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Participação do metabolismo energético lipídico miocárdio na atenuação da disfunção cardíaca pelo treinamento físico precoce em ratos com estenose aórtica

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutor em Fisiopatologia em Clínica Médica.

Orientador: Prof. Dr. Antonio Carlos Cicogna

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Palavras-chave: Estenose aórtica; Função cardíaca; Treinamento físico.

Dedicatória

Se pela necessidade de encontrar caminhos mais desafiadores e prósperos, ou simplesmente pela irreverência de alçar solos ainda não ocupados pelas minhas raízes, fato é que há muito eu tenho trilhado para além dos arredores que se podia supor. E nunca sozinho! À medida que o tempo passa, novas pessoas vão assumindo papel de importância em nossas vidas, e cada conquista é mérito também daqueles que estiveram comigo; familiares, amigos, desconhecidos que em alguns minutos de conversa somam muito aprendizado. Dedicar mais essa conquista é tarefa difícil, não pela falta de nomes, mas pelo sem-número da lista. A solução foi eleger alguns elementos-chave dos quais absorvi conhecimento humano, profissional e social até aqui...

À minha mãe, Maria do Carmo

É inenarrável o que essa mulher foi capaz de fazer para driblar as dificuldades e oferecer aos filhos a oportunidade de avançar. O ato de antecipar a nossa alfabetização, minha e de minha irmã, ainda em casa e com as limitações que eram próprias da época, ilustra bem o quão relevante foi o seu papel. Quando um único lápis era dividido em dois, o que ficava evidente não era a dificuldade financeira, mas a nobreza do compartilhar. À esta mulher eu dedicarei tantas quantas forem as etapas vencidas por mim.

Ao Professor Dr. Cicogna

O pesar de fazer parte do último grupo liderado pelo Professor Cicogna divide espaço com o prazer e honra de ter desfrutado do que há de melhor em termos de ciência e humanidade. A ciência que nos ensinou foi a ciência elementar, purista, à qual dedicou sua carreira, resistindo para que o belo da reflexão não fosse sobrepujado pelo superficial da automação e do imediatismo. Este trabalho também é dedicado a esse professor de ciência, moral e ética, sociologia, humanidade,

senso de comunidade e tantas outras disciplinas que domina como poucos, e se empenha em inseri-las no processo de formação dos seus alunos.

Às vítimas da Covid-19 (in memoriam)

Dedico esse trabalho também às pessoas que perderam suas vidas em razão da pandemia da Covid-19, pela necessidade de lembrá-las, mas principalmente pelo simbolismo das suas mortes no contexto social e da ciência. Se Mikhail Saltykoy-Stcherdrine diz, no século XIX, que de tempos em tempos a sociedade, tomada de pânico, se desvia da ciência e procura a salvação na ignorância, nos é de obrigação propagar, sobretudo em momentos de crise como o atual, que a ciência está para a vida assim como a ignorância está para a morte. Dedico o científico desse trabalho a quem não teve tempo de gozar das soluções da ciência, pela morosidade dos seus processos ou pela sua negação.

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Ao meu orientador, Professor Cicogna, pela oportunidade, paciência, e pela dedicação ímpar. Seu exemplo é escola. Muito obrigado!

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A Deus.

À minha família, especialmente à minha mãe e irmã, pelo amor incondicional, pelo apoio às minhas decisões, apesar da distância que elas impuseram entre nós, e pela inspiração de vida.

Epígrafe

Em uma nação carente de conhecimento, uma escola sem ciência estará predestinada à mediocridade. A formação de recursos humanos deve ser pautada na criticidade e reflexão, próprias da pesquisa, para que haja crescimento intelectual, social e humanista, e a sociedade se diferencie.

Antonio Carlos Cicogna

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ASPECTOS ESTRUTURAL, FUNCIONAL E METABÓLICO DA REMODELAÇÃO CARDÍACA - INFLUÊNCIA DO TREINAMENTO FÍSICO

1. INTRODUÇÃO

A sobrecarga pressórica crônica tem papel relevante como causa de insuficiência cardíaca (IC), síndrome de grande impacto na morbimortalidade da população em todo o mundo [1]. Em termos fisiopatológicos, a agressão cardíaca impõe uma nova demanda e exige do coração um processo adaptativo que inclui rearranjo estrutural, funcional e metabólico. Os mecanismos que tardiamente convertem esse remodelamento fisiológico em patológico não estão completamente compreendidos, assim como as estratégias terapêuticas seguem sendo investigadas. Nessa revisão será dado enfoque às adaptações metabólicas que ocorrem durante o processo de remodelação cardíaca, especialmente as que ocorrem no metabolismo energético lipídico miocárdico, e no papel do exercício como ferramenta não-farmacológica para o manejo dessa síndrome.

2. REMODELAÇÃO CARDÍACA: CONCEITO E EXPERIÊNCIA DO GRUPO

Em mamíferos, o miocárdio experimenta um processo hipertrófico durante o desenvolvimento pós natal, caracterizado por aumento individual da massa miocitária sem divisão celular [2]. Embora interrompido ao término do período de maturação do coração, esse padrão de crescimento hipertrófico pode ser retomado em resposta a insultos hemodinâmicos e/ou neuro-hormonais [3]. Esse recurso do miocárdio, ao que se convencionou chamar de remodelação cardíaca (RC), confere ao coração alta capacidade adaptativa frente a estímulos externos. RC foi o conceito proposto, inicialmente, para descrever alterações na anatomia ventricular esquerda após isquemia miocárdica [4]. Mais tarde, essa definição foi descrita para se referir às mudanças na expressão gênica, moleculares, celulares e intersticiais manifestadas

pelas modificações no tamanho, forma e função do coração após injúria [5]. Atualmente a RC é considerada um fenômeno que abrange mudanças estrutural, elétrica, imunológica, metabólica e funcional do coração, determinadas por alterações na expressão gênica em resposta a estresse de etiologia fisiológica ou patológica [6]. A mudança no fenótipo cardíaco, consequente à alteração do perfil proteico, tem como finalidade normalizar o estresse parietal e, consequentemente, a deformação miocárdica [7].

A RC fisiológica e a patológica compartilham alguns aspectos, como o crescimento do cardiomiócito, mas exibem características distintas, especialmente em cenários de sustentação crônica da agressão. O padrão hipertrófico fisiológico é marcado pela integridade e equilíbrio entre os diferentes constituintes do tecido miocárdico (moleculares, celulares, intersticiais e vasculares), normalidade da relação entre a espessura da parede e o diâmetro das cavidades atrial e ventricular, e preservação ou melhoria do quadro bioenergético e funcional. Em condição patológica, entretanto, a harmonia tecidual, geométrica e energética são rompidas, comprometendo a performance cardíaca. O remodelamento fisiológico compreende uma adequação do sistema cardiovascular à demanda metabólica aumentada em condições como gestação e exercício físico [8–10]; nesses casos, o aumento da massa cardíaca é sutil, na ordem de 10-20%, e a hipertrofia do cardiomiócito ocorre na ausência de fibrose e morte celular, o que a torna reversível e não potencial para a falência cardíaca [6]. A etiologia da RC patológica, entretanto, é diversa, mas está mais frequentemente relacionada à sobrecarga de volume, como a que ocorre com o infarto do miocárdio (IAM), e de pressão, observada na hipertensão arterial crônica e estenose aórtica [5,11]. Embora induzida inicialmente como uma medida compensatória ao aumento da demanda cardíaca, a hipertrofia patológica sucumbe à

natureza nociva e persistente do estímulo, acarretando deterioração da função, progressão para IC e aumento do risco de morte súbita [12–14].

No campo experimental, o estudo da RC induzida por sobrecarga pressórica é conduzido em uma variedade de modelos animais, cujos principais representantes são o rato espontaneamente hipertenso (SHR) [15] e estenose das artérias renal [16], abdominal [17–19] e estenose aórtica supravalvar (EAo) [20–26]. A EAo é empregada em função do desenvolvimento gradual e severo da hipertrofia ventricular esquerda, proporcional ao crescimento do animal [20–26].

Em nosso laboratório, estudos que utilizaram o modelo de EAo visaram uma melhor compreensão dos fatores estruturais, elétricos, moleculares e metabólicos envolvidos no prejuízo da performance cardíaca e na transição para falência [25,27–30], e contribuíram para a investigação de tratamentos capazes de atenuar os prejuízos cardíacos e sistêmicos gerados pela sobrecarga pressórica crônica [23,30–36]. Esses trabalhos traçaram uma linha temporal das alterações estruturais e funcionais a partir da indução da EAo em ratos jovens (Figura 1); seis semanas: hipertrofia acentuada do ventrículo esquerdo (VE), disfunção diastólica e aumento da função sistólica; 12 semanas: progressão da hipertrofia e da disfunção diastólica, e deterioração da função sistólica; 18 semanas: dilatação do VE, agravamento da disfunção diastólica e sistólica, e instalação da IC; 28 semanas: consolidação da IC [15,20,24,25,28,31,36–38].

Figura 1. Representação esquemática da evolução da remodelação macroestrutural e funcional cardíaca no modelo de estenose aórtica supravalvar.

O prognóstico desfavorável da remodelação patológica é determinado pela conversão do padrão genotípico e fenotípico, que passa de adaptativo a mal adaptativo, levando ao rompimento da harmonia geométrica e dos constituintes do tecido miocárdico, e deterioração progressiva da performance cardíaca. Muitos fatores estão envolvidos no processo de transição da remodelação à falência cardíaca [3,39,40]. Embora inter-relacionados, esses desajustes podem ser categorizados em 1) ventriculares: dilatação, adelgaçamento da parede, aumento da esfericidade e incompetência da valva mitral; 2) miocárdicos: perda de cardiomiócitos por necrose, apoptose e autofagia, degradação da matriz extracelular e fibrose, e rarefação da microvasculatura, e 3) miocitários: reexpressão de genes fetais, desarranjo mitocondrial, perda de miofilamentos, hipertrofia, alteração nas proteínas contráteis e no citoesqueleto, miocitólise, dessensibilização adrenérgica, alta expressão de citocinas inflamatórias, estresse oxidativo, prejuízos no acoplamento excitação/contração/relaxamento e distúrbio metabólico-energético [6,41].

Apesar de não ser possível isolar um único mecanismo responsável pela deterioração da função, a alta taxa metabólica miocárdica confere ao balanço energético um papel central na homeostasia cardíaca, de modo que desajustes no

metabolismo energético pode antecipar, iniciar ou agravar o quadro disfuncional da contratilidade [42]. Há evidências de que a deficiência na oxidação de substratos precede a instalação da IC [43]; análise transcriptômica do miocárdio na fase compensada da hipertrofia mostrou que apenas a expressão de genes da oxidação de ácidos graxos (AG) foram significativamente alterados, sugerindo que a reprogramação da oxidação de AG é a etapa inicial do remodelamento metabólico no curso da IC [44]. Nesse sentido, o tradicional conceito de que o coração em falência é um órgão em privação de energia [45] é especialmente apropriado, a julgar pela toxicidade e duração de curto prazo das terapias com agentes inotrópicos, que intensifica o trabalho mecânico sem aumento proporcional da reserva metabólica [46].

3. METABOLISMO LIPÍDICO MIOCÁRDICO

3.1. Fontes e utilização de ácidos graxos no coração normal

A adenosina trifosfato (ATP) possui atividade central no processo energético celular, atuando como reservatório de energia livre; no coração, o intenso trabalho mecânico requer constante e intensa renovação dos estoques de ATP. A geração dessa molécula pelo miocárdio passa por três etapas; captação de substratos energéticos pelo cardiomiócito, oxidação mitocondrial e transferência de fosfocreatina da mitocôndria para o local de utilização [47]. O descompasso desses estágios resulta em prejuízo para a performance cardíaca.

O coração é um órgão com alta plasticidade metabólica, podendo utilizar diferentes substratos como AG, glicose, lactato, corpos cetônicos e aminoácidos para geração de ATP, necessária para o processo de contração e estiramento cardíaco [48]. Os AG são a principal fonte de energia, enquanto a glicose é reconhecidamente a segunda escolha; no coração adulto, cerca de 50-70% da ressíntese de ATP é

normalmente oriunda da beta oxidação de AG, sendo a contribuição da via glicolítica em torno de 30-40% [49–54].

A utilização de AG pelo coração depende fundamentalmente do suprimento exógeno; as fontes incluem os AG livres do plasma e os liberados pela hidrólise das lipoproteínas plasmáticas ricas em triglicerídeos [55,56]. Devido à sua baixa solubilidade em meio aquoso, os AG são fornecidos ao coração ligados à albumina plasmática ou ligados ao núcleo de triacilglicerol das lipoproteínas circulantes. Após a dissociação do complexo albumina-AG ou hidrolise do triacilglicerol, os AG são

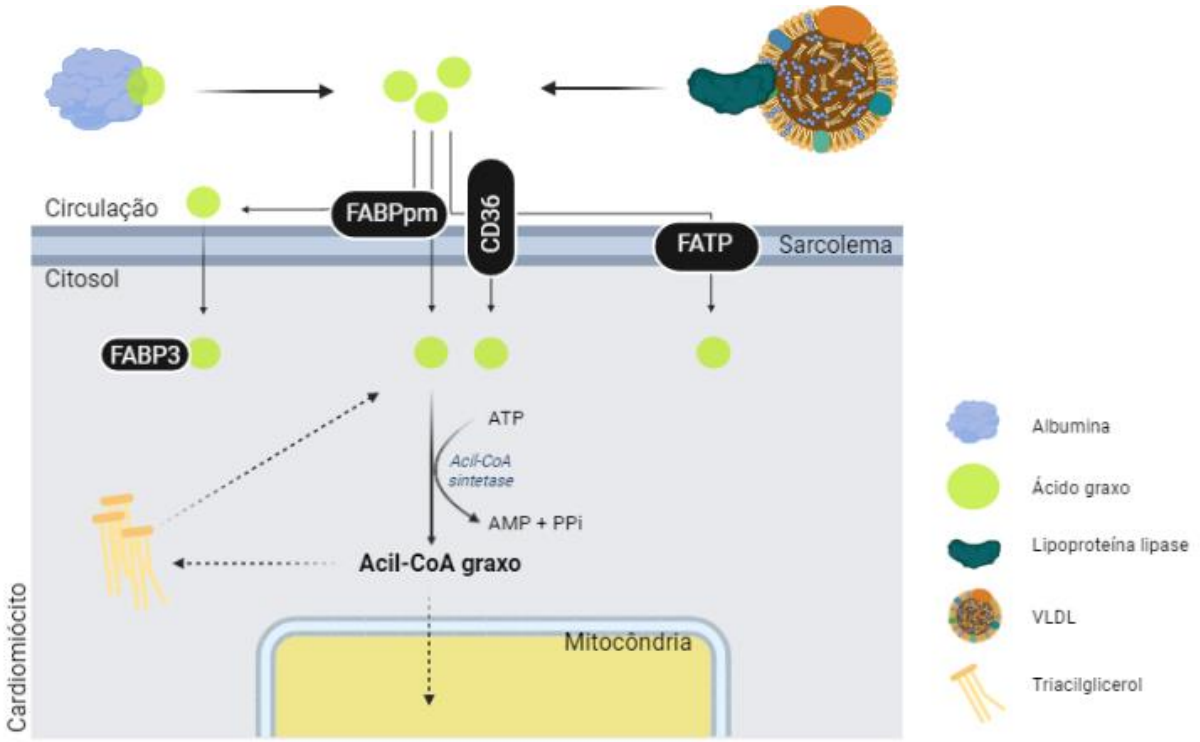

Figura 2. Fontes de ácido graxo para o miocárdio e o processo de translocação para o citosol. Liberado do complexo albumina-AG ou da lise das lipoproteínas plasmáticas pela lipoproteína lipase, o AG é translocado para o cardiomiócito por difusão passiva ou pelo transporte via proteínas, realizado pela FABPpm, CD36 e FATP. No citosol, a FABP3 atua na manutenção do fluxo de AG entre a membrana do miócito e a mitocôndria. Triacilglicerol miocárdico também fornece AG para a maquinaria energética da célula. FABPpm: proteína de ligação de ácidos graxos associada à membrana plasmática; FATP: proteína de transporte de ácidos graxos; CD36: translocase de ácidos graxos; FABP3: proteína citoplasmática de ligação de ácidos graxos; ATP: adenosina trifosfato; AMP: adenosina monofosfato; Pi: fosfato inorgânico; VLDL: lipoproteína de muito baixa densidade. Adaptado de Stanley, (2010) [49].

transferidos do lúmen capilar através do endotélio capilar e compartimento intersticial para o cardiomiócito [57]. No citoplasma, o AG é convertido em Acil-CoA graxo de cadeia longa pela ação da Acil-CoA sintetase, e poderá ser usado na síntese de intermediários lipídicos como triacilglicerol, diacilglicerol e ceramida, ou ser oxidado na mitocôndria [49] (Figura 2).

