Tese de Doutorado

BASES MOLECULARES DA REESTENOSE INTRA-STENT E NOVAS ESTRATÉGIAS TERAPÊUTICAS

Ms. Juliane da Silva Rossato

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Programa de pós-Graduação em Ciências da Saúde:

Cardiologia e Ciências cardiovasculares

BASES MOLECULARES DA REESTENOSE INTRA-STENT E NOVAS ESTRATÉGIAS TERAPÊUTICAS

Autor: Ms. Juliane da Silva Rossato

Orientador: Dr. Alexandre do Canto Zago

Tese submetida como requisito básico para obtenção do grau de doutor ao Programa de Pós-Graduação em Ciências da Saúde, Área de Concentração: Cardiologia e Ciências Cardiovasculares, da Universidade Federal do Rio Grande do Sul.

Porto Alegre

2017

CIP - Catalogação na Publicação

Rossato, Juliane da Silva
BASES MOLECULARES DA REESTENOSE INTRA-STENT E NOVAS ESTRATÉGIAS TERAPÊUTICAS / Juliane da Silva Rossato. -- 2017.
70 f.
Orientador: Alexandre do Canto Zago.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Medicina, Programa de Pós-Graduação em Ciências da Saúde: Cardiologia e Ciências Cardiovasculares, Porto Alegre, BR-RS, 2017.

1. stent. 2. reestenose. 3. aterectomia direcionada. 4. expressão gênica. I. Zago, Alexandre do Canto, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS 6
RESUMO 8
1. INTRODUÇÃO 9
2. REVISÃO DE LITERATURA 11
2.1 ATEROSCLEROSE 11
2.2 DOENÇA ARTERIAL CORONARIANA 15
2.3 BASES MOLECULARES DA HIPERPLASIA NEOINTIMAL 18
2.4 STENTS COM TERAPIA FARMACOLÓGICA 21
2.5 TERAPIA GÊNICA 22
3. JUSTIFICATIVA E OBJETIVOS 28
4. REFERÊNCIAS DA REVISÃO DE LITERATURA 29
5. ARTIGO 1 37
6. ARTIGO 2 54
7. CONSIDERAÇÕES FINAIS 70

LISTA DE ABREVIATURAS E SIGLAS

- Akt - Proteína quinase B
- ATP - Adenosina trifosfato
- AVC - Acidente vascular cerebral
- CNN – Calponina, músculo liso
- CXCR 4 - Receptor CXC de quimiocina do tipo 4
- DAC - Doença arterial coronariana
- DVC - Doenças cardiovasculares
- End 1 - Endotelina 1
- eNOS - Óxido nítrico sintase endotelial
- EROS - Espécies reativas de oxigênio
- IAM - Infarto agudo do miocárdio
- ICAM-1 - Intracellular Cell Adesion Molecule-1
- ICP – Intervenção Coronariana Percutânea
- IL 1 - Interleucina 1
- IL 10 - Interleucina 10
- IL 6 - Interleucina 26
- IFN- γ - Interferon gamma
- iNOS - Óxido nítrico sintase induzível
- MAPK - Proteína quinase ativada por mitógeno
- MMP - Metaloproteinase de matriz
- mRNA - Ácido ribonucleico mensageiro

- mTOR – mammalian target of rapamycin
- NADPH oxidase - Oxidase dependente de nicotinamida adenina dinucleotídeo fosfato
- NFκB - Nuclear Factor kappa B
- NK - células natural killer
- NO - Oxido nítrico
- $ONOO^-$ - Peroxinitrito
- PA - Pressão arterial
- PI3K - Fosfatidilinositol 3 quinase
- SMMHC – Miosina de cadeia pesada de músculo liso
- TGF β - Fator de crescimento transformante beta
- TIMP 1 - Inibidor tecidual de metaloproteinases de matriz do tipo 1
- TIMP 3 - Inibidor tecidual de metaloproteinases de matriz do tipo 3 -
- TNF α - Fator de necrose tumoral alfa
- VEGF - Fator de crescimento endotelial vascular
- VCAM-1 - Vascular Cell Adesion Molecule-1

RESUMO

As doenças cardiovasculares apresentam-se como síndromes clínicas que possuem causas relevantes como diabetes, hipertensão, obesidade, aterosclerose. O desenvolvimento de terapias medicamentosas e intervenções cirúrgicas proporcionaram uma maior sobrevivência e qualidade de vida para os pacientes. A intervenção coronariana percutânea com implante de *stent*, regular ou farmacológico, é um procedimento consagrado para restabelecimento do fluxo sanguíneo pós processo isquêmico. Entretanto, a reestenose, um fenômeno recorrente em alguns pacientes que realizam angioplastia, ainda apresenta fisiopatologia desconhecida. Esse estudo objetivou investigar o perfil genético de placas ateromatosas de pacientes que desenvolveram reestenose pós-implante de *stent*, verificando, posteriormente, se o silenciamento de genes específicos para estrutura e função das células musculares lisas vasculares poderia bloquear o processo proliferativo destas sem prejudicar o restabelecimento das células endoteliais. Como resultado do perfil genético foram identificados dois genes, *SMMHC (myosin, heavy chain) e CNN (calponin),* que apresentam expressão aumentada nos pacientes que desenvolveram reestenose. Seguiu-se o estudo *"in vitro"* de fármacos antiproliferativos e silenciadores gênicos específicos para células musculares lisas (Sh RNA SMMHC e Sh RNA CNN) na viabilidade de células endoteliais e musculares lisas de artéria coronária humana. As análises demonstraram que os silenciadores gênicos específicos para células musculares lisas apresentaram um efeito inibitório sobre a proliferação de células musculares lisas de artéria coronária humana, sem interferir na proliferação de células endoteliais.

Descritores: *Stent*; Reestenose; Expressão gênica; Aterectomia direcionada

1. INTRODUÇÃO

Doenças crônicas cardiovasculares, pulmonares, câncer e diabetes são as maiores causas de invalidez e morte na atualidade, fato que promove um impacto tanto econômico quanto social (1). As doenças cardiovasculares (DCVs) são a principal causa de morte no Brasil e, apesar de uma tendência a estabilização dos números de óbitos ocasionados por DCVs, essas continuam apresentando uma incidência elevada. Outro fator importante são as complicações que podem levar a desfechos mais severos, como o Infarto Agudo do Miocárdio e o Acidente Vascular Cerebral, normalmente associados a fatores de risco como obesidade, tabagismo, inatividade física, diabetes, hipertensão e aterosclerose (2).

A evolução da aterosclerose e o desenvolvimento da Doença Arterial Coronariana (DAC) estão relacionados com o processo de disfunção endotelial, gerada pelo aumento da produção de espécies reativas de oxigênio e ativação de um processo inflamatório cíclico, levando a um estado de estresse oxidativo celular e redução da biodisponibilidade de óxido nítrico para endotélio (3, 4). O espessamento contínuo da placa aterosclerótica promove a redução gradativa do diâmetro do vaso e consequente obstrução do fluxo sanguíneo, causando um processo isquêmico (5).

A intervenção coronária percutânea (ICP) é um procedimento estabelecido para o tratamento de pacientes com DAC, apresentando um significativo índice de sucesso no restabelecimento da homeostase vascular (6). Contudo, cerca de 15 a 30% dos pacientes, principalmente de alguns grupos especiais como os diabéticos, por exemplo, apresentam um quadro de obstrução luminal pós introdução do *stent*, denominado reestenose, fato que reduz a qualidade de vida do paciente e aumenta o risco de mortalidade (7, 8).

A reestenose é uma reação vascular a introdução de um *stent* no local de desenvolvimento de uma placa ateromatosa que estaria obstruindo o fluxo sanguíneo e gerando um processo isquêmico (9). Após a ICP, normalmente nos primeiros seis meses, inicia um processo de proliferação de células musculares lisas criando um novo tecido adjacente à túnica íntima do vaso, clinicamente denominada hiperplasia neointimal (10). Dessa limitação promovida pela reestenose, surgiram *stents* farmacológicos com propriedades antiproliferativas (11), como a rapamicina e o paclitaxel, de ação direta sobre a progressão do ciclo celular, que impediriam a proliferação desordenada das células constituintes do vaso. O uso de fármacos

antiproliferativos na confecção de *stents* farmacológicos apresenta-se como uma alternativa terapêutica consagrada na prática médica, no entanto esses procedimentos apresentaram ao longo do tempo algumas limitações, como falha na endotelização do *stent* e aumento do tempo de uso de antiagregantes plaquetários (12, 13). Dessa forma, a busca de novas perspectivas de tratamento, que considerem a proliferação de células musculares lisas como a base do desenvolvimento da reestenose, devem ser investigadas (14).

Um estudo realizado pelo nosso grupo de pesquisa sugere que cada placa ateromatosa pode apresentar diferentes níveis de predisposição ao desenvolvimento de hiperplasia neointimal, que variariam de acordo com o aumento da expressão de genes relacionados com proliferação de células musculares lisas (15). Nesse estudo, identificamos que a inibição seletiva de genes relacionados com a proliferação de células musculares lisas pode ser uma nova alternativa para diagnóstico e tratamento da reestenose. Assim, poderíamos obter um agente direto e específico no bloqueio da hiperplasia neointimal, que permitisse a proliferação fisiológica e controlada do endotélio intra-*stent* e aumentasse a probabilidade de sucesso nos procedimentos de intervenção coronária percutânea.

2. REVISÃO DE LITERATURA

2.1 ATEROSCLEROSE

Doenças crônicas não transmissíveis (DCNT), como a cardiopatia isquêmica, o acidente vascular cerebral, as doenças pulmonares obstrutivas (DPOC) são as maiores causas de invalidez e morte na atualidade, fato que promove um impacto social e econômico em diferentes países (16). A estimativa, apresentada pela Organização Mundial de Saúde (OMS), é de que, entre os anos de 2000 a 2012, ocorreram aproximadamente 17,5 milhões de óbitos relacionados à doença arterial coronariana (DAC) e ao acidente vascular cerebral (AVC), representando, assim, 31% das causas de morte no mundo (17). No Brasil, podem ser observados que os maiores índices de obesidade têm ocorrência coincidente com as regiões de maior desenvolvimento econômico do país, como São Paulo (41%), Rio de Janeiro (46%) e Porto Alegre (43%) que apresentam esses valores quanto ao percentual de excesso de peso (18, 19). Dessa forma, alterações metabólicas geradas pelo potencial inflamatório da obesidade associa-se a fatores de risco como, dislipidemias, hipertensão, diabetes, sedentarismo, tabagismo, alcoolismo, para promover o desenvolvimento de aterosclerose e doenças cardiovasculares (20).

A aterosclerose, caracterizada como uma doença inflamatória crônica promove o desenvolvimento de desfechos severos como infarto cerebral e do miocárdio (21, 22), apresentando como principais fatores de risco para a formação desse quadro a obesidade, a hipertensão, a diabetes e a inatividade física. (1, 23). Estudos revelam que a obesidade representa um estado crônico, de inflamação e nutrição excessiva associado com a infiltração do tecido adiposo por células inflamatórias. Assim, células imunológicas, como macrófagos do tecido adiposo, devem contribuir significativamente para a inflamação na obesidade e também com relação à ocorrência do processo de resistência a insulina (4, 24, 25). Processos de inflamação crônica e casos de desregulação metabólica que estão associados a doenças como diabetes, doenças cardiovasculares levam a permanente sinalização de vias que respondem a esse estresse patológico nos diferentes tecidos que constituem a parede vascular (26, 27).

O endotélio vascular exerce um importante papel na regulação do tônus vascular e na manutenção da homeostasia cardiovascular (28). O desenvolvimento da Doença Arterial Coronariana (DAC) e a evolução da ateroesclerose estão relacionados com o

processo de disfunção endotelial, gerada pelo aumento da produção de espécies reativas de oxigênio e ativação do NFκB (*Nuclear Factor kappa B*), levando a um estado de estresse oxidativo celular e redução da biodisponibilidade de óxido nítrico para endotélio (3, 29). Associado ao quadro de formação do ateroma existe uma resposta inflamatória crônica promovida por células imunológicas, como monócitos e linfócitos (28, 30).

Os monócitos/macrófagos são células de destaque no sistema imunológico, pois participam da resposta inata, realizando fagocitose e liberando espécies reativas de oxigênio em uma ação microbicida (31, 32). Um importante marcador da função imune dos macrófagos é a produção de óxido nítrico (NO), que é dependente da expressão da enzima óxido nítrico sintase indutível (iNOS) via ativação do NF-κB em resposta a variações do estado redox e estimulação por citocinas como IFN- γ (*Interferon gamma*), TNF - α (*Tumor Necrosis Factor alfa*), IL - 1β (*Interleucina 1 beta*) (33, 34). Essas citocinas induzem, nas células endoteliais, o aumento da expressão de receptores para moléculas de adesão da matriz extracelular, como ICAM-1 (*Intracellular Cell Adesion Molecule-1*) e VCAM-1 (*Vascular Cell Adesion Molecule-1*) (35), que promovem a fixação e rolamento de células imunológicas na superfície endotelial . Os monócitos são células aderentes ao endotélio, que podem penetrar na camada íntima, diferenciam-se em macrófagos capazes de expressar receptores scavenger, como CD36 (*cluster of differenciation 36*), que permitem intensa fagocitose de partículas de LDL, tornando-se, assim, uma *foam cell* (36). Os macrófagos são células capazes de acumular colesterol e ésteres de colesterol em um ciclo de hidrólise e reesterificação para uso metabólico e de síntese de membrana. No entanto, a *foam cell* fagocita LDL, provocando sua oxidação e consequentemente ampliando a lesão inflamatória tecidual (4, 37).

As partículas de LDL circulantes são permeáveis ao endotélio, acumulando-se, assim, na túnica íntima onde podem ser oxidadas por radicais livres. Esse processo gera a formação de partículas de LDL oxidadas (LDL ox), moléculas com estrutura química diferente da LDL nativa, que são responsáveis pela expressão de moléculas de adesão na superfície endotelial. No interior da parede vascular, os macrófagos não reconhecem as partículas de LDL ox como próprias do organismo e as fagocita, iniciando o processo de formação de um tipo celular com perfil altamente inflamatório, a *foam cell*. Esta é capaz de produzir mediadores inflamatórios que promovem o recrutamento de células inflamatórias para o local, a maior expressão de moléculas de adesão endoteliais e disfunção de células musculares lisas (Figura 1) (38).

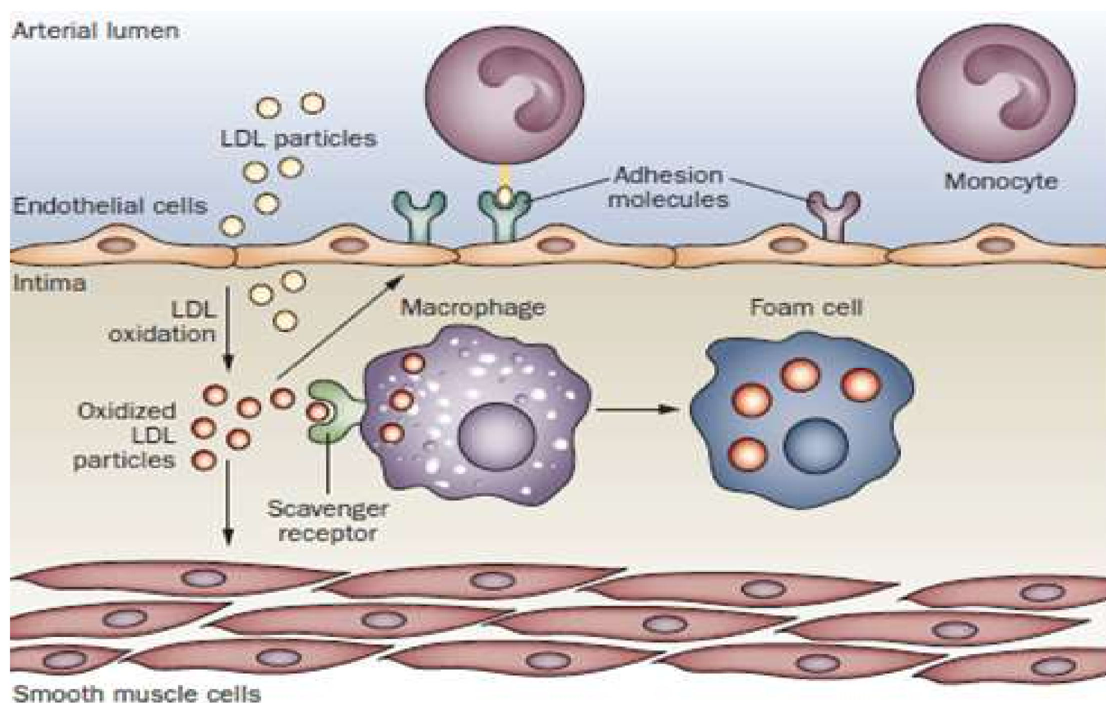

Figura 1. Efeitos das partículas de LDL na parede vascular (Rocha & Libby, 2009)

Em um quadro de formação da placa ateroesclerótica existe uma associação entre células endoteliais, musculares lisas e macrófagos ativados, com uma intensa fagocitose e oxidação de lipoproteínas de baixa densidade, com recrutamento de células imunes e produção de citocinas que levam a um processo inflamatório crônico.(30, 32)

Citocinas como o TNF- α a IL-6 são componentes pró-inflamatórios produzidos pelos macrófagos que se apresentam envolvidos no desenvolvimento de doenças vasculares. O estresse mecânico pode iniciar uma cascata de sinalização levando as células endoteliais a um ciclo de estresse. O endotélio quando tratado com inibidores do NFκB, resulta em uma inibição da IL-6 em 50%, sugerindo que a modulação deste evento contribui para a patogênese da aterosclerose (39-42). O TNF-α é um agente de fase aguda da inflamação que também contribui para o mecanismo de resistência a insulina por inibir a atividade do receptor de tirosina quinase e por produzir uma diminuição da regulação de genes do transportador de glicose (GLUT) (43). Assim, doenças como dislipidemias, aterosclerose e diabetes estão relacionadas com ao desenvolvimento de um processo inflamatório crônico, com intensa produção de citocinas pró- inflamatórias.

A aterosclerose é uma doença inflamatória crônica da parede arterial, iniciada normalmente por uma lesão vascular que gera perda da integridade endotelial e danos oxidativos, principalmente em grandes bifurcações arteriais como aorta, carótidas e

coronárias (44). Essas lesões caracterizam-se por espessamentos assimétricos focais da túnica íntima, a camada mais interna do vaso, e são mantidas pela interação entre as LDL oxidadas plaquetas, macrófagos ativados como células espumosas ("*foam cells*"), linfócitos T ativados e constituintes celulares normais da parede arterial (45).

A progressão da placa de aterosclerose ocorre a partir de uma série de alterações vasculares que incluem perturbação do fluxo sanguíneo, aumento da permeabilidade vascular, recrutamento de células imunológicas e produção de mediadores inflamatórios, proliferação de células musculares lisas e acúmulo de colesterol. (46). O espessamento contínuo da placa aterosclerótica promove a redução gradativa do diâmetro do vaso e consequente obstrução do fluxo sanguíneo, em um processo denominado isquemia. A redução do fluxo sanguíneo promove uma diminuição da disponibilidade de oxigênio e nutrientes, redução da fosforilação oxidativa e da produção de ATP, fato que leva à falência do metabolismo energético com lesão irreversível celular. (5).

Conforme a Figura 2, no processo de desenvolvimento da aterotrombose, os macrófagos *foam cells* e linfócitos, ambos com polarização inflamatória, apresentam um papel central no desenvolvimento da placa aterosclerótica e do core necrótico (NC). O estresse de cisalhamento, com consequente ruptura da placa, desencadeia um processo que envolve a ativação plaquetária e expressão de moléculas de adesão endoteliais, resultando na formação de trombos potencialmente oclusivos. A agregação plaquetária associada ao recrutamento de células inflamatórias, como monócitos e neutrófilos, gera a produção de citocinas e mediadores inflamatórios, estabelecendo a quimiotaxia de mais leucócitos para o local. O quadro de cardiopatia isquêmica, causado por obstrução da artéria coronária, contribui para o acúmulo células inflamatórias, com posterior substituição do tecido lesado por tecido fibroso, resultando em uma redução da capacidade funcional do miocárdio. A disfunção cardíaca desenvolvida promove um importante desequilíbrio hemodinâmico que frequentemente sobrecarrega outros órgãos, como por exemplo, os rins, causando flha de funcionalidade no mesmo (47).

