UNIVERSIDADE D
COIMBRA

Diogo Miguel Alves Pratas Mano

Relatórios de Estágio e Monografia intitulada "AAV pre-existing immunity: strategies to develop AAV vectors with enhanced immune evasion" referente à Unidade Curricular "Estágio", sob orientação, da Dra. Dina Lopes, da Dra. Elisa Silva e do Professor Doutor Luís Pereira de Almeida e 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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Setembro 2020

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Coimbra, 1 de setembro de 2020.

Diogo Miguel Alves Pratas Mano

(Diogo Miguel Alves Pratas Mano)

Agradecimentos

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À Faculdade de Farmácia de Faculdade de Coimbra por estes anos inesquecíveis.

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Parte I

Relatório de Estágio

Farmácia Guarda Inglesa

Lista de Abreviaturas

DCI – Denominação Comum Internacional

FFUC – Faculdade de Farmácia da Universidade de Coimbra

IMC – Índice de Massa Corporal

MICF – Mestrado Integrado em Ciências Farmacêuticas

MNSRM – Medicamento não sujeito a receita médica

MNSRM-EF – Medicamento não sujeito a receita médica de dispensa exclusiva em farmácia

MSRM – Medicamento sujeito a receita médica

PVP – Preço de Venda ao Público

SWOT – *Strengths, Weaknesses, Opportunities, Threats*

I. Introdução

O Mestrado Integrado em Ciências Farmacêuticas (MICF) da Faculdade de Farmácia da Universidade de Coimbra (FFUC) contempla, no seu plano de estudos, a realização de um estagio curricular em Farmácia Comunitária, cuja concretização é necessária para o término do curso, proporcionando aos estudantes a oportunidade de consolidar e aplicar os conhecimentos adquiridos durante o seu percurso académico e que funcionará como a primeira ponte relativamente a esta área profissional do setor farmacêutico na qual a maioria dos estudantes irão exercer sua futura função[1].

A Farmácia Comunitária é uma das áreas do setor farmacêutico com valor e relevância inegável já que, como referido anteriormente, é a área na qual a maioria dos farmacêuticos irá exercitar a sua função, mas também devido o seu papel fundamental na garantia do acesso à medicação por parte da população, como é verificado nos atos de dispensa e aconselhamento, na prestação de cuidados e serviços de saúde, na informação e educação dos utentes e na promoção da saúde[2]. Posto isto torna-se evidente o papel do Farmacêutico Comunitário como profissional de saúde, cujo principal dever é zelar pelo bem-estar da população[3], bem como a necessidade de uma formação completa que permita o adquirir das competências e valências necessárias para a correta exerção desta função.

O presente relatório relativo ao estágio realizado na Farmácia Guarda Inglesa, de 4 de maio de 2020 a 21 de agosto de 2020, sob a orientação da Dra. Elisa Silva apresenta-se sob a forma de análise SWOT (*Strengths, Weaknesses, Opportunities, Threats*), com o objetivo da identificação e análise crítica dos pontos fortes, pontos fracos, oportunidades e ameaças relativos a este. Além desta análise também serão apresentados casos clínicos vivenciados durante o período de estágio que acredito que me permitiram aplicar bem como consolidar os conhecimentos adquiridos.

2. Análise SWOT

Como já referido e descrito nas "Normas Orientadoras de Estágio do Mestrado Integrado em Ciências Farmacêuticas"[4], o presente relatório apresenta-se sob a estrutura de Análise SWOT de forma a avaliar de forma crítica a minha experiência durante o estágio realizado na Farmácia Guarda Inglesa, como membro integrante da respetiva equipa. Esta análise discriminará, numa vertente interna, os pontos fortes e fracos do estágio realizado e também, numa dimensão mais externa, as oportunidades e ameaças do mesmo.

2.1. Pontos Fortes

2.1.1. Acolhimento e Integração na Equipa

Durante o período de estágio no Farmácia Guarda Inglesa tive a oportunidade de contactar com profissionais altamente qualificados onde se realçava, em cada elemento, a motivação e dedicação clara de manter o bom funcionamento da farmácia e prestar o melhores serviços e cuidados possíveis aos utentes. Posto isto, ao longo do estágio, foram-me ensinados quais os princípios básicos da gestão de uma farmácia e de todas as tarefas associadas à vertente de Farmácia Comunitária, contribuindo assim para a minha formação nesta área. É de notar que a equipa sempre se mostrou disponível para esclarecer qualquer dúvida que surgisse no decorrer do estágio e sempre me apoiou na realização das tarefas a meu cargo, promovendo assim uma fácil integração no dia-a-dia da farmácia. Assim, o ambiente de trabalho que tive oportunidade de vivenciar na Farmácia Guarda Inglesa contribuiu de forma bastante positiva para a minha integração na esquipa de trabalho, para a aquisição e consolidação de conhecimentos relativos à área de Farmácia Comunitária e para o meu desenvolvimento e obtenção de novas valências e competências.

2.1.2. Número de Estagiários

Durante uma porção significativa do estágio fui o único estagiário presente na farmácia, possibilitando assim uma maior envolvência no conjunto de atividades realizadas na farmácia. O facto de ser o único estagiário durante grande parte do estágio permitiu também um maior acompanhamento por parte da equipa, já que este não tinha de ser distribuído por vários estagiários, bem como a sua personalização, melhorando assim a minha aprendizagem.

2.1.3. Tarefas Desempenhadas e Autonomia

No decorrer do estágio tive a oportunidade de desempenhar as várias tarefas necessárias ao bom funcionamento de uma farmácia. Numa primeira fase, as minhas principais

atividades tinham como base a receção de encomendas de medicamentos e outros produtos de saúde e respetiva arrumação. Deve ser notada a grande importância destas atividades de *back office* para a gestão e bom funcionamento da farmácia. Ao realizar estas tarefas foi-me introduzida a relevância da verificação dos prazos de validade, número de embalagens, preços de vendo ao publico (PVP) e margens de comercialização de produtos de venda livre. Só após a verificação deste parâmetros é que se poderia proceder à arrumação dos produtos onde também me foi introduzido o principio "*first in, first out*", e me foi demostrada as particularidades de medicamentos com condições especiais de conservação, como aqueles que necessitavam de ser conservados a temperaturas inferiores a 8°C, cuja arrumação era priorizada relativamente receção. Assim, estas tarefas permitiram a familiarização com os diversos medicamentos, tanto medicamentos sujeitos a receita médica (MSRM) como medicamentos não sujeitos a receita médica (MNSRM), e outros produtos passiveis de serem comercializados em farmácias, bem como a associação entre as diversas marcas de medicamentos e os respetivos princípios ativos, expressos em Denominação Internacional Comum (DCI).

Numa fase seguinte comecei a observar o atendimento aos utentes da farmácia e de posteriormente ser eu mesmo a fazê-lo, sempre com a supervisão de algum membro da equipa, o que me permitiu desenvolver as minhas capacidades de despensa de prescrições médicas e de aconselhamento ao publico. Após algum tempo já me sentia suficientemente confortável para poder desempenhar estas tarefas sozinho e sentindo-me apto para lidar com diferentes os tipos de cenários inerentes à profissão de farmacêutico comunitário, bem com apresentar um pensamento crítico relativamente a estes.

Posto isto, considero que a introdução gradual e subsequente consolidação dos conhecimentos relativos às tarefas quem me foram atribuídas, bem como a autonomia que me era concedida para a realização das mesmas contribui de forma bastante positiva para a aquisição de valências e competências fundamentais para um bom exercício da profissão de farmacêutico comunitário.

2.1.4. Preparação de Medicação

No decorrer do estágio pude constatar que nem todos os medicamentos são comercializados na forma sob a qual serão administrados, como é o caso de alguns antibióticos que, apesar de serem administrados sob a forma de suspensão oral, são comercializados sob a forma de pó liofilizado. Posto isto muitos utentes preferem que a preparação deste tipo de produtos seja feita na farmácia, para, por exemplo, evitar erros de dosagem decorrentes de uma diluição realizada incorretamente. Assim tive a oportunidade de realizar a preparação

destes produtos e de fazer as recomendações e precauções inerentes a estes, como reiterar as condições de conservação ou a necessidade de agitar o frasco vigorosamente previamente a cada administração.

2.1.5. Sifarma2000®

A Farmácia Guarda Inglesa usa o Sifarma2000® como *software* de gestão da farmácia, sendo este o sistema informativo mais usado a nível nacional pelas farmácias. De facto, o Sifarma2000® está presente numa grande porção das tarefas que são desempenhadas no dia-a-dia de um farmacêutico comunitário, tanto a nível de *back office* como a nível do atendimento aos utentes e, portanto, torna-se evidente que o conhecimento de como operar o sistema é fundamental. Apesar de já ter tido a oportunidade de contactar previamente com o sistema informático devido à realização de um estágio de verão, este contacto foi muito breve e, portanto, só neste estágio curricular consegui aprender como devidamente manuseá-lo, o que considero uma competência extremamente vantajosa já que, como já referi, uma grande maioria das farmácias portuguesas utiliza este sistema.

2.1.6. Contacto com Delegados de Informação Médica

Durante o meu estágio na Farmácia Inglesa tive a oportunidade de contactar com vários delegados de informação médica de diversas empresas, o que considero uma experiencia bastante enriquecedora já que me permitiu conhecer outra realidade profissional integrante setor farmacêutico, observar, não só de que forma é que as reuniões com os delegados eram realizadas e que temáticas eram abordadas, como por exemplo promoções e bonificações, como também de que forma é que novos produtos eram apresentados no contexto de farmácia comunitária permitindo assim a aquisição e consolidação de conhecimentos relativamente a estes.

2.2. Ponto Fracos

2.2.1. Medicamentos Manipulados

Um medicamento manipulado pode ser definido como um preparado oficinal ou formula magistral, preparado e dispensado sob a responsabilidade do farmacêutico, usando matérias-primas, bem como um locais adequados para a sua realização, tendo sempre em conta as "Boas Práticas a observar na preparação de medicamentos manipulados em farmácia de oficina e hospitalar" [5]. Este tipo de produtos torna essencial em situações em que é necessário adaptar a terapêutica de determinado utente face à inexistência de opções

devidamente adequadas no mercado. Durante o meu estágio constatei que a preparação de medicamentos manipulados não era muito requisitada, não tendo, portanto, oportunidade de contactar profundamente com esta vertente da farmácia comunitária, denotando assim uma lacuna relativamente a esta área.

2.2.2. Dimensão Cosmética

As áreas cosmética e de cuidados de pele são também vertentes que estão atualmente bastante presentes no contexto de farmácia comunitária, sendo que existem muitas farmácias têm já seções destinadas a estes tipos de produtos, bem com equipas devidamente treinadas e preparadas para aconselhar relativamente a estas temáticas. Posto isto, a dimensão cosmética da Farmácia Guarda Inglesa não era muito pronunciada e verificava-se que estes tipos de produtos não tinham muita aderência pelos utentes que a frequentavam, impossibilitando assim uma aprendizagem profunda e, por sua vez, autonomia no aconselhamento relativamente a assuntos desta natureza.

2.2.3. Serviços Farmacêuticos

A função de farmacêutico comunitário, para além do aconselhamento e despensa de medicamentos e outros produtos de saúde, pode envolver também a prestação de outros serviços aos utentes como a determinação do índice de massa corporal (IMC), medição da tensão arterial e de parâmetros bioquímicos, como a glicémia. Após a prestação destes serviços é então executada uma análise crítica dos resultados e o utente é posteriormente aconselhado em conformidade, tendo sempre em mente os fatores inerentes a este como por exemplo o seu histórico de saúde ou a medicação que lhe é prescrita. Posto isto, devido à pandemia COVID-19 vivenciada, a Farmácia Guarda Inglesa, de modo a proteger tanto a equipa como os utentes, deixou de realizar este tipo de serviços durante este período de dificuldade, originando assim lacunas na minha aprendizagem relativamente à execução dos serviços.