No processo de captura de AG pelo cardiomiócito, além da difusão passiva, uma variedade de proteínas é envolvida na transferência do compartimento microvascular para o espaço intracelular (Figura 2). Papel dominante no tráfego transmembrana de AG é desempenhado pela translocase de ácidos graxos (CD36), pela proteína de ligação de ácidos graxos associada à membrana plasmática (FABPpm), e pela proteína de transporte de ácidos graxos (FATP) [49,58]. Um tipo específico de proteína citoplasmática cardíaca de ligação de AG (*Heart*-FABP ou FABP3) é proposta como responsável pela manutenção do fluxo de AG entre o sarcolema e a membrana mitocondrial [58]. Os mecanismos propostos para a captura de AG mediada pelas proteínas incluem aumento da concentração de AG na membrana mediado pela FABPpm, facilitando a difusão passiva, transporte direto de AG pela CD36, e aceleração do transporte transmembrana por aproximação do AG à FATP, mediada por CD36 e/ou FABPpm [59]. Estudos que inibiram ou deletaram a CD36 no coração mostraram que este é o carreador de maior importância para a captação de AG pelo cardiomiócito, respondendo por cerca de 50 a 60% da captura miocárdica desse lipídio [60,61]. A translocação de AG mediada pela CD36 é estimulada pelas taxas de contração muscular e circulação de insulina, que regulam o deslocamento dessa translocase para a membrana [60]; em contraste, a isquemia tecidual sinaliza para o seu desligamento da membrana plasmática [62].

O mecanismo de translocação mediado por proteína é novamente acionado para transportar a molécula de AG para a matriz mitocondrial [47] (Figura 3). Como a membrana interna mitocondrial é impermeável ao Acil-CoA graxo, ele é transformado em acilcarnitina graxo pela carnitina palmitoiltransferase 1 (CPT1) na membrana externa da mitocôndria. A acilcarnitina graxo é então transferida para a matriz mitocondrial e sofre reconversão em acil-CoA graxo pela ação da carnitina palmitoiltransferase 2 (CPT2) na membrana interna da mitocôndria, para ser metabolizado na β-oxidação [49]. Esse sistema de transporte tem papel relevante na regulação da oxidação mitocondrial do AG [47], desde que a atividade da CPT1 é regulada pela concentração miocárdica de malonil-CoA; a taxa de oxidação de AG e

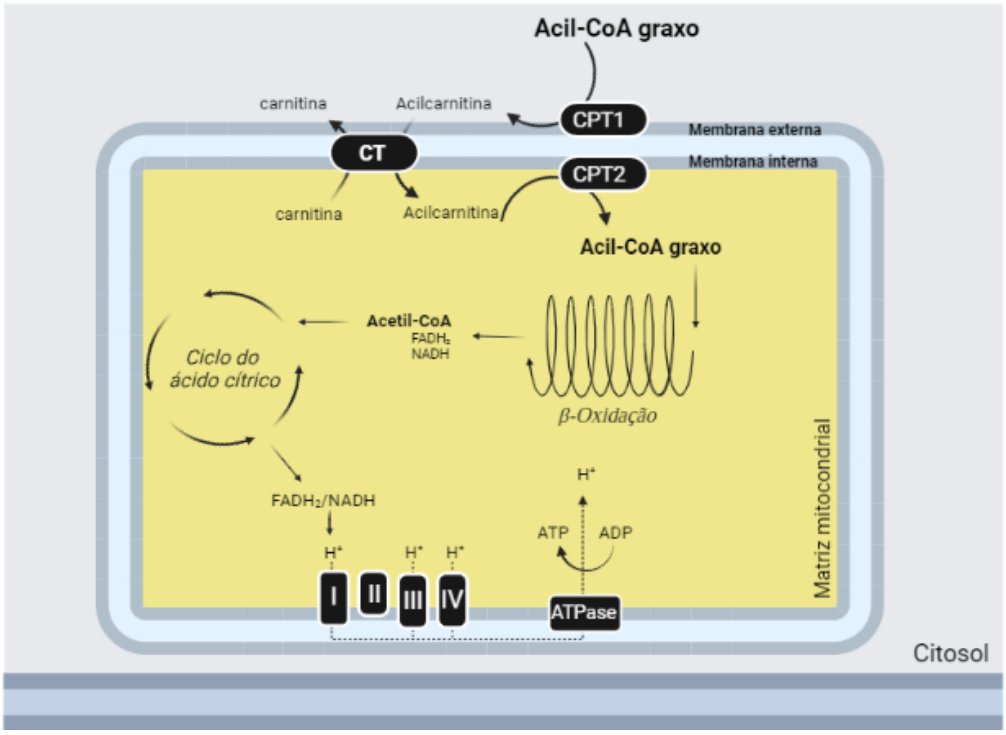

Figura 3. Transporte e oxidação do ácido graxo na mitocôndria. Para acessar a matriz mitocondrial, o Acil-CoA graxo precisa ser convertido em Acilcarnitina pela CPT1β, e então translocado do citosol pela CT. Em seguida é reconvertido em Acil-CoA graxo pela CPT2 e entra na β-Oxidação. O Acetil-CoA gerado passa pelo Ciclo de Krebs e libera FADH e NADH para a CTE. CT: carnitina transferase; CPT1 e CPT2: carnitina palmitoiltransferase do tipo 1 e 2, respectivamente; NADH: dinucleotídeo de nicotinamida e adenina; FADH: dinucleotídeo de flavina e adenina; ADP: adenosina difosfato; ATP: adenosina trifosfato; CTE: cadeia de transporte de elétrons; I, II, III e IV: complexos I, II, III e IV da CTE. Adaptado de Stanley, (2010) [49].

a concentração de malonil-CoA são inversamente proporcionais, o que reduz a oxidação quando há acúmulo celular de malonil-CoA [63,64].

A β-oxidação mitocondrial dos ácidos graxos é um processo catabólico que envolve a participação de várias enzimas para gerar acetil coenzima A (Acetil-CoA) e moléculas carreadoras de hidrogênio reduzidas – dinucleotídeo de nicotinamida e adenina ($NADH^+$) e dinucleotídeo de flavina e adenina ($FADH_2$) – que entram na cadeia respiratória para gerar ATP [65]. Esse processo consiste de repetidos giros do Acil-CoA graxo em quatro reações bioquímicas: oxidação, hidratação, segunda oxidação e tiólise, catalisadas pelas enzimas Acil-CoA desidrogenase, Enoil-CoA hidratase, β-hydroxiacil-CoA desidrogenase e Acetil-CoA tiolase, respectivamente; cada passagem pela β-oxidação elimina dois carbonos do Acil-CoA graxo, liberando uma molécula de Acetil-CoA, uma de $NADH+H^+$ e outra de $FADH_2$ [47,65] (Figura 3).

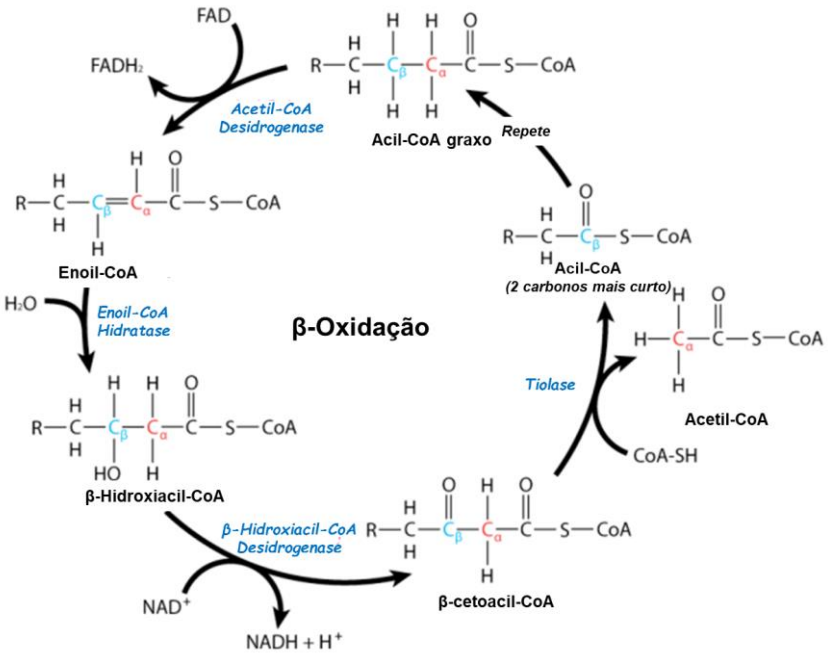

Figura 4. β-Oxidação dos ácidos graxos no miocárdio. A β-oxidação do ácido graxo envolve quatro enzimas Acil-CoA desidrogenase, Enoil-CoA hidratase, β-hydroxiacil-CoA desidrogenase e Acetil-CoA tiolase, responsáveis por remover do ácido graxo dois carbonos a cada giro no ciclo da β-oxidação. Adaptado de Stanley, (2010) [49].

O Acetil-CoA resultante da β-oxidação do AG é oxidado no ciclo de Krebs, gerando $NADH+H^+$ e $FADH_2$ para a cadeia transportadora de elétrons (CTE). A entrada do Acetil-CoA no ciclo ocorre à medida que a citrato sintase (CS), enzima responsável pela primeira etapa do ciclo, catalisa a condensação do Acetil-CoA com o oxaloacetato para formar citrato. O citrato é então oxidado em reações subsequentes, liberando CO_2; a energia oriunda dessa reação é conservada na forma das coenzimas reduzidas ($NADH+H^+$ e a $FADH_2$) que transferem elétrons para os complexos I e II da CTE, respectivamente. Dos complexos I e II, esses elétrons são transferidos para o O_2 no complexo IV no final da cadeia (Figura 3) [50].

Na cadeia de transporte de elétrons, o bombeamento de H^+ pelos complexos I, III e IV através da membrana mitocondrial interna gera um gradiente eletroquímico de prótons dentro do espaço intermembrana, acoplado à produção de ATP pela ATP-sintase. A utilização da ATP envolve o transporte de fosfocreatina; nesse sistema, o ATP deixa a matriz mitocondrial através da ATP-ADP translocase, e no espaço intermembrana transfere, sob a ação da creatina quinase (CK) mitocondrial, um fosfato para a creatina e forma fosfocreatina (PCr) [66]. A PCr, que atua como um reservatório de energia, migra para o citosol e é reconvertida em creatina pela CK citosólica após liberar o fosfato para a formação de ATP no seu local de utilização; A PCr fornece fosfato para formação de ATP que será utilizado pelas ATPases citosólicas, como proteínas contrateis e do retículo sarcoplasmático [42,67].

Para adaptar o suprimento energético às variações de demanda, o coração emprega uma complexa rede enzimática e vias de sinalização que coordena o fluxo de substratos energéticos desde sua captação pela célula até a oxidação na mitocôndria.

3.2. Regulação do metabolismo dos ácidos graxos no coração normal

O metabolismo lipídico miocárdico é regulado, principalmente, pelo receptor-α ativado por proliferador de peroxissoma (PPARα), fator transcricional membro da subfamília dos receptores ativados por proliferador de peroxissoma, sendo a isoforma de maior distribuição no músculo cardíaco. O AG de cadeia longa é descrito como o principal agonista do PPARα, embora derivados do AG como eicosanoides e leucotrienos também possam atuar como ligantes [68]. Após interação com o agonista, o PPARα é translocado para o núcleo onde se liga ao receptor do ácido 9-cis retinóico-α (RXR-α) para formar o heterodímero PPARα-RXRα, correspondente à forma transcricional ativa; posteriormente, o heterodímero se ancora ao elemento de resposta do proliferador de peroxissoma, localizado na região promotora do gene alvo. A transcrição gênica é iniciada pelo PPAR gamma coativador -1α (PGC1α), proteína necessária para a ativação da transcrição mediada pelo PPARα [69–73].

O PPARα miocárdico induz a transcrição de diversas proteínas envolvidas na captura sarcolemal (CD36) e mitocondrial (CPT1β), e na β-oxidação [coenzima A desidrogenase de cadeia média (MCAD)] dos ácidos graxos [70,72]. O PGC1α tem função relevante nas adaptações ao estado energético da célula, e sua atividade está relacionada a sensores celulares capazes de identificar perturbações metabólicas [69–73]. Dois sensores, a sirtuina 1 (SIRT1) e a proteína quinase dependente de adenosina monofosfato (AMPK), desempenham papeis importantes na regulação metabólica; ambos interagem com PGC1α para modular a transcrição gênica no sentido de manter os níveis energéticos normais [72,73]. A atividade da AMPK e da SIRT1 refletem as alterações intracelulares que ocorrem na razão entre adenosina monofosfato e adenosina trifosfato (AMP/ATP) e entre NAD+/NADH, respectivamente. No núcleo, a AMPK fosforila o PGC1α e, no citoplasma, estimula a entrada de ácidos

graxos na mitocôndria por desinibição da CPT1β. A SIRT1 é responsável por ativar o PGC1α, via desacetilação, favorecendo a geração de energia pelo miócito [73–77] (Figura 5).

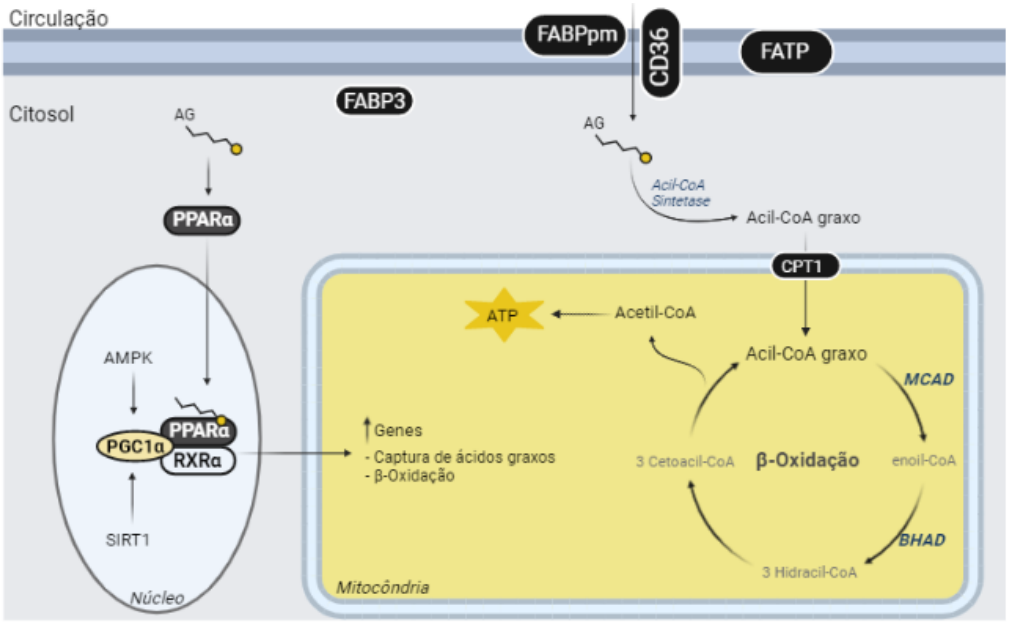

Figura 5. Representação esquemática da regulação do metabolismo lipídico miocárdico. Ativado por AG de cadeia longa, o PPARα entra no núcleo e se dimeriza com o RXRAα; o PGC1α é recrutado para ativar a transcrição de genes alvo do PPARα, envolvidos na captura e oxidação de AG. AMPK e SIRT1 atuam como sensores de estresse metabólico e interagem com PGC1α para regular a geração de energia. AG: ácido graxo; AMPK: proteína quinase dependente de adenosina monofosfato; SIRT: sirtuina 1; PGC1α: PPAR gamma coativador -1α; RXRα: receptor do ácido 9-cis retinóico; PPARα: receptor-α ativado por proliferador de peroxissoma; CD36: translocase de ácidos graxos; CPT1: carnitina palmitoiltransferase 1. Adaptado de Cantó e Auwerx, (2013) [73].