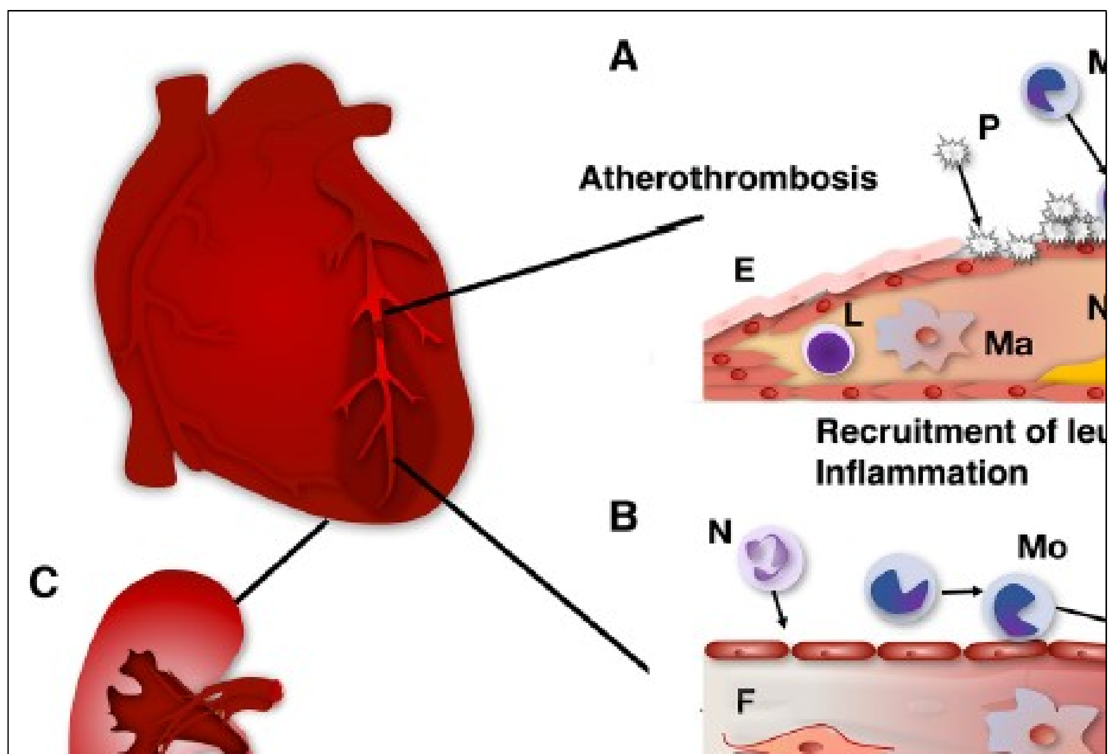

Figura 2: Síndrome Coronariana Aguda

Legenda: E (endotélio), P (plaquetas), Ma (macrófagos), Mo (monócitos), N (neutrófilos), NC (core necrótico), MF (miofibroblastos) (Adaptado de Thomas *et al*., 2017).

2.2 DOENÇA ARTERIAL CORONARIANA

A doença arterial coronariana normalmente resulta da formação de uma placa ateromatosa na superfície interna da parede de vasos que irrigam o coração (48, 49). O crescimento positivo dessa placa em direção à luz do vaso estabelece-se gradualmente, promovendo uma redução contínua do aporte de oxigênio e nutrientes para o tecido muscular cardíaco. Caso o fluxo sanguíneo cesse inteiramente, ocorrerá um infarto agudo do miocárdio, que pode significar uma grave lesão tecidual com risco de comprometimento da sobrevida do paciente (50, 51).

A doença arterial coronariana pode apresentar diferentes manifestações nos pacientes, desde silenciosa (assintomática), ao sintoma clássico de dor no peito, denominada angina *pectoris* (52). Essa consiste em um quadro de isquemia, porém sem necrose, caracterizada por uma dor subesternal, cuja duração e a gravidade não são suficientes para causar necrose no músculo cardíaco (53, 54).

A formação de placa ateromatosa vulnerável ou instável, na região intimal da parede da artéria coronária, frequentemente está relacionada ao desenvolvimento de uma capa fibrosa mais fina, que favorece a ocorrência de estresse de cisalhamento (*shear*

stress) e consequente desnudamento do endotélio, proporcionando ativação plaquetária e potencial formação de trombos (55, 56). Assim, estudos recentes destacam que uma alimentação balanceada e atividade física regular contribuiriam para a estabilização da placa aterosclerótica, evidenciando um efeito redutor sobre o colesterol total, LDL e triglicerídeos, e o aumento do HDL-c, desenvolvendo, assim, uma capa fibrosa mais espessa que protege o vaso da ruptura (57-59).

Uma placa ateromatosa vulnerável caracteriza-se um core lipídico superior a 40% do volume da mesma e um elevado acúmulo de células inflamatórias, como macrófagos, mastócitos e linfócitos T (60, 61). Essa formação na túnica íntima apresenta uma camada reduzida de células musculares lisas vasculares, no entanto o estresse de cisalhamento nesse quadro favorece a redução da biodisponibilidade de NO e a indução de hiperplasia neointimal que pode favorecer o desenvolvimento de reestenose. O aumento do estresse de cisalhamento induz a formação de uma placa aterosclerótica vulnerável através da geração de novos vasos sanguíneos na túnica íntima. Esse quadro se desenvolve pelo aumento da expressão do fator de crescimento endotelial vascular (VEGF) e do óxido nítrico endotelial (NO), que estimulam a proliferação de células endoteliais, porém induzem apoptose nas células musculares lisas vasculares, resultando em uma parede vascular mais fina e repleta de novos microvasos (Figura 3) (56). A ruptura da placa é, normalmente, a principal causa de eventos coronarianos agudos, sendo que as placas vulneráveis apresentam o maior risco de ruptura, seguidas de trombose e rápida progressão da estenose (62).

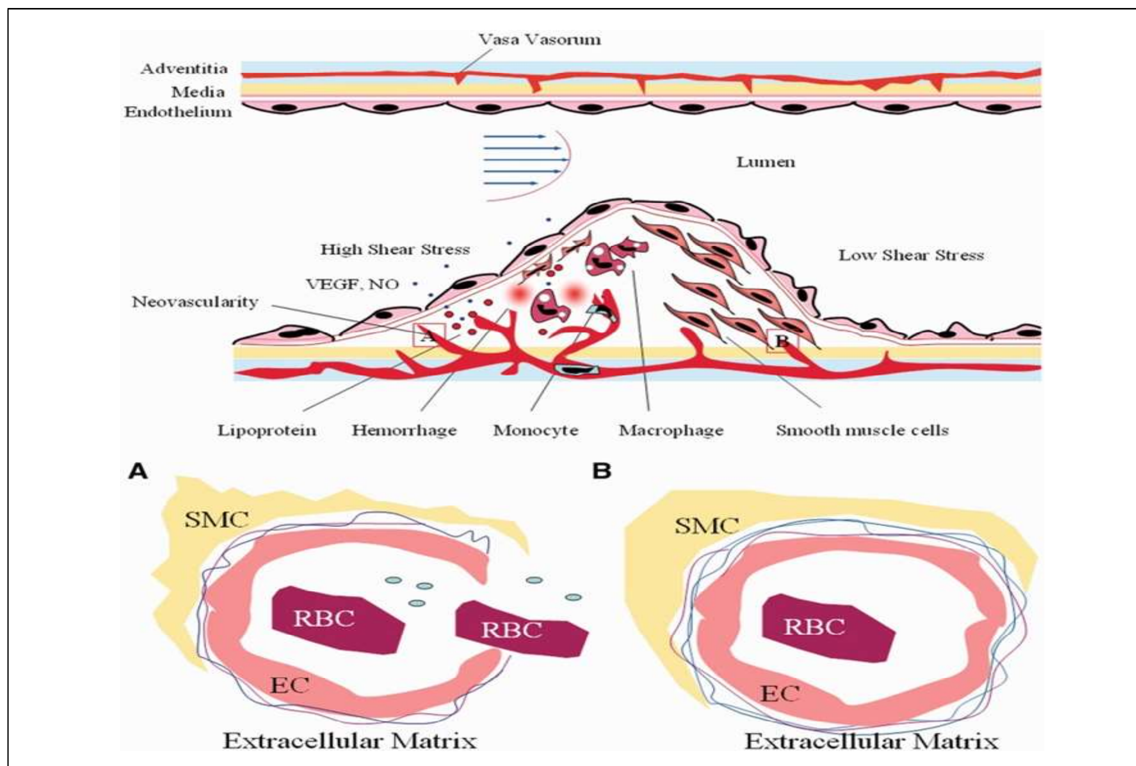

Figura 3. Formação de uma placa aterosclerótica vulnerável

Legenda: VEGF (fator de crescimento endotelial vascular); NO (óxido nítrico); SMC (células musculares lisas); RBC (glóbulos vermelhos); EC (células endoteliais) (Wang, 2016).

Numerosas estratégias vêm sendo desenvolvidas para controle dos sintomas e restabelecimento do fluxo sanguíneo pós- isquemia como, por exemplo, a intervenção coronariana percutânea (ICP), um método menos invasivo no qual são utilizados balões ou mesmo o implante de malhas metálicas (*stents*) (63-65). A ICP é um procedimento estabelecido para o tratamento de pacientes com angina *pectoris*, apresentando um significativo índice de sucesso no restabelecimento da homeostase vascular (6).

Os dispositivos desenvolvidos para tratamento da doença arterial coronariana como os *stents* do tipo metálico (*bare metal)* foram os primeiros a ser utilizados. Embora tenham apresentado inicialmente sucesso nos pacientes com DAC, ao longo dos anos demonstraram o desenvolvimento de complicações como efeito desse tratamento, pois aproximadamente 30% dos pacientes apresentam um quadro de obstrução luminal pós-introdução do stent, denominado reestenose (7, 10, 66, 67). A presença de fatores de risco como idade avançada, hipertensão, diabetes mellitus, tabagismo, proteína C reativa elevada, lesões calcificadas e difusas, parecem contribuir para a ocorrência de um novo quadro de isquemia pós-revascularização. O aprimoramento contínuo no desenvolvimento de novas técnicas intervencionistas, assim como, o desenvolvimento de

diversos dispositivos, com uma extensa variedade de materiais e fármacos associados, a reestenose intra *stent* ainda ocorre em 10 a 20% dos implantes de *stents* farmacológicos (*drug eluting stents*, DES) (68-71).

2.3 BASES MOLECULARES DA HIPERPLASIA NEOINTIMAL

A reestenose é uma reação vascular a introdução de um *stent* no local de desenvolvimento de uma placa ateromatosa que estaria obstruindo o fluxo sanguíneo e gerando um processo isquêmico (9). Após a ICP, normalmente nos primeiros seis meses, inicia um processo de proliferação de células musculares lisas criando um novo tecido adjacente à túnica íntima do vaso, clinicamente denominada hiperplasia neointimal (10).

As células musculares lisas vasculares são responsáveis pela manutenção da estrutura e tônus da parede vascular, mantendo a homeostasia de pressão vascular e perfusão tecidual (72). Essas células apresentam-se com dois fenótipos distintos, o primeiro, ativo predominantemente durante a fase de desenvolvimento embrionário, é representado por células musculares lisas vasculares com alto potencial proliferativo, caracterizadas como indiferenciadas (73); o segundo, que constitui a parede vascular funcional pós-nascimento, apresenta células musculares lisas em fase final de diferenciação, ou seja, células consideradas quiescentes. Nestas proteínas estruturais e funcionais de células musculares lisas, como, α- actina de músculo liso (*smooth muscle actin – α*, SMαA), miosina de cadeia pesada de músculo liso (*smooth muscle myosin heavy chain*, SMMHC), calponina e SM22α (*smooth muscle protein α)* apresentam uma menor expressão gênica, reduzindo-se, consequentemente, a capacidade proliferativa das mesmas. Nesse mecanismo sugere-se a participação de micro RNAs como miR-221, miR-145/143 e miocardina que aumentariam a expressão desses genes de musculatura lisa vascular (74-76).

As células musculares lisas maduras (quiescentes) apresentam-se sensíveis às mudanças no ambiente intravascular, um fenômeno denominado de troca da modulação fenotípica. Esse quadro pode permitir uma alteração das propriedades morfológicas e funcionais dessas células, ativando um processo divisional semelhante ao das células musculares lisas embrionárias (77, 78). A possível plasticidade desenvolvida pelas células quiescentes pode ser resultante de um estado de estresse oxidativo e inflamação crônicos promovidos pela placa ateromatosa, com liberação de mediadores inflamatórios e fatores de crescimento que podem induzir a migração, proliferação e acúmulo de células musculares lisas maduras na área neointimal (79).

O tecido endotelial vascular exerce um importante papel na regulação do tônus vascular e na manutenção da homeostasia cardiovascular (28). A célula endotelial também é fundamental no controle da trombólise, remodelação vascular e na resposta inflamatória pela ativação do sistema imunológico (80).

A liberação do NO, pelas células endoteliais, se dá pela entrada de cálcio no espaço intracelular, causando a abertura de canais de cálcio não seletivos, que são ativados principalmente por mediadores químicos como a acetilcolina, bradicinina e insulina, que causam a ligação da calmodulina à óxido nítrico sintase endotelial (eNOS), promovendo a quebra da ligação da enzima com uma proteína inibitória chamada caveolina. Dessa forma, a eNOS torna-se ávida por produzir NO a partir do aminoácido L-arginina (81). Além do NO, inúmeros fatores hiperpolarizantes derivados do endotélio (EDHFs), além da abertura de junções comunicantes, podem causar a hiperpolarização das células musculares lisas adjacentes (82). A hiperpolarização da célula muscular lisa vascular, seja por ação do NO ou por outro EDHF, ativa a guanilil ciclase solúvel (SoGC), aumentando a formação de guanosina monofosfato cíclico (GMPc), a partir de guanosina monofosfato (GMP), resultando em redução das concentrações de cálcio citoplasmático e consequente relaxamento da célula muscular lisa (83-85).

É importante ressaltar que a disfunção endotelial, particularmente a diminuição da capacidade vasodilatadora, tem sido relacionada à patogênese da doença vascular aterosclerótica e eventos cardiovasculares agudos (56, 86, 87). A vasodilatação dependente do endotélio não está prejudicada somente em casos clinicamente diagnosticáveis de angiopatias, mas também em situações de fatores de risco vasculares convencionais (88), a redução da vasodilatação dependente do endotélio é uma característica comum de diversos fatores de risco da aterosclerose, incluindo diabetes (89), hipertensão (90), dislipidemia (86) e envelhecimento (91).

A Akt (Proteína quinase B) é uma proteína envolvida diretamente com o controle do ciclo celular através de vias de proliferação, apoptose e migração celular, regulando a ativação ou inibição de fatores como NFκB, Caspase - 9 e mTOR. A via da Akt é ativada pela PI3K (*Phosphatidylinositide 3-kinase*), perfazendo uma via importante de sobrevivência celular denominada PI3K/Akt/ mTOR, sendo assim, responsável pelo estímulo proliferativo para as células musculares lisas na reestenose. A Akt além de regular a mTOR e p70 quinase, tem ação direta sobre as CDKs inibitórias p21 e p27 e indireta sobre a ciclina D1 e o p53 (92-94).

Os mecanismos moleculares envolvidos na proliferação de células musculares lisas são alvos importantes na busca por estratégias terapêuticas antiproliferativas. Assim, três vias inter-relacionadas poderiam induzir fatores de transcrição nucleares para estimulação da proliferação dessas células. A primeira seria através da ligação de fatores de crescimento aos receptores de tirosina quinase (TK), gerando uma sequência de fosforilações envolvendo a cascada RAS/RAF (serine/threonine-specific protein kinases, MAPKK (*mitogen-activated protein kinase kinase kinase*) e MAPK (*mitogen-activated protein kinase kinase*). A segunda via iniciaria pela ativação hormonal que resultaria no aumento do AMP (monofosfato de adenosina) intracelular e ativação da proteína quinase A (PKA). Na terceira via ocorre a ativação da proteína quinase C (PKC) que estimula a mTOR (*Mamallian target of rapamycin*), importante proteína na ativação de genes envolvidos no processo de duplicação de DNA e divisão celular (Figura 4) (95).

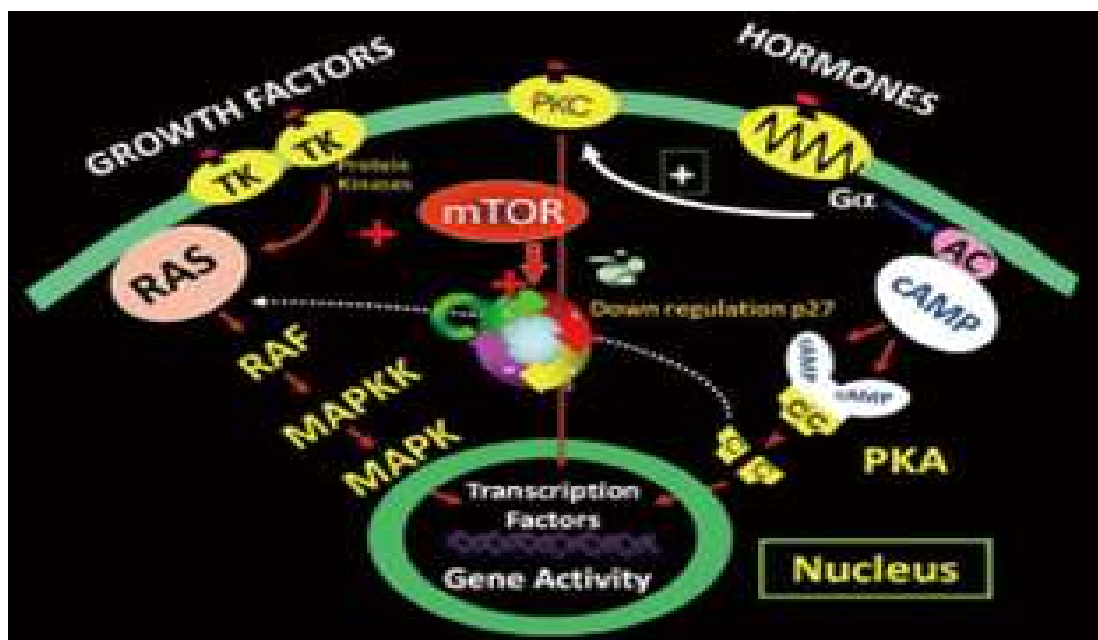

Figura 4. Mecanismos moleculares na proliferação de células musculares lisas (Curcio, 2011).

2.4 *STENTS* COM TERAPIA FARMACOLÓGICA

Diante dessa limitação promovida pela reestenose intra-*stent*, surgiram dispositivos farmacológicos com propriedades antiproliferativas (11), como a rapamicina e o paclitaxel, de ação direta sobre a progressão do ciclo celular, que impediriam a proliferação desordenada das células constituintes do vaso. Essa classe de *stents*, embora apresente um custo monetário mais elevado, demonstrou benefícios em longo prazo com uma potencial redução de complicações relacionadas à ICP, principalmente em pacientes com alto risco de morte devido à reintervenção (66, 96). *Stents* farmacológicos com liberação de sirolimus, paclitaxel, dexametasona e zotarolimus demonstraram efetiva redução nos eventos cardiovasculares como repetidas revascularizações, infarto agudo do miocárdio, trombose e óbito (65, 97-100).

O desenvolvimento de hiperplasia neointimal, com consequente geração de um quadro de reestenose intra-*sten*t, permitiu o surgimento dispositivos farmacológicos com propriedades antiproliferativas (11), como a rapamicina e o paclitaxel. Estes fármacos apresentam ação direta sobre a progressão do ciclo celular, impedindo, assim, a proliferação desordenada das células constituintes do vaso. Na interfase, momento do ciclo celular no qual não há mitose, a célula apresenta três fases distintas G1, S e G2, cuja mudança de atividade ocorre devido a proteínas denominadas ciclinas quinases dependentes (Cdks, *cyclin-dependent kinases*). Essas proteínas regulam as transições necessárias nos diferentes estágios do ciclo celular, principalmente em dois pontos considerados irreversíveis, a fase S, na qual há duplicação do DNA, e a fase M, na qual ocorre a divisão citoplasmática em duas células filhas (101, 102).

A rapamicina, princípio ativo do fármaco sirolimus, possui uma capacidade potencial de prevenir a reestenose (7) por ligar-se ao receptor intracelular FK506 *binding-protein* e consequentemente inibir a via mTOR (103). A rota da mTOR bloqueia a interfase celular, impedindo a progressão do ciclo de G1 para S, assim, sem a síntese de DNA a célula não se torna apta a realizar mitose (95, 104).

O paclitaxel é um antiproliferativo que age através de um mecanismo diferente da rapamicina, porém também gera o bloqueio do ciclo celular, impedindo que a célula passe da fase G2 da interfase para o início da mitose (105). Esse fármaco tem ação sobre a formação dos microtúbulos, que são elementos fundamentais para a formação do fuso mitótico e orientação dos cromossomos durante o processo de divisão celular (67).

O ciclo celular possui dois pontos críticos de checagem, denominados *checkpoints*, nos quais existe uma decisão celular sobre a viabilidade da progressão do

ciclo, o primeiro, na transição da fase de G1 para S e, o segundo, na transição da fase G2 para M (106). Os pontos citados são considerados de maior vulnerabilidade para a continuidade do ciclo celular, pois caso a célula apresente mutações, translocações ou deleções gênicas existe um mecanismo apoptótico que impede a progressão divisional de uma célula funcionalmente alterada (107). A apoptose, morte celular programada, normalmente ocorre através de uma via intrínseca de liberação do citocromo c, um componente presente nas cristas mitocondriais, que é liberado para o citosol e promove a ativação da cascata das caspases (108). Esta induz a fragmentação nuclear e exposição na membrana plasmática de fatores de reconhecimento para fagócitos, consequentemente gerando um corpo apoptótico passível de endocitose pelas células imunológicas.