2.3. Oportunidades

2.3.1. Dispensa de Medicamentos Hospitalares

O presente ano de 2020 foi marcado pela aparição de uma pandemia internacional, afetando o normal funcionamento dos sistemas de saúde de todo o mundo, forçando assim a sua adaptação a esta nova realidade. Portugal não é exceção e no seguimento da pandemia originada pela doença COVID-19 foi declarado estado de emergência nacional, levando também à adoção de medidas extraordinárias com o objetivo de prevenir a transmissão e

proliferação do vírus[5,6,7]. Assim, na sequência destas medidas, foi emitido o Despacho n.º 4270-C/2020 que determina as medidas de aprovisionamento medicamentos dispensados por farmácia hospitalar em regime de ambulatório através da dispensa em farmácia comunitária[8]. Apesar desta ser uma medida de caracter temporário e excecional, já decorreu um estudo piloto semelhante no contexto da dispensa de medicamentos antirretrovirais em farmácia comunitária[9]. Posto considero que a dispensa de medicamentos de regime de ambulatório em farmácia comunitária seria muito proveitosa no contexto do estágio já que possibilitaria a expansão dos conhecimentos relativos a esta vertente para além de contribuir para a melhoria do acesso dos utentes à sua medicação.

2.4. Ameaças

2.4.1. Sazonalidade

A realização do meu estágio curricular decorreu desde 4 de maio até 20 de agosto de 2020 como referido anteriormente, compreendo estes meses as estações de primavera e verão. Posto isto, as necessidades e, por conseguinte, o aconselhamento prestado, focaram-se em produtos como descongestionantes nasais, anti-histamínicos, repelentes de insetos, protetores solares, entre outros relacionados. Assim, tornou-se um pouco mais difícil contactar com outros tipos de produtos característicos de estações a períodos mais frios do ano, já que estes eram requisitados com muito menos frequência no período em que decorreu o meu estágio.

2.4.2. Homogeneidade de utentes

Durante o decorrer do meu estágio pude constatar que os utentes que usufruíam dos serviços da Farmácia Guarda Inglesa eram, na sua maioria, pessoas com uma idade mais avançada e fidelizados que, por conseguinte, já encontravam familiarizados com a equipa da farmácia. O facto de já serem clientes habituais da farmácia e de já estarem familiarizados com a equipa da farmácia fazia com que muitas vezes não se sentissem confortáveis em serem atendidos por mim e preferirem ser atendidos algum membro da equipa com que já tivessem alguma confiança, preferindo até mesmo esperar quando este se encontrava momentaneamente indisponível. Devido também ao facto dos utentes se tratarem, na sua maioria pessoas, de pessoas mais, idosas muitas vezes o atendimento passava apenas pela dispensa de prescrições médicas, onde estava contemplado a medicação crónica característica desta faixa etária, e não tanto pelo aconselhamento de outros tipos de produtos como produtos veterinários ou cosméticos afetando assim a exploração destas vertentes.

3. Casos Clínicos

Caso Clínico I

Homem, de meia idade, dirige-se à farmácia referindo que tem um "pequeno cravo" no dedo polegar, requisitando assim aconselhamento e questionando se haveria algum produto proveitoso para o respetivo tratamento. Após observação do dedo pude constatar que se tratava de uma pequena verruga. É de notar que, após questionado, o doente referiu que não sofria de nenhuma condição nem fazia qualquer tipo de medicação. Posto isto aconselhei que o utente aplicasse Verrumal®. Verrumal® trata-se de um medicamento não sujeito a receita médica de dispensa exclusiva em farmácia (MNSRM-EF), indicado para o tratamento de verrugas vulgares, plantares, juvenis planas e seborreicas. Apresenta-se como uma solução cutânea contendo 100mg de ácido salicílico e 5mg de fluorouracilo por cada mililitro. O ácido salicílico trata-se de um agente queratolítico, induzindo a descamação cutânea, enquanto o fluorourcilo é um citostático do grupo dos antagonistas da pirimidina inibindo assim a proliferação viral. No ato de dispensa informei o utente de como é que o medicamento deveria ser usado, mencionando que deveria ser aplicado 2 a 3 vezes ao dia, que deveria ser conjuntamente aplicado também um penso de modo a promover a penetração do medicamento na verruga e que o tratamento deveria ser continuado até após uma semana o desaparecimento da verruga. Referi também que, entre cada aplicação, a pelicula de laca residual deveria ser retirada. Alertei também o utente para algumas precauções que este deveria ter presente como escorrer o pincel da solução em excesso previamente à aplicação, evitar aplicar na pele vizinha (recomendando mesmo um creme gordo durante o aconselhamento com objetivo de proteção desta zona, o qual o utente acabou por recusar), evitar exposição solar, suspender o tratamento no caso de aparecimento de reações como sensação de queimadura e que, no caso de ocorrência de algum efeito secundário ou caso não se verifique melhoria da condição após alguns dias, deveria ser consultado um médico. Precavi ainda que a verruga se tratava de uma afeção contagiosa e, portanto, o utente deveria ser cuidadoso relativamente ao contacto com outras pessoas[10].

Caso Clínico II

Homem, de meia idade, dirige-se farmácia mencionando que a filha, de 20 anos, se encontrava episódios de diarreia e vómitos. O utente refere também que estes sintomas surgiram somente no dia anterior, sendo, portanto, que a condição apresentada pela filha relativamente recente, e que ela não apresenta quaisquer sintomas adicionais como febre. Posto esta situação aconselhei a que a filha do utente bebesse bastante água, com o objetivo de colmatar as perdas de água decorrentes tanto da diarreia como dos vómitos de modo a

prevenir uma possível desidratação, recomendei a toma de UL-250®, cápsulas, e de Dioralyte® e esclareci também de que maneira é que estes deveriam ser administrados. UL-250®, cápsulas, é um probiótico cuja composição consiste de células liofilizadas de *Saccharomyces boulardii*, A posologia consiste na toma de 1 cápsula, 3 vezes ao dia, e irá auxiliar na reposição da flora intestinal[11]. Por sua vez, o Dioralyte® é uma associação de glucose e eletrólitos, nomeadamente sódio e potássio, apresentado sob a forma de pó para solução oral em saquetas. A sua administração irá possibilitar a reposição de sais e líquidos descorrentes da condição da filha do utente. É de notar que cada saqueta deve ser dissolvida em 200ml de água e que deveriam ser consumidas 1 a 2 saquetas após cada ejeção diarreica[12]. Adverti também ao utente que se a sintomatologia se prolongasse por mais 2 a 3 dias então deveria consultar um médico.

4. Considerações Finais

O estágio curricular em Farmácia Comunitária, integrado no plano curricular de MICF, proporciona aos estudantes a oportunidade de contactar com a realidade profissional inerente à profissão de farmacêutico comunitário e permite a aplicação e consolidação de conhecimentos adquiridos durante o curso, bem como a obtenção de muitas outras competências que decerto serão uma grande vantagem num futuro profissional.

Verifico que de facto este estágio foi um ponto bastante marcante no meu percurso académico e que inquestionavelmente me preparou para uma profissão que poderei exercer num futuro próximo, já que me possibilitou contactar com várias realidades clínicas distintas associadas aos diversos utentes que usufruem dos serviços da farmácia e me ajudou a perceber a extrema importância do farmacêutico como profissional de saúde, cujo dever é trabalhar sempre no sentido de garantir o bem-estar dos utentes. Posto isto torna-se evidente a minha evolução ao longo do período de estágio e a aquisição de valências, no entanto é de notar que esta não é uma área profissional estática, mas sim que requer uma aprendizagem constante alicerçada no constante desenvolvimento tecnológico e científico no sentido de prestar à população o melhor serviço possível.

Assim, levo do meu estágio curricular uma experiência que sem dúvida me deixou um pouco mais preparado para enfrentar os desafios que me esperarão no mercado de trabalho e na realidade profissional, enquanto farmacêutico.

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Parte II

Relatório de Estágio

INFARMED, I.P.

Autoridade Nacional do Medicamento e Produtos de Saúde I.P.

Lista de Abreviaturas

AIM – Autorização para Introdução no Mercado

CAM – Comissão de Avaliação de Medicamentos

CTS – *Communication and Tracking System*

DAM – Direção de Avaliação de Medicamentos

FFUC – Faculdade de Farmácia da Universidade de Coimbra

FI – Folheto Informativo

GestProc – Base de dados de Gestão de Processos

GIMED – Base de dados de Gestão de Informação de Medicamentos

INFARMED, I.P. – Autoridade Nacional do Medicamento e Produtos de Saúde I.P.

MICF – Mestrado Integrado em Ciências Farmacêuticas

RCM – Resumo das Caraterísticas do Medicamento

SMUH-ALTER – Plataforma de Submissão de Pedidos de Alteração do Sistema de Gestão de Medicamentos de Uso Humano

SWOT – *Strengths, Weaknesses, Opportunities, Threats*

UAC – Unidade de Avaliação Científica

UEC – Unidade de Ensaios Clínicos

UIM – Unidade de Introdução no Mercado

UMM – Unidade de Manutenção no Mercado

1. Introdução

O plano curricula do Mestrado Integrado em Ciências Farmacêuticas (MICF) da Faculdade de Farmácia da Universidade de Coimbra (FFUC) permite a realização de um estágio adicional, para além do estágio final de caracter obrigatório em Farmácia Comunitária, numa das diversas áreas de atuação do farmacêutico, à escolha do aluno, contribuindo assim para o aprofundamento dos conhecimentos teóricos previamente adquiridos durante o decorrer do curso e para um contacto mais próximo com a realidade profissional.

A área de Assuntos Regulamentares é uma das diversas áreas abrangidas pela atividade farmacêutica, requerendo variados conhecimentos em diversas vertentes associadas ao medicamento. Nesta área os farmacêuticos praticam a sua atividade não só nos processos de desenvolvimento, registo e acesso ao mercado, mas também na monitorização da utilização de medicamentos e dispositivos médicos bem como na informação e apoio aos profissionais de saúde. Portanto, a minha decisão de estagiar na Autoridade Nacional do Medicamento e Produtos de Saúde, I.P. (INFARMED, I.P.), na Direção de Avaliação do Medicamento (DAM) assenta, não só pela grande importância subjacente a esta área, mas também pela oportunidade única de poder integrar uma agência regulamentar e, portanto, adquirir novos conhecimentos e experiências bem como uma nova visão do circuito do medicamento como parte da entidade regulamentar.

O presente relatório relativo ao estágio no INFARMED I.P., de 6 de janeiro de 2020 a 11 de março de 2020, sob a orientação da Dra. Dina Cordeiro Lopes apresenta-se sob a forma de análise SWOT (*Strengths, Weaknesses, Opportunities, Threats*), com o objetivo da identificação e análise crítica dos pontos fortes, pontos fracos, oportunidades e ameaças relativos a este.

2. INFARMED, I.P.

A Autoridade Nacional do Medicamento e Produtos de Saúde, I.P., ou abreviadamente INFARMED, I.P., criado em 1993, é um instituto publico com autonomia financeira, administrativa e próprio património integrado na administração indireta do Estado. Este instituto publico encontra-se sedeado em Lisboa, no Parque da Saúde, tutelado pelo Ministério da Saúde e com jurisdição sobre todo o território nacional e tem por missão supervisionar e regular, seguindo os mais elevados padrões de proteção e saúde pública, os setores de produtos de saúde e medicamentos de uso humano, garantindo a segurança, eficácia e qualidade destes produtos e visando a sua acessibilidade aos cidadãos e profissionais de saúde[1]. A nível organizacional, o INFARMED, I.P. é composto por cinco órgãos e por treze unidades organizacionais em que estas se subdividem relativamente às suas funções, nomeadamente de negócio ou suporte[2].

A Direção de Avaliação de Medicamentos (DAM) é uma das treze unidades orgânicas que constituem a organização do INFARMED, I.P. com funções de negócio. Esta unidade é por sua vez subdividida em 4 unidades: a Unidade de Introdução no Mercado (UIM), Unidade de Avaliação Científica (UAC), a Unidade de Manutenção no Mercado (UMM), a Unidade de Avaliação Científica (UAC) e a Unidade de Ensaios Clínicos (UEC)[3].