3.3. Metabolismo dos ácidos graxos no coração remodelado

Na remodelação patológica ocorre mudança no padrão de utilização do substrato energético miocárdico evidenciado por diminuição no metabolismo de ácidos graxos secundário à redução da expressão e atividade de proteínas envolvidas no processo de captação e oxidação dos AG; em paralelo, ocorre aumento na glicólise, sem aumento proporcional na oxidação mitocondrial desse substrato [49–53,78–82]. Embora a eficiência energética da glicose seja maior do que a do ácido graxo, 3,17 ATP/O_2 e 2,83 ATP/O_2, respectivamente, a disponibilidade energética é sensivelmente reduzida, 38 mol ATP/mol de glicose e 135 mol ATP/mol de ácido

graxo [83]. Essa mudança do perfil metabólico é evento clássico no curso da RC, entretanto os fatores envolvidos não estão completamente compreendidos.

O PPARα é proposto como o principal regulador do metabolismo energético lipídico cardíaco; vários estudos realizados em modelos de sobrecarga pressórica [69,70,84–88] reportaram diminuição da expressão do PPARα e seu coativador PGC1α. Esses resultados sugerem participação desse regulador no decréscimo das proteínas envolvidas no metabolismo dos AG, como CD36, CPT1β e MCAD. Entretanto, o papel do PPARα na desregulação do catabolismo de AG permanece controverso, desde que o aumento dessa proteína também foi documentado e associado a desfechos metabólico lipídico e funcional cardíaco negativos [89]. Além disso, na ausência de alteração do PPARα, também foi observada redução da oxidação de AG na falência cardíaca [90,91]. Adicionalmente, a ação do PPARα varia de acordo com o estágio da doença e é dependente de outros elementos que também sofrem influência da RC, como os sensores metabólicos referido anteriormente [92].

A atividade da AMPK na sobrecarga pressórica está aumentada, refletindo o estresse energético da célula que apresenta elevação na razão AMP/ATP; a ativação dessa quinase tem sido relacionada com a regulação do metabolismo de ácidos graxos e glicose, aumentando a produção de energia [93,94], entre outros mecanismos, pela ativação do PGC1α. A SIRT1 é outro elemento de interesse no cenário da hipertrofia concêntrica, com papel relevante na adaptação metabólica em situação de déficit energético, atuando na modulação da atividade do PGC1α e do PPARα [95]. Essa sirtuina foi analisada em modelo experimental de IC em cães [96] e os resultados mostraram maior expressão somente quando os animais apresentavam sinais clínicos de falência cardíaca. Entretanto, em estudos com modelos de bandagem aórtica, a expressão da SIRT1 se mostrou precocemente

aumentada após duas e quatro semanas de sobrecarga em camundongos [97] e em ratos [89], respectivamente. Apesar da atuação da SIRT1 no restabelecimento metabólico da sobrecarga pressórica, o aumento da sua expressão também é relacionado com intensificação do fenótipo da insuficiência cardíaca [89,97–99].

A transcrição de genes envolvidos com a oxidação de AG é classicamente descrita como resultante da heterodimerização do PPARα com o RXRα. Entretanto, estudos recentes mostraram que a dimerização do PPARα com a SIRT1 também pode ocorrer; essa interação está relacionada à supressão da expressão de genes alvo do PPARα na hipertrofia por sobrecarga pressórica [100]. Nesse mesmo modelo, também foi observada redução da expressão de RXRα, que compete com a Sirt1 pela interação com o PPARα. Esses estudos indicam que a maior formação de complexos PPARα-SIRT1 em detrimento de PPARα-RXRα favoreça o desarranjo metabólico lipídico miocárdico observado na sobrecarga de pressão [92,100].

4. ANGIOGÊNESE NO CORAÇÃO REMODELADO

A densidade capilar é um fator importante no curso do remodelamento fisiológico e patológico cardíaco [101]. Enquanto na hipertrofia fisiológica a rede capilar se expande, na patológica ocorre diminuição da angiogênese e rarefação capilar miocárdica [102–106]. Inicialmente, a remodelação patológica exibe um equilíbrio entre o consumo e a disponibilidade de oxigênio, isto é, entre o processo hipertrófico do miócito e a formação de novos vasos [107–109]. Com a manutenção da sobrecarga cardíaca, entretanto, ocorre desbalanço entre o crescimento dos cardiomiócitos e a capacidade proliferativa da rede capilar, limitando o abastecimento de oxigênio para o miocárdio [110–112]. A redução da microvasculatura somada ao aumento da demanda de oxigênio leva o coração a um estado de hipóxia [101,111].

A desregulação da angiogênese durante a hipertrofia envolve alterações na expressão de componentes cruciais para a formação de novos vasos, como o fator de crescimento endotelial vascular (VEGF) e seus receptores (VEGFR1 e VEGFR2), fator transcricional induzível por hipóxia 1 alpha (HIF1α), proteína de supressão tumoral (p53) e proteínas da via de sinalização PI3K/AKT/mTOR [6,101,111,113]. O VEGF é uma molécula angiogênica central na manutenção da densidade capilar no miocárdio sob estresse crônico, coordenando a angiogênese em resposta à hipertrofia miocitária [6,101–103,114]. Seu protagonismo foi testado em modelo de deficiência de VEGF, onde o estresse pressórico induziu hipertrofia severa associada com rarefação capilar e rápida transição para IC [115]. A expressão do VEGF é estimulada pelo aumento do HIF1α [116], principal fator transcricional na regulação da angiogênese; na hipertrofia patológica induzida por sobrecarga pressórica, o HIF1α torna-se mais expresso para manter a homeostase de oxigênio [111,117,118]. Entretanto, o acúmulo de p53, observado no miocárdio hipertrófico, inibe a atividade transcricional do HIF1α, comprometendo a proliferação vascular [111,119]. A via de sinalização PI3K/AKT/mTOR está envolvida em múltiplas funções celulares, incluindo a angiogênese cardíaca [13,120,121]; a ativação dessa via é reportada como fundamental na expressão de VEGF mediada por HIF1α; a perturbação dessa sinalização contribui para a desregulação da angiogênese e intensifica o processo de remodelamento patológico [122].

A redução da angiogênese e da densidade capilar é descrita como mecanismo central na transição da hipertrofia compensada para falência cardíaca [123]. Esse processo acarreta déficit de energia que pode levar a desfechos negativos relacionados ao desequilíbrio no perfil do metabólico, por exemplo, redução na oxidação miocárdica de ácidos graxos [124,125].

5. TREINAMENTO FÍSICO: CONCEITO E EFEITOS NO CORAÇÃO NORMAL E REMODELADO

O treinamento físico (TF) compreende uma rotina programada e sistematizada de atividades, resistida (ex. musculação) ou aeróbia (ex. corrida), com o objetivo de aprimorar o condicionamento físico e mental [126,127]. No mundo contemporâneo, o TF desempenha papel singular na otimização das atividades cotidianas [128], prevenção de patologias e atenuação dos efeitos da senilidade [129] e de doenças vigentes [31,32,130]. No contexto das doenças cardiovasculares, o exercício compreende estratégias diagnósticas, prognósticas e terapêuticas, sendo um componente essencial da reabilitação cardíaca inclusive para o manejo da IC com fração de ejeção reduzida [36,38,131,132]; mais recentemente, o TF foi incorporado às diretrizes das Sociedade Europeia e Brasileira de Cardiologia para o tratamento dessa síndrome como estratégia não farmacológica adjuvante [133,134]. Mecanisticamente, é sugerido que o estímulo fisiológico do TF antagoniza o estímulo patológico das cardiopatias [6,135]; nesse sentido, a literatura tem evidenciado uma relação inversa entre o TF e o risco cardiovascular, e propõe que esse hábito pode ser adotado como estratégia terapêutica e preventiva à instalação ou agravamento das cardiopatias [127,136,137].

Os efeitos positivos do TF sobre o sistema cardiovascular são evidenciados em estudos clínicos [138–140] e em modelos experimentais [31,32,37,141,142]. Os mecanismos subjacentes às adaptações benéficas do TF no coração disfuncionante decorrem de ajustes nos fatores ventriculares, miocárdicos e miocitários envolvidos na transição para falência; esses ajustamentos incluem rearranjos em compartimentos como transiente de cálcio [143,144], matriz extracelular [145], estado redox [146], *turnover* celular [147] e sistema neuro hormonal [36]. Adicionalmente, extensa

literatura revela que TF pode induzir a proliferação vascular em condição patológica [148–151], atenuando o desequilíbrio entre o processo de hipertrofia dos cardiomiócitos e a angiogênese, o que pode estar relacionado com benefícios no metabolismo energético.

O efeito pró angiogênico do exercício foi observado em modelos de cardiomiopatia chagásica [148], IAM [150,152,153], hipertensão arterial sistêmica [154–156], hiperatividade simpática [149] e estenose aórtica [151,157], sugerindo que a proliferação vascular é influenciada pelo TF independentemente da etiologia da cardiopatia. Mecanisticamente, o TF impulsiona o crescimento da rede vascular miocárdica à custa da liberação de VEGF [158]; a ligação desse fator de crescimento ao seu receptor específico de membrana (VEGFR) ativa a via de sinalização PI3K-Akt, estimulando a angiogênese [158,159]. Nosso grupo verificou que no modelo de sobrecarga pressórica por EAo, o processo hipertrófico ocorre rapidamente [28], o que poderia acarretar rarefação capilar miocárdica nas primeiras semanas pós cirurgia, levando a desajustes metabólicos que aceleram a evolução da disfunção e instalação da IC.

As adaptações metabólicas cardíacas geradas pelo TF não estão completamente elucidadas, sobretudo quanto à escolha do substrato energético. Estudos *in vivo* e *in vitro* com humanos e animais saudáveis mostraram que as taxa de utilização de glicose pelo miocárdio em resposta ao exercício pode ser mantida, diminuída ou aumentada [160–167]. Em relação ao efeito do exercício sobre metabolismo de lipídios de indivíduos saudáveis, estudos utilizando tomografia por emissão de prótons encontraram aumento no catabolismo de AG em idosos [163], enquanto em roedores a captura de AG foi encontrada aumentada [168] ou inalterada [160,162]. Em trabalhos *in vitro*, os achados são igualmente inconsistentes, indicando

manutenção [164], diminuição [166] e aumento da utilização de AG pelo miocárdio [165,167]. As variações de modelo experimental, sexo, duração e intensidade do exercício podem estar entre os fatores de inconsistência nos desfechos desses ensaios.

Os alvos mecanísticos pelos quais o exercício modula o metabolismo energético miocárdico de indivíduos saudáveis incluem proteínas envolvidas na captura e oxidação de substratos e na biogênese mitocondrial. Em modelo de natação, a atividade da CS e da BHAD, e a expressão da CPT1β, PGC1α e subunidades da CTE foram significativamente aumentadas [165]. Em estudos com treinamento em esteira também foi encontrado aumento da expressão de CPT1β e PPARα [169], e regulação positiva da expressão gênica de CD36 [170]. Em contraste, outros trabalhos não encontraram variação proteica do PPARα [162], CD36 [171] e CPT1β [171] com o exercício. Em relação ao metabolismo da glicose, avaliação metabolômica e de transcriptoma apenas detectou suprarregulação da fosfofrutoquinase como mecanismo chave na remodelação glicolítica secundária ao treinamento físico [164]. No contexto da variabilidade dos resultados observados nas adaptações metabólicas geradas pelo exercício, o aumento na utilização de AG pelo coração é a mais consistente [164,165,167,169–172]. Desde que a otimização da oxidação desse substrato preserva a função do coração agredido [173,174], o exercício pode ser um agente promissor da regulação energética no cenário das cardiopatias.

Os estudos de intervenção que investigaram o efeito do TF sobre o metabolismo miocárdico em modelos de cardiopatias são limitados [175]. Em trabalho experimental de regurgitação aórtica, o TF normalizou parcialmente a atividade de enzimas envolvidas com a beta-oxidação, glicólise e CTE [176]. Outros autores

verificaram que o TF, previamente à ligadura da artéria coronária, inibiu a inversão do metabolismo energético miocárdico, mantendo os níveis de oxidação de AG, e favoreceu a biogênese mitocondrial [177]. Ainda em modelo de IAM, foram relatados efeitos positivos do TF na atenuação da remodelação estrutural e funcional cardíaca, que ocorreu em paralelo com aumento da expressão gênica de PPARα e CPT1, do nível proteico de PGC1α e da fosforilação de AMPK [178], ou aumento nos níveis de ATP e atividade do complexo 1 da CTE [179]. Mais recentemente, também foi reportado elevação da atividade de enzimas envolvidas no metabolismo energético [piruvato desidrogenase (PDH), LDH, CS, BHAD, NADH desidrogenase e ATP sintase] em animais submetidos a TF previamente à indução de IAM [180]. Em modelo de sobrecarga pressórica os achados são mais escassos [181]; Kinney LaPier et. al., (2001) verificaram em ratos SHR, que oito semanas de exercício aeróbio restaurou a atividade da CPT1, e que após 16 semanas a atividade da HK foi aumentada em relação ao controle. Esses resultados sugerem que os prejuízos na oxidação de AG na hipertrofia patológica antecedem as adaptações no metabolismo glicolítico, e que o exercício primeiramente atua sobre o metabolismo lipídico no coração doente. Dada a carência de estudos em modelos de sobrecarga pressórica, os efeitos do TF no metabolismo energético do miocárdio concentricamente hipertrofiado continuarão sendo foco de pesquisas futuras no campo da cardiologia.

Apesar do consolidado benefício no cenário das cardiopatias, a intensidade e o momento da implementação do treinamento permanecem no centro de discussão [182]. No campo experimental, o emprego do treinamento físico precoce é pouco relatado e concentra-se principalmente em modelos de sobrecarga de volume [177,180,183,184]. Xu et al., (2015) verificaram que o pré-condicionamento cardíaco com exercício aeróbio atenuou o remodelamento patológico em ratos com estenose

aórtica [185]. Nesse sentido, para o melhor do nosso conhecimento, não há na literatura trabalhos que adotaram o exercício precoce como tratamento da remodelação induzida por sobrecarga pressórica. Em nosso laboratório, melhoria significativa da função cardíaca foi alcançada quando o TF foi introduzido previamente ao declínio da função sistólica [31,37,186], ao passo que os benefícios foram mais discretos [36,38] ou nulos [187] em roedores que apresentavam prejuízo na capacidade de ejeção. Portanto, o momento da intervenção com o treinamento é um aspecto importante a ser considerado, desde que pode determinar a magnitude dos benefícios do exercício sobre a performance cardíaca.

Embora a fisiopatologia da RC e o papel do exercício como ferramenta terapêutica sejam amplamente estudados, algumas questões ainda requerem elucidação: 1.) O TF precoce é mais efetivo em combater o remodelamento patológico? 2.) A preservação da angiogênese favorece a integridade metabólica miocárdica? 3.) Quais componentes moleculares da bioenergética estão envolvidos com os benefícios do TF no coração agredido? A condução de estudos com exercício precoce em diferentes modelos de cardiopatias poderá contribuir com respostas mais sólidas nesse campo, contribuindo para o melhor entendimento do papel das terapias não-farmacológicas no controle das cardiopatias e seus agravos.

