enzyme immunoassay. Results are reported as mean ± SD. Significant differences are p<0.05. The data present is from:* (110).

(b) *Twenty-six non-obese Japanese children (13 boys and 13 girls). The age of the subjects was 10.4 ± 0.3 years. Plasma orexin concentrations were determined by radioimmunoassay. Results are reported as mean ± SD. Significant differences are p<0.05. The data present is from:* (122).

(c) *Forty-one health prepubertal children (24 boys and 17 girls). The mean age was 7.3 ± 3.0 years. Plasma orexin concentrations were determined by enzyme-linked immunosorbent assay (ELISA) kits. Results are reported as mean ± SD. Significant differences are p<0.05. The data present is from:* (123).

(d) *Eighteen children (10 boys, with a mean age of 8.3 ± 2.5 years, and 8 girls, with a mean age of 8.2 ± 2.1 years) The age range was from 5 to 11 years. Plasma orexin concentrations were determined by ELISA kits. Results are reported as mean ± SD. Significant differences are p<0.05. The data present is from:* (124).

In adult people (between 23 and 79 years old), OX-A concentration is positively correlated with age (110,120). Therefore, elderly people have high OX-A concentration (Table 3) (110). This situation is still not understand, however it could be due to the low levels of plasmatic leptin in aging or the decrease of tissue responsiveness to leptin (120). The lack of leptin or the insensibility to it might be correlated with obesity and insulin resistance (125).

Table 3 – Plasma OX-A concentration in adult men and women.

Age	Plasma OX-A concentration (ng/mL)		
>18-39 years old	2. 61 ± 0.19 (a)	2.5 ± 0.5 Range: 1.1-3.1 (f)	0.003 ± 0.00067 (g) 0.027 ± 0.003 (h) 0.039 ± 0.015 (i) 106.56 ± 52.09 (j)
	19.2 ± 1.0 (b)		
	Men: 21.7 ± 4.2 (c) Women: 21.0 ± 10.3 (c)		
40-59 years old	0.0008 ± 0.0004 (d)		
	Men: 28.5 ± 7.8 (c) Women: 28.8 ± 9.6 (c)		
>60 years old	0.0024 ± 0.0005 (e)		
	Men: 31.0 ± 11.0 (c) Women: 35.6 ± 10.3 (c)		

(a) *Ten healthy sedentary men (mean age: 24.4 ± 2.93 years). Plasma orexin A concentrations were determined by ELISA. Results are reported as mean ± SD. Significant differences are p<0.05. The data present is from:* (126).

(b) *Ten healthy volunteers (8 males and 2 females) with mean age 30.6 ± 1.9 years. Orexin-A was measured using a radio-immunoassay kit. Results are reported as mean ± SD. Significant differences are p<0.05. The data present is from:* (127).

(c) *Younger (<39 years old), middle (40-59 years old), and older age groups (>60 years old) (in men, the n is 11, 25, and 19, respectively, while in women, is 8, 15, and 4, respectively). Orexin-A was measured using a radio-immunoassay kit. Results are reported as mean ± SD. Significant differences are p<0.05. The data present is from:* (120).

(d) *Twelve healthy volunteers with mean age = 49 ± 6 years. Plasma orexin A concentrations were determined by ELISA. Results are reported as mean ± SD. Significant differences are p<0.05. The data present is from:* (128).

(e) *Ten healthy subjects with mean age = 62.0 ± 0.5 years. Orexin-A was measured using a radio- immunoassy kit. Results are reported as mean ± SD. Significant differences are p<0.05. The data present is from:* (129).

(f) *Ten healthy subjects with a range age from 23 to 46 (mean age = 34 ± 8 years). Plasma orexin A concentrations were determined by ELISA. Results are reported as mean ± SD. Significant differences are p<0.05. The data present is from:* (130).

(g) *Fifty-one female healthy subjects with a range age from 18 to 60 years old. Plasma orexin A concentrations were determined by ELISA. Significant differences are p<0.05. The data present is from:* (131).

(h) *Twenty-four Japanese people from 19 to 68 years old. Orexin-A was measured using a radio- immunoassy kit. The data present is from:* (132).

(i) *Twenty-nine healthy subjects (23 women and 6 men) with a range age from 21 to 77 years (mean age = 47.86 ± 15.76 years). Plasma orexin A concentrations were determined with by ELISA. Results are reported as mean ± SD. Significant differences are p<0.05. The data present is from:* (133).

(j) *Thirty-nine healthy subjects with a range age from 18 to 70 years. Plasma orexin A concentrations were determined by ELISA. Significant differences are p<0.05. The data present is from:* (134).

Hence, in humans, the plasmatic orexin concentrations follow the model designed in Figure 5.