Durante o meu estágio tive a oportunidade de integrar a equipa da UIM, a unidade responsável pelas atividades de registo e autorização de introdução de medicamentos no mercado. Nesta unidade, no contexto de procedimentos nacionais ou procedimentos de reconhecimento muto e descentralizados que englobavam Portugal como *concerned member state*, realizei atividades de validação de submissões, isto é, verificação da presença da documentação e informação necessária para a iniciação do procedimento e finalização de procedimentos, ou seja, carregamento de bases de dados, emissão de certificados de Autorização de Introdução no Mercado (AIM) e verificação de textos, nomeadamente do resumo das características do medicamento (RCM), folheto informativo (FI) e rotulagem.

3. Análise SWOT

Como já referido e descrito nas "Normas Orientadoras de Estágio do Mestrado Integrado em Ciências Farmacêuticas" [4], o presente relatório apresenta-se sob a estrutura de Análise SWOT de forma a avaliar de forma crítica a minha experiência durante o estágio realizado na Autoridade Nacional do Medicamento e Produtos de Saúde, I.P, como membro integrante da respetiva equipa. Esta análise discriminará, numa vertente interna, os pontos fortes e fracos do estágio realizado e também, numa dimensão mais externa, as oportunidades e ameaças do mesmo.

3.1. Pontos Fortes

3.1.1. Acolhimento

A primeira parte do período de estágio foi caracterizada por uma fundamental formação necessária para a realização das atividades desempenhadas enquanto integrante da equipa do INFARMED, I.P. Foi introduzido, numa primeira parte, a estrutura e organização global do instituto por partes dos Recursos Humanos, o que permitiu uma melhor ambientação ao local e promoveu uma boa integração dos estagiários. Numa segunda fase foi realizada uma introdução à unidade especifica onde iria desempenhar as minhas funções bem como formação necessária para as mesmas que ajudou a consolidar conhecimentos previamente adquiridos bem como adquirir novos conhecimentos pertinentes, não só para as atividades que iria desempenhar como também para o futuro profissional, como por exemplo explicação relativamente às diferentes bases legais, preenchimento de bases de dados e tipos de pedidos, procedimentos de registo de medicamentos de uso humano e legislação. Para além das formações foi-me também atribuído um email institucional, um computador pessoal de trabalho bem como um número mecanográfico (que serviu como nome de utilizador para o uso do computador de trabalho), permitindo assim o acesso às várias bases de dados e plataformas informáticas necessárias à execução das minhas atividades.

3.1.2. Independência e Autonomia

Após as formações instruídas e integração na UIM foi-me dada completa autonomia e independência para a realização das tarefas que me eram atribuídas. Os processos para validação ou finalização eram-me atribuídos, passando eu a ser responsável por eles e com completa independência para executar todas as tarefas inerentes a estes. Apesar de ser completamente responsável pelos processos que me eram atribuídos os colaboradores da unidade sempre se mostraram completamente disponíveis para esclarecer alguma dúvida ou questão ou providenciar alguma explicação adicional. Esta abordagem permitiu que, ao longo

do estágio, fosse desenvolvido um sentimento notório de responsabilidade devido ao grande impacto das minhas funções quer a nível das Indústrias Farmacêuticas que submetem os pedidos como a nível de acesso dos medicamentos aos cidadãos e profissionais de saúde.

3.1.3. Contacto com requerentes de AIM

As tarefas desempenhadas requeriam, muitas das vezes, um contacto direto com a Indústria Farmacêutica ou com o requerente de AIM para pedido de elementos ou documentação em falta ou esclarecimentos adicionais, o que me permitiu um contacto de maior proximidade com estas entidades do setor farmacêutico e assim um maior relacionamento e conhecimento das mesmas e uma maior aproximação à sua realidade profissional.

3.1.4. Idioma

A grande maioria dos documentos e informação com que contactei durante o período de estágio encontravam-se em inglês, o que me permitiu melhorar o entendimento da língua. Uma das minhas funções era inclusivamente a verificação e retificação das traduções de inglês para português dos textos finais do produto, isto é, do RCM, FI e rotulagem, resultando assim no aprimoramento dos meus conhecimentos de inglês. Para alem disso muitos dos contactos com as Indústrias Farmacêuticas e requerentes de AIM eram realizados em inglês por *email* o que permitiu o desenvolvimento das minhas capacidades de escrita num contexto mais técnico e formal.

3.1.5. Sistema Informático

O estágio permitiu o contacto com vários programas informáticos, necessários à execução das minhas tarefas já que estas tinham uma grande componente de preenchimento de bases de dados. Este contacto permitiu o desenvolvimento de competências e experiência relativamente ao manuseamento destas bases de dados e programas informáticos, nomeadamente com o CTS (*Communication and Tracking System*), GiMed (base de dados de Gestão de Informação de Medicamentos), GiSub (base de dados de Gestão de Informação de Substancias), GiEnt (base de dados de Gestão de Informação de Entidades), GestProc (base de dados de Gestão de Processos) e SMHU-Alter (plataforma de submissão de pedidos de alteração do sistema de gestão de Medicamento de Uso Humano).

3.1.6. Competências Desenvolvidas

O decorrer do estágio permitiu não só a aplicação de conhecimentos teórico pré-adquiridos no plano curricular de MICF, mas também o desenvolvimento de competências que considero fulcrais para a minha integração no mercado de trabalho. Possibilitou o desenvolvimento de competências informáticas, dado que as minhas tarefas eram desenvolvidas maioritariamente através de bases de dados e plataformas informáticas, o desenvolvimento da minha compreensão da língua inglesa e a expansão dos meus conhecimentos relativos à área de Assuntos Regulamentares do Medicamento. O estágio ajudou-me também a desenvolver uma maior capacidade organizacional, pois muitas das vezes era necessário trabalhar com diferentes processos simultaneamente, e contribui também para o aumento do meu sentido de responsabilidade e autonomia.

3.2. Pontos Fracos

3.2.1. Falhas Informáticas

Muito do trabalho desenvolvido na unidade que integrei assentava no preenchimento de bases de dados e trabalho em programas informáticos, no entanto um dos problemas recorrentes com que me deparei durante todo o decorrer do estágio foi a sua falha destas plataformas e a sua lenta capacidade de processamento. Um dos cuidados que necessitava de ter durante o preenchimento das bases de dados era guardar após cada passo pois havia o risco de ocorrência de algum erro, ocasionando a perda da informação não guardada resultando, por conseguinte, em perda de tempo mediante da reinserção dos dados perdidos, e num sentimento de frustração do utilizador. Outra grande inconveniência dos sistemas informáticos utilizados é que, ao passar a informação para o formato *word*, grande parte da informação sofria desformatação, sendo esta proporcional à complexidade e quantidade da informação gerando assim também grandes perdas de tempo e ineficiência.

3.2.2. Período de estágio

Apesar do estágio ter sido muito proveitoso na medida de aquisição de novos conhecimentos e competências e, portanto, uma experiência muito produtiva, considero que o período total em que este decorre não é o suficiente para aquisição da formação adequada para uma autonomia completa, sendo este fator ainda mais agravado pelo seu término precoce, pelo qual foi marcada. Não obstante o término precoce do estágio foi uma medida necessária face à crise que o país apresenta relativamente à pandemia COVID-19 e tendo em vista a proteção dos estagiários contra perigos e riscos desnecessários.

3.3. Oportunidades

3.3.1. Comissão de Avaliação de Medicamentos

A Comissão de Avaliação de Medicamentos (CAM) é constituída por profissionais de diversas áreas como médico, toxicologistas, farmacêuticos, entre outros com o objetivo de discussão de assuntos relevantes do medicamento nas vertentes fundamentais e inerentes a este, nomeadamente a qualidade, eficácia e segurança[5]. Posto isto acho que seria extremamente benéfico para os estagiários o acompanhamento destas discussões dado que os assuntos discutidos iriam de encontro aos conhecimentos adquiridos durante o curso, seriam relevantes para a realização das suas funções enquanto integrantes da equipa do INFARMED, I.P. e proporcionaria aos estagiários a abordagem às matérias discutidas do ponto de vista da entidade regulamentar.

3.3.2. Laboratório de Controlo de Qualidade

O Laboratório de Controlo de Qualidade é o laboratório de referência nacional, reconhecido a nível europeu, no contexto de comprovação da qualidade de medicamentos, visando a verificação da qualidade de matérias-primas e medicamentos comercializados em Portugal[6]. Penso que a inclusão de visita ao laboratório no plano de estágio seria uma mais-valia, já que representaria uma oportunidade de aprendizagem relativamente ao seu funcionamento.

3.4. Ameaças

3.4.1. Recursos Humanos

Apesar das colaboradoras, integrantes da unidade em que desempenhei as minhas atividades, sempre se mostrarem disponíveis para me esclarecer qualquer questão e não haver qualquer tipo de falta de apoio da sua parte notou-se a sua sobrecarga com trabalho, o que fez com que por vezes atrasassem o seu próprio trabalho ou que demorassem um pouco mais a responder a questões que pudessem surgir durante o decorrer do estágio. Friso novamente não houve qualquer falta de apoio e sempre se mostraram pronto para ajudar no que pudessem, no entanto considero que o aumento do número de colaboradores nesta unidade aumentaria a produtividade tanto para a unidade em si que funcionaria de maneira mais proficiente tanto para os estagiários na medida em que amplificaria o seu desempenho.

4. Considerações Finais

Enquanto estudante torna-se difícil ter ideia relativamente ao que é realmente a atividade farmacêutica na área de Assuntos Regulamentares e quais as tarefas desempenhadas neste contexto. Posto isto, o plano curricular de MICF da FFUC, para além do estágio curricular em Farmácia Comunitária, oferece ainda a possibilidade ao aluno de realizar um estágio curricular adicional noutra área distinta da atividade farmacêutica à sua escolha possibilitando assim a aproximação do aluno a outras realidades profissionais inerentes à profissão farmacêutica.

Assim, o estágio adicional no INFARMED, I.P. revelou-se extremamente enriquecedor já que me permitiu o esclarecimento relativamente ao papel do farmacêutico na área de Assuntos Regulamentares e a observação do que é esta área do ponto de vista da entidade regulamentar. Adicionalmente este estágio constituiu uma ótima experiência de aprendizagem extremamente enriquecedora, que me permitiu a aquisição de competências que serão certamente vantajosas para o ingresso nesta área da atividade farmacêutica e exercício na mesma.

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Parte III

Monografia

AAV pre-existing immunity: strategies to develop AAV vectors with enhanced immune evasion

List of Abbreviations

AAP – Assembly activating protein

AAV – Adeno-associated virus

Ad – Adenovirus

APCs – Antigen-presenting cells

cDCs – Conventional dendritic cells

CNS – Central Nervous System

CTL – Cytotoxic T lymphocytes

DCs – Dendritic cells

EGFR – PTK – Epidermal growth factor receptor protein tyrosine kinase

ER – Endoplasmic reticulum

hFIX – Human factor IX

IFN – Interferon

IRF – Interferon-Regulatory Factor

ITRs – Inverted Terminal Repeat Sequences

IκBα – NF-κB inhibitory protein

KCs – Kupfer cells

LCO – Localized codon-optimization

LRR – Leucine-rich-repeat

MHC– Major histocompatibility complex

MyD88 – Myeloid differentiation primary response gene 88

NAbs – Neutralizing Antibodies

NF-κB – Nuclear Factor κB

ORF – Open Reading Frame

PAMPs – Pathogen-associated molecular patterns

PBMC – Peripheral blood mononuclear cell isolations

pDCs – Plasmacytoid dendritic cells

PEG – Polyethylene glycol

PRRs – Pattern recognition receptors

rAAV – Recombinant AAV

SM – Skeletal Muscle

TCR – Capsid antigen-specific soluble T cell receptors

TLRs – Toll-like receptors

TNF-α – Tumor necrosis factor α

TRAF6 – E3 ubiquitin ligase

Treg – Regulatory T cells

TRIF – TIR-domain-containing adapter-inducing interferon-β

Abstract

The adeno-associated viruses (AAV) is a nonenveloped DNA virus, first discovered as contaminant of Adenovirus (Ad) preparations. Recombinant adeno-associated viruses (rAAV), obtained through genetic engineering of AAV, have proven to be useful tools in the context of gene therapy. rAAVs are relatively less immunogenic than other viral vectors, possibly due to its poor transduction of antigen-presenting cells (APCs) and are able to achieve long-term transgene expression. Nonetheless, the viral vector can induce an immune response since it is still a foreign particle to the human body and therefore can be recognized as such. Furthermore, a memory immune response may have already been established due to previous natural *wild type* AAV infection thus impairing rAAV mediated transgene expression since the capsids of both particles are very alike or essentially similar. Additionally, not only the vector itself but also the transgene product, obtained from the modified viral genome, may also induce an immune reaction. That said, it becomes necessary to develop and apply new strategies to overcome the immune challenges that arise from gene therapy using AAV vectors.