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CAPÍTULO 2

Artigo Científico

The role of myocardial lipid energy metabolism in the attenuation of heart dysfunction by early training exercise in aortic stenosis rats

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Abstract

Background/aims. We employed a moderate training exercise (ET) program to stud the underlying mechanism involved with the amelioration of cardiac dysfunction in exercised aortic stenosis (AS) rats. Since ET induces angiogenesis and oxygen support, we tested the hypothesis that ET attenuates HF phenotype by preventing lipid energy metabolism depression. ***Methods*****:** Wistar rats were divided into four groups: Sham, trained Sham (ShamT), AS, and trained AS (AST). The ET consisted of five-week sessions of treadmill running for 16 weeks. Statistical analysis was conducted by ANOVA or Kruskal-Wallis test, and Goodman test. In addition, a global correlation between variables was performed using a 2-tailed Pearson's correlation test. All results were discussed at $p < 0.05$. ***Results*****:** AST rats displayed a higher functional capacity and a lower cardiac remodeling and dysfunction when compared to sedentary AS. Immunohistochemical analysis of the hypertrophied myocardium showed that exercise prevented capillary rarefaction. Immunoblotting and enzymatic assay raised beneficial effect of exercise also on lipid metabolic pathway, at level of fatty acid (FA) transport and oxidation, by preserving protein expression and enzymatic activity. The correlation assessment indicated a positive correlation between variables of angiogenesis and FA utilization, as well as between metabolism and echocardiographic parameters. ***Conclusion*****:** The attenuation of cardiac dysfunction is linked to the preservation of lipid energy metabolism, which in turn is related to angiogenesis restoration in rats with AS-induced heart failure.

Keywords: Aortic bandage, pressure overload, cardiac function, echocardiogram, angiogenesis, energy metabolism, fatty acid oxidation

1. INTRODUCTION

Heart failure (HF) is a syndrome with an important morbidity and mortality impact on the population worldwide and is an outcome of exposure of the heart to chronic stress, such as pressure overload (PO).[1] Chronic PO is characterized by time-dependent decline in diastolic and systolic functions, and subsequently overt HF.[2] Although several abnormalities have been raised in failing myocardium after PO, the mechanisms involved in the decompensation of performance are poorly elucidated so far.[3] At molecular level, pathological remodeling involves a set of changes including oxygen deficit, which results from reduced capillary density. [4,5] The imbalance between hypertrophy and angiogenesis, triggered by endothelial growth factor (VEGF) deficiency, limits the oxygen supply;[6] the consequent myocardial hypoxia potentially lead to a disturbance of energy substrate metabolism and subsequently fibrosis, dilatation, and dysfunction.[7]

Fat acid (FA) is the main substrate for energy generation by the healthy heart. However, the FA oxidation (FAO)[8,9] is reduced during HF, accompanied by a broad downregulation of the FA metabolic pathway genes,[10] as widely reported by studies in rodents[11,12] and humans.[13,14] Nevertheless, the molecular adaptations behind these metabolic disturbances are not entirely understood. Regarding PO, numerous studies indicate that the downregulation of the peroxisome proliferator activated receptor-α (PAPRα) plays an important role in decreased FA oxidation.[15–20] However, deficient lipid catabolism has also been found in parallel with unchanged[21,22] or upregulated PPARα expression[23]. In addition, recent studies with PO-induced HF demonstrated that PPARα heterodimerizes with Sirtuin 1 (SIRT1), and this complex (PPARα-SIRT) suppresses the transcription of PPARα target genes, which are part of myocardial FA oxidation gear.[24,25]

The high HF mortality rate despite the growing progress in the therapeutic makes the treatment of this syndrome an ongoing challenge.[1] Aerobic exercise training (ET) as a non-pharmacological method for the management of HF has been extensively described.[26–28] Studies that evaluated the influence of ET on the transition to failure in a model of aortic stenosis (AS) showed significant attenuation of cardiac disfunction,[29–31] while other investigations using the same experimental model and ET protocol did not find similar results.[32,33] The variations of cardiac dysfunction degree at which exercise training is implemented after AS surgery might be a critical factor for the different results. Moreover, the mechanisms underlying the beneficial effects of this therapy on AS still remains misunderstood.

In line with the impact of energy perturbation on HF, exercise has been demonstrated to be a potential modulator of metabolic derangements that occur in the pathological heart;[34] adjustments in the FA capture and oxidation, in addition to improved mitochondrial biogenesis, have been shown in a few experimental assays with myocardial infarction and aortic insufficiency models.[34–39] To the best of our knowledge, the influence of ET on lipid metabolism of the failing heart undergoing severe myocardial hypertrophy, such as that of AS remains unknown.

There is vast literature demonstrating that angiogenesis is stimulated by exercise, which ameliorates the VEGF signaling in a normal[40–43] or pathological condition,[44–47] including PO, especially in young animals.[48,49] ET-induced vascular capillarization could attenuate myocardial oxygen shortage and consequently improve FAO and energy production. We hypothesized that in aortic stenosis rats, the cardioprotection generated by exercise is linked to the preservation of lipid metabolism secondary to angiogenesis maintenance.

2. MATERIALS AND METHODS

2.1. Animals

Twenty-one-day-old male *Wistar* rats weighing 70-90 g were purchased from the Central Animal House, Botucatu Medical School, Unesp. Rats were housed in collective polypropylene cages in a climate-controlled environment at 23 ºC with a reverse 12:12-h light/dark cycle and free access to food and water. All experiments and procedures were supervised by a veterinarian, performed according to the Brazilian Guide for the Care and Use of Laboratory Animals, and approved by Botucatu Medical School Animal Research Ethics Committee (protocol number 1239/2017).

2.2. Pressure overload procedure and experimental design

Supra-valvar AS was surgically induced for generating cardiac PO, as described previously.[2] Briefly, rats were anesthetized with intraperitoneal ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (10 mg/kg), and the heart was exposed via a median thoracotomy. Then, a silver clip (0.60 mm internal diameter) was placed on the ascending aorta at approximately 3 mm from its root. During the surgery, the rats received 1 ml of warm saline solution intraperitoneal and manually ventilated with positive pressure on 100% oxygen. After the procedure, animals were kept warm until full consciousness was regained. Analgesia procedure consisted of intraperitoneal administration of carprofen (5mg/kg body weight) and was maintained until the disappearance of evidence of pain. Sham animals underwent the same procedure but without the constriction of the aorta.

Initially, rats underwent either supra-valvar aortic stenosis (AS) or Sham surgery (Sham). After two weeks, the animals were redistributed to be kept sedentary (Sham, n = 16 and AS, n = 16) or submitted to exercise training (ShamT, n = 16 and AST, n = 14) for 16 weeks. The mortality rate did not differ between sedentary or

trained AS groups. Two AS rats were removed from the stud because they develop unsatisfactory remodeling related to the clip placement in the aorta. Regarding the AST group, one rat had collateral circulation, and two others were unable to adapt properly to the treadmill and were also not included in the study.

Animals of all groups had the functional capacity and *in vivo* cardiac function evaluated before and after the exercise training period (i.e., two and 18 weeks after surgery, respectively), and subsequently, rats were submitted to euthanasia for additional analyzes (Figure 1).

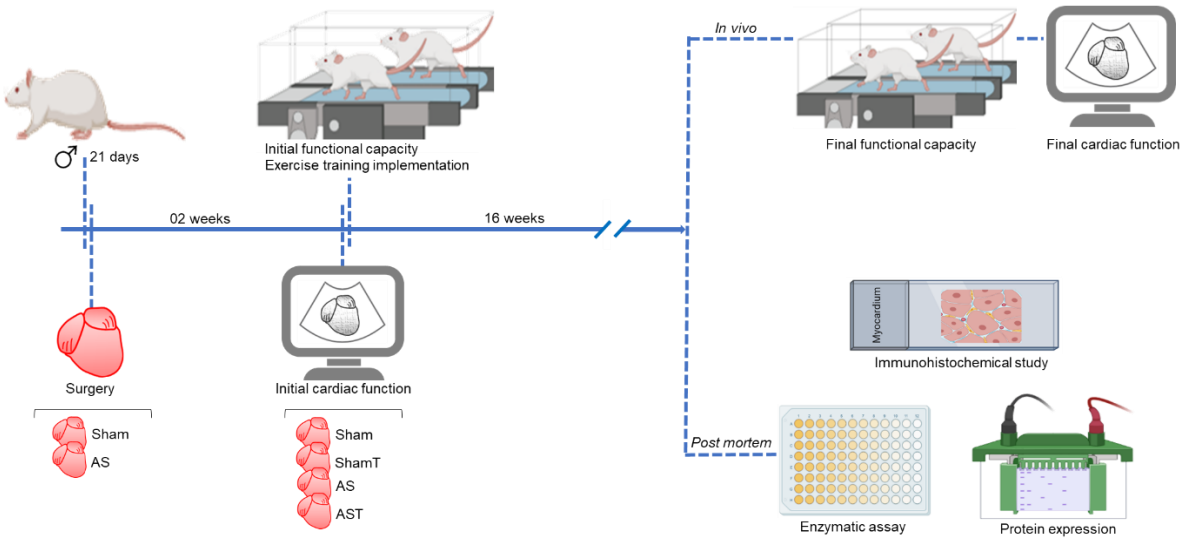

Figure 1 Schematic representation of the experimental design. Sham and ShamT, sedentary and trained control group respectively; AS and AST, sedentary and trained aortic stenosis groups.

2.3. Treadmill exercise testing (TET)

Functional capacity was assessed before and after the ET period as described previously.[50] TET was performed on a motorized treadmill for rats (AVS Projetos – São Carlos, SP, Brasil) after one week of adaptation to the treadmill environment under low speed (5m/min/day). Maximum running speed and total time were recorded. Briefly, TET began at 6 m/min and increased by 3 m/min every 3

minutes until exhaustion. Exhaustion was defined as the non-maintenance of the race at the proposed speed. The TET results were also used to prescribe the periodic ET program.

2.4. Aerobic exercise training

The ET protocol was modified from previous studies.[50,51] Rats were exercised for sixteen weeks, five days/week (Monday to Friday) at 60% of the maximal speed, achieved during the TET. The test was performed before the first week of training to initial exercise prescription and after the fourth, eighth, and eleventh week to adjust running speed. Exercise duration from the first to the fourth week was progressively added ten minutes per week until 60 min/day and then remained constant (Square S1). During the training, animals received low-voltage electrical stimulation.

2.5. Echocardiographic study

Measurements were performed after anesthesia by intramuscular injection of a mixture of ketamine (50 mg/km) and xylazine (1 mg/kg), using an apparatus (Vivid S6, General Electric Medical Systems) equipped with a 5-11.5 MHz multifrequency probe, as recently described in detail.[52] Briefly, cardiac function and morphology were measured at two and 28 weeks post-surgery. All examinations were performed blindly by a cardiologist and specialist in echocardiography. A two-dimensional parasternal short-axis view of the left ventricle (LV) was obtained at the level of the papillary muscles[53]. M-mode tracings were obtained from short-axis views of the LV at or just below the tip of the mitral valve leaflets, and at the level of the aortic valve and left atrium. M-mode images of the LV were printed on a black-and-white thermal printer (Sony UP-890MD) at a sweep speed of 100 mm/s. All LV structures were manually

measured by the same observer according to the leading-edge method of the American Society of Echocardiography. Measurements reported are the average of at least five cardiac cycles from the M-mode tracings.

2.6. Anatomical data and heart failure features

The nutritional profile was evaluated according to the following parameters: body weight, fat deposits, and food intake as previously;[54] Body weight and food intake were measured weekly.

At euthanasia, was observed the occurrence of HF features *in vivo* (tachypnea) and *post-mortem* (ascites, pleural effusion, atrial thrombi, and liver congestion) in the AS and AST groups. Two blind observers carried out a subjective evaluation of HF signals throughout the experiment as previously performed.[50] Atria (AT) and left (LV) and right (RV) ventricles were dissected and weighed to determine cardiac hypertrophy. Tibia length was measured and used to normalize total heart weight, and its separate components by the ratios HW/tibia, AT/Tibia, RV/tibia LV/tibia.

2.7. Immunohistochemistry

After the animals were killed, the heart was removed and the left ventricle separated and cut in the coronal plane. The slices were kept in buffered formalin for 12 hours at room temperature, followed by sequential dehydration in increasing concentrations of alcohol, cleared in xylene, and embedded in paraffin. The blocks were cut into 3-µm sections for immunohistochemical staining.

For immunohistochemistry, marked and pre-assembled blades were dewaxed in xylene and rehydrated in decreasing concentrations of alcohol until water. For antigen recovery, the slides were heated in a pressure cooker at 98°C with citrate buffer (pH 6.0) for 30 minutes and then incubated in 0.3% hydrogen peroxide at room

temperature for endogenous tissue peroxidase block. Then, they were washed with Tris-buffered saline/Tween 20 (TBST), pH 7.5, and incubated with primary antibodies against VEGF (ab1316, Abcam, Cambridge, MA, USA. 1:200), VEGF receptor 1 (VEGFR1; 13687-1-AP, Proteintech, Rosemont, IL, USA. 1:200), and 2 (VEGFR2; 9698s, Cell Signaling, Danvers, MA, USA. 1:200) and differentiation cluster 34 (CD34; ab81289, Abcam. 1:200) for 1 hour in a humid chamber at room temperature. Slides were washed again with TBST, incubated with a secondary peroxidase horseradish polymer-conjugated antibody for 30 minutes at room temperature, washed with TBST, incubated with 3,3′ diaminobenzidine stain for 5 minutes, and counterstained with hematoxylin. Stereological point-counting procedures were applied to perform a morphometric analysis.[55]

2.8. Protein expression by Western blot method

We analyzed the expression of cardiac lipid energy metabolism by the Western blot method.[56] As the LV hypertrophic process is related to the impaired angiogenesis pathway, we also studied the expression of proteins that regulate angiogenesis, and signals oxygen deficit. Samples were homogenized in a solution containing cold RIPA buffer (Amresco LLC, Solon, OH) and protease (Sigma-Aldrich, St. Louis, MO), and phosphatase (Roche Diagnosis, Indianapolis, IN) inhibitors. The homogenate was centrifuged at 12000 g for 20 min at 4 ºC, and the supernatant was collected. Protein concentrations were determined using the BCA Protein Assay kit (Thermo Scientific, Wilmington, DE, USA). Samples (50 µg) were subjected to SDS-PAGE in 10% polyacrylamide gel, and after that, were electrotransferred to the nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The blotted membrane was blocked with 5% nonfat dry milk (20 mmol/L Tris-HCl pH 7.4, 137

mmol/L NaCl and 1% Tween 20) for 2 h at room temperature and then incubated overnight at 4-8 ºC with primary specific antibodies: PI3 Kinase p85 (PI3K; 4292s, Cell Signaling, Danvers, MA, USA. 1:1000); Protein kinase B (AKT; sc-16646, Santa Cruz Biotechnology, Delaware Ave, USA. 1:500); phosphorylated AKT Thr308 (pAKT; sc-16646, Santa Cruz Biotechnology. 1:500); HIF1α (ab463, Abcam, Cambrige, MA, USA. 1:500); p53 tumor suppressor protein (p53; Cell Signaling. 1:1000); fatty acid transporter (CD36; ab133625, Abcam. 1:1000); carnitine palmitoyltransferase 1 (CPT1; NBP1-59576, Novus Biological, Littleton, CO, USA. 1:1000); fatty acid-binding protein (FABP3; ab133585, Abcam. 1:1000); acyl-CoA dehydrogenase (ACADL; ab196655, Abcam. 1:3000); medium-chain acyl-CoA dehydrogenase (MCAD; ab110296, Abcam. 1:1000); 2,4-dienoyl-CoA reductase (DECR1; ab198848, Abcam. 1:2000). AMP-activated protein kinase (AMPK; 2532s, Cell Signaling. 1:1000); phosphorylated AMPK (Thr 172) (pAMPK; 2535s, Cell Signaling. 1:500); SIRT1 (8469s, Cell Signaling. 1:1000); PPARα (ab8934, Abcam. 1:1000); retinoid X receptor (RXRα; 3085s, Cell Signaling. 1:1000); peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α; 20658-1-AP, Proteintech, Rosemont, IL, USA. 1:1000). The immunoblots were washed three times with TBS-T and incubated for 1.5 H with peroxidase-conjugated anti-rabbit secondary antibody (1:5000 – 1:10000; Abcam, Cambridge, MA), then washed three times again with TBS-T and incubated with ECL (Enhanced Chemi-Luminescence, Amersham Biosciences, Piscataway, NJ) for chemiluminescence detection in a western blot detection system (Image Quant™ LAS 40– GE Healthcare Life Sciences, Chalfont, UK), and quantified by densitometry using Image J Analysis software. Targeted bands were normalized to the expression of cardiac GAPDH (sc-32233, Santa Cruz Biotechnology. 1:2000).

