UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Marcadores Biológicos de Adaptação ao Treinamento Esportivo: Salivares e Sanguíneos

Aluno: Miguel Mauricio Díaz Gómez

Orientador: Prof. Dr. Foued Salmen Espindola

UBERLÂNDIA
2014

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Marcadores Biológicos de Adaptação ao Treinamento Esportivo: Salivares e Sanguíneos

Aluno: Miguel Mauricio Díaz Gómez

Orientador: Prof. Dr. Foued Salmen Espindola

Tese apresentada à Universidade Federal de Uberlândia como parte dos requisitos para obtenção do Título de Doutor em Genética e Bioquímica. Área: Bioquímica.

UBERLÂNDIA
2014

Dados Internacionais de Catalogação na Publicação (CIP)
Sistema de Bibliotecas da UFU, MG, Brasil.

D542m
2014

Díaz Gómez, Miguel Mauricio, 1983-
Marcadores biológicos de adaptação ao treinamento esportivo: salivares e sanguíneos / Miguel Mauricio Díaz Gómez. - 2014.
100 f. : il.

Orientador: Foued Salmen Espindola.
Tese (doutorado) - Universidade Federal de Uberlândia, Programa de Pós-Graduação em Genética e Bioquímica.
Inclui bibliografia.

1. Bioquímica - Teses. 2. Marcadores biológicos - Teses. 3. Saliva - Teses. 4. Sangue - Teses. I. Espindola, Foued Salmen. II. Universidade Federal de Uberlândia. Programa de Pós-Graduação em Genética e Bioquímica. III. Título.

CDU: 577.1

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Marcadores Biológicos de Adaptação ao Treinamento Esportivo: Salivares e Sanguíneos

ALUNO: Miguel Mauricio Díaz Gómez

COMISSÃO EXAMINADORA

Presidente: Prof. Dr. Foued Salmen Espindola (Orientador).

Examinadores:

Prof. Dr. Candido Celso Coimbra
Prof. Dr. Rinaldo Wellerson Pereira
Prof. Dr. Guilherme Morais Puga
Prof. Dr. Robinson Sabino

Data da Defesa: 11/02/2014

As sugestões da Comissão Examinadora e as Normas PGGB para o formato da Tese foram contempladas.

_____
Prof. Dr. Foued Salmen Espindola

Dedicatória

A Foued Salmen Espindola

Agradecimentos

Aos cidadãos brasileiros que, com seus impostos, permitem o acceso a uma educação gratuita; À CAPES, pelo investimento desses recursos financeiros no fomento de uma educação de qualidade.

SUMÁRIO

Dedicatória **iv**

Agradecimentos **v**

Apresentação **8**

Capítulo 1 **10**

Fundamentação Teórica **10**
Referências *16*

Capítulo 2 **19**

Differential Expression of Circulating miRNAs Following Resistance Exercise and Carbohydrate/Protein Supplementation **19**
Resumo *20*
Abstract *21*
Introduction *22*
Materials and Methods *24*
Subjects 24
Design 24
Diet and supplementation 25
RNA extraction 26
RT-qPCR 26
Statistical Analysis *27*
Results *27*
Discussion *28*
Acknowledgements *33*
References *34*
Figure Legends *43*
Figure 1 *44*
Figure 2 *45*
Figure 3 *46*

Capítulo 3 **47**

Salivating over Biological Markers; A Call for a Closer Collaboration between Coaches and Sports Scientists **47**
Resumo *48*
Abstract *49*
Introduction *50*
Training load *52*
Fatigue *55*
Lactate *55*
Hydration status *57*
Performance *59*
Other applications *60*
Conclusions *61*
References *63*
Table 1 *69*

Capítulo 4 **80**

Salivary Surrogates of Plasma Nitrite and Catecholamines During a 21-week Training Season in Swimmers **80**
Resumo *81*
ABSTRACT *82*
INTRODUCTION *83*
METHODS *85*
Ethics Statement 85
Design 85
Sample collection 86
Catecholamines 86
Nitrite 87
Salivary proteins 87
Determination of sAA activity 88
STATISTICAL ANALYSIS *88*
RESULTS *89*
Markers of autonomic activity 89
Nitrite 89
Correlation between biochemical markers and training outcomes 89
DISCUSSION *90*
REFERENCES *95*
Table 1 *101*
Table 2 *102*
Table 3 *103*
Figure 1 *104*
Figure 2 *105*
Figure 4 *107*
Figure 5 *108*