Keywords: Adeno-associated virus; rAAV; gene therapy; immune response; immune evasion; immune-privileged administration routes; capsid rational design; direct evolution; chemical modification; transient immunosuppression

Resumo

O vírus adeno-associado (AAV) é vírus de DNA sem envelope, inicialmente descoberto como contaminante de preparações de Adenovirus (Ad). O vírus adeno-associado recombinante (rAAV), obtidos através de engenharia genética dos AAV, provou ser uma ferramenta útil no contexto de terapia génica pois são comparativamente menos imunogénicos que outros vetores virais, possivelmente devido à sua fraca transdução de células apresentadoras de antigénio (APCs) e devido à sua capacidade de alcançar expressão transgénica prolongada no tempo. No entanto o vetor viral pode induzir uma resposta imune já que se trata de uma partícula estranha ao organismo humano e pode ser reconhecida como tal. Além disso, uma resposta de memória pode já ter sido estabelecida devido a uma prévia infeção por AAVs selvagens impedindo assim a expressão transgénica mediada pelos rAAVs já que as cápsides de ambas as partículas são muito parecidas ou essencialmente similares. Adicionalmente, não apenas o vetor, mas também o produto do transgene, obtido a partir do genoma viral modificado, pode também induzir uma reação imune. Em face destes problemas torna-se necessário desenvolver e aplicar novas estratégias para superar os desafios resultantes da resposta imunitária associada à terapia génica usando vetores AAV.

Palavras-chave: Vírus adeno-associado; rAAV; terapia génica; resposta imune; evasão imune; vias de administração imunopriviligiadas; design racional da cápside; evolução direta; modificação química; imunossupressão transitória.

1. Introduction

Gene therapy is based on the delivery of a therapeutic transgene into specific cells or tissues, with the objective of repairing, regulating, adding, replacing, or deleting of a genetic sequence, ultimately culminating in disease treatment. The therapeutic transgene can be packed in vectors or other types of formulations and delivery systems capable of releasing it into the interior of the target cells and tissues. These constructs can be defined as gene therapy medicinal products (EUROPEAN MEDICINES AGENCY, 2019). Due to the natural evolution of viruses and the mechanisms underlying viral infections its use, as vectors for gene therapy, has been the target of extensive research and investigation (NI *et al.*, 2016). However, the human body doesn't remain indifferent to the presence of viral vectors as they are still naturally perceived as strange and potentially harmful particles. The adeno-associated virus (AAV) is a non-enveloped DNA virus characterized by a low immunogenic profile, possibly due to low transduction of antigen-presenting cells (APCs) such as dendritic cells (DCs) and capable of infecting both dividing and non-dividing cells, thus presenting several advantageous and necessary properties needed to achieve a successful delivery of the therapeutic transgene (BALAKRISHNAN e JAYANDHARAN, 2014; ROSSI *et al.*, 2019). Patients treated with recombinant adeno-associated viruses (rAAV), virus-alike particles obtained through genetic engineering of AAV, in addition to the features previously mentioned, also demonstrated long-term transgene expression in clinical trials (GEORGE *et al.*, 2017; PASI *et al.*, 2020; RANGARAJAN *et al.*, 2017).

Despite having a low immunogenic profile, the immune system isn't completely blind to the presence of the therapeutic rAAV and consequently can react against it. Not only that but an immune reaction against the transgene product itself can occur. Additionally, the immune system may already possess a pre-established defense prior to rAAV administration due to natural AAV infection, since the virus and the recombinant vector capsids are fundamentally similar. For example, the presence of pre-existing neutralizing antibodies (NAbs) developed after *wild type* infection can compromise the vector cell transfection and transgene expression, thus compromising the success of the therapy (FITZPATRICK *et al.*, 2018; GARDNER *et al.*, 2019; PIEN *et al.*, 2009).

The technological development and better understanding of the AAV biology and mechanisms underlying infection enabled the development of new strategies to overcome the immune reactions following rAAV administration. These strategies include capsid modifications in an attempt to make the vector less recognizable by the immune system, co-administration of other products such as immunosuppressive molecules or administration in

immune-privileged sites, among other tactics (LEE *et al.*, 2005; MARKS *et al.*, 2008; MELIANI *et al.*, 2018).

This review summarizes the basic structure and biology of AAVs and how the rAAVs used in gene therapy are obtained, the body's immune reactions and mechanisms developed towards it, as well as strategies, and respective fundaments developed in order to overcome these reactions.

2. AAV Structure and Biology

The Adeno-associated virus (AAV) was first discovered by Astchinson and his team as a small contaminant of Adenovirus (Ad) preparations (AS *et al.*, 1965). It is a virus from the *Dependovirus* genus and *Parvoviridae* family, dependent on the co-infection with a helper virus such as Ad or Herpes Simplex to achieve proper replication and subsequent production of new virus. When in the absence of the necessary helper virus, a latent infection is established. AAV latent infection is associated with the integration of its viral genetic material into specific sites of the host cell genome, for example, AAV2 latency is accompanied by integration at a specific locus of the chromosome 19. Following co-infection with a helper virus, the provirus is excised from the host cell genome and the assembly of new virions can occur normally. AAV infection is very common in humans, however, it is not associated with any known disease. (BALAKRISHNAN e JAYANDHARAN, 2014; PAWLOWSKI *et al.*, 2013).

Regarding its structure, AAV is a non-enveloped virus composed of a linear, single DNA strain (≈4,7 Kb) encapsulated into an icosahedral capsid (consisting of 60 subunits) of approximately 25 nm in diameter. The viral genome comprises 2 open reading frames (ORF), the Rep, and Cap ORFs. The Rep ORF encodes 4 nonstructural proteins, Rep78, Rep68, Rep52, and Rep40, obtained through spliced and unspliced RNAs expressed using the promoters p5 and p19. These proteins are essential for the processes of viral replication, regulation of gene expression, integration, and excision from the host cell genome and encapsidation. The Cap ORF encodes the structural proteins VP1, VP2, and VP3, obtained through different start codons and alternative splicing, using the promotor p40. These proteins will form the capsid structure in a ratio of 1:1:10 respectively. (BALAKRISHNAN e JAYANDHARAN, 2014; PAWLOWSKI *et al.*, 2013; XIE *et al.*, 2002). Additionally, an alternative ORF overlapping the Cap ORF, upstream of the VP3 coding sequence, encodes the assembly activating protein (AAP), which also uses the promotor p40. This is a nonstructural protein required for VP protein stability, oligomerization, and capsid assembly (BALAKRISHNAN e JAYANDHARAN, 2014; MAURER *et al.*, 2018). Furthermore, the viral genome it is flanked by 2 inverted terminal repeat sequences (ITRs) on each end. ITRs are T-shaped sequences of 145 bp that mediate the replication of the viral genome (BALAKRISHNAN e JAYANDHARAN, 2014).

The recombinant AAV (rAAV) used in gene therapy is obtained by replacing the Cap and Rep ORFs with a transgene expression cassette. This expression cassette comprises not only the sequence of interest but also other transcriptional control elements such as different promoters (PAWLOWSKI *et al.*, 2013). As a matter of example, rAAVs used in hemophilia A gene therapy are designed with liver-specific promoters in order to guarantee transgene

product expression only in this tissue (PASI *et al.*, 2020; RANGARAJAN *et al.*, 2017). Likewise, control elements are necessary to modulate the level of product expression and target specificity, and therefore, an understanding of the various aspects involved in cassette design is necessary to achieve optimal product expression and a successful therapy (PAWLOWSKI *et al.*, 2013; POWELL *et al.*, 2015).

Table1. Summary of the different proteins encoded by viral genome ORF and corresponding promoters. (Balakrishnan & Jayandharan, 2014).

Open Reading Frame	Promoter	Protein
Rep	P5	Rep78
		Rep68
	P19	Rep52
		Rep40
Cap	P40	VP1
		VP2
		VP3
Aap	P40	AAP

The Rep ORF encoded the proteins Rep78 and Rep 68 using the promoter P5 and encodes Rep 52 and Rep 40 using the promoter P19. The capsid proteins (VP1, VP2 and VP3) are encoded by the Cap ORF using the promoter P40. The alternate orf also uses the promoter P40 to encode assembly activating protein (AAP).

rAAV production is typicaly based on the transient transfection of mammalian cells, such as HeLa or HEK293 cell lines, with plasmids containing the necessary elements for vector assembly. In this method the cell lines are normally transfected with 3 different plasmids, each one comprising different key elements: 1) Transfection expression cassette flanked by the ITRs; 2) Cap ORF and Rep ORF; 3) Adenoviral helper genes.

Nonetheless, rAAV can be produced using variations of the referred method or other procedures. For example, the previously described method can be modified to use only 2 plasmids by aggregating the adenoviral helper genes, Cap ORF, and Rep ORF in 1 plasmid. Cell lines, normally derived from HeLa cells, can be modified through the integration of the Cap and Rep ORFs and/or rAAV genome hence originating packaging and producer cell lines respectively. Herpes simplex type 1 can ben modified to carry either the rAAV genome or the AAV Rep and CaP ORFs, being that rAAV production is achieved through the co-infection of mammalian cells by these two types of recombinant herpes virus. Since rAAV only shares the ITRs with the wild type genome, there is no risk of viral production (CECCHINI *et al.*, 2011; CLÉMENT e GRIEGER, 2016; ROBERT *et al.*, 2017).

After rAAV administration, its genetic material does not integrate into the host cell's genome, instead, it mainly remains in an episomal state (MCCARTY *et al.*, 2004) and has shown to provide long-term transgene expression in non-dividing cells (H. *et al.*, 2006; MOUNT *et al.*, 2002; NATHWANI *et al.*, 2014; PASI *et al.*, 2020). Furthermore, the flexibility of gene therapy using AAV vectors is due to the fact that it can infect both divisible and non-divisible cells (LI *et al.*, 2012) and that different virus serotypes show tropisms for different tissues. One of the steps required to achieve a successful viral transfection is the interaction between the AAV capsid and the receptors and co-receptors located on the surface of target cells. The differences in topologies between capsids of the various serotypes lead to distinct interactions with cellular receptors, thus explaining the differences in tropism and transduction efficiency observed between them(ASOKAN *et al.*, 2012; BALAKRISHNAN e JAYANDHARAN, 2014).

Table 2. Summary of rAAV tissue tropism, as well as the cell receptors and co-receptors for each AAV serotype (SARAIVA *et al.*, 2016).