2.9. Metabolic enzymatic assays

Activities of the enzymes beta-hydroxyacyl-CoA dehydrogenase (OHADH), citrate synthase (CS), and creatine kinase (CK) that participate in fatty acid metabolism and citric acid cycle (CAC) were analyzed in the myocardium. LV samples were homogenized 1:20 (wt/vol) in 50 mM Tris-HCl, 1 mM EDTA, and protease inhibitor cocktail, pH 7.4, using a Bullet Blender storn, (Next Advanced, Troy, NY, USA). The lysate was centrifuged at 12000 rpm for 10 min at 4 ºC and the supernatant collected. All enzyme activities were determined at 25 ºC using a Spectra Max 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA), and the assay buffer without the sample was used as blank. CK was assayed in a medium consisting of 91,1 mM Tris-HCL, 1 mM ATP, 0.262 mM ß-NAD reduced form, 0.728 mM PEP, 10 mM creatine, 5 mM KCl, 2 mM $MgSO_4$, 10 units lactic dehydrogenase, 0.4 to 0.6 unit pyruvate kinase and 0.05% Triton X-100. pH 7.4. The total assay volume was 165 and 2 microliter of homogenate was added. The assay was initiated by the addition of creatine. The absorbance of CS and CK assays was monitored at 340 nm. For OHADH, the reaction contained 100 mM PBS pH 7.3, 0.45mM NADH and 1.0 mM acetoacetyl CoA. CS activity was measured in a reaction containing 100 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.2 mM dithio-bis(2-nitrobenzoic acid), 0.3mM acetyl CoA and 0.5 mM oxaloacetate, pH 8.2. The rate of change in absorbance was monitored at 412 nm (ε = 13.6 µmol _ ml^{-1}).

2.10. Adenosine triphosphate content

ATP content was measured as the method described previously.[57] Briefly, LV was homogenized in perchloric acid (0.6 N) and the supernatant was neutralized using potassium bicarbonate aqueous solution (1 M). The assay medium consisted of

75 mM Tris-HCl, 7.5 mM $MgCl_2$, 0.8 mM EDTA, 1.5 mM KCl, 4 mM 2 mercaptoethanol, 0.4 mM $NADP^+$, 1m mM glucose, 1.4 units glucose-6-phosphate dehydrogenase, 0.05% Triton X-100, pH 7.2. The total assay volume was 165 µL and 10 µL of fresh homogenate was added. The assay was initiated by the addition of glucose and monitored at 340 nm.

2.11. Statistical analysis

Data are expressed as mean ± standard deviation or median and percentiles. Kolmogorov-Smirnov normality test was used to test data normal distribution. Two-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test (parametric distribution) or Kruskal-Wallis followed by Dunn's (non-parametrical distribution) was used to analyze differences between groups. Goodman test was used to test differences in heart failure features between AS and AST groups. The correlation matrix with all data was analyzed and generated using a 2-tailed Pearson's correlation test.[58] All statistical analyses were performed using Sigma Plot 12.0 (Systat Software, Inc., San Jose, CA, USA), except for the correlation matrix that was performed in GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). All graphics were generated using GraphPad Prism 8. Differences between groups or moments were accepted as statistically significant at a $p < 0.05$.

3. RESULTS

3.1. Exercise prevents reduced ingestion during heart failure

Nutritional data are shown in Table 1. Body weight of ShamT and AS groups were lower than Sham, while AST presented lower body weight than AS. Except by epididymal, all fat deposits were reduced in aortic stenosis rats regardless of exercise training. Additionally, food ingestion was reduced in AS rats, and this scenario was attenuated by the exercise.

Table 1 Nutritional profile

	Sham	Sham-T	AS	AS-T
Boddy weight (g)				
Initial	77.1 ± 14.3	82.8 ± 16.4	80.3 ± 9.76	72.0 ± 18.7
Final	492 ± 64.8	430 ± 58.9*	420 ± 47.9*	368 ± 49.9&#
Fat deposits (g)				
Epididymal	4.09 ± 1.45	5.07 ± 1.85	2.70 ± 1.63	2.75 ± 0.73&
Retroperitoneal	6.57 ± 2.61	6.28 ± 1.78	4.06 ± 2.31*	4.03 ± 0.87&
Visceral	4.07 ± 1.26	4.12 ± 0.84	2.33 ± 1.48*	2.44 ± 0.46&
Total	14.7 ± 4.86	15.5 ± 3.77	9.10 ± 4.98*	9.22 ± 1.40&
Food intake (g)				
Initial	16.8 ± 2.96	18.1 ± 7.71	16.0 ± 6.34	18.0 ± 5.93
Final	32.5 ± 3.24	35.6 ± 2.65	25.0 ± 1.65*	30.4 ± 3.39&#

ShamT, trained Sham; AS, sedentary aortic stenosis; AST, trained aortic stenosis. Data expressed as mean ± SD. $p < 0.05$. * *vs*. Sham; & vs ShamT; # *vs*. AS. (n = 14-16 each group).

3.2. Exercise sustains functional capacity in aortic stenosis rats

Exercise tolerance was assessed by exhaustion speed and total time during

treadmill exercise testing. The final test showed that PO impaired exercise tolerance while ET improved the functional capacity in both normal or pathological condition (Figure 2).

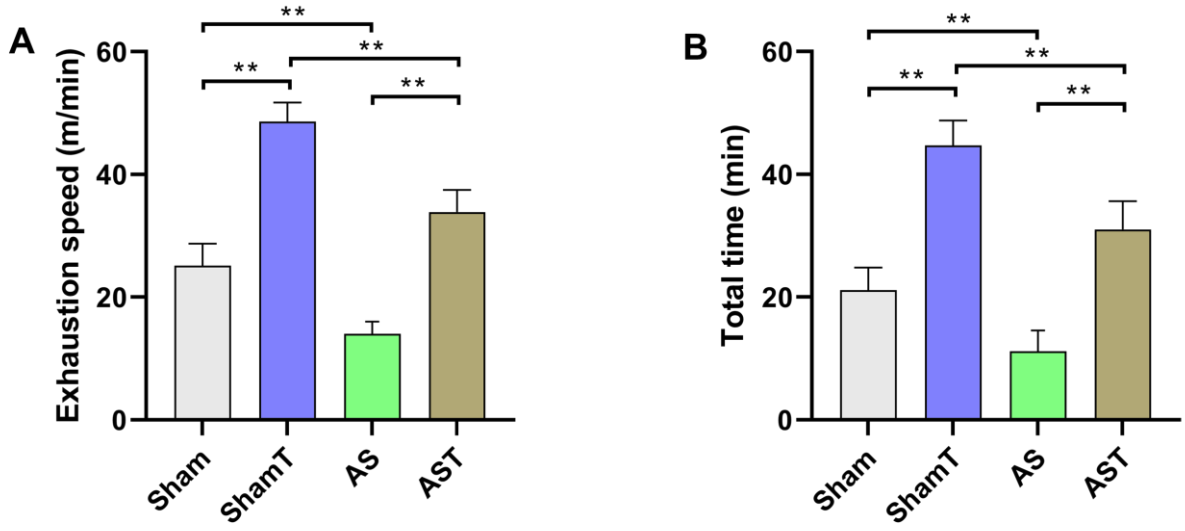

Figure 2 Final treadmill exercise testing. ShamT, trained Sham; AS, sedentary aortic stenosis; AST, trained aortic stenosis. Data are individual values. * $p < 0.05$. ** $p < 0.001$. (n = 14-16 each group).

3.3. Exercise training inhibits cardiac hypertrophy induced by aortic stenosis and heart failure features occurrence

Cardiac anatomical parameters are presented in Table 2. Atria, RV, and LV weights were greater in AS group than in Sham, both in absolute and normalized to tibia length values, indicating cardiac hypertrophy. Compared to AS, the AST group displayed a lower weight in all parameters studied. Symptoms related to HF were observed in all sedentary animals while the exercised group had these features occurrence substantially reduced (Figure 3).

Table 2. Cardiac anatomical data

	Sham	ShamT	AS	AST
Tibia (cm)	4.41 ± 0.08	4.28 ± 0.08	4.27 ± 0.12	4.19 ± 0.17
Heart (g)	1.18 ± 0.11	1.11 ± 0.18	2.47 ± 0.29*	1.81 ± 0.29&#
LV (g)	0.82 ± 0.08	0.78 ± 0.13	1.50 ± 0.16*	1.26 ± 0.16&#
RV (g)	0.24 ± 0.02	0.23 ± 0.04	0.53 ± 0.08*	0.32 ± 0.08&#
Atria (g)	0.11 ± 0.01	0.11 ± 0.01	0.44 ± 0.09*	0.27 ± 0.09&#
Heart/Tibia	0.27 ± 0.02	0.26 ± 0.04	0.58 ± 0.06*	0.44 ± 0.07&#
LV/Tibia	0.19 ± 0.02	0.18 ± 0.03	0.35 ± 0.03*	0.30 ± 0.04&#
RV/Tibia	0.05 ± 0.005	0.05 ± 0.008	0.12 ± 0.01*	0.07 ± 0.02&#
Atria/Tibia	0.03 ± 0.003	0.03 ± 0.004	0.10 ± 0.02*	0.06 ± 0.02&#

ShamT, trained Sham; AS, sedentary aortic stenosis; AST, trained aortic stenosis. LV, left ventricle; RV, right ventricle. Data are mean ± SD. $p < 0.05$. * *vs*. Sham; & vs ShamT; # *vs*. AS. (n = 14-16 each group).

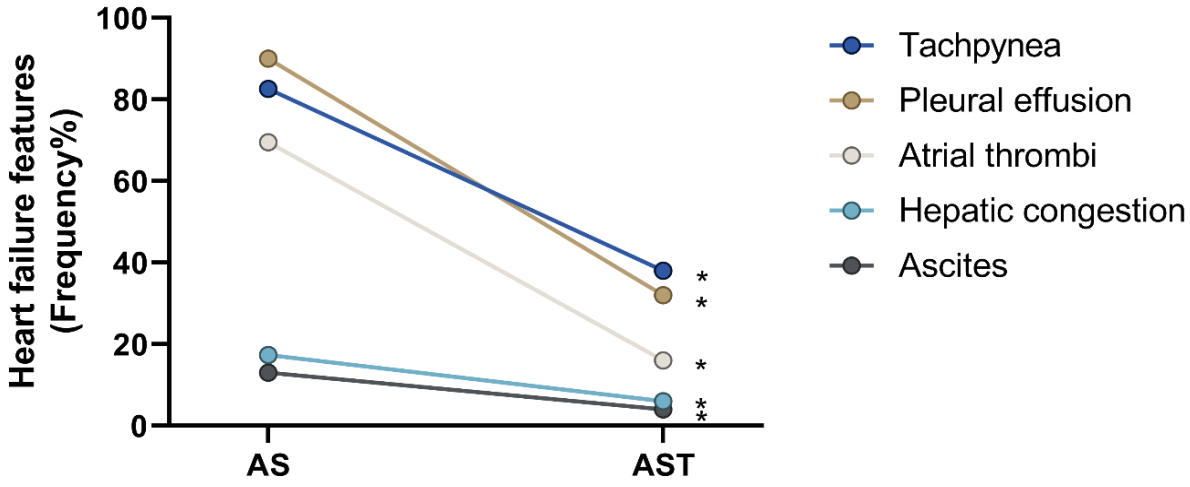

Figure 3 Heart failure features frequency. ShamT, trained Sham; AS, sedentary aortic stenosis; AST, trained aortic stenosis. Data are relative frequency. * $p < 0.05$ *vs*. AS. (n = 14-16 each group).

3.4. Exercise attenuates cardiac structural and functional deterioration induced by pressure overload

Before exercise program, an initial echocardiogram was performed to ensure homogeneity between groups (Table S1). The final echocardiographic assessment, after the experimental period (18th-week post-surgery), is presented in

Table 3. AS groups displayed LV hypertrophy, visualized by higher LV diastolic (LVDD) and systolic diameter (LVSD), and relative wall thickness (RWT), accompanied by LA enlargement. Additionally, PO induced systolic dysfunction characterized by lower Mesocardial (MFS) and endocardial (EFS) fractional shortening, posterior wall thickness systolic velocity (PWSV), Tei index, and tissue Doppler image (TDI) of the systolic velocity of mitral annulus (TDI S'). Furthermore, diastolic dysfunction was also diagnosed by lower isovolumetric relaxation time and E-wage deceleration time, and by the higher following ratios: E-wave (E) to A-wave, E to TDI early (E') velocity of mitral annulus, and TDI E' to TDI late velocity of mitral annulus. However, the AST group in comparison to AST displayed an attenuated status of structural remodeling, characterized by reduced LVDD, LVSD, PWDT/Tibia, and LA/AO ratio, and cardiac dysfunction as visualized in systolic (MFS, ESF, PWSV, and TDI S') and diastolic (IVRT, EDT, and E/A,) parameters. Heart rate did not influence the results of cardiac function since its values were similar between groups.

3.5. Exercise prevents myocardial capillary rarefaction and angiogenesis disturbance in aortic stenosis rats

The effect of PO and early exercise on cardiac angiogenesis was examined by immunohistochemistry using anti-VEGF, -VEGFR-1, -VEGFR-2, and -CD34 antibodies. Figures 4 A-E reveal that VEGF expression was down-regulated in AS group while it was maintained unaffected in AST. Moreover, VEGFR-1 expression was also impaired by AS surgery and preserved by ET. Interestingly, the VEGFR-2 level was up-regulated in AS group relative to the Sham group and down-regulated in AST relative to ShamT and AS groups. It was found that CD34 expression, a tissue vessel density marker, was markedly reduced after PO and maintained at control levels in AST hearts. Since VEGF signaling is regulated by other agents, such as HIF1α and

PI3K/AKT pathway, we studied the protein expression of these regulators in LV (Figures 4 F-J). HIF1α, the master hypoxia marker, was found to be overexpressed in AS rats, and not in AST rats; this result corroborates the higher capillary density visualized in immunohistochemical assay and, together, suggest a lower oxygen deficit in AST hearts. Both PI3K, AKT, and p53 did not present a significant difference between groups.