Apresentação

Os estudos apresentados nesta tese foram delineados com o intuito de 1) examinar a o potencial de microRNAs (miRNAs) circulantes no sangue na identificação de processos moleculares associados ao exercício físico e 2) o potencial da saliva como ferramenta na valoração da adaptação ao treinamento esportivo em atletas profissionais. Por um lado, a recente identificação de miRNAs tornou possível um melhor entendimento de processos moleculares que controlam diversos estados fisiopatológicos. A pesquisa envolvendo miRNAs é de grande relevância clínica já que os miRNAs regulam quase dois terços do genoma de mamíferos. Os miRNAs podem ser secretados no sangue em microvesículas e da mesma forma que hormônios, podem exercem funções em células alvo. Infelizmente, embora o exercício físico seja uma das ferramentas mais eficazes para a manutenção da saúde, a pesquisa sobre miRNAs nesta área ainda é limitada. Assim, no capitulo 1 são resumidos os estudos sobre miRNAs circulantes e exercício físico enquanto que no capitulo 2 são descritos os resultados experimentais da mensuração de 12 miRNAs circulantes em sujeitos fisicamente ativos submetidos a uma sessão de exercício resistido seguido de suplementação nutricional.

Por outro lado, a saliva representa uma alternativa interessante como matriz para a busca de marcadores biológicos na medicina do esporte. Principalmente devido à facilidade na sua coleta, manipulação e armazenamento. A identificação de biomarcadores salivares de adaptação ao treinamento esportivo tem sido uma das linhas de pesquisa do nosso laboratório durante os últimos. Assim, na segunda parte desta tese são apresentados dois manuscritos que discutem o raciocínio cientifico por trás da busca de marcadores biológicos e sua aplicação durante uma temporada de treinamento em atletas profissionais. O Capitulo 3 é um manuscrito de opinião e revisão sobre o estado atual da literatura em marcadores salivares aplicados ao esporte. Na nossa opinião, é necessário um melhor entendimento sobre a informação que um biomarcador deve oferecer. Infelizmente a maioria dos estudos

publicados ate agora carecem do potencial de aplicação à medicina do esporte pois não identificam condições fisiológicas de adaptação, recuperação, ou desempenho durante o treinamento. Além disso, uma interpretação incorreta sobre o comportamento de moléculas alvo tem levado a muitos autores a considerar como biomarcador a qualquer molécula que apresente uma diferença estatística significativa em resposta ao exercício quando comparada aos níveis de repouso. Assim, neste capitulo chamamos à reflexão sobre como abordar a busca de biomarcadores na saliva discutindo os estudos que na nossa opinião tem mostrado de fato potencial na preparação de atletas profissionais. No Capitulo 4, apresentamos evidencia de como proteínas e metabolitos na saliva podem ser utilizados para monitorar a carga de treinamento em nadadores de elite durante cinco meses de treinamento. Além de apresentar uma forte correlação com a variação na intensidade e carga de treinamento, estes marcadores salivares, principalmente as proteínas, apresentaram a mesma dinâmica que clássicos marcadores de adaptação ao exercício como as catecolaminas no sangue.

Capítulo 1

Fundamentação Teórica

microRNAs e Exercício Físico[1]

A recente identificação de microRNAs (miRNAs) tornou possível o melhor entendimento de processos moleculares que controlam diversos estados fisiopatológicos[1]. Os miRNAs são pequenas moléculas de RNA endógeno não codificadores de proteínas que possuem aproximadamente de 18 a 24 nucleotídeos em extensão. Estes miRNAs atuam na célula regulando a expressão gênica de RNAs mensageiros (mRNA) alvos ao nível pós transcricional[2]. A regulação gênica mediada por miRNAs ocorre através do silenciamento do gene alvo ou através de sua degradação. Estes processos são direcionados devido a uma complementaridade entre as moléculas efetoras, miRNAs, e regiões específicas dos respectivos mRNA alvos, mais precisamente na região 3' UTRs (3' não traduzidas)[3]. Os miRNAs também podem atuar ao nível de cromatina levando a metilação do DNA em regiões promotoras e, portanto, podendo afetar a expressão de genes-alvo [3]. Os miRNAs são considerados reguladores essenciais de processos intracelulares de expressão gênica inerentes à adaptação ao exercício tais como angiogênese[4], inflamação[5], metabolismo mitocondrial[6] e regeneração muscular [7,8].