O uso de fármacos antiproliferativos na confecção de *stents* farmacológicos apresenta-se como uma alternativa terapêutica consagrada na prática médica, no entanto esses procedimentos apresentaram ao longo do tempo algumas limitações, como falha na endotelização do *stent* e aumento do tempo de uso de antiagregantes plaquetários (12).

O desenvolvimento de novos dispositivos farmacológicos permitiu uma importante redução nos casos de reestenose. *Stents* eluídos com glicocorticoides (*dexamethasone –eluting vascular stents*) apresentam uma ação anti-inflamatória e antiproliferativa nas células musculares lisas vasculares. *Stents* de segunda geração, como, por exemplo, XIENCE V (*everolimus-eluting stent*) , apresentaram, em modelos animais e ensaios clínicos em humanos, uma redução na hiperplasia neointimal, do quadro inflamatório e da formação de granulomas, quando comparados a *stents* de primeira geração, como o TAXUS (*paclitaxel-eluting stents*) e o CYPHER (*sirolimus-eluting stents*) (65, 100, 109, 110).

O uso de fármacos antiproliferativos na confecção de stents farmacológicos apresenta-se como uma alternativa terapêutica consagrada na prática médica, no entanto esses procedimentos apresentaram ao longo do tempo algumas limitações, como falha na endotelização do *stent* e aumento do tempo de uso de antiagregantes plaquetários (12).

2.5 TERAPIA GÊNICA

A implantação de *stents* farmacológicos, principalmente com antiproliferativos, reduziu significativamente a ocorrência hiperplasia neointimal em pacientes com risco de desenvolvimento de reestenose. No entanto, os efeitos antiproliferativos desses fármacos apresentam ação concomitante sobre as células endoteliais, resultando em retardo do

restabelecimento do endotelial. Dessa forma, protocolos inovadores baseados em alvos gênicos como inibição da expressão de genes, reparo com células tronco, ou mesmo a entrega local de genes podem representar importantes técnicas no tratamento da reestenose (111, 112).

O endotélio apresenta um papel importante na produção de NO, uma molécula responsável por diferentes funções como inibição da adesão e agregação plaquetária, inibição da quimiotaxia de leucócitos, inibição da proliferação de células musculares lisas, vasodilatação e re-endotelização. Por apresentar-se fundamental para o restabelecimento vascular, dispositivos de liberação de NO se tornaram alvos terapêuticos para aplicação clínica em paciente com reestenose. Devido ao curto tempo de meia vida do NO in vivo (2 a 5 segundos), a alternativa para entrega deste poderia ser através da terapia gênica (113, 114). Brito *et. al*. (2010) testaram a eficácia de um dispositivo não viral para administração do gene da eNOS (oxido nítrico sintase endotelial) para recobrir *stents* em modelo de reestenose na artéria ilíaca de coelhos. Após 14 dias do implante os animais que haviam recebido o vetor não viral apresentaram redução da proliferação das células musculares lisas e a promoção da re-endotelização quando comparados aos animais controle (115).

Em modelos suínos (*mini pig)* houve a implantação de *stents* recobertos com VEGF e paclitaxel. Conforme demonstrado na figura 5, o *stent* metálico (*bare metal*) foi pré - tratado para gerar nanoporos, pois estes favoreceriam a estabilidade da superfície do dispositivo ao recobri-lo com plasmídeo, contendo o gene VEGF, e com paclitaxel. O modelo demonstrou uma re-endotelização completa e uma significativa supressão de reestenose intra *stent*, quando comparado ao DES, no período de um mês após o implante. Dessa forma, a liberação de VEGF promoveria a recuperação do endotélio e o paclitaxel bloquearia a proliferação das células musculares lisas vasculares (116).

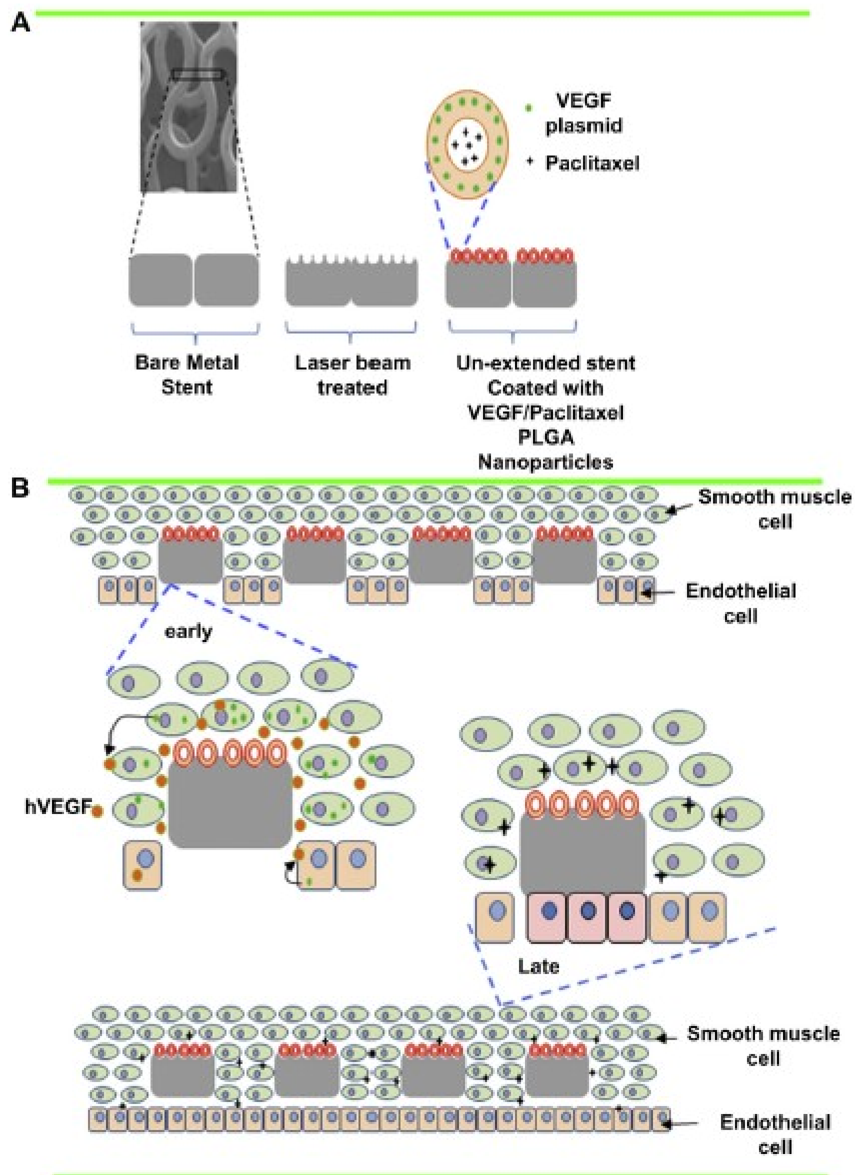

Figura 5. Diagrama esquemático do stent recoberto com VEGF e Paclitaxel (Wang, 2016).

O papel do VEGF foi testado com o uso da rapamicina, que tem como alvo a via da mTOR, resultando na supressão do crescimento de células do endotélio vascular. Nesse processo, a mTOR bloqueia a AP-1 (*activator protein-1*), uma proteína que ativa a transcrição de VEGF, consequentemente, inibindo a expressão de VEGF que poderia permitir a reconstituição do endotélio. A terapia com rapamicina, combinada com uso de silenciadores gênicos para AP-1, elucidou que o uso desse fármaco promove uma redução da proliferação de células musculares lisas, com menor expressão da mTOR e maior expressão de VEGF, permitindo o restabelecimento das células endoteliais da aorta de ratos (117).

Li e colaboradores (2016) sugeriram que o receptor de secretagogo do hormônio do crescimento (GHSR) poderia ser um regulador importante no processo de desenvolvimento de hiperplasia intimal, quando em resposta ao dano vascular causado pelo implante *stent*. Assim, em modelo de camundongos, o estudo demonstrou que a deficiência de GHSR atenua a formação de neoíntima, reduzindo a progressão do quadro de reestenose após injúria vascular. Acredita-se, então, que o efeito inibitório sobre GHSR, bloquearia a proliferação de células musculares lisas através da supressão da cascata de fosforilação da Akt e da ERK1/2. Essas vias apresentam um importante papel regulatório para ativação da mTOR, sendo essa o alvo terapêutico da rapamicina (118). Nanopartículas com silenciadores gênicos para Akt1 estão sendo testadas como alvos terapêuticos nos modelos de reestenose em coelhos com implante de *stent* na artéria ilíaca, resultando na supressão da ação pró-proliferativa da Akt1 em células musculares lisas vasculares, impedindo a reestenose intra *stent*. Esse estudo sugere que esse tipo de dispositivo poderia ser um eficiente substituto dos *stents* farmacológicos por não apresentar os efeitos adversos observados nos mesmos (92).

A modulação fenotípica das células musculares lisas, promovendo o estímulo à proliferação celular apresenta diversos estudos que confirmam a via Akt 1 e mTOR como principais envolvidas na ativação de fatores de transcrição que induziriam o processo de hiperplasia (8, 92, 117, 118). No entanto, Cidad e colaboradores (2014) verificaram, que a expressão de Kv1.3 e Kv1.5, canais de potássio voltagem dependente responsáveis pela contração e proliferação de células musculares lisas, apresentaria uma via tardia de ativação modulada por fatores de crescimento. Esse estudo sugere, portanto, a existência de outras vias responsáveis pelo desenvolvimento da hiperplasia neointimal, independentes da mTOR, gerando um quadro de resistência ao efeito antiproliferativo da rapamicina (119).

A terapia gênica como tratamento da reestenose apresenta-se como uma nova alternativa, no entanto, a maioria dos estudos está em fase pré-clínica, pois precisam elucidar questões importantes como a seleção do vetor para entrega do gene, que possa garantir uma transfecção eficiente para as células alvo. De acordo com a Tabela 1, pode-se verificar principalmente o adenovírus como principal tipo de vetor, pois, apesar de vetores não virais serem mais seguros em termos de risco biológico, estes não apresentam uma transfecção tão eficiente quanto os virais, resultando em um efeito terapêutico a curto prazo por induzir uma importante degradação celular (14). Alguns estudos clínicos em humanos como o REGENT I (120) e a terapia gênica com VEGF (121), com entrega

de genes como iNOS (óxido nítrico sintase indutível) e VEGFA respectivamente, apresentam resultados bastante promissores, no entanto, ainda não são capazes de esclarecer se há uma redução relevante na ocorrência de eventos cardiovasculares mais graves .

Tabela 1. Estudos pré-clínicos recentes baseados em terapia gênica para reestenose

Vetor	*Gene/SiRNA*	*Função*	*Modelo pré- clinico*	*Ref*
Adenovírus	SERCA2a	Controle de canais de cálcio, vias de sinalização de células musculares lisas	Modelo de artéria mamária esquerda humana (*ex- vivo*)	(122)
Adenovírus	NF-Y	Fator de transcrição	Artéria femoral (ratos)	(123)
Adenovírus	Peptídeo natriurético atrial	Inibidor do crescimento de células musculares lisas	Artéria carótida (ratos)	(124)
Plasmídeo/ Lipofectamina	EPC1	Regulador da diferenciação de células musculares lisas	Artéria carótida (ratos)	(125)
Adenovírus	p53	Gene suppressor tumoral	Artéria carótida (ratos)	(126)
Adenovírus	TIMP-3	Inibidor de MMP	Enxerto autólogo (suínos)	(127)

Legenda: SERCA2a (*sarco/endoplasmatic reticulum Ca* $^{2+}$ *ATPase*); NF-Y (*nuclear factor Y*); EPC1 (*Enhancer Polycomb 1)*; TIMP-3 (*tissue inhibitor of metalloproteinases-3.*); MMP *(matrix metalloproteinases)*

Fonte: Adaptado de Forte e colaboradores (2014).

Mesmo com diferentes técnicas e dispositivos sendo criados, a efetividade encontrada em modelos animais (ratos, camundongos, porcos, coelhos) não se reproduz em ensaios clínicos. Nos modelos pré-clínicos, utiliza-se um cateter balão que induz um espessamento neointimal, mas obtém-se como resultado mínima inflamação e formação de trombos. Os modelos suínos são considerados os mais confiáveis para estudo da reestenose, especialmente para avaliação de DES (128-130). Contudo, existe outra limitação importante, os testes são realizados em um intervalo de curta duração (28 dias em porcos, 14 dias em ratos), resultando na impossibilidade da avaliação das técnicas e dispositivos a longo prazo, apenas realizando a extrapolação para humanos (131).

Os estudos com terapia gênica apresentaram inúmeras vantagens em modelos animais, no entanto, os benefícios ainda não foram efetivamente comprovados em humanos. Existem muitos fatores a serem determinados para uma maior eficiência dessa prática terapêutica, principalmente na escolha da aplicação de *stents*, tipos de carreadores gênicos, duração do efeito e o método de entrega do gene à parede vascular.

3. JUSTIFICATIVA E OBJETIVOS

A intervenção coronariana percutânea é o tratamento recomendado para pacientes com cardiopatia isquêmica e aterosclerose, no entanto, esse procedimento pode resultar em uma complicação, a reestenose. No desenvolvimento desse quadro, estão envolvidas células inflamatórias, citocinas, formação de trombos, fatores de crescimento e mudanças fenotípicas nas células musculares lisas. O resultado desse processo é a proliferação e migração de células musculares lisas, deposição de peptideoglicano e matriz extracelular, levando a uma hiperplasia neointimal e remodelamento vascular (14, 110, 112).

O desenvolvimento de reestenose vascular ocorre em aproximadamente 30 a 50% dos pacientes que realizam procedimentos de revascularização. Assim, representa um importante fator limitante para o sucesso do implante de *stent*, apresentam-se necessária a criação de dispositivos inovadores e novas terapias a serem administradas aos pacientes. Os tratamentos atuais, que visam o implante ótimo do dispositivo, podem ser por imagem como IVUS (ultrassom intravascular) e OCT (tomografia de coerência ótima), ou *stents* biodegradáveis, com polímeros bioabsorvíveis, com novos fármacos antiproliferativos, com terapia gênica (100, 103, 110).

Nesse estudo visamos investigar se a placa aterosclerótica dos pacientes que desenvolveram reestenose intra-*stent* apresenta genes preditores que estimulam a hiperplasia neointimal. Assim, após a identificação de genes chave constituintes das placas ateromatosas, verificamos se a inibição seletiva destes, *in vitro*, poderia apresentar-se como um agente direto e específico no bloqueio da proliferação e migração das células musculares lisas, mas que concomitantemente permitiria a proliferação fisiológica e controlada do endotélio. O controle do crescimento das células musculares lisas e a preservação do endotélio poderiam, portanto, aumentar a probabilidade de sucesso nos procedimentos de intervenção coronária percutânea, sendo uma nova alternativa para diagnóstico e tratamento da reestenose.

Dessa forma, o objetivo geral desse estudo foi investigar o perfil genético de placas ateromatosas de pacientes que desenvolveram reestenose pós-implante de *stent*, verificando, posteriormente, se o silenciamento de genes específicos para estrutura e função das células musculares lisas vasculares poderia bloquear o processo proliferativo destas sem prejudicar o restabelecimento das células endoteliais.

4. REFERÊNCIAS DA REVISÃO DE LITERATURA

1. Pedersen BK, Fischer CP. Beneficial health effects of exercise--the role of IL-6 as a myokine. Trends Pharmacol Sci. 2007 Apr;28(4):152-6.
2. Ishitani LH, Franco Gda C, Perpetuo IH, Franca E. [Socioeconomic inequalities and premature mortality due to cardiovascular diseases in Brazil]. Rev Saude Publica. 2006 Aug;40(4):684-91.
3. Saotome M, Katoh H, Yaguchi Y, Tanaka T, Urushida T, Satoh H, et al. Transient opening of mitochondrial permeability transition pore by reactive oxygen species protects myocardium from ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol. 2009 Apr;296(4):H1125-32.
4. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest. 2007 Jan;117(1):175-84.
5. Fok PW. Growth of necrotic cores in atherosclerotic plaque. Math Med Biol. 2012 Dec;29(4):301-27.
6. Okada T, Yoshikawa D, Ishii H, Matsumoto M, Hayakawa S, Matsudaira K, et al. Impact of the first-generation drug-eluting stent implantation on periprocedural myocardial injury in patients with stable angina pectoris. J Cardiol. 2012 Oct;60(4):264-9.
7. Zago AC, Raudales JC, Attizzani G, Matte BS, Yamamoto GI, Balvedi JA, et al. Local delivery of sirolimus nanoparticles for the treatment of in-stent restenosis. Catheter Cardiovasc Interv. 2012 May 4.
8. Woods TC. Dysregulation of the Mammalian Target of Rapamycin and p27Kip1 Promotes Intimal Hyperplasia in Diabetes Mellitus. Pharmaceuticals (Basel). 2013;6(6):716-27.
9. Natarajan MK, Strauss BH, Rokoss M, Buller CE, Mancini GB, Xie C, et al. Randomized trial of insulin versus usual care in reducing restenosis after coronary intervention in patients with diabetes. the STent Restenosis And Metabolism (STREAM) study. Cardiovasc Revasc Med. 2012 Mar-Apr;13(2):95-100.
10. Simon DI, Dhen Z, Seifert P, Edelman ER, Ballantyne CM, Rogers C. Decreased neointimal formation in Mac-1(-/-) mice reveals a role for inflammation in vascular repair after angioplasty. J Clin Invest. 2000 Feb;105(3):293-300.
11. Tarantini G, Facchin M, Capodanno D, Musumeci G, Saia F, Menozzi A, et al. Paclitaxel versus sirolimus eluting stents in diabetic patients: Does stent type and/or stent diameter matter?: Long-term clinical outcome of 2,429-patient multicenter registry. Catheter Cardiovasc Interv. 2012 Apr 18.
12. Inoue T, Croce K, Morooka T, Sakuma M, Node K, Simon DI. Vascular inflammation and repair: implications for re-endothelialization, restenosis, and stent thrombosis. JACC Cardiovasc Interv. 2011 Oct;4(10):1057-66.
13. Piccolo R, Galasso G, Piscione F, Esposito G, Trimarco B, Dangas GD, et al. Meta-analysis of randomized trials comparing the effectiveness of different strategies for the treatment of drug-eluting stent restenosis. Am J Cardiol. 2014 Nov 1;114(9):1339-46.
14. Forte A, Rinaldi B, Berrino L, Rossi F, Galderisi U, Cipollaro M. Novel potential targets for prevention of arterial restenosis: insights from the pre-clinical research. Clin Sci (Lond). 2014 Dec;127(11):615-34.
15. Zago ACMarco AC, Alcides J. Zago, Juliane S. Rossato, Bruno S. Matte,German Iturry-Yamamoto, Márcio Mossmann, Ricardo Savaris, Mariano Albertal, Cristiane S. Rocha,, Aileen M. Healy Russell W, Satoko Tahara, Daniel I. Simon. Identification of Genes Involved in Smooth Muscle Cell Protein Synthesis with Increased Expression in Atheromatous Plaques Associated with Neointimal Hyperplasia after Bare-Metal Stenting: A GENESIS-R Study. Revista Brasileira de Cardiologia Invasiva. [original]. 2012 junho 2012;20(2):140-5.
16. The top 10 causes of death [database on the Internet]. WHO. Available from: http://www.who.int/mediacentre/factsheets/fs310/en/.