Table 3 Final echocardiographic data

	Sham	Sham-T	AS	AS-T
HR (bpm)	292 ± 27.1	317 ± 35.9	318 ± 52.0	323 ± 52.9
LVDD (mm)	7.44 ± 0.50	7.42 ± 0.51	8.42 ± 0.59*	7.56 ± 0.95$^{\#}$
LVDD/Tibia	1.69 ± 0.12	1.74 ± 0.12	1.97 ± 0.12*	1.74 ± 0.24$^{\#}$
LVSD (mm)	2.94 (2.81 - 3.32)	3.07 (2.81 – 3.32)	4.09 (4.09 – 4.60)*	3.41 (3.01 – 3.90)$^{\#}$
PWDT (mm)	1.53 (1.53 – 1.53)	1.53 (1.51 – 1.58)	2.81 (2.69 – 2.92)*	2.50 (2.30 – 2.62)$^{\&}$
PWDT/Tibia	0.35 ± 0.01	0.37 ± 0.06	0.66 ± 0.04*	0.60 ± 0.07$^{\&\#}$
AO (mm)	3.83 (3.64 – 4.09)	3.96 (3.83 – 4.09)	4.09 (3.90 – 4.31)	4.09 (3.83 – 4.34)
LA (mm)	4.85 (4.60 – 5.05)	4.85 (4.60 – 5.11)	8.05 (7.73 – 8.63)*	7.15 (6.39 – 8.05)$^{\&}$
LA/AO (mm/g)	1.23 ± 0.10	1.27 ± 0.12	2.00 ± 0.18*	1.78 ± 0.32$^{\&\#}$
RWT (%)	0.41 (0.40 – 0.44)	0.41 (0.39 – 0.44)	0.69 (0.63 – 0.72)*	0.68 (0.57 - 0.78)$^{\&}$
MFS (%)	27.1 ± 3,78	27.5 ± 3.37	21.2 ± 4.16*	24.1 ± 2.76$^{\&\#}$
EFS (%)	59.7 ± 3.07	59.4 ± 4.05	47.9 ± 5.73*	54.5 ± 5.21$^{\&\#}$
PWSV (mm/s)	73.5 ± 5.33	70.8 ± 8.77	44.8 ± 6.48*	56.4 ± 11.4$^{\&\#}$
Tei index	0.21 ± 0.08	0.26 ± 0.09	0.34 ± 0.16*	0.26 ± 0.11
TDI S (cm/s)	5.74 ± 0.33	5.73 ± 0.23	4.18 ± 0.50*	4.78 ± 0.43$^{\&\#}$
IVRT (ms)	23.5 ± 2.31	22.9 ± 2.69	13.3 ± 2.69*	17.8 ± 4.58$^{\&\#}$
EDT (ms)	48.6 ± 2.71	47.7 ± 3.93	25.9 ± 6.30*	37.4 ± 10.5$^{\&\#}$
Mitral E (cm/s)	83.2 (82 – 87.0)	81.4 (78.6 – 91.1)	133 (116 – 143)*	104 (93.0 – 118)$^{\&}$
Mitral A (cm/s)	50.3 (48.2 – 53.6)	55.4 (47.1 – 62.7)	23.1 (20.3 – 26.6)*	34.8 (19.7 – 64.5)$^{\&}$
E/A (cm/s)	1.60 ± 0.18	1.83 ± 1.34	5.63 ± 0.96*	3.88 ± 2.48$^{\&\#}$
TDI E' (average, cm/s)	5.65 ± 0.69	6.12 ± 0.71	5.11 ± 0.69*	5.52 ± 0.71$^{\&}$
TDI A' (average, cm/s)	3.84 ± 0.67	3.99 ± 0.37	2.78 ± 0.81*	3.51 ± 0.96$^{p=0.07}$
E/E' (cm/s)	15.3 (14.3 – 16.4)	14.0 (12.4 – 15.3)	24.8 (22.6 – 29.4)*	19.5 (16.6 – 23.0)$^{\&}$
E'/A' (cm/s)	1.47 (1.40 – 1.55)	1.54 (1.44 – 1.59)	1.98 (1.73 – 2.13)*	1.64 (1.53 – 1.95)$^{\&}$

ShamT, trained Sham; AS, sedentary aortic stenosis; AST, trained aortic stenosis; HR, heart rate; LVDD and LVSD, left ventricular (LV) diastolic and systolic diameters; PWDT, posterior wall diastolic thickness; AO, aorta; LA, left atrial diameter; RWT, relative wall thickness; MFS, Mesocardial fractional shortening; EFS, endocardial fractional shortening; PWSV, posterior wall shortening velocity; Tei index, myocardial performance index; TDI S, tissue doppler imaging (TDI) systolic velocity of mitral annulus; IVRT, isovolumetric relaxation time; EDT, E-wave deceleration time; E/A, ratio between early (E) to late (A) diastolic mitral inflow; TDI E' and A', TDI of early (E') and late (A') diastolic velocity of mitral annulus. Data are mean ± SD or median and interquartiles. $p < 0.05$. * *vs*. Sham; $^{\&}$ vs ShamT; $^{\#}$ *vs*. AS. (n = 14-16 each group).

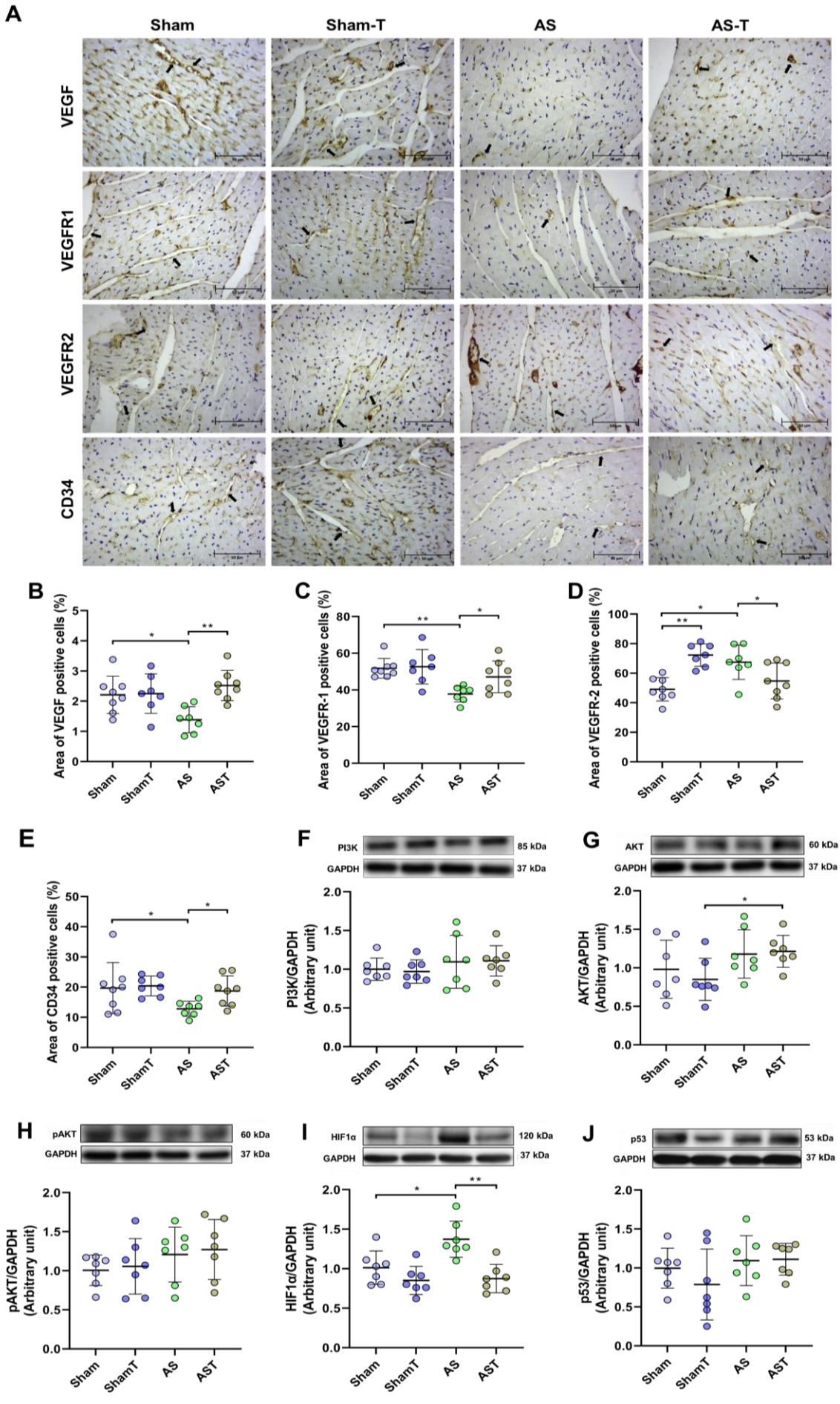
A
Sham
Sham-T
AS
AS-T
VEGF
VEGFR1
VEGFR2
CD34
B
Area of VEGF positive cells (%)
C
Area of VEGFR-1 positive cells (%)
D
Area of VEGFR-2 positive cells (%)
E
Area of CD34 positive cells (%)
F
PI3K
GAPDH
85 kDa
37 kDa
PI3K/GAPDH (Arbitrary unit)
G
AKT
GAPDH
60 kDa
37 kDa
AKT/GAPDH (Arbitrary unit)
H
pAKT
GAPDH
60 kDa
37 kDa
pAKT/GAPDH (Arbitrary unit)
I
HIF1α
GAPDH
120 kDa
37 kDa
HIF1α/GAPDH (Arbitrary unit)
J
p53
GAPDH
53 kDa
37 kDa
p53/GAPDH (Arbitrary unit)
Sham
ShamT
AS
AST

Figure 4 (A-E) Representative photographs of immunohistochemistry staining (A) and quantitation of the expression level of the VEGF, vascular endothelial growth factor (B); VEGFR-1, VEGF receptor type 1 (C) and 2 (D); and CD34, differentiation cluster 34 (E) in myocardium sections (indicated by black arrows). Scale bar, 50 µm. **(F-J)** Representative western blots and quantification of phosphoinositol 3 kinase, PI3K (F); protein kinase B, AKT (G); AKT phosphorylated at threonine 308, pAKT (H); hypoxia inducible factor 1-α (I); p53 tumor suppressor protein, p53 (J). AS, sedentary aortic stenosis; ShamT, trained Sham; AS, sedentary aortic stenosis; AST, trained aortic stenosis. Data are mean ± SD. * $p < 0.05$. ** $p < 0.001$. (n = 6-8 each group).

3.6. Exercise partially prevents disturbance in fatty acid metabolism pathway

The expression of a set of proteins involved in lipid uptake, translocation and oxidation was evaluated and is shown in Figure 5-I. The sedentary rats undergoing PO presented a reduction in the translocation of FA across the sarcolemma and mitochondrial membrane, and FA intracellular transport, as suggested by lower protein expression of CD36, CPT1β, and FABP3, respectively, in comparison to Sham. On the other hand, the AST rats displayed to be less influenced by PO than the sedentary ones, as visualized in the CD36 expression; however, ET did not alter the transport intracellular and mitochondrial of FA when compared with AS group (Figure 5-I A – C). We also evaluated the protein levels of enzymes that catalyze the steps of β-oxidation of saturate and unsaturated fatty acids. ACADL, an enzyme involved with the oxidation of unsaturated FA was found to be markedly diminished in aortic stenosis rats regardless of exercise. MCAD and DECR1, which act on the oxidation of medium-chain and polyunsaturated fatty acids, respectively, had their levels reduced by PO and recovered after training in AST rats, indicating a favorable impact of exercise in preserving myocardial mitochondrial β-oxidation (Figure 5-I D – F). As impaired β-oxidation contributes to energy imbalance status, we carried out the expression study of metabolic sensors AMPK and SIRT, and results are shown in Figure 5-II A-C; there was no difference between sedentary or exercised groups. Lastly, to study the regulatory mechanism underlying the FA utilization during HF, we

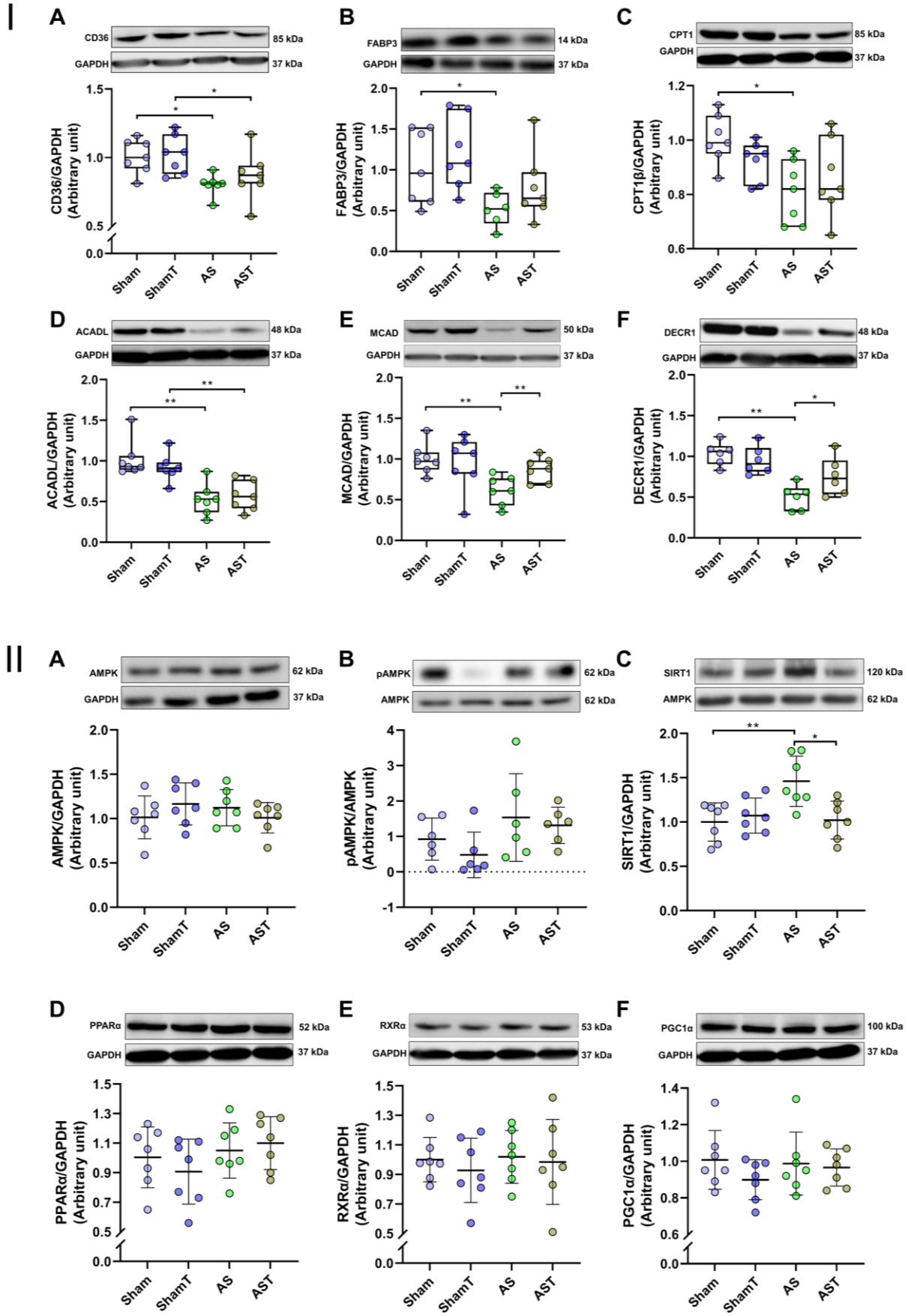
I
A
CD36
GAPDH
85 kDa
37 kDa
CD36/GAPDH (Arbitrary unit)
B
FABP3
GAPDH
14 kDa
37 kDa
FABP3/GAPDH (Arbitrary unit)
C
CPT1
GAPDH
85 kDa
37 kDa
CPT1β/GAPDH (Arbitrary unit)
D
ACADL
GAPDH
48 kDa
37 kDa
ACADL/GAPDH (Arbitrary unit)
E
MCAD
GAPDH
50 kDa
37 kDa
MCAD/GAPDH (Arbitrary unit)
F
DECR1
GAPDH
48 kDa
37 kDa
DECR1/GAPDH (Arbitrary unit)
Sham
ShamT
AS
AST
II
A
AMPK
GAPDH
62 kDa
37 kDa
AMPK/GAPDH (Arbitrary unit)
B
pAMPK
AMPK
62 kDa
62 kDa
pAMPK/AMPK (Arbitrary unit)
C
SIRT1
AMPK
120 kDa
62 kDa
SIRT1/GAPDH (Arbitrary unit)
D
PPARα
GAPDH
52 kDa
37 kDa
PPARα/GAPDH (Arbitrary unit)
E
RXRα
GAPDH
53 kDa
37 kDa
RXRα/GAPDH (Arbitrary unit)
F
PGC1α
GAPDH
100 kDa
37 kDa
PGC1α/GAPDH (Arbitrary unit)
Sham
ShamT
AS
AST

Figure 5 (I) Representative western blots and quantification of proteins involved with cardiac fatty acids translocation and intracellular transport, and β-oxidation. CD36, fatty acid (FA) translocase (A); FA-biding protein, FABP3 (B); carnitine palmitoyltransferase 1, CPT1β (C); acetyl-CoA dehydrogenase, ACADL (D); medium-chain acyl-CoA dehydrogenase, MCAD (E); 2,4-dienoyl-CoA reductase, DECR1 (F). **(II)** Representative western blots and quantification of metabolic sensors and transcriptional effectors. AMP-activated protein kinase, AMPK (A); phosphorylated AMPK, pAMPK (B); sirtuin 1, SIRT1 (C); peroxisome proliferator activated receptor-α, PPARα (D); receptor X retinoid, RXR (E); peroxisome proliferator-activated receptor gamma coactivator 1α, PGC1α (F). ShamT, trained Sham; AS, sedentary aortic stenosis; AST, trained aortic stenosis. Data are mean ± SD. * $p < 0.05$. ** $p < 0.001$. (n = 6-8 each group).

analyzed the expression level of proteins related to the transcription of genes linked to FA transport and oxidation. Interestingly, the downregulation of PPARα proposed as the mechanism to reduced FAO in hypertrophied hearts did not occur in our investigation; the protein level of the transcriptional effectors RXRα and PGC1α also remained unaffected in AS groups (Figure 5-II D – F). However, the downregulation of the downstream targets cited above might be related to a plausible influence of SIRT1 on PPARα signaling, which will be discussed below.