Neste contexto, foi comprovado por exemplo, que o miR-696 é altamente sensível à prática de exercício aeróbico em ratos [9]. A expressão do miR-696

[1] A fundamentação teórica aqui apresentada descreve de forma sucinta o papel de microRNAs circulantes como reguladores de processos moleculares e fisiológicos em resposta ao exercício, assim como seu potencial como marcadores de adaptação física e recuperação. O presente capitulo introduz ao leitor ao estudo experimental conduzido com microRNAs (capitulo 2). O leitor encontrará que a fundamentação teórica para o uso de marcadores salivares no monitoramento do treinamento esportivo professional é apresentada como manuscrito de revisão no capitulo 3 e, em consequência, não será abordada aqui.

diminui após o exercício aeróbico enquanto que após imobilização unilateral dos membros inferiores esta expressão encontra-se aumentada. Interessantemente, o miR-696 controla a expressão do gene do receptor ativado por proliferadores de peroxissoma gama (PPARg), regulador central da biogênese mitocondrial [9]. Devido à função dos miRNAs no silenciamento de genes alvo, a expressão do mRNA e da proteína PPARg foi maior quando os animais foram submetidos ao exercício físico. Estes resultados fornecem fortes evidências, ao nível molecular, da importância dos miRNAs no controle da expressão gênica em diferentes situações fisiológicas geradas pelo exercício físico e treinamento.

Recentemente, foi descoberto que os miRNAs são secretados na corrente sanguínea no repouso, em resposta à lesão muscular assim como a diferentes condições patológicas[10–12]. Estes miRNAs circulantes (c-miRNAs) encontram-se principalmente em microvesículas e, em consequência, são mais resistentes a degradação por nucleases [13]. Além disso, os c- miRNAs podem ser transportados desde o corrente sanguíneo e regular funções no interior de células alvo[13]. Os exosomas podem ser formados por dobras da membrana celular ao interior da célula levando à formação de microvesículas que subsequentemente se fundem com a membrana plasmática liberando os exosomas na circulação[14]. Além do sangue, exosomas ricos em miRNA são encontrados em outros fluidos como a saliva, lagrimas e leite materno[15].

Evidencia recente sugere que o empacotamento de miRNA em exosomas não é aleatório e os níveis de expressão de c-miRNAs são diferentes aos da célula de origem[13]. Quase o 30% dos miRNA secretados não reflete o perfil de expressão das células de origem, o que sugere que miRNAs específicos são selecionados para serem mantidos no meio interior ou secretados em exosomas[16]. O mecanismo detalhado da secreção de c-miRNAs ainda não foi estabelecido. No entanto, foi comprovado que a esfingomielinase neutra tipo 2, a enzima limitante da taxa de conversão na biossíntese de ceramida, é responsável pela secreção dos exosomas. Além das microvesículas, outras

vesículas extracelulares como exosomas, corpos apoptóticos assim como as proteínas HDL, LDL e argonauta estão envolvidos no processo de secreção e transporte de c-miRNAs[17]. Assim, considera-se que os c-miRNAs, de forma similar aos hormônios, determinam varias interações não somente entre células mas também entre diferentes tecidos.