AAV Serotype	Tissue(s) Tropism	Primary Receptor	Secondary Receptor	
AAV1	SM, CNS, Heart, Lung, Eye, Pancreas	α2–3/ α2–6 N-linked sialic acid		AURICCHIO *et al.*, 2001; CHAO *et al.*, 2000; DODIYA *et al.*, 2010; FLOTTE *et al.*, 2010; KAWASE *et al.*, 2008; LOILER *et al.*, 2005; WANG *et al.*, 2003; WU *et al.*, 2006
AAV2	Kidney, SM, CNS, Liver, Eye	HSPG	FGFR1, HGFR, Integrins, LamR	AKACHE *et al.*, 2006; BARTLETT *et al.*, 1998; KASHIWAKURA *et al.*, 2005; MACLAREN *et al.*, 2014; MANNO *et al.*, 2003; PONNAZHAGAN *et al.*, 1997; QING *et al.*, 1999; SUMMERFORD *et al.*, 1999; SUMMERFORD e SAMULSKI, 1998; TAKEDA *et al.*, 2004
AAV3	SM, HCC	HSPG	FGFR1, HGFR, LamR	AKACHE *et al.*, 2006; BLACKBURN *et al.*, 2006; CHAO *et al.*, 2000; GLUSHAKOVA *et al.*, 2009; LING *et al.*, 2010; RABINOWITZ *et al.*, 2004
AAV4	Eye, CNS	α2–3 O-linked sialic acid		DAVIDSON *et al.*, 2000; KALUDOV *et al.*, 2001; WEBER *et al.*, 2003
AAV5	CNS, Lung, Eye, SM	α2–3 N-linked sialic acid	PDGFR	CHAO *et al.*, 2000; DAVIDSON *et al.*, 2000; DODIYA *et al.*, 2010; KALUDOV *et al.*, 2001; LOTERY *et al.*, 2003; PASQUALE, DI *et al.*, 2003; SEILER *et al.*, 2006
AAV6	Lung, Heart, SM	HSPG, α2–3/ α2–6 N-linked sialic acid	EGFR	CHAO *et al.*, 2000; HALBERT *et al.*, 2001; NG *et al.*, 2010; WELLER *et al.*, 2010; WU *et al.*, 2006; ZINCARELLI *et al.*, 2010
AAV7	SM, Eye, CNS			ALLOCCA *et al.*, 2007; GAO *et al.*, 2002; TAYMANS *et al.*, 2007
AAV8	Liver, SM, CNS, Eye, Pancreas, Heart		LamR	AKACHE *et al.*, 2006; ALLOCCA *et al.*, 2007; DODIYA *et al.*, 2010; GAO *et al.*, 2002; NAKAI *et al.*, 2005; TAYMANS *et al.*, 2007; WANG *et al.*, 2005
AAV9	Liver, lung, SM, Heart, CNS, Pancreas, Eye, Kidney	N-linked galactose	LamR	AKACHE *et al.*, 2006; BOSTICK *et al.*, 2007; FOUST *et al.*, 2009; INAGAKI *et al.*, 2006; SHEN *et al.*, 2011; VANDENDRIESSCHE *et al.*, 2007; YUE *et al.*, 2011

Abbreviations: CNS - Central nervous system; EGFR - Epidermal growth factor receptor, FGFR1 - Fibroblast growth factor receptor 1; HCC - Hepatocellular carcinoma; HGFR - hepatocyte growth factor receptor; HSPG - Heparan sulfate proteoglycan; LamR - Laminin receptor; PDGFR - Platelet-derived growth factor receptor; SM - Skeletal muscle;
Adapted from: Saraiva, Nobre and Pereira de Almeida, 2016

3. AAV Immunogenicity and Immune Responses

AAV has been observed to be generally less immunogenic than other viruses, possibly due to its poor transduction of APCs such as DCs (ROSSI *et al.*, 2019). Additionally, the vector lacks viral genes meaning that there is no active viral expression to amplify a possible immune response.

However, the immune system does not remain indifferent to the presence of the rAAV, recognizing it as a foreign particle, thus developing an immune response to it and compromising transfection efficiency, transgene expression, and therapy success (PIEN *et al.*, 2009). Furthermore, most humans have been previously exposed to *wild type* AAV and consequently developed some kind of immune response to it, such as pre-existent NAbs (LI *et al.*, 2012). Although rAAV are modified versions of *wild type* AAV, their capsid retains a high level of similarity with the *wild type* capsid, thus the human body may already have a pre-conceived defense against the viral vector (KRUZIK *et al.*, 2019; KURANDA *et al.*, 2018). Besides that, there is a significant level of homology between capsids from different AAV serotypes (BALAKRISHNAN e JAYANDHARAN, 2014), which in turn can lead to cross-reactivity and subsequent impairment of rAAV therapy using a particular serotype even when the host has not developed a specific response against it (ARRUDA and XIAO, 2007). During treatment, the immune system can react not only against the vector itself but also against the transgene product expressed upon cell infection, which means that successful transfection of target cells may not be enough to successful therapy (GARDNER *et al.*, 2019; H. *et al.*, 2006)

3.1. Innate Immunity

Innate response constitutes the human body's first line of defense against viral infections. During the development of the innate immune response, the rAAV is perceived through its unique motifs and structures, as a foreign particle by the immune system. After rAAV administration, these unique motifs and unique structures, referred to as pathogen-associated molecular patterns (PAMPs), are recognized by pattern recognition receptors (PRRs). PAMPs are essential to pathogen survival and remain relatively invariable among a wide range of pathogenic agents (AKIRA *et al.*, 2006). PRRs stimulation triggers signaling pathways that enable reactions against pathogenic agents, such as the activation of Nuclear Factor κB (NF-κB) and Interferon-Regulatory Factor (IRF) transcription factor which in turn promote the expression of pro-inflammatory cytokines and type I interferons (IFN) (AKIRA *et al.*, 2006; KAWASAKI e KAWAI, 2014).

Mammals have different families of PRRs, one of them being Toll-like receptors (TLRs), which are type I integral membrane glycoproteins, composed by an ectodomain comprised of

leucine-rich-repeat (LRR) motifs, that enables the recognition of PAMPs, and a cytoplasmic signaling domain, similar to the interleukin 1 receptor, referred to as Toll/IL-1R (TIR) domain, responsible for signal initiation (BOWIE e O'NEILL, 2000; KAWASAKI e KAWAI, 2014). The TLRs family is comprised of 10 elements in humans that can be expressed both in immune cells, such as DCs, or non-immune cells, such as epithelial cells and can be located either on the surface of the cell or in internal organelles such as endosomes or lysosomes. Normally the receptors that recognize membrane structures such as lipoproteins are located at the surface of cells and the ones that recognize nucleic acids can be found in endosomal compartments (AKIRA *et al.*, 2006; KAWASAKI e KAWAI, 2014).

The interaction between the viral vector and the receptor results in the activation of signaling pathways, such as the myeloid differentiation primary response gene 88 (MyD88) dependent pathway, which culminates in the degradation of the NF-κB inhibitory protein (IκBα), thus enabling the translocation of the NF-κB to the nucleus and subsequent expression of pro-inflammatory genes and in the activation of AP1 transcription factors; and the TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway, which culminates, in the expression of type I IFN genes and the activation of NF-κB (KAWASAKI e KAWAI, 2014; TRINCHIERI e SHER, 2007).

The TLR9 is one of the receptors involved in viral recognition. This receptor is located in the endosomal compartments of plasmacytoid dendritic cells (pDCs), a subtype of dendritic cells characterized by a high capacity of type I IFN secretion, and is stimulated by the DNA CpG motifs (KAWASAKI e KAWAI, 2014). Zhu *et al.* observed that, upon stimulation, pDCs produced manly IFNs, such as IFN-α and IFN-β, mediated by the MyD88 pathway (Figure 1), since IFN production was compromised in bone marrow cells from MyD88-/- mice but not from TRIF-/- mice (ZHU *et al.*, 2009). TLR9 viral recognition has also been linked to adaptative immune responses (ZHIQUAN *et al.*, 2019; ZHU *et al.*, 2009). Hepatic infiltration of CD8+T cells was observed to increase in mice administered with viral vectors containing DNA with a high number of CpG motifs, however, mice administered with low CpG motifs vectors failed to demonstrate any increase (ZHIQUAN *et al.*, 2019). Likewise, antibody titters were substantially reduced in Tlr9-/- and MyD88-/- mice (ZHU *et al.*, 2009).

An innate immune response can also be induced by recognition of the viral capsid by the cell surface receptor TLR2. It has been demonstrated that TLR2, present at the cell surface of Kupffer cells and liver sinusoidal endothelial cells, can interact with the viral capsid, and induce a response consisting of inflammatory cytokines up-regulation, via NF-κB activation, as

incubation of these cells with anti-TLR2 antibodies caused a significant decrease in cytokine secretion in response to AAV2 and AAV8 capsids (HÖSEL *et al.*, 2012).

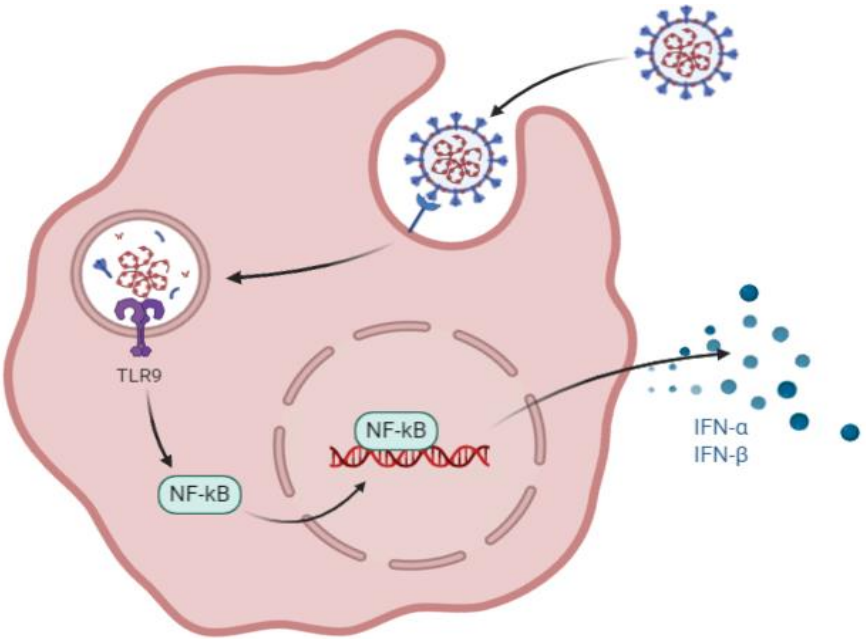

Figure 1. Signaling pathway following TLR9 recognition of viral CpG motifs (ZHU *et al.*, 2009).

Another immune mechanism relies on NK cells. It has been demonstrated that exposure of AAV2 in seronegative individuals leads to the transient secretion of Tumor necrosis factor α (TNF-α) and IFN-γ, peaking 24 hours after stimulation, mediated by NK activation. Secretion failed after stimulation with other viral particles other than AAV, thus suggesting a capsid specific response (KURANDA *et al.*, 2018). Studies also suggest that the complement system may also be involved in innate immune responses since it has been observed that AAV2 can bind to C3 complement proteins, which can, in turn, cause macrophage activation and vector opsonization (ZOU *et al.*, 2019).

3.2. Pre-existing Humoral Immunity

A major issue regarding rAAV therapy is based on the fact that a large part of the population has already been infected with the *wild type* AAV and therefore has already developed NAbs that can jeopardize cell transduction, even when present at low concentrations (FITZPATRICK *et al.*, 2018; LI *et al.*, 2012; MONTEILHET *et al.*, 2010; SCALLAN *et al.*, 2006). Kuranda *et al.* stimulated peripheral blood mononuclear cell cultures with AAV2 and observed a 7-fold higher frequency of AAV2-specific antibody-secreting cells in cultures from seropositive donors compared to cultures from seronegative donors, thus suggesting that previous AAV infection can stimulate specific memory B cells. (KURANDA *et*

al., 2018). Furthermore, simply switching to a different *wild type* serotype may not be enough to circumvent a pre-existing humoral immunity, since there is a high co-prevalence of anti-AAV NAbs specific for different serotypes and cross-reactivity (KRUZIK *et al.*, 2019).

Additionally, it has been observed that new-borns present natural protection against the virus and subsequently against the viral vector due to the mother's vertical transmission of NAbs. The prevalence of NAbs decreases over time and is followed by a gradual growth with age, leaving a window for optimal administration from about 7 to 11 months of age (CALCEDO *et al.*, 2011).

That said, it is worth noting the great importance of quantifying assays protocols to assess, not only the presence of neutralizing antibodies but also previously established cellular immune responses, in gene therapy and clinical trials using rAAV (CALCEDO *et al.*, 2018; MARTINO *et al.*, 2012).