3.7. Metabolic enzyme activity is recovered in exercised aortic stenosis rats

Enzymatic assays were conducted to explore whether exercise could attenuate the impact of the cardiac PO on the activity of enzymes involved with FAO, citric acid cycle, and energy transfer. Significant reduction was observed in the activity of OHADH, CS, and CK of AS hearts (Figure 6 A – C). The AST group, however, presented all enzyme activities normalized at the level of their control and differed significantly from the operated sedentary group. Interestingly, creatine kinase and citrate synthase activities were found reduced in the ShamT.

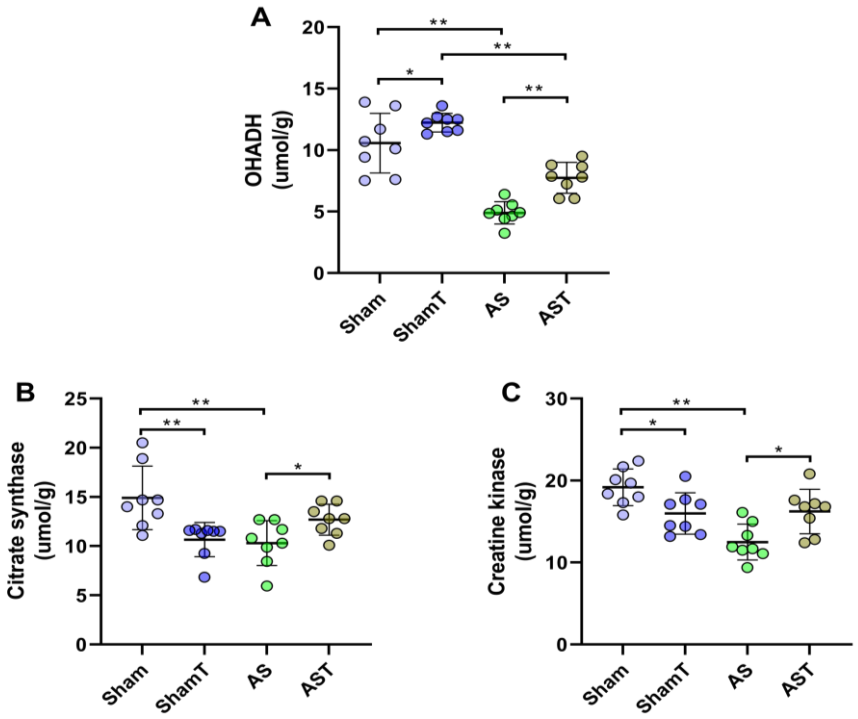

Figure 6. ShamT, trained Sham; AS, sedentary aortic stenosis; AST, trained aortic stenosis. OHADH, beta-hydroxyacyl-CoA dehydrogenase. Data are mean ± SD. * $p < 0.05$. ** $p < 0.001$. (n = 08 each group).

3.8. Left ventricle adenosine triphosphate content is maintained in both sedentary and exercised aortic stenosis rats

Interestingly, the myocardial ATP content did not differ between groups (Figure 7).

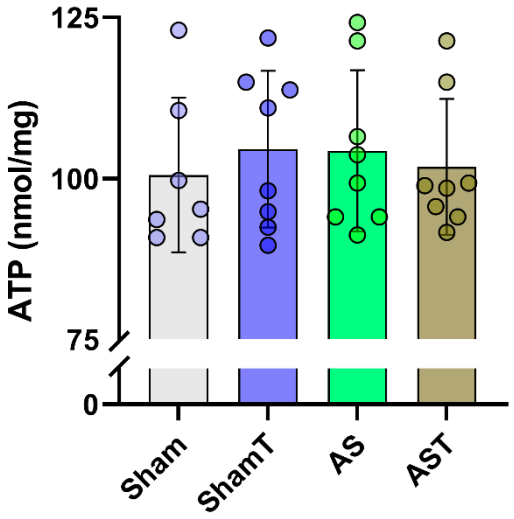

Figure 7 ShamT, trained Sham; AS, sedentary aortic stenosis; AST, trained aortic stenosis. ATP, adenosine triphosphate. Data are mean ± SD. $p < 0.05$.

3.9. Global correlation map reveals a panel of correlated parameters

To correlate the variables of myocardial angiogenesis, lipid energy metabolism, and cardiac function, we used a correlation matrix via Pearson's correlation, followed by clustering of variables. Figure 8 displays the major association results in the format of a heatmap. White squares indicate non-significant correlation coefficients and stronger coloration reflects more association and higher r2 values; blue and red colors indicate positive and negative correlation, respectively.

As expected, angiogenesis/capillary density was positively correlated with metabolic parameters; VEGF with *PPARα, FABP3, ACADL, MCAD, DECR1, OHADH, CK,* and *CS*; CD34 with *FABP3, CPT1β*, *ACADL, MCAD, DECR1,* and *OHADH.* We also found a negative correlation between angiogenic markers (VEGF, CD34) and sensors of the cellular metabolic stress (AMPK, SIRT1). In addition, HIF1α, as a hypoxia marker, was found negatively correlated with the lipid energy metabolic pathway (ACADL, DECR1, MCAD, OHADH, and CK), which suggests a high relationship between oxygen deficit and energy perturbation during HF.

Regarding the involvement of the parameters of lipid metabolism and cardiac function, the heatmap showed a positive correlation of proteins linked to FA transport and oxidation (CPT1β, FABP3, ACADL, DECR1, MCAD, and OHADH) with systolic variables (MFS, EFS, PWSV, TDI S'). Conversely, metabolic variables were inversely correlated with cardiac structural and diastolic parameters, except by SIRT1 that showed a positive correlation with hypertrophy and diastolic dysfunction indicators. Based on these results, lipid energy metabolism is primarily linked to systolic performance, while FAO decline occurs in parallel with pathological hypertrophy and diastolic dysfunction.

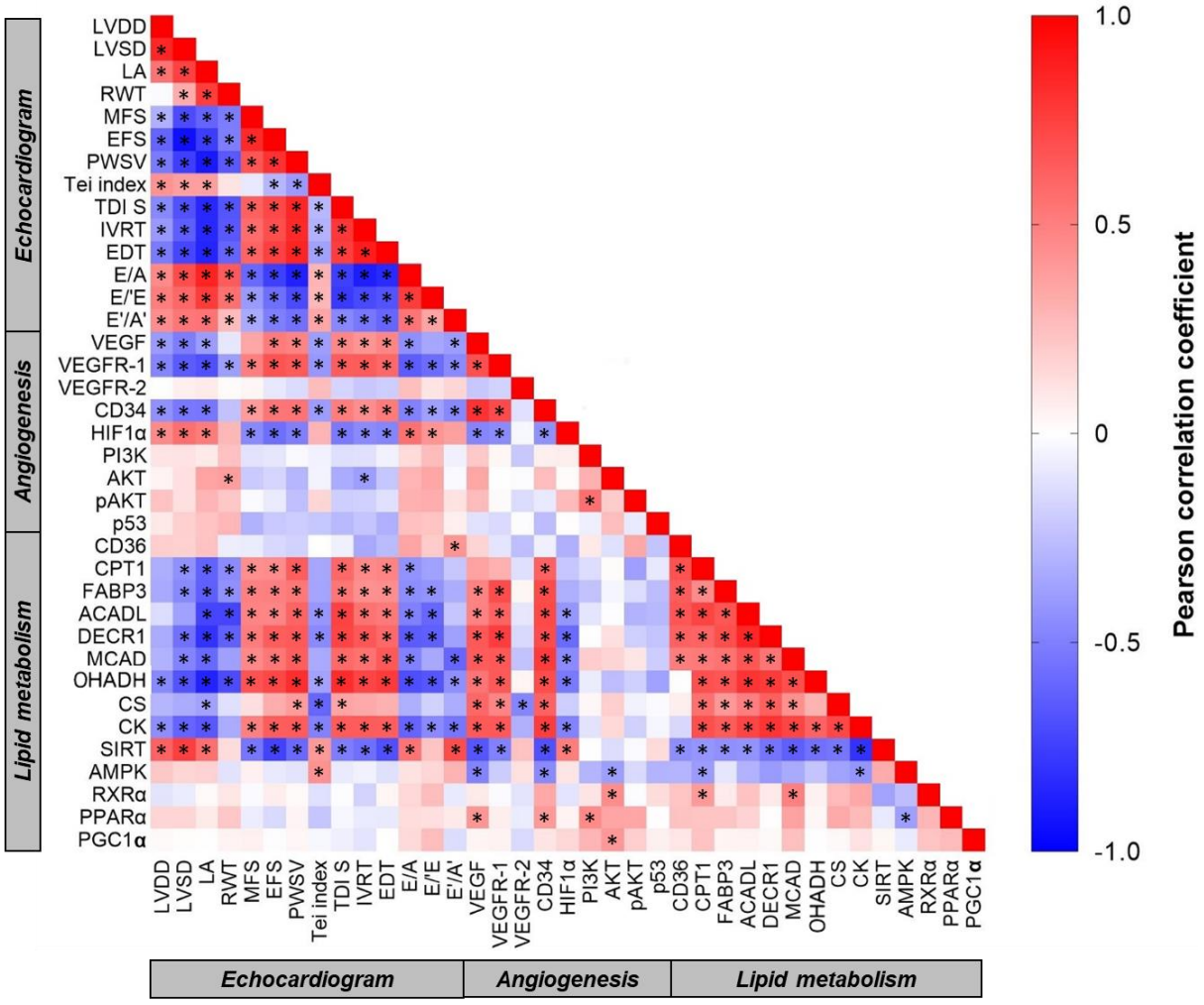

Figure 8 Global correlation map of angiogenesis, lipid energy metabolism, and cardiac function. Pairwise correlation of all parameters, resulting in a matrix of correlation coefficients where each variable is compared to all others. Red squares represent variables with a positive correlation with each other. The negative correlation is indicated in blue squares. LVDD and LVSD, left ventricular (LV) diastolic and systolic diameters; LA, left atrium; RWT, relative wall thickness; MFS, Mesocardial fractional shortening; EFS, endocardial fractional shortening; PWSV, posterior wall shortening velocity; Tei index, myocardial performance index; TDI S, tissue doppler imaging (TDI) systolic velocity of mitral annulus; IVRT, isovolumetric relaxation time; EDT, E-wave deceleration time; E/A, ratio between early (E) to late (A) diastolic mitral inflow; E/E', ratio between E and TDI of early diastolic velocity of mitral annulus (E'); E'/A', ratio between E' to TDI of late diastolic velocity of mitral annulus; VEGF, vascular endothelial growth factor; VEGFR1 and VEGFR2, VEGF receptor type 1 and 2; CD34, differentiation cluster 34; HIF1α; hypoxia inducible factor 1-α; PI3K, phosphoinositol 3 kinase, AKT, protein kinase B; p53, p53 tumor suppressor protein; CD36, fatty acid (FA) translocase; CPT1, carnitine palmitoyltransferase, FABP3, FA-binding protein; ACADL, acyl-CoA dehydrogenase; DECR1, 2,4-dienoyl-CoA reductase; MCAD, medium-chain acyl-CoA dehydrogenase; OHADH, beta-hydroxyacyl-CoA dehydrogenase; CS, citrate sintase; CK, creatine kinase; AMPK, AMP-activated protein kinase; PPARα, peroxisome proliferator activated receptor-α; RXRα, receptor X retinoid-α; SIRT1, sirtuin 1; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1α. The data were analyzed by Graphpad Prism 8 using 2-tailed Pearson's correlation test. Symbol (*) indicates a statistically significant correlation ($p < 0.05$).

4. DISCUSSION

Searching for the therapeutic approaches for the management of heart diseases remains a challenge and non-pharmacological strategies are emerging as an effective tool for prevention and treatment.[59] Here, we showed that 1) early exercise training had a protective effect against AS-induced pathological remodeling and cardiac dysfunction in rats, 2) corrected vascular rarefaction in the myocardium, and 3) re-established myocardial lipid metabolism during HF. These benefits were accompanied by a significant alleviation of the HF profile including functional capacity maintenance.

For this report, we adapted a training protocol from previous studies that found benefits on functional capacity and cardiac function with different experimental models of cardiac disease.[50,52,60,61] Consistent with the earlier investigations,[31,50,62–64] both trained groups presented higher functional capacity than sedentary Sham and AS groups. These data reinforce the efficacy of the used exercise protocol in improving exercise tolerance in normal or pathological conditions.

Numerous previous studies attempt to rescue deficient cardiac function in aortic stenosis rats using a late exercise program intervention that resulted in inconsistent conclusions.[31,33,50,63–66] These studies started the training after the worsening of the structural and functional cardiac disturbance, and different degrees of systolic disability probably determined controversial results. Thus, the timing of the ET after aortic stenosis has not yet been optimized. As in sustained PO the remodeling becomes maladaptive early on[67] leading to incompetence of adaptive molecular signaling pathways,[5,68] for this study, we started the ET program before severe cardiac hypertrophy and dysfunction took place. The assumption that early ET could alleviate the dysfunctional scenario of PO heart raises from the recognized potential of the

physiological signaling of exercise in counteracting the pathological signaling cascades involved with adverse heart remodeling and failure.[43]

Regarding cardiac structure, our data demonstrate that AST rats displayed a consistent alleviation of the hypertrophic process as visualized in the weight of chambers and structural parameters of echocardiographic assessment. Following the effect on cardiac structure, both diastolic, and systolic functional impairment were significantly attenuated by ET. Additionally, exercise reduced the occurrence of tachypnea, pleural effusion, atrial thrombi, liver congestion, and ascites, classical symptoms of HF observed in experimental trials.[50,61,63] Collectively, the outcomes confirm our first assumption that early ET can alleviate PO-induced HF, as well as allow us to suggest that early moderate aerobic exercise is safe in young AS rats.

We mainly aimed to investigate the underlying mechanisms involved with the benefits of exercise on the failing heart. Several studies have documented that transition from hypertrophy towards HF is associated with impaired myocardial capillary density,[4,69–71] which is then unable to support oxygen and nutrient demands imposed by chronic PO.[72] We presently hypothesized that early exercise would lead to the reactivation of the angiogenic process. As expected, aortic stenosis rats presented a significant reduction of myocardial capillary density assessed by local CD34 expression, which was associated with impaired expression of VEGF, as largely reported.[72,73] In contrast, exercise prevented the blunting of capillarization after AS by maintaining a normal expression of VEGF and its receptors. Our findings corroborate lines of evidence showing that ET induces a myocardial angiogenic phenotype characterized by activation of the VEGF pathway in several experimental models,[44,46,47,74,75], and improve the knowledge about the angiogenic scenario in failing AS heart.