miRNAs específicos a um tecido são aqueles com um nível de expressão maior a 20 vezes sua expressão em outros tecidos[18]. Recentemente, foi reportado que vários miRNAs altamente expressos no músculo, mioMIRs, podem ser detectados no plasma e no soro e cujos níveis mudam em desordens musculares. Por exemplo, os níveis séricos de miR-1, miR-133a, e miR-206 são maiores na síndrome de distrofia muscular de Duchenne (DMD), no modelo de distrofia muscular deficiente de distrofina em camundongos (mdx), assim como na distrofia muscular canina ligada ao cromossomo X quando comparados com animais normais[19]. A maior expressão de miR-1, miR-133a, miR-133b, e miR-206 também tem sido demostrada em pacientes com DMD quando comparados a sujeitos controles da mesma idade[20,21]. Finalmente, o grupo de Karolina e colaboradores demonstrou no 2011 que os níveis circulantes de miR-144, são maiores em humanos e outros animais com diabetes tipo 2. Este aumento esta negativamente correlacionado com o substrato de receptor de insulina 1e em consequência, a elevação de c-miR-144 pode estar associada ao desenvolvimento da resistência à insulina no musculo esquelético[22].

Ainda que vários c-miRNAs tenham sido propostos como biomarcadores de doenças, poucos estudos tem avaliado a dinâmica de c-miRNAs em função do exercício. O primeiro relato foi publicado no 2011 e neste os autores reportaram a expressão diferenciada dos c-miRNAs -20a, -21, -146a, -221 e 222 após três meses de treinamento[23]. Embora o origem destes miRNA ainda não seja conhecido, especula-se que além do músculo, o endotélio e células do sistema imune possam contribuir ao seu aumento no plasma. Correlações positivas entre os níveis de miR-146 e o consumo máximo de oxigênio (VO_{2max})

também foram relatadas[23]. Resultados similares foram reportados pelo grupo de Bye e colaboradores no 2013, onde c-miR-21, -210 e -222 apresentaram maiores níveis de expressão no grupo de sujeitos com baixo VO_{2max}[24]. Embora nenhuma correlação foi encontrada entre fatores de risco de doenças cardiovasculares com os níveis de c-miRNAs, os autores sugeriram que estes c-miRNAs podem ser considerados como marcadores de aptidão física e futuro risco de desenvolvimento de doença cardiovascular.

Recentemente, o grupo de Aoi e colaboradores investigaram o efeito de um programa de exercício sobre os níveis circulantes de miRNAs específicos ao músculo esquelético em sujeitos sedentários. Foi encontrado que um grande numero de mioMIRs (miR-1, -133a, -133b, -206, -208b, e -499) apresenta uma baixa expressão no soro. Além disso, os níveis de c-miR-486 diminuíram após uma sessão de exercício de 60 min ao 70% do VO_{2max} e após 4 semanas de treinamento[25]. Devido a que segundo os autores, 60 minutos de exercício não é tempo suficiente para provocar mudanças na expressão (diminuição) e subsequente secreção de mioMIRs no sangue, a redução em c-miR-486 pode ser explicada por uma maior captação do miRNA induzida pelo exercício.

Alguns estudos tem sugerido que a maior expressão de mioMIRs na circulação em resposta ao exercício é consequência de extravasamento do conteúdo celular após dano à membrana plasmática. Isto principalmente devido a que só o exercício excêntrico, mas não concêntrico, provocou a liberação de miR-1, -133a, -133b, e -208b[26] e que o perfil de expressão no sangue é similar ao do músculo em resposta ao exercício[27]. Além disso, foi reportado que mioMIRs e outros miRNAs expressos no musculo cardíaco e no endotélio vascular aumentam em resposta a uma maratona em sujeitos fisicamente ativos[28,27,29]. No entanto, três horas de exercício em bicicleta (exercício concêntrico) aumentaram a expressão de miR-1, -133a, -133-b, e -181a, é de forma interessante também a expressão de proteínas relevantes à síntese de miRNA como Drosha, Dicer e exportina-5. Uma concomitante redução em

mioMIRs relacionados a miopatias como miR-9, -23a, -23b, e -31[30]. Isto pode estar associado a uma melhoria na função muscular subsequente ao exercício[30].