17. McAloon CJ, Boylan LM, Hamborg T, Stallard N, Osman F, Lim PB, et al. The changing face of cardiovascular disease 2000-2012: An analysis of the world health organisation global health estimates data. Int J Cardiol. 2016 Dec 01;224:256-64.
18. Taxa de prevalência de excesso de peso [database on the Internet]. Ministério da Saúde/Secretaria de Vigilância em Saúde (SVS)/Instituto Nacional do Câncer (Inca): Inquérito

Domiciliar de Comportamentos de Risco de Morbidade Referida de Doenças e Agravos Não Transmissíveis. 2008 [cited 24/05/2008].
19. Iser BP, Vigo A, Duncan BB, Schmidt MI. Trends in the prevalence of self-reported diabetes in Brazilian capital cities and the Federal District, 2006-2014. Diabetol Metab Syndr. 2016;8:70.
20. Nascimento BR, Brant LC, Moraes DN, Ribeiro AL. Global health and cardiovascular disease. Heart. 2014 Nov;100(22):1743-9.
21. Gutierrez LL, Maslinkiewicz A, Curi R, de Bittencourt PI, Jr. Atherosclerosis: A redox-sensitive lipid imbalance suppressible by cyclopentenone prostaglandins. Biochem Pharmacol. 2008 Mar 14.
22. Muscogiuri G, Annweiler C, Duval G, Karras S, Tirabassi G, Salvio G, et al. Vitamin D and cardiovascular disease: From atherosclerosis to myocardial infarction and stroke. Int J Cardiol. 2016 Dec 26.
23. Turan TN, Nizam A, Lynn MJ, Egan BM, Le NA, Lopes-Virella MF, et al. Relationship between risk factor control and vascular events in the SAMMPRIS trial. Neurology. 2016 Dec 21.
24. Bender SB, McGraw AP, Jaffe IZ, Sowers JR. Mineralocorticoid receptor-mediated vascular insulin resistance: an early contributor to diabetes-related vascular disease? Diabetes. 2013 Feb;62(2):313-9.
25. Franssens BT, Westerink J, van der Graaf Y, Nathoe HM, Visseren FL. Metabolic consequences of adipose tissue dysfunction and not adiposity per se increase the risk of cardiovascular events and mortality in patients with type 2 diabetes. Int J Cardiol. 2016 Nov 01;222:72-7.
26. Kramer HF, Goodyear LJ. Exercise, MAPK, and NF-kappaB signaling in skeletal muscle. J Appl Physiol. 2007 Jul;103(1):388-95.
27. Tabas I. 2016 Russell Ross Memorial Lecture in Vascular Biology: Molecular-Cellular Mechanisms in the Progression of Atherosclerosis. Arterioscler Thromb Vasc Biol. 2016 Dec 15.
28. Glasser SP, Selwyn AP, Ganz P. Atherosclerosis: risk factors and the vascular endothelium. Am Heart J. 1996 Feb;131(2):379-84.
29. Teasdale JE, Hazell GG, Peachey AM, Sala-Newby GB, Hindmarch CC, McKay TR, et al. Cigarette smoke extract profoundly suppresses TNFalpha-mediated proinflammatory gene expression through upregulation of ATF3 in human coronary artery endothelial cells. Sci Rep. 2017 Jan 06;7:39945.
30. Chistiakov DA, Melnichenko AA, Orekhov AN, Bobryshev YV. How do macrophages sense modified low-density lipoproteins? Int J Cardiol. 2016 Dec 28.
31. Maestroni GJ, Conti A. Modulation of hematopoiesis via alpha 1-adrenergic receptors on bone marrow cells. Exp Hematol. 1994 Mar;22(3):313-20.
32. Bobryshev YV, Ivanova EA, Chistiakov DA, Nikiforov NG, Orekhov AN. Macrophages and Their Role in Atherosclerosis: Pathophysiology and Transcriptome Analysis. Biomed Res Int. 2016;2016:9582430.
33. Niess AM, Dickhuth HH, Northoff H, Fehrenbach E. Free radicals and oxidative stress in exercise--immunological aspects. Exerc Immunol Rev. 1999;5:22-56.
34. Droge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002 Jan;82(1):47-95.
35. Blankenberg S, Barbaux S, Tiret L. Adhesion molecules and atherosclerosis. Atherosclerosis. 2003 Oct;170(2):191-203.

36. Li CY, Chou TC, Lee CH, Tsai CS, Loh SH, Wong CS. Adrenaline inhibits lipopolysaccharide-induced macrophage inflammatory protein-1 alpha in human monocytes: the role of beta-adrenergic receptors. Anesth Analg. 2003 Feb;96(2):518-23, table of contents.
37. Nedeljkovic ZS, Gokce N, Loscalzo J. Mechanisms of oxidative stress and vascular dysfunction. Postgrad Med J. 2003 Apr;79(930):195-9; quiz 8-200.
38. Rocha VZ, Libby P. Obesity, inflammation, and atherosclerosis. Nat Rev Cardiol. 2009 Jun;6(6):399-409.
39. Zaldivar F, Wang-Rodriguez J, Nemet D, Schwindt C, Galassetti P, Mills PJ, et al. Constitutive pro- and anti-inflammatory cytokine and growth factor response to exercise in leukocytes. J Appl Physiol. 2006 Apr;100(4):1124-33.
40. Ostrowski K, Rohde T, Asp S, Schjerling P, Pedersen BK. Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. J Physiol. 1999 Feb 15;515 (Pt 1):287-91.
41. Drenth JP, Krebbers RJ, Bijzet J, van der Meer JW. Increased circulating cytokine receptors and ex vivo interleukin-1 receptor antagonist and interleukin-1beta production but decreased tumour necrosis factor-alpha production after a 5-km run. Eur J Clin Invest. 1998 Oct;28(10):866-72.
42. Zampetaki A, Zhang Z, Hu Y, Xu Q. Biomechanical stress induces IL-6 expression in smooth muscle cells via Ras/Rac1-p38 MAPK-NF-kappaB signaling pathways. Am J Physiol Heart Circ Physiol. 2005 Jun;288(6):H2946-54.
43. Foss-Freitas MC, Foss NT, Donadi EA, Foss MC. In vitro TNF-alpha and IL-6 production by adherent peripheral blood mononuclear cells obtained from type 1 and type 2 diabetic patients evaluated according to the metabolic control. Ann N Y Acad Sci. 2006 Oct;1079:177-80.
44. Yan ZQ, Hansson GK. Innate immunity, macrophage activation, and atherosclerosis. Immunol Rev. 2007 Oct;219:187-203.
45. Pereira EC, Ferderbar S, Bertolami MC, Faludi AA, Monte O, Xavier HT, et al. Biomarkers of oxidative stress and endothelial dysfunction in glucose intolerance and diabetes mellitus. Clin Biochem. 2008 Dec;41(18):1454-60.
46. Hristov M, Weber C. Differential role of monocyte subsets in atherosclerosis. Thromb Haemost. 2011 Nov;106(5):757-62.
47. Thomas MR, Lip GY. Novel Risk Markers and Risk Assessments for Cardiovascular Disease. Circ Res. 2017 Jan 06;120(1):133-49.
48. Nissen SE, Yock P. Intravascular ultrasound: novel pathophysiological insights and current clinical applications. Circulation. 2001 Jan 30;103(4):604-16.
49. Haft JI. Multiple atherosclerotic plaque rupture in acute coronary syndrome. Circulation. 2003 Mar 11;107(9):e65-6; author reply e-6.
50. Wentzel JJ, Chatzizisis YS, Gijsen FJ, Giannoglou GD, Feldman CL, Stone PH. Endothelial shear stress in the evolution of coronary atherosclerotic plaque and vascular remodelling: current understanding and remaining questions. Cardiovasc Res. 2012 Nov 01;96(2):234-43.
51. Chatzizisis YS, Coskun AU, Jonas M, Edelman ER, Feldman CL, Stone PH. Role of endothelial shear stress in the natural history of coronary atherosclerosis and vascular remodeling: molecular, cellular, and vascular behavior. J Am Coll Cardiol. 2007 Jun 26;49(25):2379-93.
52. Berry JD, Liu K, Folsom AR, Lewis CE, Carr JJ, Polak JF, et al. Prevalence and progression of subclinical atherosclerosis in younger adults with low short-term but high lifetime estimated risk for cardiovascular disease: the coronary artery risk development in young adults study and multi-ethnic study of atherosclerosis. Circulation. 2009 Jan 27;119(3):382-9.
53. Pizzi C, Xhyheri B, Costa GM, Faustino M, Flacco ME, Gualano MR, et al. Nonobstructive Versus Obstructive Coronary Artery Disease in Acute Coronary Syndrome: A Meta-Analysis. J Am Heart Assoc. 2016 Dec 16;5(12).
54. Dalal JJ, Ponde CK, Pinto B, Srinivas CN, Thomas J, Modi SK, et al. Time to shift from contemporary to high-sensitivity cardiac troponin in diagnosis of acute coronary syndromes. Indian Heart J. 2016 Nov - Dec;68(6):851-5.

55. Pozo E, Agudo-Quilez P, Rojas-Gonzalez A, Alvarado T, Olivera MJ, Jimenez-Borreguero LJ, et al. Noninvasive diagnosis of vulnerable coronary plaque. World J Cardiol. 2016 Sep 26;8(9):520-33.
56. Wang Y, Qiu J, Luo S, Xie X, Zheng Y, Zhang K, et al. High shear stress induces atherosclerotic vulnerable plaque formation through angiogenesis. Regen Biomater. 2016 Dec;3(4):257-67.
57. Sanchez-Aguadero N, Alonso-Dominguez R, Garcia-Ortiz L, Agudo-Conde C, Rodriguez-Martin C, de Cabo-Laso A, et al. Diet and physical activity in people with intermediate cardiovascular risk and their relationship with the health-related quality of life: results from the MARK study. Health Qual Life Outcomes. 2016 Dec 07;14(1):169.
58. Dussaillant C, Echeverria G, Urquiaga I, Velasco N, Rigotti A. [Current evidence on health benefits of the mediterranean diet]. Rev Med Chil. 2016 Aug;144(8):990-7.
59. Tong TY, Wareham NJ, Khaw KT, Imamura F, Forouhi NG. Prospective association of the Mediterranean diet with cardiovascular disease incidence and mortality and its population impact in a non-Mediterranean population: the EPIC-Norfolk study. BMC Med. 2016 Sep 29;14(1):135.
60. Michel JB, Martin-Ventura JL, Nicoletti A, Ho-Tin-Noe B. Pathology of human plaque vulnerability: mechanisms and consequences of intraplaque haemorrhages. Atherosclerosis. 2014 Jun;234(2):311-9.
61. Russell CJ, Exley AR, Ritchie AJ. Widespread coronary inflammation in unstable angina. N Engl J Med. 2003 May 08;348(19):1931.
62. Schaar JA, Muller JE, Falk E, Virmani R, Fuster V, Serruys PW, et al. Terminology for high-risk and vulnerable coronary artery plaques. Report of a meeting on the vulnerable plaque, June 17 and 18, 2003, Santorini, Greece. Eur Heart J. 2004 Jun;25(12):1077-82.
63. Pleva L, Kukla P, Kusnierova P, Zapletalova J, Hlinomaz O. Comparison of the Efficacy of Paclitaxel-Eluting Balloon Catheters and Everolimus-Eluting Stents in the Treatment of Coronary In-Stent Restenosis: The Treatment of In-Stent Restenosis Study. Circ Cardiovasc Interv. 2016 Apr;9(4):e003316.
64. Kufner S, Cassese S, Valeskini M, Neumann FJ, Schulz-Schupke S, Hoppmann P, et al. Long-Term Efficacy and Safety of Paclitaxel-Eluting Balloon for the Treatment of Drug-Eluting Stent Restenosis: 3-Year Results of a Randomized Controlled Trial. JACC Cardiovasc Interv. 2015 Jun;8(7):877-84.
65. Dangas GD, Serruys PW, Kereiakes DJ, Hermiller J, Rizvi A, Newman W, et al. Meta-analysis of everolimus-eluting versus paclitaxel-eluting stents in coronary artery disease: final 3-year results of the SPIRIT clinical trials program (Clinical Evaluation of the Xience V Everolimus Eluting Coronary Stent System in the Treatment of Patients With De Novo Native Coronary Artery Lesions). JACC Cardiovasc Interv. 2013 Sep;6(9):914-22.
66. Hill R, Bagust A, Bakhai A, Dickson R, Dundar Y, Haycox A, et al. Coronary artery stents: a rapid systematic review and economic evaluation. Health Technol Assess. 2004 Sep;8(35):iii-iv, 1-242.
67. Pires NM, Eefting D, de Vries MR, Quax PH, Jukema JW. Sirolimus and paclitaxel provoke different vascular pathological responses after local delivery in a murine model for restenosis on underlying atherosclerotic arteries. Heart. 2007 Aug;93(8):922-7.
68. Kastrati A, Mehilli J, von Beckerath N, Dibra A, Hausleiter J, Pache J, et al. Sirolimus-eluting stent or paclitaxel-eluting stent vs balloon angioplasty for prevention of recurrences in patients with coronary in-stent restenosis: a randomized controlled trial. JAMA. 2005 Jan 12;293(2):165-71.
69. Kong J, Deng Y, Dong Q, Liu W, Lu Y. Colchicine reduces restenosis after balloon angioplasty treatment for in-stent restenosis. Arch Med Res. 2015 Feb;46(2):101-6.
70. Bonaa KH, Mannsverk J, Wiseth R, Aaberge L, Myreng Y, Nygard O, et al. Drug-Eluting or Bare-Metal Stents for Coronary Artery Disease. N Engl J Med. 2016 Sep 29;375(13):1242-52.

71. Ferrante G, Niccoli G, Biasucci LM, Liuzzo G, Burzotta F, Galiuto L, et al. Association between C-reactive protein and angiographic restenosis after bare metal stents: an updated and comprehensive meta-analysis of 2747 patients. Cardiovasc Revasc Med. 2008 Jul-Sep;9(3):156-65.
72. Alexander MR, Owens GK. Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease. Annu Rev Physiol. 2012;74:13-40.
73. Salmon M, Gomez D, Greene E, Shankman L, Owens GK. Cooperative binding of KLF4, pELK-1, and HDAC2 to a G/C repressor element in the SM22alpha promoter mediates transcriptional silencing during SMC phenotypic switching in vivo. Circ Res. 2012 Aug 31;111(6):685-96.
74. Owens GK. Regulation of differentiation of vascular smooth muscle cells. Physiol Rev. 1995 Jul;75(3):487-517.
75. Owens GK. Role of mechanical strain in regulation of differentiation of vascular smooth muscle cells. Circ Res. 1996 Nov;79(5):1054-5.
76. Owens GK, Wise G. Regulation of differentiation/maturation in vascular smooth muscle cells by hormones and growth factors. Agents Actions Suppl. 1997;48:3-24.
77. Cheung C, Bernardo AS, Pedersen RA, Sinha S. Directed differentiation of embryonic origin-specific vascular smooth muscle subtypes from human pluripotent stem cells. Nat Protoc. 2014 Apr;9(4):929-38.
78. Wang G, Jacquet L, Karamariti E, Xu Q. Origin and differentiation of vascular smooth muscle cells. J Physiol. 2015 Jul 15;593(14):3013-30.
79. House SJ, Potier M, Bisaillon J, Singer HA, Trebak M. The non-excitable smooth muscle: calcium signaling and phenotypic switching during vascular disease. Pflugers Arch. 2008 Aug;456(5):769-85.
80. LUZ PL. Endotélio e doenças cardiovasculares. 1, editor. São Paulo: Ateneu; 2005.
81. Brovkovych V, Dobrucki LW, Brovkovych S, Dobrucki I, Do Nascimento CA, Burewicz A, et al. Nitric oxide release from normal and dysfunctional endothelium. J Physiol Pharmacol. 1999 Dec;50(4):575-86.
82. Busse R, Edwards G, Feletou M, Fleming I, Vanhoutte PM, Weston AH. EDHF: bringing the concepts together. Trends Pharmacol Sci. 2002 Aug;23(8):374-80.
83. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature. 1980 Nov 27;288(5789):373-6.
84. Furchgott RF, Vanhoutte PM. Endothelium-derived relaxing and contracting factors. FASEB J. 1989 Jul;3(9):2007-18.
85. Tahir H, Bona-Casas C, Hoekstra AG. Modelling the effect of a functional endothelium on the development of in-stent restenosis. PLoS One. 2013;8(6):e66138.
86. Creager MA, Cooke JP, Mendelsohn ME, Gallagher SJ, Coleman SM, Loscalzo J, et al. Impaired vasodilation of forearm resistance vessels in hypercholesterolemic humans. J Clin Invest. 1990 Jul;86(1):228-34.
87. Tahir H, Niculescu I, Bona-Casas C, Merks RM, Hoekstra AG. An in silico study on the role of smooth muscle cell migration in neointimal formation after coronary stenting. J R Soc Interface. 2015 Jul 06;12(108):20150358.
88. Celermajer DS, Sorensen KE, Bull C, Robinson J, Deanfield JE. Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interaction. J Am Coll Cardiol. 1994 Nov 15;24(6):1468-74.
89. Johnstone MT, Creager SJ, Scales KM, Cusco JA, Lee BK, Creager MA. Impaired endothelium-dependent vasodilation in patients with insulin-dependent diabetes mellitus. Circulation. 1993 Dec;88(6):2510-6.
90. Taddei S, Virdis A, Mattei P, Ghiadoni L, Fasolo CB, Sudano I, et al. Hypertension causes premature aging of endothelial function in humans. Hypertension. 1997 Mar;29(3):736-43.

91. Taddei S, Virdis A, Mattei P, Ghiadoni L, Gennari A, Fasolo CB, et al. Aging and endothelial function in normotensive subjects and patients with essential hypertension. Circulation. 1995 Apr 1;91(7):1981-7.
92. Che HL, Bae IH, Lim KS, Song IT, Lee H, Lee D, et al. Therapeutic Effect of Akt1 siRNA Nanoparticle Eluting Coronary Stent on Suppression of Post-Angioplasty Restenosis. J Biomed Nanotechnol. 2016 Jun;12(6):1211-22.
93. Yu X, Takayama T, Goel SA, Shi X, Zhou Y, Kent KC, et al. A rapamycin-releasing perivascular polymeric sheath produces highly effective inhibition of intimal hyperplasia. J Control Release. 2014 Oct 10;191:47-53.
94. Lu S, Liu H, Lu L, Wan H, Lin Z, Qian K, et al. WISP1 overexpression promotes proliferation and migration of human vascular smooth muscle cells via AKT signaling pathway. Eur J Pharmacol. 2016 Oct 05;788:90-7.
95. Curcio A, Torella D, Indolfi C. Mechanisms of smooth muscle cell proliferation and endothelial regeneration after vascular injury and stenting: approach to therapy. Circ J. 2011;75(6):1287-96.
96. Hartwell D, Colquitt J, Loveman E, Clegg AJ, Brodin H, Waugh N, et al. Clinical effectiveness and cost-effectiveness of immediate angioplasty for acute myocardial infarction: systematic review and economic evaluation. Health Technol Assess. 2005 May;9(17):1-99, iii-iv.
97. Serruys PW, Farooq V, Kalesan B, de Vries T, Buszman P, Linke A, et al. Improved safety and reduction in stent thrombosis associated with biodegradable polymer-based biolimus-eluting stents versus durable polymer-based sirolimus-eluting stents in patients with coronary artery disease: final 5-year report of the LEADERS (Limus Eluted From A Durable Versus ERodable Stent Coating) randomized, noninferiority trial. JACC Cardiovasc Interv. 2013 Aug;6(8):777-89.
98. Piccolo R, Stefanini GG, Franzone A, Spitzer E, Blochlinger S, Heg D, et al. Safety and efficacy of resolute zotarolimus-eluting stents compared with everolimus-eluting stents: a meta-analysis. Circ Cardiovasc Interv. 2015 Apr;8(4).
99. Qi-Hua L, Qi Z, Yu Z, Xiao-Long L, Hai-Gang J, Jian-Feng Y, et al. Long-term effect of second-generation drug-eluting stents for coronary artery disease, everolimus-eluting versus zotarolimus-eluting stents: a meta-analysis. Coron Artery Dis. 2015 May;26(3):259-65.
100. Hamalainen M, Nieminen R, Uurto I, Salenius JP, Kellomaki M, Mikkonen J, et al. Dexamethasone-eluting vascular stents. Basic Clin Pharmacol Toxicol. 2013 May;112(5):296-301.
101. Murray AW. Recycling the cell cycle: cyclins revisited. Cell. 2004 Jan 23;116(2):221-34.
102. Haberichter T, Madge B, Christopher RA, Yoshioka N, Dhiman A, Miller R, et al. A systems biology dynamical model of mammalian G1 cell cycle progression. Mol Syst Biol. 2007;3:84.
103. Abe M, Kimura T, Morimoto T, Taniguchi T, Yamanaka F, Nakao K, et al. Sirolimus-eluting stent versus balloon angioplasty for sirolimus-eluting stent restenosis: Insights from the j-Cypher Registry. Circulation. 2010 Jul 6;122(1):42-51.
104. Yano H, Horinaka S, Yagi H, Ishimitsu T. Comparison of inflammatory response after implantation of sirolimus- and paclitaxel-eluting stents in patients on hemodialysis. Heart Vessels. 2012 Apr 22.
105. Cheng H, An SJ, Zhang XC, Dong S, Zhang YF, Chen ZH, et al. In vitro sequence-dependent synergism between paclitaxel and gefitinib in human lung cancer cell lines. Cancer Chemother Pharmacol. 2011 Mar;67(3):637-46.
106. Chen T, Stephens PA, Middleton FK, Curtin NJ. Targeting the S and G2 checkpoint to treat cancer. Drug Discov Today. 2012 Mar;17(5-6):194-202.
107. Hui-qing X, Jian-da Z, Xin-min N, Yan-zhong Z, Cheng-qun L, Quan-yong H, et al. HSP70 inhibits burn serum-induced apoptosis of cardiomyocytes via mitochondrial and membrane death receptor pathways. J Burn Care Res. 2008 May-Jun;29(3):512-8.
108. Simon DK, Johns DR. Mitochondrial disorders: clinical and genetic features. Annu Rev Med. 1999;50:111-27.