The myocardial hypertrophy causes hypoxia and HIF1-α overexpression, triggering angiogenesis.[5] However, the prolongation of hypertrophy leads to inhibition of angiogenesis secondary to HIF1-α inactivation via p53 overexpression.[76] The role of raised HIF1-α in the activation of VEGF gene transcription in hypoxic tissue is well established.[77] Our data reveal that the preservation of VEGF levels and capillary density in hearts of AST occurred in parallel with reduced HIF1-α protein content compared to the sedentary AS group, which presented HIF1-α overexpression. These results strengthen the assumption that early exercise, by angiogenesis supporting, can mitigate the PO-induced myocardial hypoxia, as the normalized HIF1-α expression points out. In contrast to previous literature,[76] AS hearts did not display p53 overexpression, which could be involved with HIF1-α disability, blunting the angiogenic signaling in the AS group; earlier studies have also demonstrated that protein expression of HIF1-α is mitigated by exercise in the failing myocardium.[78,79] Additionally, PI3K and AKT, predicted to be involved with ET proangiogenic effect,[43,80] were also unaffected by PO or exercise. Overall,

The disturbances of myocardial hypoxia are numerous, and dysregulation of energy substrate metabolism is an important factor underlying such consequences.[81] Based on the above findings related to angiogenesis and hypoxia, we tested whether myocardial lipid metabolism is also associated with cardioprotection promoted by early exercise. This approach is essential since increasing evidence demonstrates that energy metabolic derangements contribute to the pathogenesis of cardiac functional decompensation,[25] and that impaired FAO precedes the onset of the PO-induced HF.[82]

To the best of our knowledge, we provide the first scientific literature about the effects of early ET on cardiac lipid metabolism in AS-induced HF. Our results

showed that pathological hypertrophy was accompanied by a significant disturbance in the FA utilization pathway. AS rats presented a reduced expression of key components involved with fatty acid transport and β-oxidation. Conversely, the impact of PO on the FA pathway was less important in the exercised group, as visualized in the results session, which suggests that ET in course of HF attenuates the pathological pattern of energy generation from FA. In line with present data, the downregulation of genes involved in FA metabolism, from uptake to cleavage within the mitochondrial β-oxidation cycle, is widely shown.[83–85] However, although studies conducted in rats revealed that treadmill running triggered a distinct cardio-metabolic gene profile compared to PO or myocardial infarction,[86,87] the paucity of data studying the influence of exercise on the modulation of myocardial metabolism in the AS failing heart does not allow us to compare our results. Nevertheless, the metabolic coordination induced by ET has been also shown by experimental studies with myocardial infarction,[36,37,39,88] aortic regurgitation,[35] and spontaneously hypertensive rats.[38]

The mechanisms associated with the decreased FAO pathway appear to be related to a transcriptional disorder that includes transcription factors, especially PPARα.[22,25] PPARα heterodimerizes with RXRα and recruits PGC1α to promoving transcription of PPARα targets involved with lipid metabolism, including FA uptake, transport, and β-oxidation.[89] It has been described that decreased expression of PPARα enrolls part in the downregulation of genes of FA metabolism, however, its decreased expression is not uniformly reported in PO-induced HF.[25] In the present stud, PPARα protein level was similar between groups, as well as RXRα and PGC1α. Our data even indicate that concomitant maintenance of PPARα and RXRα in the AS group did not prevent damage in the cardiac FAO pathway, as described by others,[21,22] suggesting that protein level of PPARα *per se* is not the only factor that leads to

metabolic imbalance during HF. Noteworthy, downregulation of the lipid metabolism has been linked to a mechanism related to an atypical interaction between PPARα and Sirt1;[24,25] Sirt1 overexpression favors the increased formation of PPARα-Sirt1 complexes over PPARα-RXRα, which has a detrimental influence on the transcription of some target genes involved in FAO.[24] As we found overexpressed Sirt1 in AS hearts, it is likely that PPARα-RXRα activity was impaired, responding, at least in part, by the diminished FAO. This hypothesis is plausible since the exercised group presented SIRT1 expression at the level of control, accompanied by a less impairment of lipid metabolism. Thus, ET might be involved with the balancing of transcriptional regulation of FAO signaling genes throughout pathological heart remodeling in aortic stenosis.

It is widely accepted that one characteristic of the failing heart is persistent and progressive loss of ATP as well as phosphocreatine.[90] Interestingly, in our experiment the myocardial ATP pool did not differ between groups, which would be expected due to important cardiac dysfunction and energy metabolism stress. On the other hand, the activity of CK was significantly reduced in AS group, which can bring down the phosphocreatine supply and consequently increase the free adenosine diphosphate (ADP), which is involved with the inhibition of a set of intracellular enzymes, including that of contractile mechanism.[91] In AS rats, despite ATP maintenance, it is highly likely that reduced CK activity and damaged lipid metabolic behavior contributed to the HF installation. In contrast, attenuated cardiac dysfunction in AST group occurred together with CK activity maintenance and significantly preserved FAO.

Global correlation map adds weight to the results that revealed a beneficial influence of the exercise on the restoration of angiogenesis and lipid energy metabolism. It was demonstrated a positive correlation between variables of

angiogenesis and FA signaling, as well as the negative correlation between cardiac structure and function with both angiogenesis and metabolism. In a simple way, as hypertrophy progress, capillary rarefaction occurs and lipid metabolism declines. Our data, on the other hand, suggest that exercise rescues capillary density and FAO, even in the setting of important hypertrophy.

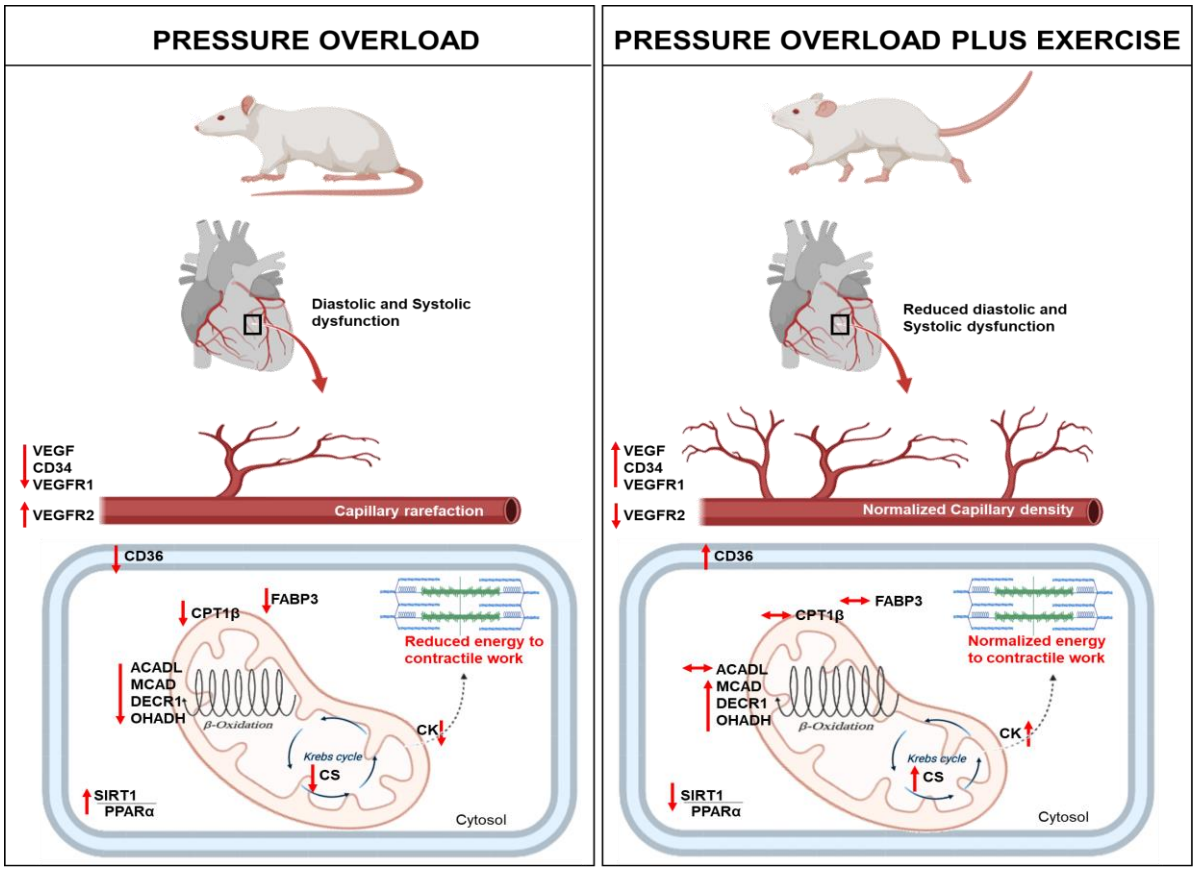

Figure 9. Overview of the effects of pressure overload (PO) and PO plus exercise on cardiac function, angiogenesis and lipid energy metabolism. VEGF, vascular endothelial growth factor; VEGFR-1 and VEGFR2, VEGF receptor type 1 and 2; CD34, differentiation cluster 34; CD36, fatty acid (FA) translocase; CPT1, carnitine palmitoyltransferase, FABP3, FA-binding protein; ACADL, acyl-CoA dehydrogenase; DECR1, 2,4-dienoyl-CoA reductase; MCAD, medium-chain acyl-CoA dehydrogenase; OHADH, beta-hydroxyacyl-CoA dehydrogenase; CS, citrate sintase; CK, creatine kinase; PPARα, peroxisome proliferator activated receptor-α; SIRT1, sirtuin 1.

5. Conclusions

In conclusion, early exercise training expressively attenuated cardiac structural and functional remodeling, reduces HF symptoms, and improve functional capacity of aortic

stenosis rats. For these results, we demonstrate that early exercise triggers a more consistent effect on the management of AS-induced HF. Regarding the underlying mechanisms involved with the beneficial effects of ET in AS rats, we provide evidence that attenuation of cardiac dysfunction is linked to the preservation of lipid energy metabolism, which in turn is related to angiogenesis restoration.

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Disclosure statement

The authors declare that there are no conflicts of interest regarding the publication of this article.

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SUPPLEMENTARY INFORMATION

Supplemental Table 1. Initial echocardiogram (2th-week post-surgery)

	Sham	Sham-T	AS	AS-T
HR (bpm)	389 ± 59.7	396 ± 59.5	434 ± 56.8	395 ± 64.3
LVDD (mm)	6.02 ± 0.38	5.91 ± 0.53	5.23 ± 0,52	5.72 ± 0.88
LVSD (mm)	2.30 ± 0.52	2.18 ± 0.09	2.10 ± 0.23	2.13 ± 0.47
AO (mm)	2.96 ± 0.13	3.00 ± 0.22	3.25 ± 0.23	3.02 ± 0.12
LA (mm)	3.29 ± 0.47	3.40 ± 0.40	3.87 ± 0.21*	4.16 ± 0.54$^{\&}$
LA/AO (mm/g)	1.11 ± 0.14	1.13 ± 0.10	1.19 ± 0.07	1.37 ± 0.15$^{\&\#}$
RWT (%)	0.44 ± 0.03	0.46 ± 0.07	0.71 ± 0.13†	0.62 ± 0.09$^{\&}$
MFS (%)	29.7 ± 2.33	31.2 ± 4.49	25.7 ± 3,41	28.6 ± 3.36
EFS (%)	62.1 ± 6.06	62.8 ± 2.92	59.9 ± 1.39	62.9 ± 5.02
PWSV (ms)	80.0 ± 4,55	78.0 ± 8.55	71.1 ± 5.61	72.0 ± 6.83
Tei index	0.26 ± 0.07	0.24 ± 0.09	0.25 ± 0.08	0.25 ± 0.07
TDI S (average, cm/s)	5.25 ± 0.10	5.60 ± 0.45	5.51 ± 0.46	5.74 ± 0.45
TRIV (ms)	20.2 ± 3.50	21.8 ± 1.98	19.4 ± 4.28	21.4 ± 2.19
EDT (ms)	42.2 ± 3.77	47.4 ± 5.48	42.9 ± 7.99	44.8 ± 0.83
Mitral E (cm/s)	103 ± 13.8	107± 6.82	108 ± 12.9	107 ± 11.6
Mitral A	69.8 ± 20.0	72.3 ± 7.58	74.8 ± 13.4	78.0 ± 19.6
E/A (cm/s)	1.53 ± 0.26	1.48 ± 0,11	1.45 ± 0.11	1.43 ± 0.29
TDI E' (average, cm/s)	5.85 ± 0.68	6.44 ± 1.43	5.75 ± 0.32	6.86 ± 0.51
TDI A' (average, cm/s)	3.75 ± 0.52	4.36 ± 0.43	5.41 ± 1.60	4.34 ± 0.79
E/E' (cm/s)	17.6 ± 1.06	17.5 ± 5.02	18.7 ± 2.06	15.6 ± 1.00
E'/A' (cm/s)	1.57 ± 0.07	1.50 ± 0.38	1.32 ± 0.42	1.61 ± 0.21

ShamT, trained Sham; AS, sedentary aortic stenosis; AST, trained aortic stenosis; HR, heart rate; LVDD and LVSD, left ventricular (LV) diastolic and systolic diameters; PWDT, posterior wall diastolic thickness; AO, aorta; LA, left atrial diameter; RWT, relative wall thickness; MFS, Mesocardial fractional shortening; EFS, endocardial fractional shortening; PWSV, posterior wall shortening velocity; Tei index, myocardial performance index; TDI S, tissue doppler imaging (TDI) systolic velocity of mitral annulus; IVRT, isovolumetric relaxation time; EDT, E-wave deceleration time; E/A, ratio between early (E) to late (A) diastolic mitral inflow; TDI E' and A', TDI of early (E') and late (A') diastolic velocity of mitral annulus. Data are mean ± SD or median and interquartiles. $p < 0.05$. * *vs*. Sham; $^{\&}$ vs ShamT; $^{\#}$ *vs*. AS. (n = 14-16 each group).

Adaptation period (days)	Intensity (m/min)	Time (min)
1º	5	10
2º	5	15
3º	5	20
4º	5	25
5º	5	30
Treadmill exercise test 1		
Exercise Training Program		
Weeks	Intensity (% ES)	Time (min)
1ª	60	30
2ª	60	40
3ª	60	50
4ª	60	60
Treadmill exercise testing 2		
5ª	60	60
6ª	60	60
7ª	60	60
8ª	60	60
Treadmill exercise testing 3		
9ª	60	60
10ª	60	60
11ª	60	60
12ª	60	60
Treadmill exercise testing 4		
13ª	60	60
14ª	60	60
15ª	60	60
16ª	60	60
Final treadmill exercise test		

Supplemental square 1. Training exercise protocol. ES, exhaustion speed achieved during treadmill exercise testing.

ANEXO A – Parecer da CEUA (Comissão de Ética no Uso de Animais) da Faculdade de Medicina de Botucatu, Unesp.

unesp

UNIVERSIDADE ESTADUAL PAULISTA
CAMPUS DE BOTUCATU
FACULDADE DE MEDICINA

FMB

FACULDADE DE MEDICINA
BOTUCATU

CEUA

Comissão de Ética no Uso de Animais

Criada através da Portaria DFM nº 611 de 13/12/2012

CERTIFICADO Nº 1239/2017-CEUA

Certificamos que a proposta intitulada "**Influência do treinamento físico na atenuação da disfunção cardíaca de ratos com estenose aórtica supravalvar: participação da densidade capilar e metabolismo enérgico lipídico miocárdico**", registrada com o nº 1239/2017, sob a responsabilidade de Sérgio Luiz Borges de Souza, orientado pelo Prof. Dr. Antonio Carlos Cicogna, com a colaboração de Vitor Loureiro da Silva, Profa. Dra. Silméia Garcia Zanati Bazan, Gustavo Augusto Ferreira Mota, Dijon Henrique Salomé de Campos – que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei n. 11.794, de 8 de outubro de 2008, do Decreto n. 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais da Faculdade de Medicina de Botucatu, em reunião de 31 de agosto de 2017.

Finalidade ( ) Ensino (X) Pesquisa Científica	
Vigência da autorização	31/03/2018
Espécie/Linhagem/Raça	Ratos Wistar
Nº de animais	90
Peso/Idade	70 gramas/ 21 dias
Sexo	Macho
Origem	Biotério Central da UNESP - FMB

Prof. Dr. Guilherme Antônio Moreira de Barros
Presidente da CEUA

Alberto Santos Capelluppi
Secretário da CEUA

Distrito Rubião Junior, s/nº - Botucatu – S.P. CEP: 18.618-970 Fone: (14) 3880-1608/3880-1609 e-mail secretaria: ceua@fmb.unesp.br

ANEXO B – Artigos publicados durante o doutorado

- Da Silva, V.L, **de Souza, S.L.B**, Mota, G.A.F, Melo, A.B, Vileigas, D.F, Coelho, P.M, Sant'Ana, P.G, Campo, D.H.S, Bazan, S.G.Z, Leopoldo, A.S, Cicogna, A.C. The dysfunctional scenario of the major componentes responsible for myocardial calcium balance in heart failure by aortic stenosis. *Archivos Brasileiros de Cardiologia. 2021.*

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