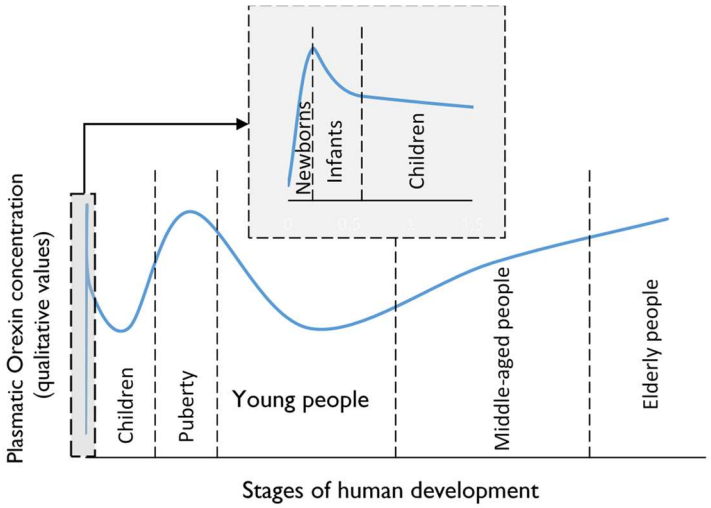

Figure 5 – Graphical representation of plasmatic OX concentration throughout human life *(based on data previously mentioned).*

As orexin neurons, the orexin receptors, in the human brain, are reduced during the aging process and this reduction might be observed in one or both receptors. Their expression has species-specific differences throughout life (7,121).

Similarly to humans, in rats, their development is also characterized by alterations in OX system.

In rats, before birth (perinatal period), PPO mRNA was found in lower levels in every cerebral zone, except in hypothalamus, and it was increased gradually during neonatal (0 to 7 days) and infancy periods (8 to 20 days) until reaching the maximum at 20th day after birth and continued to be highly expressed in adults (28,62,76,81,92,117). The levels were not detected until 15th day after birth, in rats, as well as the immunoreactivity to OX (81) because the PPO cleavage only occurs since 10th to 14th day after birth (62,135).

The number of OX-A cells gets higher from 2 weeks to 9 weeks with a significate increase between 6 and 9 weeks. From 9 weeks, the number of OX-A and OX-B cells in rats and the intensity of immunoreactivity continues to increase. From 4 months up to 8 months, the levels remain stable and, between 8 and 24 months, the number of OX cells and the quantity of OX decrease (135). The biggest decrease of OX happens at 12-month old, despite the fact that aging is associated to a continuous deterioration of physiological functions (between 3 and 24 months) (6,111,136). This decrease might be due to a reduction in the number of cells that express OX or in the number of OX fibres (135).

The total number of OX-A cells is about 3998 ± 459 in young animals (3–4 months) (3). In aged animals (26–28 months), the number is 2323 ± 271 (3) and the expression of PPO suffer a decrease of 28% (6). However, OX levels do not influence sexual maturation in mice and the OX-A levels in CSF remain unchanged (93).

In face of the reduction of OX cells, OD of OX-A and OX-B, that increases between 2th and 9th weeks and is higher at 9th week, shows a decrease between 4th and 24th months (135).

Comparatively to Hcrt gene, the expression of Hcrtr1 gene increases gradually between day 0 to day 20 and remains up until 10th week (117). From 20th day, the levels are similar to adults (76). Therefore, OX1R and OX2R are highly expressed at the day of the birth and then decrease gradually with age (62).

6. Orexin: a possible biomarker?

The WHO has defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (137).

OX has associated to several physiological and regulatory changes as sleep diseases (such as narcolepsy) and metabolic and endocrine disorders (such as obesity) (4,93). Considering these facts and the fact that orexin has an important role in physiological processes, it was considered the hypothesis of orexin as a biomarker.

Initially, in narcolepsy, it was studied the possibility of orexin to be a biomarker. However, it was disregarded, because, despite the fact that narcolepsy is characterized by a decrease in OX-immunoreactivity neurons (4,65) and a destruction of OX neurons (64,87,104), the plasma OX levels were normal (7,138). Therefore, it is not possible to test using a blood test (139). However, the OX might be detected in the brain by imaging technique (as single photon emission computed tomography, SPECT, Positron emission tomography, PET, or functional magnetic resonance imaging, fMRI (140,141)). Thereby, with the evolution of these imaging techniques, OX might become a biomarker of narcolepsy.

In obesity, it was also thought that OX could be a biomarker because the plasma OX levels are decreased in obese people (7,110). However, it was not observed in all cases and sometimes the decrease of OX levels might be linked to other physiological changes.

Besides these, OX has been yet defined as a biomarker in some disorders as fatigue and cognitive performance (142) and more studies are being done in neurodegenerative diseases (as Alzheimer's disease or multiple sclerosis) in order to understand if OX is a good biomarker (143).

Aging is a very heterogeneous and complex physiological process (2) and the mechanism underlying this process is still under investigation. However, currently, it is still not possible to define OX as a biomarker of aging because not all the changes observed in hypothalamus during the aging process are translated into changes in plasma OX concentration. Although, with the development of robust and high-performance imaging techniques, OX might be considered a biomarker of aging and age-related diseases.