109. Yeh JS, Oh SJ, Hsueh CM. Frequency of Vascular Inflammation and Impact on Neointimal Proliferation of Drug Eluting Stents in Porcine Coronary Arteries. Acta Cardiol Sin. 2016 Sep;32(5):570-7.
110. Doostzadeh J, Clark LN, Bezenek S, Pierson W, Sood PR, Sudhir K. Recent progress in percutaneous coronary intervention: evolution of the drug-eluting stents, focus on the XIENCE V drug-eluting stent. Coron Artery Dis. 2010 Jan;21(1):46-56.
111. Walker JP, Owens CD. Current Status of Drug-Eluting Stents and Drug-Eluting Balloons for the Superficial Femoral Artery. Curr Surg Rep. 2013 Jun 01;1(2):90-7.
112. Inoue T, Node K. Molecular basis of restenosis and novel issues of drug-eluting stents. Circ J. 2009 Apr;73(4):615-21.
113. Chen AF, Ren J, Miao CY. Nitric oxide synthase gene therapy for cardiovascular disease. Jpn J Pharmacol. 2002 Aug;89(4):327-36.
114. Iwata A, Sai S, Moore M, Nyhuis J, de Fries-Hallstrand R, Quetingco GC, et al. Gene therapy of transplant arteriopathy by liposome-mediated transfection of endothelial nitric oxide synthase. J Heart Lung Transplant. 2000 Nov;19(11):1017-28.
115. Brito LA, Chandrasekhar S, Little SR, Amiji MM. Non-viral eNOS gene delivery and transfection with stents for the treatment of restenosis. Biomed Eng Online. 2010 Sep 27;9:56.
116. Yang J, Zeng Y, Zhang C, Chen YX, Yang Z, Li Y, et al. The prevention of restenosis in vivo with a VEGF gene and paclitaxel co-eluting stent. Biomaterials. 2013 Feb;34(6):1635-43.
117. Wang S, Lu J, You Q, Huang H, Chen Y, Liu K. The mTOR/AP-1/VEGF signaling pathway regulates vascular endothelial cell growth. Oncotarget. 2016 Aug 16;7(33):53269-76.
118. Li J, Zhang M, Wang M, Wang Z, Liu Y, Zhang W, et al. GHSR deficiency suppresses neointimal formation in injured mouse arteries. Biochem Biophys Res Commun. 2016 Oct 14;479(2):125-31.
119. Cidad P, Miguel-Velado E, Ruiz-McDavitt C, Alonso E, Jimenez-Perez L, Asuaje A, et al. Kv1.3 channels modulate human vascular smooth muscle cells proliferation independently of mTOR signaling pathway. Pflugers Arch. 2015 Aug;467(8):1711-22.
120. von der Leyen HE, Mugge A, Hanefeld C, Hamm CW, Rau M, Rupprecht HJ, et al. A prospective, single-blind, multicenter, dose escalation study of intracoronary iNOS lipoplex (CAR-MP583) gene therapy for the prevention of restenosis in patients with de novo or restenotic coronary artery lesion (REGENT I extension). Hum Gene Ther. 2011 Aug;22(8):951-8.
121. Muona K, Makinen K, Hedman M, Manninen H, Yla-Herttuala S. 10-year safety follow-up in patients with local VEGF gene transfer to ischemic lower limb. Gene Ther. 2012 Apr;19(4):392-5.
122. Lipskaia L, Hadri L, Le Prince P, Esposito B, Atassi F, Liang L, et al. SERCA2a gene transfer prevents intimal proliferation in an organ culture of human internal mammary artery. Gene Ther. 2013 Apr;20(4):396-406.
123. Silvestre-Roig C, Fernandez P, Esteban V, Pello OM, Indolfi C, Rodriguez C, et al. Inactivation of nuclear factor-Y inhibits vascular smooth muscle cell proliferation and neointima formation. Arterioscler Thromb Vasc Biol. 2013 May;33(5):1036-45.
124. Larifla L, Deprez I, Pham I, Rideau D, Louzier V, Adam M, et al. Inhibition of vascular smooth muscle cell proliferation and migration in vitro and neointimal hyperplasia in vivo by adenoviral-mediated atrial natriuretic peptide delivery. J Gene Med. 2012 Jul;14(7):459-67.
125. Joung H, Kwon JS, Kim JR, Shin S, Kang W, Ahn Y, et al. Enhancer of polycomb1 lessens neointima formation by potentiation of myocardin-induced smooth muscle differentiation. Atherosclerosis. 2012 May;222(1):84-91.
126. Jacob T, Hingorani A, Ascher E. p53 gene therapy modulates signal transduction in the apoptotic and cell cycle pathways downregulating neointimal hyperplasia. Vasc Endovascular Surg. 2012 Jan;46(1):45-53.
127. Zee RY, Hoh J, Cheng S, Reynolds R, Grow MA, Silbergleit A, et al. Multi-locus interactions predict risk for post-PTCA restenosis: an approach to the genetic analysis of common complex disease. Pharmacogenomics J. 2002;2(3):197-201.

128. Russo RJ, Silva PD, Yeager M. Coronary artery overexpansion increases neointimal hyperplasia after stent placement in a porcine model. Heart. 2007 Dec;93(12):1609-15.
129. Ma X, Hibbert B, McNulty M, Hu T, Zhao X, Ramirez FD, et al. Heat shock protein 27 attenuates neointima formation and accelerates reendothelialization after arterial injury and stent implantation: importance of vascular endothelial growth factor up-regulation. FASEB J. 2014 Feb;28(2):594-602.
130. Oyamada S, Ma X, Wu T, Robich MP, Wu H, Wang X, et al. Trans-iliac rat aorta stenting: a novel high throughput preclinical stent model for restenosis and thrombosis. J Surg Res. 2011 Mar;166(1):e91-5.
131. Schwartz RS, Chronos NA, Virmani R. Preclinical restenosis models and drug-eluting stents: still important, still much to learn. J Am Coll Cardiol. 2004 Oct 06;44(7):1373-85.

5. ARTIGO 1

Journal of Molecular and Cellular Cardiology

GENE EXPRESSION PATTERN OF *DE NOVO* CORONARY ATHEROMATOUS PLAQUE IS CORRELATED WITH IN-STENT NEOINTIMAL VOLUME IN PATIENTS WITH RESTENOSIS.

Juliane S. Rossato[1], Marco A. Costa[2], Alcides J. Zago[1], Bruno S. Matte[1], German Iturry-Yamamoto[1], Ricardo Savaris[1], Mariano Albertal[3], Cristiane S. Rocha[4], Aileen M. Healy[5], Russell Walker[5], Tahara Satoko[2], Daniel I. Simon[2], Alexandre C. Zago[1,2]

1. Hospital de Clínicas de Porto Alegre – Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
2. UH Hospitals – Case Western University, Cleveland, OH, USA
3. Instituto Cardiovascular de Buenos Aires, Buenos Aires, Argentine
4. Campinas State University (Unicamp), Campinas, SP, Brazil
5. Millennium Pharmaceuticals, Cambridge, MA, USA

Word count: 4893

Corresponding author:

Alexandre do Canto Zago, MD, PhD

Hospital de Clínicas de Porto Alegre

Rua Ramiro Barcelos, 2350 (zip code: 90.035-930)

Porto Alegre, Rio Grande do Sul, Brazil

Phone: +55 (51) - 999168888

E- mail: aczago@hcpa.edu.br

ABSTRACT

In-stent restenosis (ISR) is a poorly understood phenomenon that remains a challenge even in the drug eluting stent era. There is limited data about its genetic basis and practically null evaluating the link between *de novo* lesions and ISR. This study sought to identify overexpressed genes in human *de novo* coronary plaques in relation to ISR that may work as novel therapeutic targets. Forty *de novo* coronary atheromatous plaques were retrieved from patients with symptomatic obstructive coronary artery disease through directional atherectomy prior to bare-metal stent implantation. RNA profiling analysis of more than 22,000 genes was successfully performed in 28 of 40 samples using the Affymetrix GeneChip microarray system. All patients underwent intravascular ultrasound assessment at six-month follow-up for stent volumetric analysis. The correlation between gene expression of *de novo* coronary atheromatous plaque and in-stent neointimal volume was evaluated. Several overexpressed genes related to oxidative stress and inflammation (MAOA, r = 0.83, p = 0.000; MFAP4, r = 0.77, p = 0.0001; AOC3, r = 0,65, p = 0.0018), cell proliferation (FLNA A, r = 0.76, p = 0.0001; AVEN, r = 0,63, p = 0.0031; MAP4, r = 0,60, p = 0.0049), and structure and function of smooth muscle cell (CNN1, r = 0,78, p = 0.000; MYH11, r = 0,67, p = 0.0012) were identified having correlation with in-stent neointimal volume. *De novo* coronary lesions present several overexpressed genes associated to future in-stent neointimal proliferation emerging as novel therapeutic targets.

Keywords: gene expression, stent, restenosis, intravascular ultrasound, directional atherectomy

1. INTRODUCTION

Devices developed for coronary artery disease (CAD) treatment, as bare metal stents (BMS), were the first to be used and, although initially well succeeded in patients with CAD, they were shown to be primarily responsible for serious complications over the years. The main adverse effect of this treatment is the luminal obstruction after stent implant. This process is called restenosis and it is presented in about 30% [1-4]. Risk factors as advanced age, hypertension, diabetes, tabagism, high C reactive protein levels, calcified lesions, seem to contribute to the occurrence of a new post-revascularization

ischemia event. Even the continuous improvement in the development of new interventional techniques, as well as the development of several devices with an extensive variety of materials and associated drugs, intra stent restenosis still occurs in 10 to 20% of patients (*drug eluting stents*, DES) [5-9].

Restenosis is one of the main complications after percutaneous coronary intervention (PCI). It is characterized by smooth muscle cell proliferation and migration to vascular intimal region. [2, 10, 11]. This condition is developed as result of the injury generated by angioplasty procedure, which induces a cascade of events such as endothelial denudation, release of growth factors and inflammatory mediators, which can modulate the vascular smooth muscle cell phenotype, thus stimulating neointimal hyperplasia [12-16].

Initially, inflammatory response plays a key role in the restenosis process. A wealth of cell molecular adhesion, inflammatory cytokines, and many others mediate a wide range of immune and inflammatory responses and have been found to be involved in the development of restenosis and atherosclerosis [17-20].

Neointimal proliferation stands as the main mechanism for coronary restenosis after stent implantation. [14] Even after the introduction of drug-eluting stents (DES), restenosis is not abated. [10, 21] In brief, neointimal proliferation constitutes a healing process attributed to vessel trauma due to stent implantation, in which medial smooth muscle cells migrate from the media to the intima, proliferate and produce abundant extracellular matrix. [14, 22] Several angiographic and intravascular ultrasound (IVUS) studies have indicated that baseline plaque characteristics are predictors of restenosis. [9, 23, 24] In contrast, studies designed to identify genetic predictors of restenosis only evaluated neointimal tissue. [13, 25].

Here we aimed to verify genetic predictors of neointimal hyperplasia in atherosclerotic plaque of patients who developed restenosis. This study sought to establish a relationship between genetic expressions at human *de novo* plaques and the degree of neointimal formation following bare-metal stent implantation.

2. METHODS

2.1 Study Population

Forty patients with symptomatic *de novo* coronary artery disease who underwent coronary stenting with adjunctive directional atherectomy were included in the study. Patients were excluded if they required treatment for: (a) saphenous vein lesions, (b) ostial

right coronary artery lesions, (c) in-stent restenotic lesions, or (d) if they had a history of cardiac transplant, dilated or restrictive cardiomyopathy. The study was conducted following its approval by the ethics committee of Hospital de Clínicas de Porto Alegre (nº 02374) and all patients gave written informed consent.

2.2 Procedure

IVUS was performed just before percutaneous coronary intervention to achieve plaque and vessel data to help in the decision making of the stent size. Next, directional atherectomy was performed with the Flexicut directional atherectomy device (Guidant Corp., Temecula, USA) through an 8Fr guiding catheter as previously described. [25] This atherectomy catheter has a 6Fr shaft and an urging balloon available in 3 different diameters (2.5, 3.0, and 3.5 mm) that was inflated to low pressure (about 0.5 atm) to allow proper apposition of the cutting window against the atheromatous plaque. Once it is activated, the cutter rotates at a speed of 2,000 rpm and should be slowly advanced to the distal end of the window to suck the excised material into the nosecone located at the distal portion of the catheter. In 40 study patients, adequate number of samples was retrieved. After atherectomy, all lesions received bare-metal stents according to standard techniques followed by IVUS to confirm satisfactory stent expansion and apposition. When necessary, stent post dilation followed by IVUS control was performed.

2.3 Quantitative Measurements

Quantitative coronary angiography and IVUS imaging were performed after bolus infusion of intracoronary nitrates during the index procedure and at follow-up assessments. IVUS images were acquired with a motorized pullback at a constant speed of 0.5 mm/s. Quantitative angiographic analysis was performed by independent investigators at Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil) and volumetric IVUS analyses was performed by independent core laboratory at UH Hospitals (Cleveland, USA). In-stent restenosis was defined as $\geq$ 50% diameter stenosis (DS) at follow-up within the stent. Minimal lumen diameter and percent DS were measured for each segment. In-stent late lumen loss was calculated as postprocedural minimal lumen diameter minus follow-up minimal lumen diameter. Intimal hyperplasia volume (IH) was calculated as stent volume minus luminal volume. Percent intimal hyperplasia (PIH) was defined as intimal hyperplasia volume divided by stent volume. The PIH was chosen for the gene correlation because it is the most representative parameter of neointima formation and allows equalizing the different diameters and lengths of the stents analyzed.

2.4 Genetic analysis and gene expression profiling

Atherectomy specimens were placed in RNA Stat 60, stored in liquid nitrogen and sent to Millennium Pharmaceuticals (Cambridge, USA) for gene profiling analysis of more than 22,000 genes. RNA was isolated from atherectomy lesion samples by homogenization with a PowerGen model 125 homogenizer (Thermo Fisher Scientific, Waltham, USA), followed by extraction with 0.2 volume chloroform, precipitation with 0.5 volume isopropanol, wash in 75% ethanol, and suspension in RNase-free water. RNA concentration was measured by use of the NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA quality and purity were assessed with the use of the RNA 6000 pico or nano assay (Agilent 2100 Bioanalyzer; Agilent Technologies, Santa Clara, USA).

Samples were labeled for gene expression profiling using the Affymetrix GeneChip microarray system (Affymetrix, Santa Clara, USA). A standard T7-based amplification protocol (Affymetrix) was used to convert RNA to biotinylated cRNA. Fragmented, biotin-labeled cRNA was hybridized to Affymetrix GeneChip Human Genome U133A arrays according to standard Affymetrix protocol. Operators, chip lots, and scanners (GeneArray 3000, Affymetrix) were controlled throughout. Quality controls of arrays that used GeneChip Operating Software included scaling factor and percentage of genes present. Arrays with a scaling factor difference of less than 3 and more than 20% genes present were analyzed further. (Figure 1).

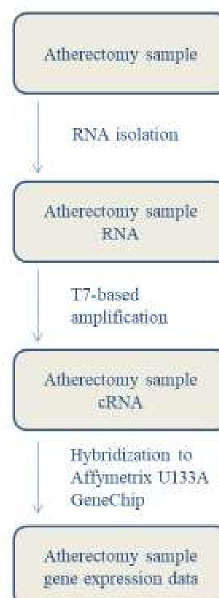

Figure 1. Genetic analysis and gene expression profiling

2.5 Statistical analysis

Continuous variables are expressed as mean ± SD. Pearson´s correlation was used to evaluate the relationship between gene expression and the amount of neointimal hyperplasia expressed as PIH, as well as the relationship between stent length and PIH, and between stent diameter and PIH. A sample size of 29 atheromatous plaques was calculated to be necessary for *rho* (r) ≥ 0.5, statistical power of 80%, and alpha error (p) < 0.05 (two-tails), while a sample size of 19 atheromatous plaques was required for r ≥ 0.6, statistical power of 80%, and p < 0.05 (two-tails). The T Student test was used to evaluate the association between diabetes and PIH.

3. RESULTS

From 40 patients who underwent single bare-metal stenting with adjunctive directional atherectomy, 28 had adequate tissue sampling for RNA analysis. Baseline and procedural characteristics of these patients are summarized in table 1. Mean age was 60.2 ± 11.4 and diabetes proportion 35.7%. Mean IH and PIH at follow-up were 52.8 ± 28.2 mm^3 and 29.9 ± 18.7%, respectively (Table 2).

Table 1. Baseline and Procedural Characteristics (n= 28)

Age, year ± SD	60.2 ± 11.41
Men	24 (85.71)
Diabetes mellitus	10 (35.71)
Hypertension	18 (64.28)
Hyperlipidemia	17 (60.71)
Smoking	15 (53.57)
Acute Coronary Syndrome	22 (78.57)
Vessel	
Left anterior descending	18 (64.28)
Left circumflex	4 (14.28)
Right	6 (21.42)
Preinterventional angiographic findings	
Reference segment diameter	3.49 ± 0.22
Lesion length. mm	16.28 ± 4.82
Percentage diameter stenosis	76.23 ± 10.09
Minimum luminal diameter. mm	0.83 ± 0.36
Postinterventional angiographic findings	
In-stent minimal lumen diameter. mm	3.33 ± 0.29
In-stent diameter stenosis	6.00 ± 4.43
Procedure details	
Stent length. mm	20.15 ± 4.63
Stent/artery ratio	1.01 ± 0.02

Values are mean ± SD and n (%).

Table 2. Angiographic and IVUS assessment at follow-up (n=28)

Absolute late loss, mm	1,07 ± 0,75
Late loss index	0,43 ± 0,24
Stent Volume, mm^3	197,98 ± 56,31
Lumen Volume, mm^3	145,29 ± 51,84
Intimal hyperplasia volume, mm^3	52,84 ± 28,16
Percent Intimal hyperplasia volume, %	29,91 ± 18,74

Values are mean ± SD

The degree of neointimal proliferation did not vary between patients with and without diabetes (PIH: diabetics 29.5% versus 30.7%, p=0.89). There was not significant correlation between PIH and stent length (r= -0.26, p=0.26) or stent diameter (r= 0.14, p=0.56).

As shown in table 3, several genes involved in atherosclerosis cascade (6 genes), cell proliferation (9 genes) and smooth muscle cell structure and function (8 genes) showed a positive correlation with PIH, while the protector gene TRAF family member-associated NFκB activator had an inverse relationship with PIH (r= -0.57, p = 0.0074).

Table 3. Correlation between Genes in atheromatous plaques and Percentage of Intimal Hyperplasia.

Gene	*Symbol*	*Genebank acess (ID)*	*Function*	*Correlation with % IH*	*p*
Atherosclerosis Cascade					
Monoamine oxidase A	MAOA	4128	Oxidative stress	0.83	0.0000
Microfibrillar-associated protein 4	MFAP4	4239	Cell adhesion	0.77	0.0001
Tumor necrosis factor receptor superfamily, 11b	TNFRSF11B	4982	TNF-receptor	0.65	0.0018
Amine oxidase, copper containing 3	AOC3	543808	Oxidative stress	0.65	0.0018
Mature T-cell proliferation 1 neighbor	MTCP1NB	100272147	T cell proliferation	0.63	0.0028
Adhesion regulating molecule 1	ADRM1	3383	Cell adhesion	0.50	0.0244
Cell Proliferation					
Filamin A, alpha	FLNA	2316	Cell migration	0.76	0.0001
Apoptosis, caspase activation inhibitor	AVEN	57099	Inhibitor of apoptosis	0.63	0.0031
Microtubule-associated protein 4	MAP4	4134	Microtubule protein	0.60	0.0049
Cyclin D1	CCND1	595	Cell cycle regulator	0.59	0.0061
TNFAIP3 interacting protein 2	TNIP2	7128	Inhibit NF-kB	0.56	0.0100
Growth arrest-specific 6	GAS6	2621	Cell proliferation	0.56	0.0097
Modulator of apoptosis 1	MOAP1	6688	Cell cycle regulation	0.55	0.0122
Tubulin, beta 2A	TUBB2A	7280	Microtubule protein	0.52	0.0256
Palladin, cytoskeletal associated protein	PALLD	23022	Control of cell shape, adhesion and contraction	0.51	0.0231

Smooth Muscle Cell					
Calponin 1, basic, smooth muscle	CNN1	1264	Structural protein	0.78	0.0000
Myosin, heavy chain 11, smooth muscle	MYH11	4629	Structural protein	0.67	0.0012
Leiomodin 1	LMOD1	25802	Structural protein	0.61	0.0046
Myosin, light chain 9. regulatory	MYL9	10398	Structural protein	0.54	0.0148
Tropomyosin 1 alpha	TPM1	7168	Contractile	0.53	0.0152
Myosin light chain kinase	MYLK	4638	Cell contraction	0.53	0.0157
Smoothelin	SMTN	6525	Structural protein	0.51	0.0258
Caldesmon 1	CALD1	800	Regulation	0.50	0.0232

Accordingly, the present study found a relationship between neointimal growth and upregulation of cell adhesion molecules such as adhesion regulating molecule 1 (ICAM) and microfibrillar-associated protein 4 ($r = 0.78$), which is found in the extracellular matrix that constitutes one of the key components of neointima hyperplasia.

The intracellular oxidative stress status is involved in the release of inflammatory cytokines (TNF-α, IL-β e INF-γ) and, consequently, for the amplification of the inflammatory response. In our findings, there was a direct association between neointima growth and the expression of genes related to intracellular oxidative stress, such as oxidase monoamine ($r = 0.83$) and oxidase amine. In addition, we observed an overexpression of genes related to the inflammatory process, such as TNF receptor superfamily and mature T-cell proliferation 1 neighbor that induce INF-γ production, according to IH.