Table 3. Prevalence of NAbs against different AAV serotypes, according to a variety of studies.

Serotype	KRUZIK *et al.*, 2019	ANDRZEJEWSKI *et al.*, 2019	MONTEILHET *et al.*, 2010[1]	CORDEN *et al.*, 2017	MIMURO *et al.*, 2014
AAV1	27%		50.5%		36.5%
AAV2	47-74%	61-81%	59%	45%	35.3%
AAV4				11%	
AAV5	20-59%	2-10%	3.2%	11%	37.6%
AAV6		37-31%	37%		
AAV8	32.63%	2-10%	19%		32.9%
AAV9			33.5%		36.5%

[1]The data comprises healthy patients and patients with type 1 diabetes mellitus.

3.3. Pre-existing Cellular Immunity

Prior exposure to the *wild type* AAV not only leads to the development of a humoral response but also induces a memory cellular immune response. (GHENASSIA *et al.*, 2017; KURANDA *et al.*, 2018). CD8+T cells were found in peripheral blood mononuclear cell cultures from AAV-2 seropositive donors, but not in cultures from seronegative donors, which suggests capsid-specific memory CD8+T cell response (KURANDA *et al.*, 2018). Other studies showed similar results, thus confirming the hypothesis that pre-existing cellular immune response is mediated mainly by memory CD8+T cells. Similarly to humoral response, serotype

switch has limited potential since this response is also characterized by cross-reactivity due to high levels of epitope conservation between AAV serotypes (HUI *et al.*, 2015; MANNO *et al.*, 2006; MINGOZZI *et al.*, 2007; VERON *et al.*, 2012). Stimulation of memory CD8+T leads to the elimination of transduced cells (MANNO *et al.*, 2006), secretion of TNF-α, IFN-γ, IL-2 and is characterized by granzyme B expression and CD107a degranulation markers (HUI *et al.*, 2015; KURANDA *et al.*, 2018; MARTINO *et al.*, 2012).

Furthermore, it appears that the presence of CD4+T cells and the route by which the viral vector is administered have an impact on the induction of memory CD8+T cells (GHENASSIA *et al.*, 2017; GROSS *et al.*, 2018). Mice CD4+T cells+ and CD4+T cells- were intramuscularly and intradermally immunized with rAAV2/1. After intradermal administration, it was observed a significant increase of memory CD8 + T cells in helper + mice comparatively to helper- mice. However, such a marked difference was not observed in intramuscularly administered mice, thus evidencing the synergistic effect of CD4+T cells and the administration route in the modulation of memory cellular immune response (GHENASSIA *et al.*, 2017).

Another point that should be noted is the fact that the presence of Nabs against the virus, doesn't automatically indicate the presence of memory T cells and vice-versa, thus suggesting that individuals exposed to the same virus may react differently and develop unlike immune responses (KURANDA *et al.*, 2018; VERON *et al.*, 2012). Further studies will be required to fully understand the relation between the two responses and what are the necessary conditions to develop each one of these in the context of AAV exposition.

3.4. Adaptative immune response and APC interactions

The understanding of how the immune cells interact is essential to comprehend the responses that may compromise rAAV therapy. Antigen presentation plays a major role in the modulation of immune responses against potentially harmful agents. This process consists of the exposure of antigens or processed antigens, in the context of major histocompatibility complexes (MHC), to competent cells of the immune system such as T cells and ultimately resulting in the triggering of an immune reaction (NEEFJES *et al.*, 2011). Particles formed endogenously, from such newly produced virion, inside of the host cell, are processed into products, such as small peptides, apt to be exposed in class I MHC, triggering a predominantly CD8+T cell response. Activated CD8+T cells scan the cell surface, search for similar products exposed in class I MHC, and eliminate infected cells (PIEN *et al.*, 2009). All nucleated cells can display class I MHC and therefore be eliminated by activated CD8+T cells, however, product presentation through APCs is needed to activate CD8+T cells (NEEFJES *et al.*, 2011; SEI *et al.*, 2015). Unlike class I MHC, only APCs can display class 2 MHC. Exogenous products are

processed and subsequently presented in class 2 MHC to CD4+T cells. Nonetheless, it has been demonstrated that endogenous particles can also be present in these complexes (NEEFJES *et al.*, 2011; STERN e SANTAMBROGIO, 2016).

Moreover, antigen presentation can also be achieved through cross-presentation, a process widely studied in the context of AAV infection and rAAV therapy, namely observed in DCs, which, contrary to the classical pathway previously described, consists in the internalization of extracellular antigens followed by its presentation in the context of class I MHC (EMBGENBROICH e BURGDORF, 2018; LI *et al.*, 2013; ROGERS *et al.*, 2017). Studies observed a CD8+T cell response in vector-transfected cells, however, the AAV vector lacks the genetic sequences necessary to achieve proper replication, and thus presentation in class I MHC through the classical pathway is unlikely. Instead, this suggests that viral vectors are internalized and processed and that the resulting peptides are later exposed through cross-presentation (MANNO *et al.*, 2006; ROGERS *et al.*, 2017). Furthermore, the blockade of class I MHC by engineered capsid antigen-specific soluble T cell receptors (TCR) prevented a cytotoxic T lymphocyte (CTL) mediated lysis of vector-transfected cells, hence confirming the need for the MHC/T cell interaction to occur an effective immune response (PIEN *et al.*, 2009).

The AAV vector is internalized via a clathrin-dependent receptor-mediated endocytic process. The use of substances that inhibit proteasome action, such as MG132 and bortezomib, and ubiquitination impairment has resulted in increased transgene expression, due to a reduction in antigen presentation hence evidencing that, prior to antigen exposition, the vector is submitted to processing. (GABRIEL *et al.*, 2013; LI *et al.*, 2013). The newly formed peptides now undergo translocation into the endoplasmic reticulum (ER) mediated by the transporter associated with antigen presentation (TAP), where they will bind to class I MHC and be presented to the immune system cells. The reduction of transduction after the use of Brefeldin A, a substance that inhibits protein transport from ER to the Golgi complex and blocks protein secretion, suggests that, after class I MHC binding in the ER, the Golgi complex has a role in peptide transport to the cell surface and consequent display (GROMMÉ *et al.*, 1999; LI *et al.*, 2013).

Nonetheless, instead of undergoing proteasome degradation, rAAV can be processed in the endosomal/lysosomal organelle and then loaded into recycled class I MHC, in a TAP independent and proteasome inhibitor-resistant manner (LI *et al.*, 2013). The use of NH_4Cl, a lysosomotropic agent, that is, a compound capable of entering lysosomes and cause an increase in pH (ASHFAQ *et al.*, 2011), has impaired immune response in TAP-deficient cells after infection with the measles virus, thus showing that the interference with acidic compartments,

and therefore with the antigenic processing in them, compromises the cross-presentation in class I MHC (GROMMÉ *et al.*, 1999).

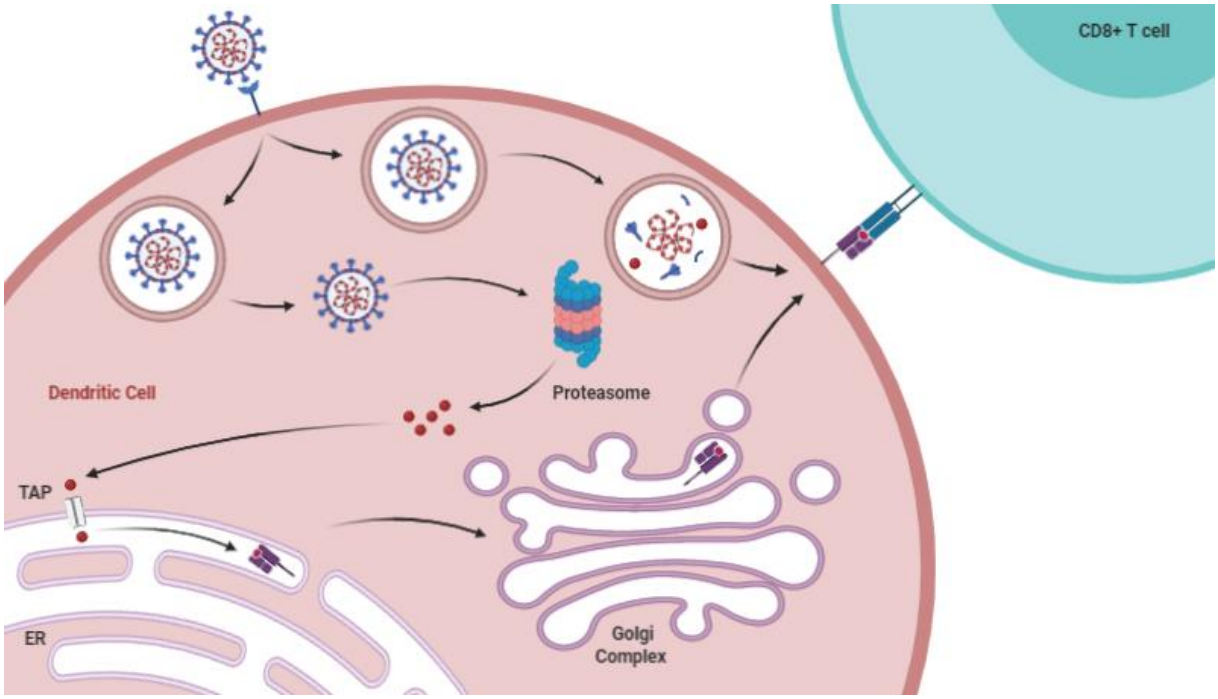

Figure 2. Antigen cross-presentation schematic. Upon internalization, the vector can escape the endosomal compartment, and undergo ubiquitination and subsequent proteasome degradation. The newly formed peptides are then translocated to the ER in a transporter associated with antigen presentation (TAP) dependent manner, followed by class I MHC biding. This complex will then be transported to the Golgi Complex and later displayed at the surface of the cell, becoming able to interact with other receptors and promote an immune response. Antigen cross-presentation can also be achieved in a proteasome and TAP independent manner, through vector processing in the endosomal/lysosomal organelle followed by the loading of the peptides formed into recycled class I MHC (GROMMÉ et al., 1999; LI et al., 2013).

As mentioned previously, innate immunity seems to be linked to the adaptative responses facing rAAV therapy. The TLR9 and MyD88 dependent pathway, activated upon viral interaction, seems to play a role in the modulation of the immune response since TLR9-/- and MyD88-/- mice failed to induce a response after AAV vector stimulation, contrary to what happened in wild type mice that presented capsid-specific CD8+T cells in peripheral blood and spleen, 7 days after administration. The same study that showed the previous correlation also demonstrated that pDCs provide the activation signal necessary to initiate a capsid-specific CD8+ T cell response, this response was recovered in TLR9-/- mice receiving *wild type* pDCs but not *wild type* conventional dendritic cells (cDCs) and the block of type I IFN receptors resulted in the caused the abolition of the CD8+ T cell response. It should be noted that type I IFN production is one of the characteristics of pDCs (ROGERS *et al.*, 2017).

Furthermore, stimulation of capsid-specific CD8+ T cells depends not only on the TLR9 stimulation and signaling pathway, expressed in pDCs, but also on the cooperation of cDCs (BREWITZ *et al.*, 2017; ROGERS *et al.*, 2017; SHIRLEY *et al.*, 2019). The activation of TLR9, present in pDCs, leads to the instigation of an immune response through activation of

capsid-specific CD8+T cell, while the antigenic presentation is primarily achieved through the action of cDCs (ROGERS *et al.*, 2017; SHIRLEY *et al.*, 2019). It appears that the cross-presentation by the cDC is promoted by the secretion of type I IFN by the pDCs after viral stimulation and that this compound also upholds the maturation of the cDCs. Moreover, following viral immunization of mice, cell migration patterns were observed in their lymph nodes. pDCs migrated into 2 different areas: to the infected macrophages located at the subcapsular sinus and to CD8+T cell clusters formed around infected cDCs in the interfollicular area, referred to as CD8+T cell priming sites. CD8+ T cell activation led to the attraction of resident XCR1+DCs, a subtype of cDC, to the CD8+ T cell priming sites. This migration pattern allows the creation of an optimal environment to immune response development (BREWITZ *et al.*, 2017; GUTIÉRREZ-MARTÍNEZ *et al.*, 2015).