Hence, with this experimental study we expect to shed light on how accelerated aging impacts on OX and their function and whether these alterations contribute to Hutchinson-Gilford Progeria Syndrome (HGPS) and other premature aging disorders, unravelling a role for hypothalamus on premature aging progression. We also expect to provide OX as an innovative therapeutic strategy for the treatment of HGPS and other premature aging disorders. Moreover, given the similarities between HGPS phenotype and normal aging phenotype, the results will also contribute to a better understanding of OX as an anti-aging molecule and a possible biomarker of aging.

II. Experimental study: Is orexin a potential biomarker or therapeutic target in mouse model of human aging?

A. Introduction

HGPS, commonly known as Progeria, is a rare genetic condition characterized by premature and accelerated aging (144,145). This disease was first described in 1886 by Jonathan Hutchinson, in a 3 and half-year old patient with alopecia and mammary glands with atrophic condition and some other symptoms (146). Later, a second case was described, in more detail, by Hastings Gilford, in 1897 (147). Afterwards, Gilford crossed the information between these two patients and designated the disease as a premature aging syndrome based on the overall resemblance of patients to aged individuals (148). For about one century, the characterization of this syndrome was extremely difficult due to the fact that it is one of the rarest diseases in the world and, only in 2003, the cause for HGPS was found. Two independent groups showed that HGPS is caused by mutations on the Lamin A/C (LMNA) gene, that encodes for Lamin A/C proteins, resulting in the distortion of the nuclear envelope (149-151). Since then, this disease has gained more attention, especially due to the similarity with normal aging.

Progerin, the truncated protein that results from the mutation in the LMNA gene, is expressed in several tissues including skin, bone, skeletal muscle, adipose tissue, heart and large and small arteries. Their expression induces defects in cell mechanisms, alterations in stability and structural and mechanical changes in nucleus which might result in a cellular decline and an impairment of cell division (151,152). These lead to differentiation defects and premature cellular aging (153).

Initially, it was very difficult to access to samples due to the fact that HGPS is an extremely rare disease and the affected children were fragile. A part of this problem is solve by the development of induced pluripotent stem cells (iPSCs) from HGPS patient's fibroblasts (154,155). These cells have HGPS phenotype and, after their differentiation, they express progerin and exhibit some HGPS characteristics as cell senescence, shortening of telomeres and other signs of accelerated aging (154). This *in vitro* model is a great advantage and allow a better understanding of the mechanism of the disease (156). However, these findings need to be confirmed by *in vivo* models. Despite of the fact that several *in vivo* models were proposed (157,158) (for example the *Zmpste24*-deficient mice (159)) and they reproduced most of the phenotypic alterations of HGPS, none of them was capable of reproducing the molecular alterations, namely progerin accumulation. More recently, a new

model was produced and it is the one that most accurately represents progeria. This model is known as $Lmna^{G609G/G609G}$ mice and, apart from carries a mutation in LMNA gene analogous to the one found in HGPS patients, it also expresses progerin. As consequence of progerin accumulation, these model show nuclear abnormalities with higher amounts of DNA double-strand breaks and premature cellular senescence (160).

$Lmna^{G609G/G609G}$ seems healthy until 3 weeks of age but then start to show clinical signs of HGPS, such as reduction in growth rates, progressive loss of weight, cardiovascular, bone and skin abnormalities and reduced lifespan (average life of 103 days) (160).

In the present study, the *Lmna*$^{G609G/G609G}$ mice was used as a model of a premature aging disease in order to understand the relation between the molecular mechanisms underlying HGPS pathology and the aging process.

The aim of the study is the evaluation of the alterations in OX system in hypothalamus in a mouse model of progeria.

B. Methods and materials

1. Animals

In the present study, wild-type C57BL/6 mice (*Lmna*$^{+/+}$) and *Lmna*$^{G609G/G609G}$ mice (C57BL/6 genetic background) were used to perform all experiments. These different genotypes were obtained through heterozygous males and females crossings, as *Lmna*$^{G609G/G609G}$ mice are infertile. Mice were housed two to four *per* cage, in a 12-hour light/dark, with controlled temperature and humidity with *ad libitum* access to water and standard chow diet.

All the experiments were performed under the European Community Directive for the Care and Use of Animals in Laboratory (2010/63/EU) which was translated to the Portuguese law in 2013 (Decree-law 113/2013). The animals were housed in our licensed animal facility (International Animal Welfare Assurance number 520.000.000.2006). All investigators who handled the mice have received an appropriate education (FELASA course) and are credited to perform animal experimentation as required by the Portuguese authorities. The present study is included in projects approved and financed by the Progeria Research foundation that approved the utilization of animals for these projects (PRF2014-53 and PRF2015-60).