After an initial inflammatory phase, growth factors are released from platelets, leukocytes and smooth muscle cells, which stimulate migration and proliferation of smooth muscle cells from the media to the neointima [26]. In the present study, the expression of several cell cycle regulator genes involved in G_1/S (cyclin D1; modulator of apoptosis 1; caspase-dependent mediator of nuclear apoptosis; growth arrest-specific 6; filamin A; TNFAIP3 interacting protein 2, paladin, cytosketal associated protein) and

G_2/M transitions (microtubule-associated protein 4; tubulin, beta 2) correlated with the magnitude of the neointima formation.

An increased expression of several genes related to the structure and the function of smooth muscle cells, which constitutes the cellular basis of restenosis, such as myosin, heavy chain 11, calponin 1, leiomodin 1, myosin, light chain 9, tropomyosin 1, myosin light chain kinase, caldesmon 1 e smoothelin, was a rather unexpected finding. The high expression of cell cycle proliferation genes and vascular smooth muscle genes strongly suggests that there is a genetic predisposition to smooth muscle proliferation in plaques that will develop restenosis.

4. DISCUSSION

The development of restenosis represents a significant limitation to coronary stenting. Although, the introduction of drug-eluting stents (DES) has substantially reduced restenosis rates, it still occurs at low rates (7-8%), particularly in high risk cases (i.e. long lesions, small vessels, patients with diabetes mellitus or chronic renal insufficiency).[10, 21] For these reasons and others, such as DES thrombosis rates and need for prolonged dual antiplatelet therapy, there is continued interest for a better understanding of restenosis biology. After mechanical arterial injury, a series of events lead to smooth muscle proliferation and abundant synthesis of extracellular matrix, key components of neointima thickening.[14] The present study findings indicate that human *de novo* plaques capable of mounting an enhanced neointimal response after bare-metal stenting possess an augmented expression of several genes involved in (1) the restenosis inflammatory cascade, (2) cellular differentiation and proliferation, and (3) production of structural and functional proteins of smooth muscle cells. Consequently, our results suggest that atherosclerotic plaques hold an inherent predisposition to cell hyperproliferation and neointima formation that predates stent implantation.

Sirolimus is an immunosuppressant drug used to prevent rejection after organ transplantation that binds the cytosolic protein FK-binding protein 12 (FKBP12). The sirolimus-FKBP12 complex inhibits the mammalian target of rapamycin (mTOR) pathway and, consequently, interrupts the cell cycle at the G_1/S transition in vascular smooth muscle cells.[27, 28] Therefore, sirolimus and its analogs inhibit, through mTOR pathway, several cell cycle intermediates (cyclin D1; modulator of apoptosis 1; caspase-dependent mediator of nuclear apoptosis; growth arrest-specific 6; filamin A; TNFAIP3

interacting protein 2, paladin, cytosketal associated protein) that were correlated with neointimal proliferation. Sirolimus also inhibits other cell cycle regulators such as the NFκB/Bcl-xl pathway that induces cell proliferation.[15] In our study, an inhibitor of NFκB activation via TNF-α gene named TRAF family member-associated NFκB activator was found, which presented negative correlation (r = - 0.57) with neointimal proliferation.

Paclitaxel, another effective drug against restenosis, binds specifically to the ß-tubulin subunit of microtubules, stabilizing the structure and compromising the microtubules dynamics, which causes mitosis arrest in G_2/M phase. [16, 17] Consistent with paclitaxel effect, the present study found correlation between in-stent neointimal proliferation and upregulation of three genes associated with the production of tubulin, microtubules formation and cytoskeletal remodeling – tubulin ß 2A, microtubule-associated protein 4, and filamin Aα, respectively.

Several specific smooth muscle cell promoters involved in smooth muscle structure and function were increasingly expressed according to neointimal response. For that reason, targeting these genes may constitute a novel therapeutic strategy to prevent restenosis. Based on our findings, genetic therapy raises as a novel paradigm for future DES that may exert direct inhibition of smooth muscle cell proliferation by targeting specific smooth muscle cell regulators and/or structural proteins. Data from animal and human studies indicate that either sirolimus- (and its analogs) or paclitaxel-eluting stents can lead to delayed arterial healing characterized by incomplete reendothelization and fibrin persistence at the stented site.[18-21] Therefore, novel generation of DES based on genetic therapy driven to smooth muscle cells shall reduce neointimal formation while allowing more physiological endothelial cell proliferation and, consequently, early endothelial reendothelialization.

Isoforms are several forms of mRNA with distinct nucleotide sequence formed by alternative splicing, capable of producing the same protein. Multiple mRNA and proteins isoforms are produced by each gene. [18] In our results, the microarray analysis identified two mRNA isoforms of myosin, heavy chain 11 that support the role of increased production of proteins related to the structural development of smooth muscle cells. We also found two isoforms of mature T cell proliferation 1 neighbor gene, two isoforms of growth arrest-specific 6, and four isoforms of gene microtubule-associated protein 4 related to inflammatory response, cell proliferation and microtubules production in G2/M phase of the cell cycle, respectively. The identification of mRNA isoforms in specific

overexpressed genes points towards a non-random finding and further strengthens our results.

Through the years, a number of clinical baseline and procedural predictors of restenosis have been identified. Several studies have examined circulating genetic markers/predictors of ISR, demonstrating allelic variations associated with the restenotic response. On the other hand, human pathological data with regard to restenosis are limited and controversial. Previous attempts focused mainly on tissue characterization of human in-stent neointima. Gene expression profiling with RNA microarray technology is becoming a popular tool to monitor the expression level of thousands of genes simultaneously and has been applied extensively in cardiovascular research to detect patterns of gene expression indicative of underlying disease states. Previously, few studies have used this technique to evaluate the genetic expression of human *de novo* plaques and in-stent neointima. In one study, 10 neointima specimens retrieved by an atherectomy device were compared to 4 coronary atherosclerotic plaques and 7 gastrointestinal artery plaques. [25] The authors reported a distinct genetic profile in the neointima compared to the control plaques. In another study, Ashley et al. compared *de novo* and restenotic samples through histology and gene expression with a dual-dye 22,000 oligonucleotide microarray. [13] By histology, greater cellularity and fewer inflammatory infiltrates and lipid pools were found in the restenosis group compared to *de novo* atherosclerosis. Gene ontology analysis demonstrated the prominence of cell proliferation programs in ISR and inflammation/immune programs in *de novo* atherosclerosis. By network analysis, which combines semantic mining of the published literature with the expression signature of restenosis, two gene expression modules were revealed as candidates for selective inhibition of restenotic disease (i.e. procollagen type 1 α2 gene and the ADAM17/tumor necrosis factor-converting enzyme gene). [13] Our study further extends these analysis, demonstrating a relationship between the genetic profile of coronary *de novo* plaques and the amount of neointimal formation. To our knowledge, the present study is the first to report this relationship.

Prior studies have demonstrated that baseline clinical characteristics like diabetes mellitus as well as angiographic characteristics such as lesion length, lesion type (i.e. restenotic lesions, chronically occluded or at vein grafts) and vessel size predispose to restenosis. [21, 29-31] In addition, procedural-related variables have also been implicated. For instance, the longer the stented length [29], the number of stents used and the postprocedural minimal lumen diameter are also associated with increased restenosis

rates [32]. In our study, there was no association between diabetes, stent length or stent diameter values and IH. Thus, patients´ specific clinical and anatomical characteristics are unlikely confounders and further multivariate statistical analysis does not seem necessary in the present study.

During routine clinical practice, restenosis occurrence may vary even at the same patient or same coronary artery. Thus, restenosis appears to be more plaque-prone dependent than patient or artery dependent. Some plaque related factors like a large plaque burden, the presence of soft plaque or evidence of positive remodeling have all been linked with restenosis. [9, 24] In agreement with these observations, our findings further support the concept of a local predisposition for the development of restenosis in addition to the presence of smooth muscle cells promoters as key players of restenosis process.

Limitations of the Study

In the present study, some limitations should be considered. First, as we analyzed a large number of genes, many genes that bear no relationship with the biology of restenosis were screened and found to have a random positive or negative relationship with neointimal formation. Such random correlation may also be explained by the non-uniform neointimal proliferation inside the stent as seen on focal restenosis, which bias the IH volume results. Secondly, RNA extraction and testing of each atherosclerotic plaque, which also constituted a strong point of methodology, led to the analysis of insufficient quantities of RNA in 12 out of the 40 tissue samples causing their exclusion. This probably was due to the presence of large amounts of lipid and the small number of cells in the atheromatous plaques. Finally, the amount of tissue retrieved did not allow for further validation of study results by direct proteins measurement through Western-Blot test.

The above-mentioned technical limitations regarding tissue sampling and genetic testing do not compromise the present study, because complete elucidation of all physiopathological mechanisms of restenosis was out of its scope. However, the study exploratory design allowed to point out new features for better understanding of the complex processes involved in restenosis as well as new genetic therapeutic targets.

5. CONCLUSION

De novo coronary lesions present several overexpressed genes involved in oxidative stress, inflammation, cell proliferation, and structure and function of smooth muscle cell associated to future in-stent neointimal proliferation emerging as novel therapeutic targets.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial conflict of interests.

ACKNOWLEDGMENTS

Fundo de Incentivo à Pesquisa e Eventos of Hospital de Clínicas de Porto Alegre (FIPE-HCPA), Grant nº 02/374, Porto Alegre, Brazil.

6. REFERENCES

[1] Zago AC, Raudales JC, Attizzani G, Matte BS, Yamamoto GI, Balvedi JA, et al. Local delivery of sirolimus nanoparticles for the treatment of in-stent restenosis. Catheter Cardiovasc Interv. 2012.

[2] Hill R, Bagust A, Bakhai A, Dickson R, Dundar Y, Haycox A, et al. Coronary artery stents: a rapid systematic review and economic evaluation. Health Technol Assess. 2004;8:iii-iv, 1-242.

[3] Simon DI, Dhen Z, Seifert P, Edelman ER, Ballantyne CM, Rogers C. Decreased neointimal formation in Mac-1(-/-) mice reveals a role for inflammation in vascular repair after angioplasty. J Clin Invest. 2000;105:293-300.

[4] Pires NM, Eefting D, de Vries MR, Quax PH, Jukema JW. Sirolimus and paclitaxel provoke different vascular pathological responses after local delivery in a murine model for restenosis on underlying atherosclerotic arteries. Heart. 2007;93:922-7.

[5] Kastrati A, Mehilli J, von Beckerath N, Dibra A, Hausleiter J, Pache J, et al. Sirolimus-eluting stent or paclitaxel-eluting stent vs balloon angioplasty for prevention of recurrences in patients with coronary in-stent restenosis: a randomized controlled trial. JAMA. 2005;293:165-71.

[6] Kong J, Deng Y, Dong Q, Liu W, Lu Y. Colchicine reduces restenosis after balloon angioplasty treatment for in-stent restenosis. Arch Med Res. 2015;46:101-6.

[7] Bonaa KH, Mannsverk J, Wiseth R, Aaberge L, Myreng Y, Nygard O, et al. Drug-Eluting or Bare-Metal Stents for Coronary Artery Disease. N Engl J Med. 2016;375:1242-52.

[8] Ferrante G, Niccoli G, Biasucci LM, Liuzzo G, Burzotta F, Galiuto L, et al. Association between C-reactive protein and angiographic restenosis after bare metal stents: an updated and comprehensive meta-analysis of 2747 patients. Cardiovasc Revasc Med. 2008;9:156-65.

[9] Hong YJ, Jeong MH, Lim SY, Lee SR, Hong SN, Kim KH, et al. Relation of soft plaque and elevated preprocedural high-sensitivity C-reactive protein levels to incidence of in-stent restenosis after successful coronary artery stenting. Am J Cardiol. 2006;98:341-5.

[10] Dangas GD, Claessen BE, Caixeta A, Sanidas EA, Mintz GS, Mehran R. In-stent restenosis in the drug-eluting stent era. J Am Coll Cardiol. 2010;56:1897-907.

[11] Granada JF, Stenoien M, Buszman PP, Tellez A, Langanki D, Kaluza GL, et al. Mechanisms of tissue uptake and retention of paclitaxel-coated balloons: impact on neointimal proliferation and healing. Open Heart. 2014;1:e000117.

[12] Inoue T, Node K. Molecular basis of restenosis and novel issues of drug-eluting stents. Circ J. 2009;73:615-21.

[13] Ashley EA, Ferrara R, King JY, Vailaya A, Kuchinsky A, He X, et al. Network analysis of human in-stent restenosis. Circulation. 2006;114:2644-54.

[14] Costa MA, Simon DI. Molecular basis of restenosis and drug-eluting stents. Circulation. 2005;111:2257-73.

[15] Curcio A, Torella D, Indolfi C. Mechanisms of smooth muscle cell proliferation and endothelial regeneration after vascular injury and stenting: approach to therapy. Circ J. 2011;75:1287-96.

[16] Li J, Zhang M, Wang M, Wang Z, Liu Y, Zhang W, et al. GHSR deficiency suppresses neointimal formation in injured mouse arteries. Biochem Biophys Res Commun. 2016;479:125-31.

[17] Berry JD, Liu K, Folsom AR, Lewis CE, Carr JJ, Polak JF, et al. Prevalence and progression of subclinical atherosclerosis in younger adults with low short-term but high lifetime estimated risk for cardiovascular disease: the coronary artery risk development in young adults study and multi-ethnic study of atherosclerosis. Circulation. 2009;119:382-9.

[18] Chatzizisis YS, Coskun AU, Jonas M, Edelman ER, Feldman CL, Stone PH. Role of endothelial shear stress in the natural history of coronary atherosclerosis and vascular remodeling: molecular, cellular, and vascular behavior. J Am Coll Cardiol. 2007;49:2379-93.

[19] Tabas I. 2016 Russell Ross Memorial Lecture in Vascular Biology: Molecular-Cellular Mechanisms in the Progression of Atherosclerosis. Arterioscler Thromb Vasc Biol. 2016.

[20] Rocha VZ, Libby P. Obesity, inflammation, and atherosclerosis. Nat Rev Cardiol. 2009;6:399-409.

[21] Lemos PA, Hoye A, Goedhart D, Arampatzis CA, Saia F, van der Giessen WJ, et al. Clinical, angiographic, and procedural predictors of angiographic restenosis after sirolimus-eluting stent implantation in complex patients: an evaluation from the Rapamycin-Eluting Stent Evaluated At Rotterdam Cardiology Hospital (RESEARCH) study. Circulation. 2004;109:1366-70.

[22] Moreno PR, Palacios IF, Leon MN, Rhodes J, Fuster V, Fallon JT. Histopathologic comparison of human coronary in-stent and post-balloon angioplasty restenotic tissue. Am J Cardiol. 1999;84:462-6, A9.

[23] Ishikawa T, Hatakeyama K, Imamura T, Date H, Shibata Y, Hikichi Y, et al. Involvement of C-reactive protein obtained by directional coronary atherectomy in plaque instability and developing restenosis in patients with stable or unstable angina pectoris. Am J Cardiol. 2003;91:287-92.

[24] Sahara M, Kirigaya H, Oikawa Y, Yajima J, Nagashima K, Hara H, et al. Soft plaque detected on intravascular ultrasound is the strongest predictor of in-stent restenosis: an intravascular ultrasound study. Eur Heart J. 2004;25:2026-33.

[25] Zohlnhofer D, Klein CA, Richter T, Brandl R, Murr A, Nuhrenberg T, et al. Gene expression profiling of human stent-induced neointima by cDNA array analysis of microscopic specimens retrieved by helix cutter atherectomy: Detection of FK506-binding protein 12 upregulation. Circulation. 2001;103:1396-402.

[26] Welt FG, Rogers C. Inflammation and restenosis in the stent era. Arterioscler Thromb Vasc Biol. 2002;22:1769-76.

[27] Marx SO, Jayaraman T, Go LO, Marks AR. Rapamycin-FKBP inhibits cell cycle regulators of proliferation in vascular smooth muscle cells. Circ Res. 1995;76:412-7.

[28] Sabatini DM, Erdjument-Bromage H, Lui M, Tempst P, Snyder SH. RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell. 1994;78:35-43.

[29] Kereiakes D, Linnemeier TJ, Baim DS, Kuntz R, O'Shaughnessy C, Hermiller J, et al. Usefulness of stent length in predicting in-stent restenosis (the MULTI-LINK stent trials). Am J Cardiol. 2000;86:336-41.

[30] Kobayashi Y, De Gregorio J, Kobayashi N, Akiyama T, Reimers B, Finci L, et al. Stented segment length as an independent predictor of restenosis. J Am Coll Cardiol. 1999;34:651-9.

[31] Mercado N, Boersma E, Wijns W, Gersh BJ, Morillo CA, de Valk V, et al. Clinical and quantitative coronary angiographic predictors of coronary restenosis: a comparative analysis from the balloon-to-stent era. J Am Coll Cardiol. 2001;38:645-52.

[32] Rathore S, Terashima M, Katoh O, Matsuo H, Tanaka N, Kinoshita Y, et al. Predictors of angiographic restenosis after drug eluting stents in the coronary arteries: contemporary practice in real world patients. EuroIntervention. 2009;5:349-54.

6. ARTIGO 2

Journal of Molecular and Cellular Cardiology

EFFECT OF SMOOTH MUSCLE CELLS GENE SILENCERS ON THE VIABILITY OF HUMAN CORONARY ARTERY ENDOTHELIAL AND SMOOTH MUSCLE CELLS

Juliane Rossato[1], Maximiliano Schaun [2], Bruno S. Matte[1], Ana Maria Krepsky[1], Gabriel Zago[1], Alexandre C. Zago[1,2]

1. Hospital de Clínicas de Porto Alegre – Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.
2. Instituto de Cardiologia do Rio Grande do Sul/Fundação Universitária de Cardiologia, Porto Alegre, RS, Brazil.

Word count: 4811

Corresponding author:

Alexandre do Canto Zago, MD, PhD

Hospital de Clínicas de Porto Alegre

Rua Ramiro Barcelos, 2350 (zip code: 90.035-930)

Porto Alegre, Rio Grande do Sul, Brazil

Phone: +55 (51) - 999168888

E- mail: aczago@hcpa.edu.br

ABSTRACT

The implantation of drug-eluting stents (DES) substantially reduced the occurrence of in-stent restenosis, however neointimal hyperplasia still occurs in approximately 5 to 15% of percutaneous coronary intervention cases. Usually, this condition is developed by patients with more complex lesions and occurs in approximately five months after implantation of bare metal stent and 12 months after the DES. This study aimed to compare the *in vitro* effect of specific gene silencers for smooth muscle cells and antiproliferative drugs on the viability of human coronary artery endothelial and smooth muscle cells. Primary cultures of human coronary artery endothelial and smooth muscle cells were cultivated in a fetal bovine serum enriched medium for the addition of antiproliferative drugs Rapamycin (100 μg/mL), Paclitaxel (100 μg/mL) and both combined, and the gene silencers Sh RNA SMMHC and Sh RNA CNN. Cell viability was determined by MTT (*4,5-dimethylthiazol- 2-yl]-2,5 diphenyltetrazolium bromide*) and the effectiveness of gene silencing was determined by ELISA (pg/mL). Continuous variables were presented in mean and standard deviation, and the comparison between groups performed using one-way ANOVA. The proliferation of smooth muscle cells was also reduced under the effect of Rapamycin (21.3 ± 9.2%; $p<0.001$), Paclitaxel (14.5 ± 3.8; $p<0.001$) and both combined (Rapamycin – Paclitaxel, 25.2% ± 4.4; $p<0.001$), when compared to control. Gene silencers Sh RNA SMMHC and Sh RNA CNN showed inhibitory effect only on smooth muscle cell lineage (9.8 ± 2.7% and 25.3 ± 4.4%, respectively; $p<0.001$). Antiproliferative drugs isolated reduced the proliferation of endothelial cells (Rapamycin 16.8 ± 3.2% and Paclitaxel 13.5 ± 3.0%; $p<0.001$). The same effect was found when Rapamycin and Paclitaxel were combined (18.4% ± 1.9; $p<0.001$). The *in vitro* analyzes demonstrated that gene silencers specific for smooth muscle cells have selective inhibitory effect on the proliferation of human coronary artery smooth muscle cells, without interfering in the proliferation of endothelial cells.

Keywords: Silencing genes; neointimal hyperplasia; endothelialization; restenosis.

1. INTRODUCTION

The advent of bare metal stents (BMS) changed completely the clinical results of the Interventional Cardiology. Although it initially presented good results in the treatment of coronary artery disease (CAD), over the years 15-30% of treated patients develop luminal re-narrowing after stent deployment, which is called in-stent restenosis (ISR) [1-4]. Risk factors such as advanced age, hypertension, diabetes mellitus, smoking, elevated C-reactive protein, and calcified and diffuse vascular lesions seem to contribute to the occurrence of a new post-revascularization ischemia due to ISR or neo-atherosclerosis [5-8].