Helper CD4+T cells appear to have a noteworthy contribution to the modulation of the CD8+T cell response, since class 2 MHC-/- mice or suffered a significant reduction in this response after viral immunization. The blockage of the CD40L receptor present in CD4+T cells hinders the CD40L/CD40 interaction between them and DCs resulting in a diminished response compared to controls thus indicating that CD4+T cells are in fact necessary to achieve a proper CD8+T cell response (SHIRLEY *et al.*, 2019). Not only that but CD4+T cells produce cytokines which lead to the proliferation of B cells and antibody secretion targeting the viral particle (CROTTY, 2015).

3.5. Immune response towards the transgene product

During rAAV gene therapy, an immune response may be stimulated, not only against the vector itself but also against the expressed product, meaning that successful cells transduction may not be enough to therapy success (ASHLEY *et al.*, 2019; GARDNER *et al.*, 2019; HERZOG *et al.*, 2019)**.** For example, a robust specific T cell mediated immune response, leading to necrosis of liver tissue, cellular inflammation, and loss of product expression after AAV administration packaging peptide chicken ovalbumin in mice (ASHLEY *et al.*, 2019). Another case in which an immune response was detected against the transgene product consisted of the intramuscular administration of an AAV vector comprising an IgG sequence (as a method for HIV transmission prevention) in rhesus macaques followed by observation of the correlation between the therapeutic anti-HIV IgG and anti-IgG levels. The anti-HIV IGg levels were shown to decrease accompanied by an increase in anti-IgG levels, potentially leading to loss of its preventive action (GARDNER *et al.*, 2019).

Furthermore, it seems that the balance between the activation of TLR9 and regulatory T cells (Treg), a subtype of CD4+T cell expressing Forkhead box P3, defines the response

level. TLR9 activation (via ODN-1826, a potent TLR9 agonist) and Treg depletion, separately, caused a reduction of human factor IX (hFIX) expression correlated with an increase in the anti-hFIX IgG response in the context of AAV1-hFIX intramuscular administration in mice. CD8+T cell tissue infiltration was also observed in Treg depleted mice. However, the combination of TLR9 activation and Treg depletion failed to sustain an anti-hFIX response. Nevertheless, it induced a strong CD8+T cell muscle infiltration, correlated with an IFN-γ response (HERZOG *et al.*, 2019). One point that should be noted is that whether it is humoral or cellular, TLR9 and innate immunity once again appear to be linked and have a role in stimulating the immune reactions (ASHLEY *et al.*, 2019; HERZOG *et al.*, 2019).

Table 4. Summary of immune responses against rAAV in gene therapy.

Immune Responses	
Innate Immunity	Activation of TLR9 and TLR2, after interaction with DNA CpG motifs or capsid surface structures respectively, resulting in the expression of type I IFN and inflammatory cytokines (HÖSEL *et al.*, 2012; KAWASAKI e KAWAI, 2014; ZHU *et al.*, 2009).
	Expression of TNF-α and IFN-γ mediated by the interaction between the viral vector and NK cells (KURANDA *et al.*, 2018).
	Binding to C3 complement proteins, which lead to macrophage activation and vector opsonization (ZOU *et al.*, 2019).
Pre-existing Humoral Immunity	High prevalence of Nabs as a significant part of the population has been infected with *wild type* AAV (FITZPATRICK *et al.*, 2018; LI *et al.*, 2012; MONTEILHET *et al.*, 2010; SCALLAN *et al.*, 2006).
	Cross-reactivity and high co-prevalence of NAbs directed at different serotypes limit the potential of using different serotypes (KRUZIK *et al.*, 2019).
Pre-existing Cellular Immunity	Presence of memory CD8+T cells patients previously exposed to wild type, which when stimulated by the vectors secret TNF-α, IFN-γ, IL-2 and lead to the expression of granzyme B and CD107a degranulation markers (HUI *et al.*, 2015; KURANDA *et al.*, 2018; MARTINO *et al.*, 2012).
	CD4+T cells play a role in the modulation of the memory CD8+T response, and this response can vary depending on the vector administration route (GHENASSIA *et al.*, 2017; GROSS *et al.*, 2018).
	Cross-reactivity between different serotypes due to a great level of capsid conservation between them limits the potential of using different serotypes (HUI *et al.*, 2015; MANNO *et al.*, 2006; MINGOZZI *et al.*, 2007; VERON *et al.*, 2012).
Adaptative humoral immunity	Cytokine production by CD4+T cells leading to the proliferation of B cells and antibody secretion (CROTTY, 2015).
Adaptative cellular immunity	Stimulation of a cytotoxic response through cross-presentation and cross-priming mediated by cDCs and pDCs respectively (ROGERS *et al.*, 2017; SHIRLEY *et al.*, 2019).
	Cooperation between cDCs, pDCs, CD4+T cells, and TLR9 activation is needed for an effective immune response (BREWITZ et al., 2017; GUTIÉRREZ-MARTÍNEZ et al., 2015; ROGERS et al., 2017; SHIRLEY et al., 2019).
Transgene Immunity	The immune system can not only target the vector itself but also the transgene product (ASHLEY *et al.*, 2019; GARDNER *et al.*, 2019; HERZOG *et al.*, 2019).

4. Strategies to immune evasion

As previously discussed, immune responses against either the vector itself or the transgene product are an obstacle to the effective transfection of target cells or tissues, long-term product expression and, therapy success. Therefore, it becomes necessary to develop and deploy new strategies to circumvent the host reaction against the AAV vector. These include procedures such as the exploration of immune-privileged administration routes, the use of immunosuppressive drugs, or rational viral capsid design (CRAMER *et al.*, 2017; MARKS *et al.*, 2008; SELOT *et al.*, 2017).

4.1. Administration route

The human body has some compartments where the development of an immune response against the viral vector is less likely, probably to prevent inflammatory reactions in tissues with relatively low regenerative capabilities, hence being potentially beneficial routes for vector administration. It has been observed, in multiple studies, that administration in these immune-privileged tissues, such as the eye and the central nervous system, contributes to positive outcomes in rAAV gene therapy (BARTUS *et al.*, 2013; BOUQUET *et al.*, 2019; HAUSWIRTH *et al.*, 2008; LEWITT *et al.*, 2011; MARKS *et al.*, 2016; WORGALL *et al.*, 2008). For example, none of 12 patients, diagnosed with Parkinson's Disease, present in phase I clinical trial had significant humoral responses to neurturin (the neurotrophic factor packed into the viral vector, able to protect against neurodegeneration and to improve neuronal function) and only 4 of them had significant changes in AAV2 serology after intraputaminal administration. It should be noted that the patients with meaningful variations in anti-AAV2 antibodies titers were part of the group receiving a higher dose of vector while none of the patients receiving a low vector dose presented any significant changes and that this humoral response was not correlated with pre-existing immunity to AAV2 but with the dose of vector administered. Additionally, the patients presented good tolerability, safety, and improvement of their condition (MARKS *et al.*, 2008).

There also appears to be an immunotolerance when the therapy targets the liver, due to the immunosuppressive environment mediated by Treg and Kupfer cells (KCs) (BREOUS *et al.*, 2009; HEYMANN *et al.*, 2015). After vector administration, there is an increase in hepatic Treg levels accompanied by a production of immunosuppressive cytokines such as TGF-β and IL-10. In Treg depleted mice a pronounced hepatic CTL response was observed, while the same was not detected in Treg non-depleted mice. In turn, KCs seem to contribute to immunosuppression through the production of IL-10 (BREOUS *et al.*, 2009), activation, and promotion of Treg proliferation (HEYMANN *et al.*, 2015; WIEGARD *et al.*, 2005). Likewise,

there are long term positive results in rAAV gene therapy targeting the liver (GEORGE *et al.*, 2017; PASI *et al.*, 2020; RANGARAJAN *et al.*, 2017).

Furthermore, the simple change in the intravascular site by which the vector is administered can affect the immune response developed. The levels of NAbs observed in macaques, in which AAV8-FIX was administered differed according to the injection site. Macaques injected in the mesenteric vein showed much greater anti-AAV8 NAbs titters that the ones injected in the saphenous vein, and, as expected, much higher levels of vector genome were found on the liver of these last ones. Macaques in which the vector was administered via the mesenteric vein had approximately 1% of the hepatic vector DNA levels observed in macaques administered through the saphenous vein (MIMURO *et al.*, 2013).

Hereupon, it is important to study and access the various routes of administration by which the viral vector can be administered, as there are differences in the immune response that is induced depending on which one is used, since routes where there is a lack of response or immunosuppression prove to be advantageous for the success of gene therapy.

4.2. Transient immunosuppression

Gene therapy using rAAV can be accompanied by transient immunosuppression, that is, the induced impairment of the host's immune response during treatment, in order to improve its effectiveness. Several strategies are thought to be able to reduce the reactivity of the host's immune system towards the viral vector and/or the transgene product such as the use of antibodies directed at receptors involved in the modulation of the response or immunosuppressive medicines (FLANIGAN *et al.*, 2013; NATHWANI *et al.*, 2011; RECINO *et al.*, 2019).

In diabetic non-obese diabetic mice, the delivery of proinsulin-encoding AAVs and co-treatment with repeated nondepleting anti-CD4 antibody YTS177 injections resulted in a decrease of adaptative immune responses, expressed in diminished anti-AAV levels, allowed the achievement of normal glucose levels and the use of a lower effective dose, which wasn't possible in mice treated only with the viral vector (RECINO *et al.*, 2019).

Inhibition of the proteasome with inhibitors namely bortezomib and MG132 in mice HepG2/H-2Kb cells transfected by AAV2 packaging ovalbumin sequence lead to a great decrease in ovalbumin antigen presentation by these cells (LI *et al.*, 2013). As discussed before, the proteasome seems to be necessary for the processing of the vector in smaller peptides, amenable to being presented to competent immune cells in the context of class I MHC and subsequent loss of transgene expression (FINN *et al.*, 2010; GROMMÉ *et al.*, 1999; LI *et al.*, 2013).

In a clinical trial whose participants suffered from haemophilia B, prednisolone treatment allowed long-term expression of hFIX. The administration of the immunosuppressive medicine prevented the destruction of AAV-transduced hepatocytes, evidenced by the normalization of the increased aminotransferase plasma levels, and preserved transgene expression (NATHWANI *et al.*, 2014). Prednisone administration has shown to decrease skeletal muscle T-cell infiltrates and reduced serum anti-AAV levels in macaques treated with rAAVrh74 encoding GALGT2 (CRAMER *et al.*, 2017). Likewise, the administration of nanoencapsulated rapamycin resulted in an overall reduction in the immune responses towards the vector and allowed re-administration in both mice and non-human primates (MELIANI *et al.*, 2018). Oral prophylactic administration of the immunogenic protein before AAV-therapy can also affect the immune reactions presented. Oral tolerization with ovalbumin before muscle AAV gene transfer prevented both cellular and humoral responses and CD8+T cells infiltration in the transduced muscles, thus favouring long-term product expression (HARDET *et al.*, 2016).

Another interesting immunosuppression strategy is the use of immunoadsorption. This procedure consists of the removal of circulating immunoglobulins from the patient's plasma using a suitable matrix (LAZARIDIS *et al.*, 2015, 2017). The patient's plasma passes through a matrix composed of AAV-virions, to which the respective immunoglobulins will bind, causing its retention. The patient then receives the processed plasma, disproved of anti-AAV, resulting in a diminished humoral response (ORLOWSKI *et al.*, 2020; SALAS *et al.*, 2019). A simpler method for overcoming a pre-existing immune response is to use empty capsids as a decoy when administering the therapeutic product since both are similarly effective at adsorbing antibodies (MINGOZZI *et al.*, 2013).