1.1. Impact of premature aging on the hypothalamus of *Lmna*$^{G609G/G609G}$ mice

To characterize the alterations that occur in the hypothalamus of *Lmna*$^{G609G/G609G}$ mice along the aging process, mice of the two genotypes were sacrificed at different time points: *Lmna*$^{+/+}$ and *Lmna*$^{G609G/G609G}$ mice were sacrificed at 1.5 and 3 months of age.

1.2. Tissue Collection

Animals from each experimental group were randomly selected either for whole brain removal for immunohistochemistry experiments, or for collection of blood, hypothalamic tissue for RNA extraction and peripheral organs extraction for histological and RNA/protein analysis.

For immunohistochemistry, mice were euthanazied with an overdose of avertin (tribromoethanol; 2.5 times 14 uL/g; 250 mg/kg intraperitoneal) and then intracardially perfused with 4% (w/v) paraformaldeheyde/0.1 M phosphate buffered saline (PBS; 137 mM NaCl; 2.7 mM KCl; 10 mM Na_2PO_4; 1.8 mM KH_2PO_4; pH 7.4) fixative solution. After decapitation, the whole brain was collected and fixed for 48 hours in 4% paraformaldehyde (w/v) in PBS and then cryopreserved in 30% sucrose/PBS solution (w/v) for 72 hours. Brains were dried and stored at -80°C until use.

For hypothalamus analysis, mice were first anesthetized with isofluorane (Abbot) and then decapitated. After decapitation, brain was removed and the hypothalamus dissected and stored at -80°C for posterior processing. A total of 3-6 animals were used *per* time point, for each genotype.

2. Gene expression analysis

2.1. Purification and quantification of total RNA

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Briefly, cells were lysed, the total RNA was adsorbed to a silica matrix, washed with the recommended buffers and eluted with 30 µL of RNase-free water by centrifugation. Total RNA amount was quantified by OD measurements using a ND-1000 Nanodrop Spectrophotometer (Thermo Scientific), and the purity was evaluated by measuring the ratio of OD at 260 and 280 nm. RNA samples were treated with RNase-free DNAse (Qiagen) to eliminate any contamination with genomic DNA. RNA samples were kept at -80°C until use.

2.2. Reverse transcription

Reverse transcription into cDNA was carried out using the iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's instructions. Briefly, 1 μg of total RNA from each sample was reverse transcribed into cDNA in a 30 μL reaction containing 1x iScript reaction buffer, and 1 μL of iScript reverse transcriptase. Reverse transcription reactions were performed in a thermocycler at 25°C for 5 minutes, 46°C for 30 minutes, 95°C for 5 minutes, and 4°C for 5 minutes. cDNA samples were then stored at -20°C until use.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was performed in the StepOne Plus Real-Time PCR System (Applied Biosystems) using 96-well microliter plates and the SsoAdvanced SYBR Green Supermix (Bio-Rad). All primers used were designed using PrimerBlast Software and produced by Invitrogen. The following primers sequences were used: OX (Fw: 5'-TGGGTATTTGGACCACTGCAC-3'; Rv: 5'-CCAGGGAACCTTTGTAGAAGGAA-3'), OX1R (Fw: 5'-AGATGTGCTGGTGACTGCC-3'; Rv: 5'-ACCGACACAGCCTGGAGATA-3'), OX2R Fw: 5'-CACTGGGGCGGTGAAGTTTA-3'; Rv: 5'-TGTCGGCACCAGAGTTTACG-3') and mouse HPRT (Fw: 5'-GCTTACCTCACTGCTTTCCG-3'; Rv: 5'-CATCATCGCTAATCACGACGC-3'). For each primer set, qPCR reactions were carried out in 10 μL reaction volume containing 5 μL of 2× SsoAdvanced SYBR Green Supermix, 0.5 μL of forward primer (500 nM), 0.5 μL of reverse primer (500 nM) and 4 μL of template cDNA. Appropriate negative controls were also prepared. All reactions were performed in duplicate and according to the manufacturer's recommendations: 95°C for 30 seconds, followed by 45 cycles at 95°C for 5 seconds and 56 – 60°C for 30 seconds. The melting curve protocol started immediately after amplification with the following protocol: 65°C to 95°C with 0.5°C increments, 5 seconds /step and 95°C for 10 seconds. For each primer set, the specific annealing temperature and the quantity of cDNA used: 56°C for OX, 60°C for OX1R and OX2R and 58°C for HPRT. The amplification efficiency for each gene and the threshold values for threshold cycle determination (Ct) were determined automatically by the StepOne Software (Applied Biosystems). Relative mRNA quantification was performed using the ΔCt method for genes with the same amplification efficiency using hypoxanthine-guanine phosphoribosyltransferase (HPRT) as the endogenous housekeeping gene.