The emergence of drug-eluting stents (DES), which are devices coated with pharmacological agents such as rapamycin (or analogs) and paclitaxel, allowed a direct therapeutic action on the progression of the cell cycle, thus preventing a disordered proliferation of human coronary artery smooth muscle cells (HSMCs) [9]. Rapamycin and its analogs has a potential ability to prevent restenosis by binding to the cellular receptor FKBP12, therefore inhibiting the mammalian target of rapamycin (mTOR) pathway [10]. The mTOR pathway blocks cellular interphase, preventing the progression of the cell cycle from gap 1 (G1) to synthesis (S), and without DNA synthesis the cell is not able to perform mitosis [11, 12]. Paclitaxel is another antiproliferative agent that acts through a mechanism different from rapamycin, but also causes cell cycle blockade, preventing the progression from gap 2 (G2) to mitosis [13]. This drug acts on the formation of microtubules, which are key elements for the formation of the mitotic spindle and the orientation of chromosomes during the process of cell division [4].

The use of antiproliferative drugs in the manufacture of DES is a therapeutic alternative well established in the current medical practice, besides the remaining restenosis rates of 10-20% [5-8]. However, it has presented some specific limitations, since the antiproliferative drugs promote non selective inhibitory effect on HSMCs, that is they also inhibit endothelial cells proliferation which leads to incomplete stent endothelialization and increased time of use of antiplatelet agents [14].

The endothelium plays an important role in the regulation of vascular tone and in the maintenance of cardiovascular homeostasis, which are determinant for the control of thrombolysis, for vascular remodeling and in the inflammatory response by the activation of the immune system [15, 16]. The lack of a protective mechanism in the vascular wall enables the development of neointimal hyperplasia, as the antiproliferative effect of these

drugs results in delayed endothelial healing [9]. Thus, innovative protocols based on gene targets such as inhibition of gene expression, stem cell repair, or even local delivery of genes may represent important techniques for the prevention and treatment of ISR [17, 18].

In a previous study conducted by our group, we observed that the atheromatous plaque of a patient who developed a *de novo* lesion showed a greater expression of genes related to smooth muscle cell structural and functional protein synthesis. The myosin heavy chain (SMMHC) and calponin (CNN) genes demonstrated a positive correlation (0.68 and 0.78, respectively) with the percentage of neointimal hyperplasia measured by intravascular ultrasound (IVUS) six months after BMS implantation. Such findings indicate that these genes are potentially activated in the atheromatous plaque prior to stent implantation, which suggests that they may be considered predictors of the development of ISR [19].

The SMMHC protein belongs to the family of motor molecules that interact with actin filaments in the process of muscle contraction. This class of proteins is quite variable and includes a domain with adenylpyrophosphatase (ATPase) and actin-binding subdomains [20]. The CNN protein participates in the process of smooth muscle contractility through modulation of the calcium-calmodulin complex and functions as a regulator of the actin cytoskeleton [21].

Genetic therapy is a new alternative for the prevention and treatment of ISR, being most studies reported in the literature still in the pre-clinical phase [22-26]. However, there is no study regarding the Sh RNA SMMHC and Sh RNA CNN silencing genes and restenosis. Thus, the objective of the present study was to compare the *in vitro* effect of smooth muscle cell-specific genes silencers (Sh RNA SMMHC and Sh RNA CNN) with antiproliferative drugs on the viability of human coronary artery endothelial and smooth muscle cells.

2. METHODS

Cell culture

Primary cultures of human coronary artery endothelial cells (HECs) and human coronary artery smooth muscle cells (HSMCs) were purchased from Invitrogen, Calsbad, CA, USA. Cells were obtained at passage 2 and cultured in endothelial cell growth medium (Gibco, Medium 200) or smooth muscle cell growth medium (Gibco, Medium 231), supplemented with 10% fetal bovine serum (FBS, Invitrogen, Calsbad, CA, USA),

streptomysin (100 μg/mL) and penicillin (100 U/mL) at 37°C in a humidified incubator (5% CO_2). The cells used in this study were from passages 5-6.

Lentiviral particles transduction

HSMCs were placed in 12-well plate 24 hours prior to viral infection. Gene silencers Sh RNA SMMHC (gene ID: 4629) and Sh RNA CNN (gene ID: 1264) (Santa Cruz Biotechnology, USA) were added to a medium with serum and antibiotics, and cells were incubated overnight. On the day of infection, cells were approximately 50% confluent. They were transfected using the protocol for Sh RNA lentiviral particles transduction, according to the manufacturer's instructions. Several colonies were picked and expanded for the quantitative determination of SMMHC and CNN concentrations in cell cultures.

***In vitro* assay**

Primary cultures of HECs and HSMCs and a co-culture were separately seeded in 6-well plates at a density of 3×10^5 cells per well in 2 mL of grown medium. After 24 hours, the culture medium was replaced with 500 mL of fresh medium. The cultures were divided into six groups (n = 3 each): control (no drugs or gene silencers), rapamycin (100 μg/mL, Invitrogen, Calsbad, CA, USA), paclitaxel (100 μg/mL, Invitrogen, Calsbad, CA, USA), combination of antiproliferative drugs (rapamycin + paclitaxel), Sh RNA CNN and Sh RNA SMMHC. They were incubated for 24 hours.

Cell viability and proliferation assay

Cell proliferation was determined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Vybrant^R, Eugene, OR, USA). Prior to treatments with rapamycin, paclitaxel and gene silencers, HECs and HSMCs were plated at 30-40% confluence on a 96-well plate and incubated overnight with 100 mL of Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. MTT solution (10 mL; 12 mM) in phenol red-free medium was added to each well and incubated at 37ºC for 4 hours, followed by addition of 100 mL of hydrogen chloride (HCl) solution. After incubation of the plate at 37ºC for 4 hours, absorbance was measured at 570 nm (Bio-Rad, USA). All proliferation assays were performed in triplicate.

Quantitative determination of SMMHC and CNN concentrations in cell cultures

HSMC lysates were quantified using a capture assay (enzyme-linked immunosorbent assay, ELISA) for SMMHC and CNN (Cusabio Biotech, USA) in all groups. The assays were performed according to manufacturers' instructions. Optical density (absorbance) was measured using a spectrophotometer (Anthos Zenyth 200,

Biochorm. Harvard Bioscience Company, USA) at 25ºC. Data were expressed as picograms of protein per millilitre (pg/mL), and the detection range was 25-1600 pg/mL.

Statistical analysis

Data were expressed as mean ± standard deviation except where noted. The treatments with rapamycin, paclitaxel and gene silencers were compared by one-way analysis of variance (one-way ANOVA) with *post hoc* analysis for multiple comparisons; $p < 0.05$ was considered significant. All statistics were calculated with SPSS, version 22.0.

3. RESULTS

The cell proliferation assay was evaluated considering the percentage of the control group (100%) and performing the relative calculation of the other groups. Figure 1 shows a co-culture of HECs and HSMCs. There was no difference between Sh RNA SMMHC (92.6 ± 9.4%) and controls ($p = 0.702$). The antiproliferative drugs groups did not show a statistically significant difference between them (rapamycin: 15.7 ± 2.8%; paclitaxel: 11.4 ± 3.4%; rapamycin + paclitaxel: 12.9 ± 1.3%; $p = 0.999$), but they had a significant inhibitory effect on both cell lines when compared with control, Sh RNA CNN and Sh RNA SMMHC ($p < 0.0001$ for all comparisons). Sh RNA CNN (62.9 ± 8.3%) also showed an inhibitory effect when compared with control ($p < 0.0001$) and Sh RNA SMMHC ($p = 0.0009$), but a higher significant proliferative percentage was observed when it was compared with the antiproliferative drugs ($p < 0.0001$ for all comparisons).

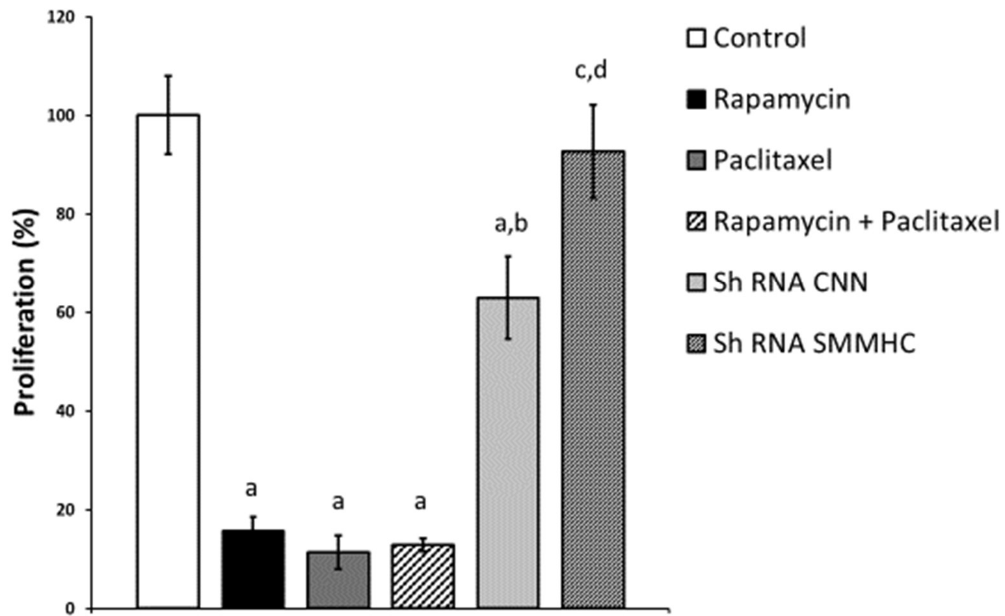

Figure 1. Human Endothelial and Smooth Muscle Cells proliferation. Values are presented as mean and standard deviation. (a) Comparisons between the Control sample and Rapamycin, Paclitaxel, Rapamycin/Paclitaxel, Sh RNA CNN ($p<0.0001$); (b) comparisons between Sh RNA CNN sample and Rapamycin, Paclitaxel, Rapamycin/Paclitaxel ($p<0.0001$); (c) comparisons between Sh RNA SMMHC sample and Rapamycin, Paclitaxel, Rapamycin/Paclitaxel ($p<0.0001$); (d) comparisons between Sh RNA SMMHC sample and Sh RNA CNN ($p=0.0009$).

As shown in Figure 2, Sh RNA SMMHC (9.8 ± 2.7%) and Sh RNA CNN (25.3 ± 4.4%) had an important inhibitory effect on the proliferation of HSMC when compared with controls ($p < 0.0001$ for both comparisons). There was no difference between gene silencers and antiproliferative drugs, alone or in combination, in terms of HSMC proliferation.

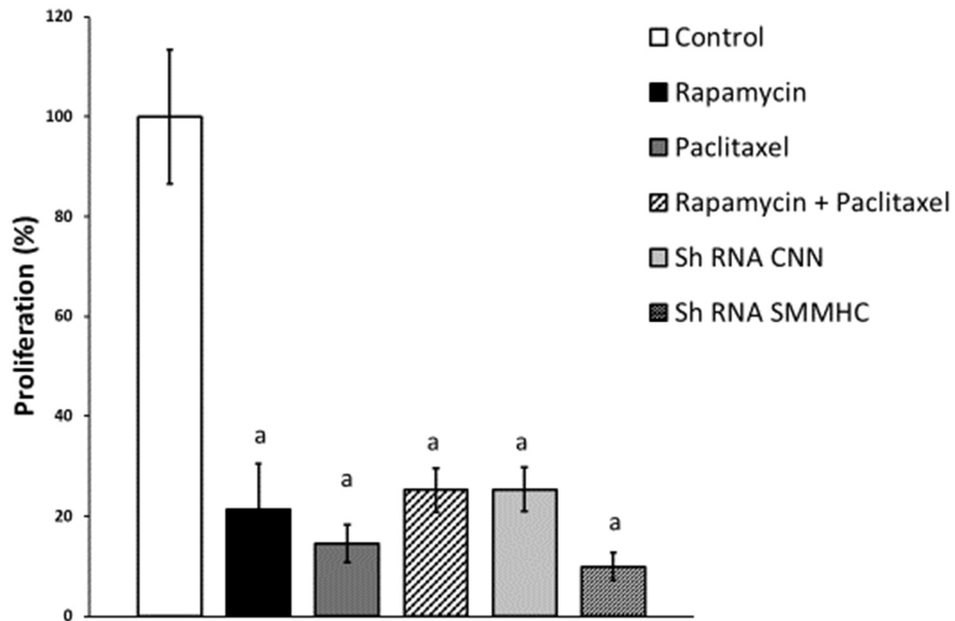

Figure 2. Human Vascular Smooth Muscle Cells proliferation. Values are presented as mean and standard deviation. (a) Comparisons between the Control sample and all other groups, they were considered different when p<0.0001.

The percentage of proliferation of HECs is shown in Figure 3. Samples treated with antiproliferative drugs showed lower values of endothelial cell proliferation (rapamycin: 16.8 ± 3.2%; paclitaxel: 13.5 ± 3.0%; rapamycin + paclitaxel: 18.5 ± 1.9%; p < 0.0001) than controls and gene silencer groups. Sh RNA SMMHC was an effective blocker of smooth muscle cell proliferation, but did not affect the proliferation rate of endothelial cells (93.9 ± 9.6%; p = 0.604). On the other hand, Sh RNA CNN (78.1 ± 4.1%) presented a small but statistically significant inhibitory effect in comparison with control (p< 0.05), no significant difference in comparison with Sh RNA SMMHC (p = 0.590), and a much lesser inhibitory effect on HEC proliferation in comparison with the antiproliferative drugs (p<0.0001).

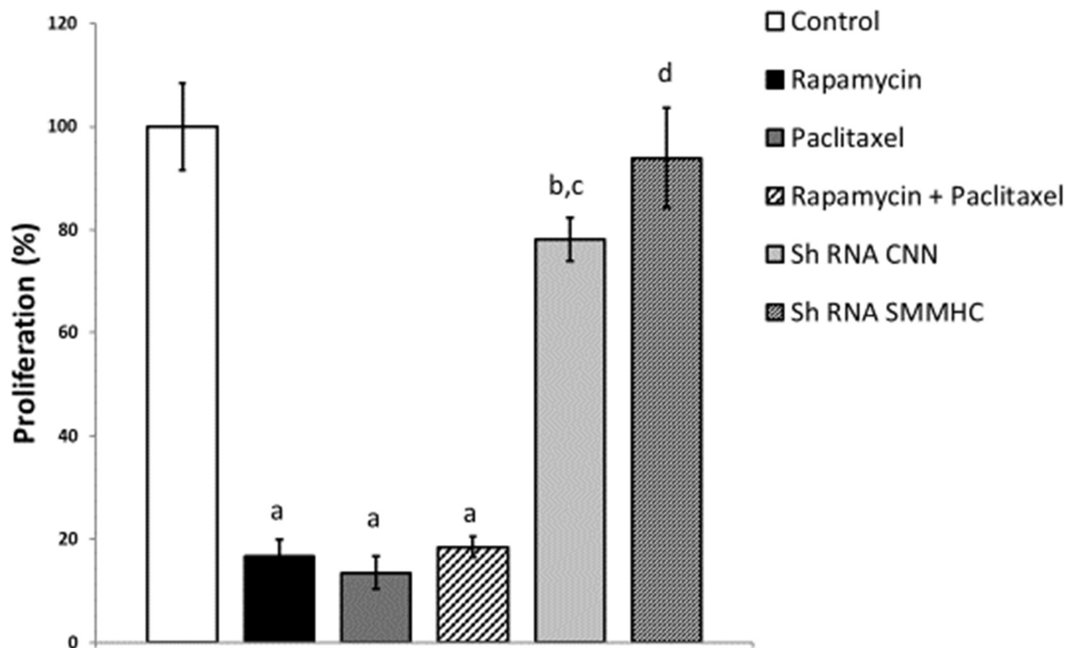

Figure 3. Human Endothelial Cells proliferation. Values are presented as mean and standard deviation. (a) Comparisons between the Control sample and Rapamycin, Paclitaxel, Rapamycin/Paclitaxel, they were considered different when $p<0.0001$; (b) comparisons between Sh RNA CNN sample and Rapamycin, Paclitaxel, Rapamycin/Paclitaxel ($p<0.0001$); (c) comparisons between Control sample and Sh RNA CNN ($p<0.05$); (d) comparisons between Sh RNA SMMHC and Rapamycin, Paclitaxel, Rapamycin/Paclitaxel ($p<0.0001$).

Figure 4 shows CNN concentration in a smooth muscle cell culture. The control group presented higher values than the other groups (1386.1 ± 70.1 pg/mL; $p < 0.0001$ for all comparisons). This result demonstrates that without the interference of antiproliferative drugs or gene silencers, there is a higher expression of CNN protein in the HSMC culture. The combination of antiproliferative drugs (27.8 ± 6.3 pg/mL) and Sh RNA CNN (4.9 ± 1.7 pg/mL) showed lower levels of CNN protein concentration when compared with paclitaxel alone (140.1 ± 16.3; $p < 0.05$).

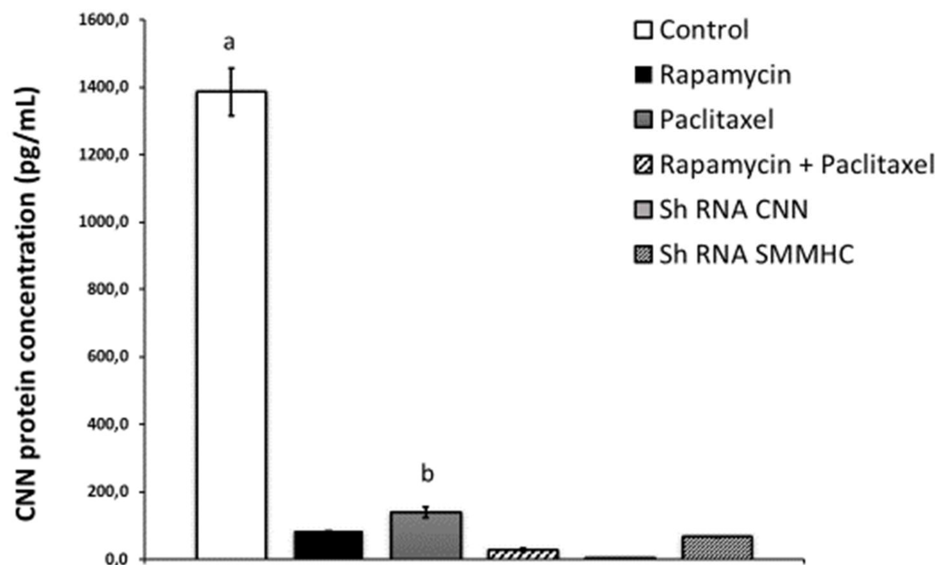

Figure 4. Human Smooth Muscle Cells CNN protein concentration. Values are presented as mean and standard deviation. (a) Comparisons between the Control sample and all other groups, they were considered different when $p < 0.0001$. (b) comparisons between Paclitaxel and Rapamycin/Paclitaxel ($p = 0.006$) and Sh RNA CNN ($p=0.001$).

In the analysis of SMMHC concentration (Figure 5) in the HSMC culture, a higher concentration of this protein in controls (1240.5 ± 79.7 pg/mL) than in the other groups was observed. The antiproliferative drugs and gene silencers led to a reduction in SMMHC levels ($p < 0.0001$), being the treatment with Sh RNA SMMHC resulted in almost undetectable protein levels (3.7 ± 1.7 pg/mL).

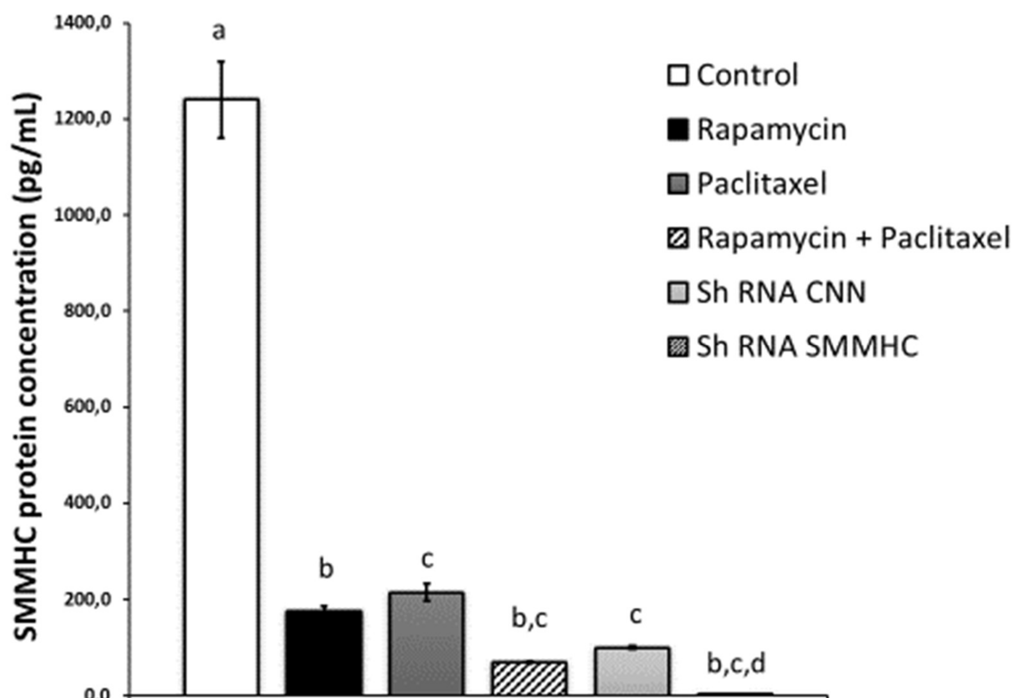

Figure 5. Human Smooth Muscle Cells SMMHC protein concentration. Values are presented as mean and standard deviation. (a) Comparisons between the Control sample and all other groups, they were considered different when p<0.0001; (b) comparisons between Rapamycin and Rapamycin/paclitaxel (p=0.02), Sh RNA SMMHC (p=0.0005); (c) comparisons between Paclitaxel and Rapamycin/paclitaxel (p=0.002), Sh RNA SMMHC (p<0.0001), Sh RNA CNN (p=0.001); comparisons between Sh RNA SMMHC and Sh RNA CNN (p=0.04).