4.3. Direct Evolution

Direct evolution strategies are based on AAV genetic diversification through the attainment of potentially beneficial mutation and subsequent creation of libraries comprising of different capsid sequences. Selective pressure is then applied in order to select the AAV-variants most advantageous and apt to overcome therapy barriers (BARTEL *et al.*, 2012). These strategies don't require previous mechanistic or structural knowledge to be successful and allow quick obtention of a large number of different genetic sequences (CABANES-CREUS *et al.*, 2019; MCCULLUM *et al.*, 2010).

4.3.1. Error-prone PCR mutagenesis

Error-prone PCR mutagenesis is a method consisting of a PCR procedure in conditions that affect the polymerase activity, allowing errors to occur during DNA chain synthesis, leading to the occurrence of random point mutations during amplification of the AAV capsid sequence. At the end of the amplification, a wide range of different capsid sequences is obtained (Mccullum *et al.*, 2010). Using error-prone PCR mutagenesis, Maheshri et *al.* generated a library comprised of AAV2-variants and subsequently tested its antibody evasion and gene delivery capabilities. Mice presented an increased haematocrit following erythropoietin delivery via the AAV-variants r2.15 and 2.4 compared to mice where gene delivery was mediated by *wild type* vectors. It should be noted that gene delivery and expression persisted even when preincubated with antiserum capable of neutralizing the *wild type* capsid vector (MAHESHRI *et al.*, 2006).

4.3.2. DNA shuffling

The DNA shuffling method consists on the digesting and fragmentation of AAV capsid sequences from two or more different capsid sequences and subsequent primerless PCR reassembly. The reassembly process is based on the homology between the capsid sequences of different serotypes, which allows the fragments to self-prime, leading to the creation of new capsid sequences. The final product consists of several chimeric capsids with characteristics from the different parental serotypes used in the procedure (CABANES-CREUS *et al.*, 2019; HERRMANN *et al.*, 2019). Chimeric capsids have been created through DNA shuffling using capsid sequences from parental serotypes 1, 2, 3b, 4, 5, 6, 8, 9_hu14, avian, and bovine. The assays performed demonstrated greater liver transduction in humanized mice treated with the variants NP40 and NP59 in comparison to control serotypes. It should also be noted the great specificity for human hepatocytes since little or no transduction was seen in non-humanized rat livers. Furthermore, human serum from 50 healthy US adults was used to access the seroreactivity of the variants *versus* controls, in which the variants NP84, NP59, NP40, and DJ presented significantly diminished seroreactivity profiles (PAULK *et al.*, 2018).

As this method is based on capsid sequence digestion followed by reassembly, a high degree of homology between the fragments from the parental serotypes is necessary to achieve successful recombination, therefore, fragments from more distinct paternal capsids have less effective annealing, thus reducing library diversity (CABANES-CREUS *et al.*, 2019; HERRMANN *et al.*, 2019; KIENLE *et al.*, 2012). Cabanes-Creus *et al.*, developed the localized codon-optimization (LCO) strategy, consisting of localized optimization at each codon, independently of the rest of the sequence. The application of the LCO algorithm resulted in

the optimization of paternal capsid sequences and greater library diversity (Cabanes-Creus *et al.*, 2019).

4.3.3. Peptide Insertion

Capsid diversity can be achieved by inserting peptide sequences into the capsid sequence, altering the capsid, and potentially assisting in immune response evasion (BARTEL *et al.*, 2012). Random peptides can be introduced into specific sites of the viral capsid through the insertion of random oligonucleotides into the cap ORF (BARTEL *et al.*, 2012; MÜLLER *et al.*, 2003). Applying this method, a novel random peptide library was created, by inserting random peptides into the A589 residue of the AAV9 capsid, in which mutant species with greater antibody evasion abilities that the *wild type* were identified (VARADI *et al.*, 2012). Contrarywise, a specific peptidic sequence can be inserted in a random site of the capsid sequence mediated by transposon-based mutagenesis. This method involves a transposase mediated transfer of a defined oligonucleotide, encoding the peptide of interest, into random sites of the Cap ORF (BARTEL *et al.*, 2012; KOERBER e SCHAFFER, 2008).

Table 5. Summary of strategies for creating AAV-variants libraries

Direct Evolution		
Error-prone PCR mutagenesis		PCR procedure in conditions that promote the occurrence and accumulation of errors during DNA amplification, potentially resulting in beneficial capsid mutations (Mccullum et al., 2010).
DNA shuffling		Digestion of capsid sequences of different serotypes and subsequent primerless PCR recombination of the generated fragments, hence creating new chimeric capsid sequences with characteristics of the ones that originated them (CABANES-CREUS et al., 2019; HERRMANN et al., 2019).
Peptide Insertion	Insertion of random peptides	Insertion of random oligonucleotides into a specific AAV cap ORF site (BARTEL et al., 2012; MÜLLER et al., 2003).
	Transposon-based mutagenesis	Insertion of a specific oligonucleotide into a random AAV cap ORF site via transposase mediated transfer (BARTEL et al., 2012; KOERBER e SCHAFFER, 2008).

Virions originating from these sequences subsequently undergo selective pressure to determine which ones are most apt to overcome therapy barriers and to achieve optimal gene delivery (BARTEL et al., 2012).

4.4. Rational Design

Xie *et al.*, using x-ray crystallography, studied the AAV2 structure (XIE *et al.*, 2002), Lerch *et al.* determined the AAV3B crystalline structure and identified its binding site to heparin, an analogue of the heparan sulfate proteoglycan receptor, using a combination of

biochemical and structural approaches (LERCH *et al.*, 2010; LERCH e CHAPMAN, 2012). Using cryo-electron microscopy and image reconstruction, a 3D reconstruction of the AAV8: ADK8 (a neutralizing monoclonal antibody) complex was accomplished (GURDA *et al.*, 2012). The novel information attained regarding the AAV structure, biology, and mechanisms underlying infection (DIMATTIA *et al.*, 2012; NAM *et al.*, 2007; PADRON *et al.*, 2005; WALTERS *et al.*, 2004) allows a new approach to issues of immunologic nature. This newly acquired knowledge allows the rational manipulation of the virus in order to bypass immune mechanisms that otherwise would be unapproachable. Giles et al. reconstructed the complex between AAV9 and the antibody PAV9.1, followed by an examination of the epitopes that participated in this interaction. Taking into account the structural analysis, the viral capsid was then modified, leading to interaction disruption and diminished binding (GILES *et al.*, 2018).

4.4.1. Site-directed Mutagenesis

Based on the knowledge previously acquired regarding the immune mechanisms underlying AAV infection and rAAV therapy, a greater evasion of the immune response can be achieved, through site-directed mutagenesis. As discussed earlier, proteasome degradation is an important process for antigenic presentation and subsequent immune response (FINN *et al.*, 2010; LI *et al.*, 2013). The process appears to be mediated by capsid tyrosine phosphorylation via epidermal growth factor receptor protein tyrosine kinase (EGFR-PTK) since its inhibition led to a decrease in the ubiquitination of AAV2 capsids and consequently to a diminished AAV2 degradation. Tyrosine-phosphorylated AAV2, despite still being infectious, had a significant reduction in transduction efficiency (ZHONG *et al.*, 2007, 2008). Petrs-Silva *et al*. produced tyrosine-to-phenylalanine capsid mutants of the AAV serotypes 2, 8, and 9 leading to an increase in cell transduction up to almost 20 times compared to *wild type* (PETRS-SILVA *et al.*, 2009). Furthermore, AAVrh.10 were similarly modified (serine/threonine to alanine and lysine to arginine) at phosphodegron like regions, that is, regions prone to phosphorylation. Transgene expression was relatively high in pre-immunized mice in which mutant AAVrh.10 was administered, however, in *wild type* AAVrh.10 treated mice was either negligible or abrogated (SELOT *et al.*, 2017).

4.4.2. Peptide Insertion

Another strategy to rational design relies on the insertion of known peptides into the AAV capsid. The atomic structure of AAV2 was determined by x-ray crystallography as previously mentioned. It seems that each subunit consists of a β-barrel motif and between them, there are large loop insertions, responsible for direct interaction with cellular receptors

and antibodies (XIE *et al.*, 2002). Furthermore, Huttner *et al* inserted a 14 aminoacid peptide of the laminin fragment P1 into the positions 261, 381, 447, 534, 573, or 587 of the VP3 capsid protein to examine the antigenic domains implicated in the humoral immune response. Following modification, the developed AAV variants were then tested in human serum samples from 65 different participants. Mutations at the positions 534 or 573 reduced antibody binding up to 70% (HUTTNER *et al.*, 2003).

4.5. Chemical Modifications

It is possible to enhance immune evasion through chemical modifications onto the AAV vector surface. Polyethylene glycol (PEG) is a low immunogenic polymer, lacking toxicity, and regularly used in the food, cosmetic and pharmaceutical industries (ROBERTS *et al.*, 2012). PEGylation, that is, the addition of PEG to the viral capsid surface, appears to have beneficial properties in the context of immune evasion and gene therapy (LE *et al.*, 2005; LEE *et al.*, 2005; YAO *et al.*, 2017). PEG conjugation with lysine capsid residues results in a moderate cell transduction increase in the presence of Nabs compared to the unprotected vector. Factors such as PEG chain molecular weight or PEG:lysine ratios should be carefully considered due to the risk of cell transduction impairment (LEE *et al.*, 2005). Capsid genetic engineering can facilitate the insertion of surface ligands. Yao *et al.* genetically incorporated unnatural aminoacids, namely the lysine mimic Nε-2-azideoethyloxycarbonyl-L-lysine, enabling capsid site-specific PEG coupling. PEGylated rAAV2 presented a reduced antibody recognition of approximately 50% *in vitro. In vivo,* the PEGylated vector presented a delayed blood clearance and a reduction in antibody induction (YAO *et al.*, 2017). Using a similar method, Katrekar *et al.* incorporated oligonucleotides into the AAV capsid surface and later incubated the resulting product with lipofectamine, a commercial lipid based transfection reagent, leading to the formation of a "cloaked AAV". The "cloaked AAV" retained activity in pig serum concentrations that fully neutralized the *wild type* vector (KATREKAR *et al.*, 2018).

5. Conclusion

Scientific and technological advances have enabled a great development of new therapeutic strategies, better adapted to patients and their needs, namely in the field of gene therapy. The use of AAV as a transgenic delivery system in the context of gene therapy emerged due to its advantageous properties, such as its ability to provide long-term transgene expression, its low immunogenic profile, the fact that transgene expression can be localized in specific tissues depending on the AAV serotype used, among other features and has provided some noteworthy results in clinical trials.

However, AAV mediated gene therapy still faces some challenges, namely the immune response induced following vector administration. Despite having a low immunologic profile, the immune system can still perceive the AAV vector as a strange and potentially hazardous particle and does not remain indifferent to its presence. Both innate and adaptative immune responses can be induced following vector administration. There is evidence that such reactions are not independent of each other, and for example, stimulation of a cellular cytotoxic response depends on TLR9 and MyD88 dependent pathway activation, as previously discussed. Additionally, the immune system may already have pre-established defenses, such as NAbs or memory CD8+T cells, due to natural *wild type* AAV infection and to the fact that the *wild type* and vector capsids and essentially similar. Furthermore, an immune reaction can also take place in response to the transgene product and not only against the vector itself, meaning that successful transgene delivery may not be enough to achieve a successful therapy.

The scientific advanced and new knowledge acquired concerning AAV biology and mechanisms underlying infection enabled the development of new strategies to overcome the immune responses induced following AAV vector administration. These strategies include vector administration at immune-privileged tissues where the development of an immune response is unlikely, such as the eye or central nervous system, transient immunosuppression during treatment to reduce the reactivity of the immune system towards the AAV vector or diminishment of vector recognition by immune cells through capsid modifications. These modifications can be accomplished at a superficial/structural level through the binding of specific ligands, such as PEG, at the capsid surface, or a genetic level, for example, through the insertion of mutations or new sequences in the capsid sequence, that can later translate in capsid structure differences.

In conclusion, despite being one of the most promising transgenic delivery systems and a very useful tool in the gene therapy kit the AAV vector sill faces some challenges, namely of an immunological nature, however, the new developments in this matter raise the expectancy of further success in AAV mediated gene therapy.

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