3. Protein expression analysis

3.1. Immunohistochemistry

Brains collected for the immunohistochemistry studies were included in Tissue-Tek optimum cutting temperature (OCT) compound (Sakura Finetek) and then sectioned into 25 µm coronal sections at -20°C using a cryostat-microtome Leica CM3050S (Leica Microsystems Nussloch GmbH, Nußloch, Germany). Sections were collected and stored in 48-well plates, free floating in 0.1 M PBS supplemented with 0.05% (v/v) sodium azide, and stored at 4°C until use. The Paxino's Mouse Brain Atlas was used to define the coordinates for the beginning (1.18 mm Bregma) and end (-2.92 mm Bregma) of the sectioning in order to assure the representation of the whole hypothalamus.

Hypothalamic Orexin-A protein levels were assessed by immunohistochemistry on mice brain sections. For that, coronal sections of approximately equal spacing were sampled over the anterior–posterior extent of the hypothalamus (approximately between Bregma -0.46 mm to -2.80 mm using The Paxino's Mouse Brain Atlas). For immunohistochemistry processing, brain coronal sections were washed three times with PBS (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2PO_4; 1.8 mM KH_2PO^4; pH 7.4) and then blocked and permeabilized for 1 hour at room temperature, in PBS supplemented with 10% goat serum (Sigma) and 0.3% (v/v) triton X-100 (Merk Millipore). Afterwards, brain coronal sections were incubated with the following primary antibodies: rabbit anti-Orexin A (1:1000; Phoenix Pharmaceuticals) and mouse anti-NeuN (1:500; Chemicon) diluted in the blocking solution, overnight at 4°C. For immunofluorescence antibody detection, sections were incubated with the following secondary antibodies: anti-rabbit Alexa Fluor 488, anti-anti-mouse Alexa Fluor 568 (1:500; Invitrogen) and nuclei were stained with Hoechst 33342 (2 µg/mL; Invitrogen). After incubation, brain sections were washed four times in PBS and mounted in slides with Mowiol mounting medium (Sigma-Aldrich).

Brain coronal sections were analyzed on an Axio Imager Z2 (Carl Zeiss). For hypothalamic protein immunoreactivity determination, tiles images encompassing the hypothalamus were acquired using a Plan-Apochromat 20x/0.8 M27 objective.

3.2. Quantification of OX immunoreactivity in the hypothalamic nuclei

For Orexin-A immunoreactivity, ten coronal sections of approximately equal spacing were sampled over the anterior–posterior extent of the hypothalamic ARC using the Paxino's Mouse Brain Atlas as reference. Orexin-A immunostaining cell number was counted manually using Image J software and values obtained for each mouse were added and it was

determined the average for which group, before group mean was determined. Immunostaining is expressed as the average value/hypothalamus/group.

4. Statistical analysis

All the results are expressed as mean ± standard error of the mean (SEM). The statistical analysis was performed using two-way analysis of variance (ANOVA), one-way ANOVA followed by or Bonferroni's multiple comparisons test or unpaired Student's t test, depending on the number of experimental groups or the number of variables to be analysed in each experiment. For the statistical analysis Prism 8.4.2 (GraphPad Software) was used.

C. Results

1. Impact of premature aging on the hypothalamus of *Lmna*$^{G609G/G609G}$ mice

Hypothalamus has a key role in whole-body aging (20,56). This organ is responsible for the regulation of body homeostasis, through the integration of central and peripheral signals, and coordination of several physiological functions as sleep, food intake, reproduction, neuroendocrine axis and metabolism (22,25,161). Therefore, a dysregulation or a decline in these neurons with age affect the whole body and might lead to homeostatic imbalance and accelerating aging (20,162-164).

Considering these facts and that HGPS is characterized by a premature aging, we investigate the impact of premature aging on the hypothalamus of *Lmna*$^{G609G/G609G}$ mice. Brains of *Lmna*$^{+/+}$ and *Lmna*$^{G609G/G609G}$ mice were collected at 1.5 and 3.0 months and processed for determination of protein immunoreactivity by immunohistochemistry or mRNA levels (qRT-PCR) of orexin. Protein immunoreactivity was evaluated in arcuate nucleus (ARC). Gene expression analysis was performed in whole hypothalamus.

1.1. Premature aging on hypothalamic orexin neurons and orexin receptors

At 1.5 and 3.0 months of age, the *Lmna*$^{G609G/G609G}$ and *Lmna*$^{+/+}$ mice have similar number of OX-positive neurons in hypothalamus (Figure 6.A and B).