4. DISCUSSION

The development of different medical devices, such as BMS, DES and DES with biodegradable polymers, allowed myocardial revascularization and increased patient survival [27]. DES decreased significantly ISR but long studies demonstrated incomplete endothelialization due to inappropriate inhibition of endothelial cells proliferation, concomitant with inhibition of smooth muscle cells, a scenario that favors in-stent thrombosis and requires prolonged drug antiplatelet pharmacological therapy (DAPT) [28-31]. Thus, we aimed to evaluate the effect of gene silencers specific to smooth muscle cells on the proliferation of endothelial and smooth muscle cells in comparison with the antiproliferative drugs widely used in the DES.

In the present study, the antiproliferative drugs rapamycin and paclitaxel as well as their combination demonstrated an inhibitory effect on HSMC, but also had the same effect on HEC, both in isolated culture and in co-culture with the two cell lineages. These

two antiproliferative drugs have distinct pathway mechanisms as well as act in different phases of the cell cycle, but both have direct action on the progression of the cell mitosis, thus preventing the disordered proliferation of human coronary artery smooth muscle cells.

In the isolated culture of HSMC, the inhibitory effect of the gene silencers Sh RNA SMMHC and Sh RNA CNN was similar to the antiproliferative drugs. However, unlike rapamycin and paclitaxel, Sh RNA SMMHC did not inhibit HEC proliferation, demonstrating proliferation rates not different from those found in the control group. Although Sh RNA CNN inhibitory effect on HEC proliferation was similar to the Sh RNA SMMHC, it was small but statistically different when compared to the control group. It is worth noting that CNN is not exclusive of smooth muscle tissue, since it has been previously detected in other tissues, including the endothelial tissue [32]. Thus, the results of the present study show a potentially inhibitory effect of the gene silencers on the proliferation of smooth muscle cells, without compromising significantly endothelial cell viability.

In the analysis of CNN and SMMHC concentrations in smooth muscle cells, we found that these proteins were not expressed when there was proliferative blockade caused by the cell antiproliferative drugs or the gene silencers. The phenotypic modulation of smooth muscle cells, which induces the onset of cell proliferation by hyperplasia, has been directly associated with the activation of specific transcription factors by the Akt and mTOR pathways in several studies [33-36]. However, Cidad et al. (2014) observed that the expression of Kv1.3 and Kv1.5, voltage-dependent potassium channels responsible for contraction and proliferation of smooth muscle cells, would present a late pathway of activation modulated by growth factors. This study, therefore, suggests the existence of other pathways responsible for the development of neointimal hyperplasia, regardless of mTOR, generating resistance to the antiproliferative effect of rapamycin [37]. Thus, this finding can be an important cell cycle alternative pathway that explains DES failure due to restenosis.

HSMCs present two different phenotypes: the first one, predominantly active during the embryonic development, is represented by cells with high proliferative potential and characterized as undifferentiated [38] and presenting high expression of SMMHC and CNN genes; the second one, which is the functional postnatal vascular wall, is represented by cells in late-phase of differentiation, or quiescent cells. In the latter, structural and functional proteins such as smooth muscle myosin heavy chain (SMMHC)

and CNN have a lower gene expression, which reduces their proliferative capacity. In the present study, smooth muscle cells with proliferative potential, observed in the control group (Figure 2), showed high SMMHC and CNN concentrations, demonstrating that a reactivation of these genes could have occurred for the formation of constitutive muscle proteins in these cells. The dedifferentiation process of smooth muscle cells is suggested as the main pathway in intimal thickening, thus, after vascular damage, cells would proliferate and migrate to the neointima, showing immature cellular characteristics [39-41]. Thus, our data suggest that gene therapy is a potential tool in the development of new devices for the prevention and treatment of ISR.

The literature reports that the greater expression of the SMMHC and CNN genes is used as a HSMC marker, frequently associated with the non-proliferative phenotype [42]. However, mature HSMCs with a quiescent phenotype are sensitive to changes in the intravascular environment, a phenomenon representing the phenotypic modulation. This scenario may allow changes in the morphological and functional properties of these cells, activating a divisional process similar to that of the embryonic smooth muscle cells [42, 43]. The possible plasticity developed by the quiescent cells may be a result of the state of oxidative stress and chronic inflammation caused by the atheromatous plaque, with the release of inflammatory mediators and growth factors that may induce the migration, proliferation and accumulation of mature smooth muscle cells in the neointimal area [44].

Regardless of the gene silencer, the fact that there is no proliferative activity of HSMCs is sufficient for both CNN and SMMHC to have low protein concentration. Protein synthesis in these cases seems to be triggered by an activating stimulus of transcription factors related to the production of proteins essential for the formation of new HSMCs. Accordingly, to our results, the selective blockade of either SMMHC or CNN inhibits the other one gene expression and, consequently, the formation of the final protein structure.

Therefore, a DES that elutes gene silencers such as Sh RNA SMMHC and Sh RNA CNN, could be a novel therapeutic strategy capable of providing an effective solution for ISR with concomitant early endothelialization. The results of our *in vitro* study show that Sh RNA SMMHC and Sh RNA CNN inhibited significantly HSMC proliferation without promoting an antiproliferative effect on the endothelial cells, thus having a selective effect on HSMC not observed with rapamycin and paclitaxel. Additionally, the risk for ISR establishment is reduced if a functional endothelium

appears much earlier, being so a probable stronger inhibition on HSMC proliferation might be observed in a future clinical study. Thus, the local delivery of Sh RNA SMMHC or Sh RNA CNN through a DES may be an important strategy in the reduction of the current rates of both in-stent thrombosis and restenosis.

5. CONCLUSION

The smooth muscle cell-specific genes silencers Sh RNA SMMHC and Sh RNA CNN have selective inhibitory effect on the proliferation of human coronary artery smooth muscle cells, without interfering in the proliferation of endothelial cells.

Conflicts of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. All authors have participated and approved the final version of this manuscript.

Acknowledgments

Funding was provided by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), grant number 14/2013, Brasília, Brazil, and by Fundo de Incentivo à Pesquisa e Eventos of Hospital de Clínicas de Porto Alegre (FIPE-HCPA), grant number 13/0248, Porto Alegre, Brazil.

6. REFERÊNCIAS

[1] Zago AC, Raudales JC, Attizzani G, Matte BS, Yamamoto GI, Balvedi JA, et al. Local delivery of sirolimus nanoparticles for the treatment of in-stent restenosis. Catheter Cardiovasc Interv. 2012.
[2] Hill R, Bagust A, Bakhai A, Dickson R, Dundar Y, Haycox A, et al. Coronary artery stents: a rapid systematic review and economic evaluation. Health Technol Assess. 2004;8:iii-iv, 1-242.
[3] Simon DI, Dhen Z, Seifert P, Edelman ER, Ballantyne CM, Rogers C. Decreased neointimal formation in Mac-1(-/-) mice reveals a role for inflammation in vascular repair after angioplasty. J Clin Invest. 2000;105:293-300.
[4] Pires NM, Eefting D, de Vries MR, Quax PH, Jukema JW. Sirolimus and paclitaxel provoke different vascular pathological responses after local delivery in a murine model for restenosis on underlying atherosclerotic arteries. Heart. 2007;93:922-7.
[5] Ferrante G, Niccoli G, Biasucci LM, Liuzzo G, Burzotta F, Galiuto L, et al. Association between C-reactive protein and angiographic restenosis after bare metal stents: an updated and comprehensive meta-analysis of 2747 patients. Cardiovasc Revasc Med. 2008;9:156-65.
[6] Kong J, Deng Y, Dong Q, Liu W, Lu Y. Colchicine reduces restenosis after balloon angioplasty treatment for in-stent restenosis. Arch Med Res. 2015;46:101-6.
[7] Bonaa KH, Mannsverk J, Wiseth R, Aaberge L, Myreng Y, Nygard O, et al. Drug-Eluting or Bare-Metal Stents for Coronary Artery Disease. N Engl J Med. 2016;375:1242-52.

[8] Kastrati A, Mehilli J, von Beckerath N, Dibra A, Hausleiter J, Pache J, et al. Sirolimus-eluting stent or paclitaxel-eluting stent vs balloon angioplasty for prevention of recurrences in patients with coronary in-stent restenosis: a randomized controlled trial. JAMA. 2005;293:165-71.
[9] Tarantini G, Facchin M, Capodanno D, Musumeci G, Saia F, Menozzi A, et al. Paclitaxel versus sirolimus eluting stents in diabetic patients: does stent type and/or stent diameter matter?: long-term clinical outcome of 2,429-patient multicenter registry. Catheter Cardiovasc Interv. 2013;81:80-9.
[10] Abe M, Kimura T, Morimoto T, Taniguchi T, Yamanaka F, Nakao K, et al. Sirolimus-eluting stent versus balloon angioplasty for sirolimus-eluting stent restenosis: Insights from the j-Cypher Registry. Circulation. 2010;122:42-51.
[11] Yano H, Horinaka S, Yagi H, Ishimitsu T. Comparison of inflammatory response after implantation of sirolimus- and paclitaxel-eluting stents in patients on hemodialysis. Heart Vessels. 2012.
[12] Curcio A, Torella D, Indolfi C. Mechanisms of smooth muscle cell proliferation and endothelial regeneration after vascular injury and stenting: approach to therapy. Circ J. 2011;75:1287-96.
[13] Cheng H, An SJ, Zhang XC, Dong S, Zhang YF, Chen ZH, et al. In vitro sequence-dependent synergism between paclitaxel and gefitinib in human lung cancer cell lines. Cancer Chemother Pharmacol. 2011;67:637-46.
[14] Inoue T, Croce K, Morooka T, Sakuma M, Node K, Simon DI. Vascular inflammation and repair: implications for re-endothelialization, restenosis, and stent thrombosis. JACC Cardiovasc Interv. 2011;4:1057-66.
[15] LUZ PL. Endotélio e doenças cardiovasculares. São Paulo: Ateneu; 2005.
[16] Glasser SP, Selwyn AP, Ganz P. Atherosclerosis: risk factors and the vascular endothelium. Am Heart J. 1996;131:379-84.
[17] Walker JP, Owens CD. Current Status of Drug-Eluting Stents and Drug-Eluting Balloons for the Superficial Femoral Artery. Curr Surg Rep. 2013;1:90-7.
[18] Inoue T, Node K. Molecular basis of restenosis and novel issues of drug-eluting stents. Circ J. 2009;73:615-21.
[19] Zago ACMarco AC, Alcides J. Zago, Juliane S. Rossato, Bruno S. Matte,German Iturry-Yamamoto, Márcio Mossmann, Ricardo Savaris, Mariano Albertal, Cristiane S. Rocha,, Aileen M. Healy Russell W, Satoko Tahara, Daniel I. Simon. Identification of Genes Involved in Smooth Muscle Cell Protein Synthesis with Increased Expression in Atheromatous Plaques Associated with Neointimal Hyperplasia after Bare-Metal Stenting: A GENESIS-R Study. Revista Brasileira de Cardiologia Invasiva. 2012;20:140-5.
[20] Matsuoka R, Yoshida MC, Furutani Y, Imamura S, Kanda N, Yanagisawa M, et al. Human smooth muscle myosin heavy chain gene mapped to chromosomal region 16q12. Am J Med Genet. 1993;46:61-7.
[21] Liu R, Jin JP. Calponin isoforms CNN1, CNN2 and CNN3: Regulators for actin cytoskeleton functions in smooth muscle and non-muscle cells. Gene. 2016;585:143-53.
[22] Lipskaia L, Hadri L, Le Prince P, Esposito B, Atassi F, Liang L, et al. SERCA2a gene transfer prevents intimal proliferation in an organ culture of human internal mammary artery. Gene Ther. 2013;20:396-406.
[23] Silvestre-Roig C, Fernandez P, Esteban V, Pello OM, Indolfi C, Rodriguez C, et al. Inactivation of nuclear factor-Y inhibits vascular smooth muscle cell proliferation and neointima formation. Arterioscler Thromb Vasc Biol. 2013;33:1036-45.
[24] Larifla L, Deprez I, Pham I, Rideau D, Louzier V, Adam M, et al. Inhibition of vascular smooth muscle cell proliferation and migration in vitro and neointimal hyperplasia in vivo by adenoviral-mediated atrial natriuretic peptide delivery. J Gene Med. 2012;14:459-67.
[25] Joung H, Kwon JS, Kim JR, Shin S, Kang W, Ahn Y, et al. Enhancer of polycomb1 lessens neointima formation by potentiation of myocardin-induced smooth muscle differentiation. Atherosclerosis. 2012;222:84-91.

[26] Jacob T, Hingorani A, Ascher E. p53 gene therapy modulates signal transduction in the apoptotic and cell cycle pathways downregulating neointimal hyperplasia. Vasc Endovascular Surg. 2012;46:45-53.
[27] Garg S, Serruys PW. Coronary stents: current status. J Am Coll Cardiol. 2010;56:S1-42.
[28] Shuchman M. Debating the risks of drug-eluting stents. N Engl J Med. 2007;356:325-8.
[29] Dangas GD, Serruys PW, Kereiakes DJ, Hermiller J, Rizvi A, Newman W, et al. Meta-analysis of everolimus-eluting versus paclitaxel-eluting stents in coronary artery disease: final 3-year results of the SPIRIT clinical trials program (Clinical Evaluation of the Xience V Everolimus Eluting Coronary Stent System in the Treatment of Patients With De Novo Native Coronary Artery Lesions). JACC Cardiovasc Interv. 2013;6:914-22.
[30] Doostzadeh J, Clark LN, Bezenek S, Pierson W, Sood PR, Sudhir K. Recent progress in percutaneous coronary intervention: evolution of the drug-eluting stents, focus on the XIENCE V drug-eluting stent. Coron Artery Dis. 2010;21:46-56.
[31] Kufner S, Cassese S, Valeskini M, Neumann FJ, Schulz-Schupke S, Hoppmann P, et al. Long-Term Efficacy and Safety of Paclitaxel-Eluting Balloon for the Treatment of Drug-Eluting Stent Restenosis: 3-Year Results of a Randomized Controlled Trial. JACC Cardiovasc Interv. 2015;8:877-84.
[32] Wu KC, Jin JP. Calponin in non-muscle cells. Cell Biochem Biophys. 2008;52:139-48.
[33] Che HL, Bae IH, Lim KS, Song IT, Lee H, Lee D, et al. Therapeutic Effect of Akt1 siRNA Nanoparticle Eluting Coronary Stent on Suppression of Post-Angioplasty Restenosis. J Biomed Nanotechnol. 2016;12:1211-22.
[34] Li J, Zhang M, Wang M, Wang Z, Liu Y, Zhang W, et al. GHSR deficiency suppresses neointimal formation in injured mouse arteries. Biochem Biophys Res Commun. 2016;479:125-31.
[35] Wang S, Lu J, You Q, Huang H, Chen Y, Liu K. The mTOR/AP-1/VEGF signaling pathway regulates vascular endothelial cell growth. Oncotarget. 2016;7:53269-76.
[36] Woods TC. Dysregulation of the Mammalian Target of Rapamycin and p27Kip1 Promotes Intimal Hyperplasia in Diabetes Mellitus. Pharmaceuticals (Basel). 2013;6:716-27.
[37] Cidad P, Miguel-Velado E, Ruiz-McDavitt C, Alonso E, Jimenez-Perez L, Asuaje A, et al. Kv1.3 channels modulate human vascular smooth muscle cells proliferation independently of mTOR signaling pathway. Pflugers Arch. 2015;467:1711-22.
[38] Salmon M, Gomez D, Greene E, Shankman L, Owens GK. Cooperative binding of KLF4, pELK-1, and HDAC2 to a G/C repressor element in the SM22alpha promoter mediates transcriptional silencing during SMC phenotypic switching in vivo. Circ Res. 2012;111:685-96.
[39] Owens GK. Regulation of differentiation of vascular smooth muscle cells. Physiol Rev. 1995;75:487-517.
[40] Owens GK. Role of mechanical strain in regulation of differentiation of vascular smooth muscle cells. Circ Res. 1996;79:1054-5.
[41] Owens GK, Wise G. Regulation of differentiation/maturation in vascular smooth muscle cells by hormones and growth factors. Agents Actions Suppl. 1997;48:3-24.
[42] Cheung C, Bernardo AS, Pedersen RA, Sinha S. Directed differentiation of embryonic origin-specific vascular smooth muscle subtypes from human pluripotent stem cells. Nat Protoc. 2014;9:929-38.
[43] Wang G, Jacquet L, Karamariti E, Xu Q. Origin and differentiation of vascular smooth muscle cells. J Physiol. 2015;593:3013-30.
[44] House SJ, Potier M, Bisaillon J, Singer HA, Trebak M. The non-excitable smooth muscle: calcium signaling and phenotypic switching during vascular disease. Pflugers Arch. 2008;456:769-85.

7. CONSIDERAÇÕES FINAIS

O presente trabalho teve como objetivo geral investigar o perfil genético de placas ateromatosas de pacientes que desenvolveram reestenose pós-implante de *stent*, verificando, posteriormente, se o silenciamento de genes específicos para estrutura e função das células musculares lisas vasculares poderia bloquear o processo proliferativo destas sem prejudicar o restabelecimento das células endoteliais.

O artigo intitulado "GENE EXPRESSION PATTERN OF *DE NOVO* CORONARY ATHEROMATOUS PLAQUE IS CORRELATED WITH IN-STENT NEOINTIMAL VOLUME IN PATIENTS WITH RESTENOSIS" demonstrou que as placas ateromatosas de pacientes que irão desenvolver reestenose apresentam genes que predispõem ao desenvolvimento da hiperplasia neointimal. Os resultados indicaram que houve correlação positiva entre o percentual de hiperplasia neointimal e uma maior expressão de genes relacionados com a cascata aterosclerótica, proliferação celular e proteínas estruturais e funcionais de células musculares lisas. Esse estudo, portanto, permitiu avaliar que genes específicos de células musculares lisas, como o SMMHC (*myosin heavy chain*) e o CNN (*calponin*), apresentam-se como elementos chave no desenvolvimento do processo proliferativo que envolve o quadro de reestenose.

A literatura e a prática clínica na prevenção e tratamento da reestenose baseia-se, atualmente, no desenvolvimento de diferentes dispositivos que permitam a revascularização local após um evento isquêmico, com o mínimo de risco de proliferação neointimal. Assim, os *stents* com drogas antiproliferativas, como rapamicina (e análogos) e paclitaxel, são utilizados como elementos terapêuticos usuais. No entanto, os dispositivos farmacológicos são eficientes em impedir a proliferação de células musculares lisas, assim como de células endoteliais. Esse efeito pode ser responsável por retardar a reendotelização local, fato que prejudica o reparo tecidual e estimula a proliferação de células musculares lisas.

Ao considerar o embasamento desenvolvido o estudo acima, a placa ateromatosa do indivíduo parece apresentar preditores gênicos que poderiam iniciar a estimulação proliferativa das células musculares lisas após o implante de *stent*. Dessa forma, identificamos genes específicos estruturais de células musculares lisas para realizar o silenciamento gênico e verificar a efetividade desse bloqueio em um estudo in vitro com células musculares lisas de artéria coronária humana.

No artigo “EFFECT OF SMOOTH MUSCLE CELLS GENE SILENCERS ON THE VIABILITY OF HUMAN CORONARY ARTERY ENDOTHELIAL AND SMOOTH MUSCLE CELLS” foi testado o efeito de silenciadores gênicos, Sh RNA SMMHC e Sh RNA CNN, em células musculares lisas e endoteliais de artéria coronária humana. Os resultados demonstraram que os silenciadores gênicos específicos para células musculares lisas foram capazes de impedir a proliferação destas, sem interferir na viabilidade das células endoteliais. Os fármacos antiproliferativos testados, rapamicina, paclitaxel ou a combinação ambos, apresentaram atividade inibitória sobre ambas as linhagens celulares.

Nesse estudo observamos, portanto, o efeito seletivo de silenciadores gênicos específicos para células musculares lisas, que não apresentam influência sobre a viabilidade de células endoteliais. Assim, os silenciadores gênicos testados poderiam inibir o desenvolvimento da hiperplasia neointimal, contribuindo para um eficaz restabelecimento endotelial e favorecendo a manutenção da homeostasia vascular.