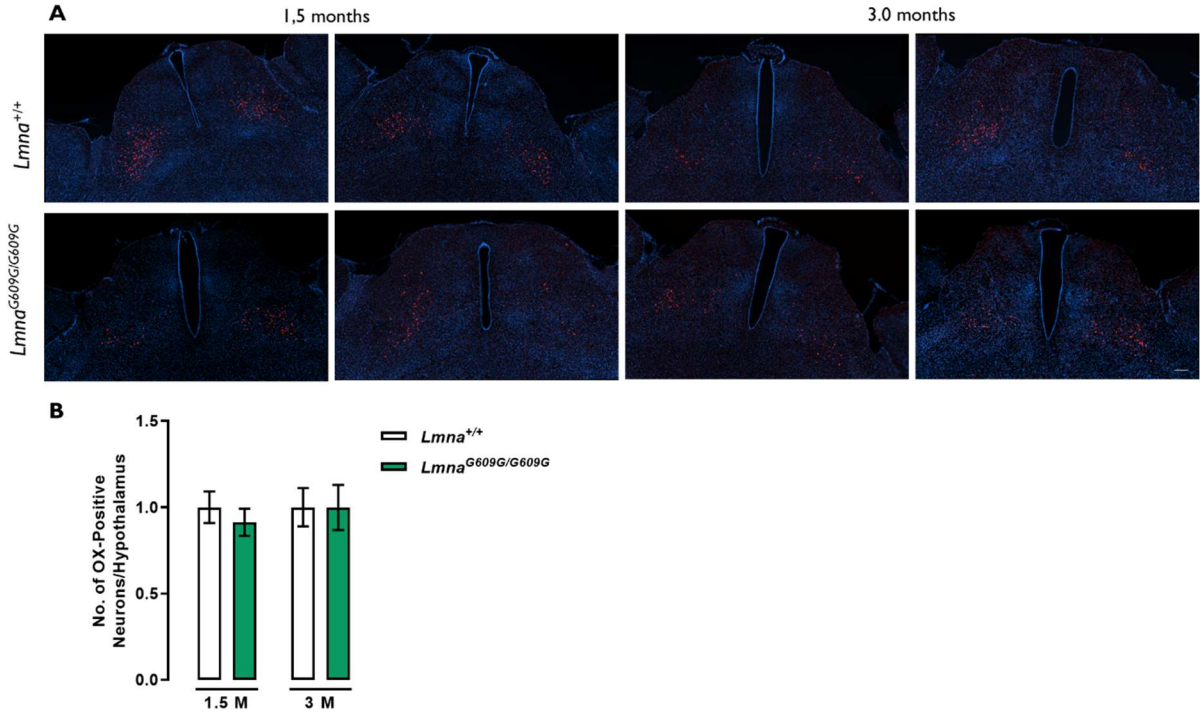

Figure 6 – OX immunoreactivity in the hypothalamus of Lmna$^{+/+}$ and Lmna$^{G609G/G609G}$ mice along time. *(A) Representative images of OX immunoreactivity in the hypothalamus of 1.5 and 3.0 months-old Lmna$^{+/+}$ and Lmna$^{G609G/G609G}$ mice. OX (Red) and nuclei are stained with Hoescht 33342 (blue). Scale bar, 200 µm. (B) Quantification of OX immunoreactivity in the hypothalamus through the anterior–posterior extent of the hypothalamic mouse ARC. The results represent the mean ± SEM of the total integrated density normalized to OX-positive neurons of Lmna$^{+/+}$* mice *(n=3-8 mice per group).*

We also assessed whether premature aging alters hypothalamic gene expression in *Lmna$^{G609G/G609G}$* mice. The whole hypothalamus was collected from *Lmna$^{G609G/G609G}$* and *Lmna$^{+/+}$* mice at 1.5 and 3.0 months of age for RNA extraction. Gene expression analysis was assessed by qRT-PCR.

As shown in Figure 7A, the OX mRNA levels in *Lmna$^{G609G/G609G}$* mice were lower at 3.0 months comparing with *Lmna$^{G609G/G609G}$* mice with 1.5 months of age. Furthermore, *Lmna$^{G609G/G609G}$* mice showed a decrease in OX mRNA levels at both ages (1.5 and 3.0 months of age) comparatively with *Lmna$^{+/+}$* mice.

We also analysed the changes of OX receptors expression with age. The OX1R mRNA levels in *Lmna$^{G609G/G609G}$* mice were lower, comparing to wild-type mice (Figure 7B). The OX2R mRNA levels are similar in *Lmna$^{G609G/G609G}$* and *Lmna$^{+/+}$* mice at 1.5 or 3.0 months of age (Figure 7C).

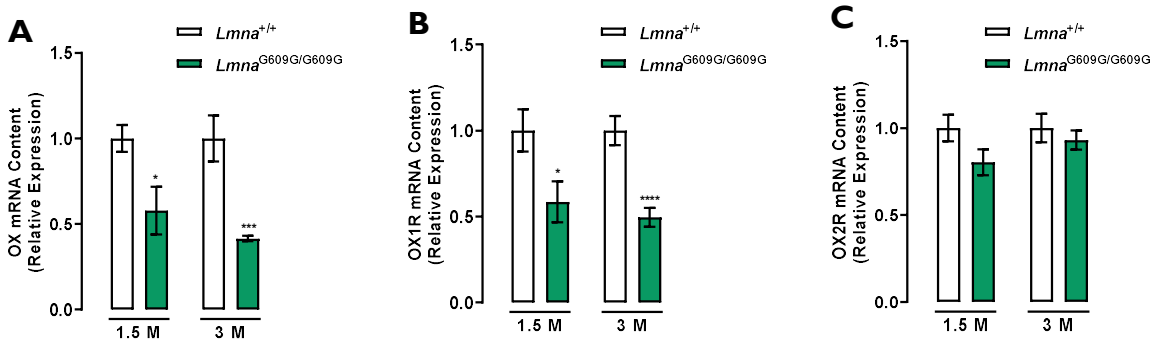

Figura 7 – Comparative analysis of hypothalamic gene expression of 1.5 and 3.0 month-old Lmna$^{+/+}$ and Lmna$^{G609G/G609G}$ mice. *Gene expression analysis of (A) OX neuropeptide; (B) OX1R; (C) OX2R in hypothalamus of 1.5 and 3.0 months-old Lmna$^{+/+}$ and Lmna$^{G609G/G609G}$ mice. The results represent the mean ± SEM of the relative expression normalized to Lmna$^{+/+}$ mice. (n=8-10 mice per group). *p<0.05, ***p<0.001 significantly different.*

III. Discussion/Conclusion

Aging is characterized by a time-dependent progressive decline in cellular and tissue function. Therefore, the risk of development several diseases, as neurodegenerative and metabolic diseases, are increased (2).

The desynchronization and coordination defect of autonomic, metabolic, endocrine and vigilance functions (6), that define the aging process, might be related to the decline of OX signalling (3). The OX system expression, in aging, is altered through the decrease of OX expression (3), the reduction of OX immunoreactivity (98) and the decrease of OX receptors' mRNA (65,111).

HGPS is a rare genetic disorder characterized by premature and accelerated aging, causing premature death in children at an average age of 14.6 years old (152,156,165,166). It has features of premature aging and affects multiple organs/tissues with some similar features observed in natural aging (153). Due to these similarities, new therapeutic compounds has been studied and discovered in order to delay this fatal disease but also to delay natural aging (152,156).

In order to investigate OX as a potential therapeutic strategy and a possible biomarker to thwart the progression of premature aging, we used *Lmna$^{G609G/G609G}$* mice as experimental model.

Firstly, we analysed the OX levels in hypothalamus, since the hypothalamic function is strongly related to the action of this neuropeptide. From previous reports, we know that OX levels are decreased with age in rodents (6,111,135,136) and elderly humans (65).

Although by immunohistochemistry analysis, we did not observe differences in both genotypes, by qRT-PCR, the OX mRNA levels in progeria mice were significant lower (about 50 %) when compared to wild-type mice at 1.5 and 3.0 months of age.

Lmna$^{G609G/G609G}$ mice seem normal until 3 weeks after birth, when they start to present the main clinical features of the disorder, including cardiovascular and bone abnormalities, impaired growth rate, low body weight, lipodystrophy and alopecia. Their lifespan is, on average, 103 days, while the wild type mice usually live for more than 2 years (160). The abnormalities culminate in some molecular and cellular alterations, namely loss of nuclear architecture, downregulating of gene expression and alteration of DNA structure (153,165) and these might explain the decrease of OX immunoreativity in *Lmna*$^{G609G/G609G}$ mice, through the downregulation of Hcrt gene.

However, at 3.0 months of age, the OX levels were similar between *Lmna*$^{+/+}$ and *Lmna*$^{G609G/G609G}$ mice. Besides these unexpected results, they might be explained by the fact that *Lmna*$^{+/+}$mice, at this age, start the natural aging process follow by a decrease in the cells that express OX, with or without neuronal loss (6,111,135,136) or by the protection given by microRNA-9 (miR-9) in *Lmna*$^{G609G/G609G}$ mice. Since miR-9 limits the high expression of progerin and, consequently may prevent the risk of neuronal loss (167), it could explain the similar number of orexin neurons in both genotypes.

To support our results, and further explore the alterations occurring in OX neuropeptide in the hypothalamus of *Lmna*$^{G609G/G609G}$ mice, we analysed OX receptors (OX1R and OX2R) gene expression within hypothalamus by qRT-PCR. In *Lmna*$^{G609G/G609G}$ mice, comparatively to *Lmna*$^{+/+}$mice, we observed lower levels of OX1R mRNA levels at 1.5 months and 3.0 months of age. This lower levels might be due to the fact that *Lmna*$^{G609G/G609G}$ mice have a premature aging and that the aging process is characterized by a decrease in OX synthesis (3,7,65), but also by a decrease in the number of mRNA copies *per* cell or in the number of cells producing mRNA (94,108). The wild-type mice, *Lmna*$^{+/+}$mice, have higher levels than *Lmna*$^{G609G/G609G}$ mice because, at the age of 1.5 and 3.0 months, the normal aging process is still not started or it is in the beginning.

Relatively to OX2R mRNA levels in *Lmna*$^{G609G/G609G}$ mice comparing to *Lmna*$^{+/+}$mice, we observed a decrease in these levels at 1.5 months of age. However, at 3.0 months-old, the decrease was not statically significant. These results were also observed by other authors (121), although it is still not known the underlying mechanism for this observation.

To summarize, it is possible to establish an association between orexin system and HGPS. This lethal genetic disorder has some similarities with the normal aging process such as the decrease of OX and OX receptors mRNA levels.

Hence, OX might be considered as a future therapeutic strategy for aging-related diseases, including for HGPS.

IV. Future perspectives

OX is a neuropeptide produced mainly in hypothalamus and has a role in several and diverse functions. Thereby, it is named as "physiological regulator" and guarantees the homeostatic state of human body (4).

Its interaction with several physiological functions and its role in many diseases lead to one question: Is orexin a biomarker in these situations? Due to its relation in fatigue and cognitive performance, it was considered as a biomarker (142). The possibility of orexin being a biomarker in aging was investigated, however the alterations in plasma OX levels are not always related to the aging process. Nevertheless, the development of robust and high-performance imaging techniques might be the basis for the designation of OX as a biomarker of aging and age-related diseases.

In HGSP, OX could not be defined as a biomarker since this disorder has a genetic cause (151,166,168). Although, OX was showed that might be a future therapeutic strategy for this disorder and other aging-related diseases because the orexin neurons activity and/or their receptor function might increase, through the raised levels of OX during the aging process (7).

The ectopic expression of orexin might also allow the control of the natural loss of 40-50 % of these neurons, although this expression is limited to areas with orexin receptors and MCH neurons (4).

Therefore, more research should be done in order to have a better understand of the relation between OX and premature aging diseases and aging-related diseases.

In the other hand, the routes of administration for OX also need to be more investigated.

In narcoleptic canines, the systemic OX delivery had therapeutic efficacy, however OX suffered peripheral degradation and had a low capacity to cross blood–brain barrier (BBB) and significant secondary effects. Besides that, brain penetration and a distribution of peptides and proteins can be affected by multiple factors as molecular weight, tertiary

structure, lipophilia and receptor localization and, when OX is in CNS, it can spread to various rostral and caudal brain regions.

Therefore, the systemic administration of OX has still many limitations. For example, OX-B has low lipophilia and it is rapidly degraded by metabolization and inactivation by peptidases, then it is not capable of crossing the BBB. To solve this problem, an insertion of 2 amino acids is made in OX-B peptide and originate [Ala^{11}, D-Leu^{15}]-OX-B, which has an increase in the affinity to OX2R in 400 times and also increases the neuronal activation.

The intranasal administration might also be considered another solution for the limitations of systemic administration, since it allows the delivery to CNS, decreases peripheral complications and crosses BBB. The time and the extension of peptide delivery depends on the size of the peptide, its lipophilia and the transportation methods from olfactory mucous to the brain. The transportation mechanisms of OX to brain are not completely known but it is thought that carrier proteins present in olfactory and trigeminal nerve of nasal epithelium proceed to olfactory bulb and sensory/spinal trigeminal regions of the pons (origin of chemosensory and somatosensory innervation of nasal mucous).

The evidence shows that intranasal administration of OX-A increases neuronal activation and glutamatergic and cholinergic neurotransmission. Then, it could increase attentional processing and might be useful to cognitive disorders' treatment.

Besides all advantages of intranasal administration, this also have side effects as amyloid plaque formation (88).

The administration of OX might be also a pharmacological treatment in sleep disorders, such as narcolepsy. It has been studied and the systemic administration of OX-A showed effects in the reversion of sleepiness and disrupted night-time sleep and in the reduction of cataplexy in narcoleptic dogs (104).

The discovery of OX agonist and antagonists and their importance in sleep disorders was revealed as promising therapeutic innovation. The agonist of OX-B receptor no-peptide (YNT-185) is a possible solution for narcoleptic patients (88) and the suvorexant, in 2014, became the first US Food and Drug Administration (FDA) approved dual OX receptor antagonist for the treatment of insomnia (107). Other two selective OX2R antagonists have been tested in humans, MK-1064 and JNJ-42847922, and both demonstrated a dose-dependent reduction in alertness/increased sleepiness (169).

Sleep disorders like excessive daytime sleep or fragmentation of sleep (8) are commons situations in elderly (3,6) and are associated to a dysfunction in OX system (65,70,94).

Thereby, these new approaches developed for sleep disorders might also be a future pharmaceutic strategy for aging and aging-related diseases due to the relation between these disorders and the aging process, *per se*.

In conclusion, the results may suggest that OX administration could be a potential therapeutic strategy for HGPS and other age-related diseases. However, it is needed further understand about the mechanisms underlying orexin's effect in order to establish orexin as a promising therapeutic strategy to block HGPS or aging-related diseases progression. The routes of administration for OX and the effects of OX agonists in diseases related to aging need also to be under investigation.

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