Os estudos mencionados acima sugerem que a secreção de miRNAs pelas fibras musculares, endoteliais e/ou sanguíneas esta associada à sua habilidade de influenciar o ambiente para seu próprio beneficio. Após serem transcritos no núcleo e exportados no citoplasma nas células de origem, as moléculas de miRNA são empacotadas em microvesículas que quando ligadas à membrana celular, secretam exosomas na circulação. Estes exosomas, a sua vez, são captados por endocitose e doam os miRNAs na células alvo promovendo e/ou regulando um amplo leque de processos moleculares[31]. Infelizmente, os estudos sobre a resposta de c-miRNAs em função ao exercício ainda são escassos. Considerando o anterior, no próximo capitulo o leitor encontrará evidencia experimental sobre a expressão de miRNAs no plasma de sujeitos fisicamente ativos submetidos a uma sessão de exercício resistido seguida de suplementação nutricional.

Referências

1. Güller, I. & Russell, A. P. MicroRNAs in skeletal muscle: their role and regulation in development, disease and function. *J. Physiol.* **588,** 4075–4087 (2010).
2. Reinhart, B. J. *et al.* The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature* **403,** 901–906 (2000).
3. Ebert, M. S. & Sharp, P. A. Roles for microRNAs in conferring robustness to biological processes. *Cell* **149,** 515–524 (2012).
4. Zhang, C. MicroRNAs in vascular biology and vascular disease. *J Cardiovasc. Transl. Res.* **3,** 235–240 (2010).
5. Davidson-Moncada, J., Papavasiliou, F. N. & Tam, W. MicroRNAs of the immune system: roles in inflammation and cancer. *Ann. N. Y. Acad. Sci.* **1183,** 183–194 (2010).
6. Chan, S. Y. *et al.* MicroRNA-210 controls mitochondrial metabolism during hypoxia by repressing the iron-sulfur cluster assembly proteins ISCU1/2. *Cell Metab.* **10,** 273–284 (2009).
7. Williams, A. H. *et al.* MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. *Science* **326,** 1549–1554 (2009).
8. Davidsen, P. K. *et al.* High responders to resistance exercise training demonstrate differential regulation of skeletal muscle microRNA expression. *J. Appl. Physiol. Bethesda Md 1985* **110,** 309–317 (2011).
9. Aoi, W. *et al.* The microRNA miR-696 regulates PGC-1{alpha} in mouse skeletal muscle in response to physical activity. *Am. J. Physiol. Endocrinol. Metab.* **298,** E799–806 (2010).
10. Mitchell, P. S. *et al.* Circulating microRNAs as stable blood-based markers for cancer detection. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 10513–10518 (2008).
11. Laterza, O. F. *et al.* Plasma MicroRNAs as sensitive and specific biomarkers of tissue injury. *Clin. Chem.* **55,** 1977–1983 (2009).

12. Heneghan, H. M., Miller, N. & Kerin, M. J. Circulating miRNA signatures: promising prognostic tools for cancer. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **28,** e573–574; author reply e575–576 (2010).

13. Valadi, H. *et al.* Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* **9,** 654–659 (2007).

14. Théry, C., Zitvogel, L. & Amigorena, S. Exosomes: composition, biogenesis and function. *Nat. Rev. Immunol.* **2,** 569–579 (2002).

15. Michael, A. *et al.* Exosomes from human saliva as a source of microRNA biomarkers. *Oral Dis.* **16,** 34–38 (2010).

16. Pigati, L. *et al.* Selective release of microRNA species from normal and malignant mammary epithelial cells. *PloS One* **5,** e13515 (2010).

17. Turchinovich, A., Weiz, L., Langheinz, A. & Burwinkel, B. Characterization of extracellular circulating microRNA. *Nucleic Acids Res.* **39,** 7223–7233 (2011).

18. Lee, E. J. *et al.* Systematic evaluation of microRNA processing patterns in tissues, cell lines, and tumors. *RNA* **14,** 35–42 (2008).

19. Mizuno, H. *et al.* Identification of Muscle-Specific MicroRNAs in Serum of Muscular Dystrophy Animal Models: Promising Novel Blood-Based Markers for Muscular Dystrophy. *PLoS ONE* **6,** e18388 (2011).

20. Vignier, N. *et al.* Distinctive Serum miRNA Profile in Mouse Models of Striated Muscular Pathologies. *PLoS ONE* **8,** e55281 (2013).

21. Cacchiarelli, D. *et al.* miRNAs as serum biomarkers for Duchenne muscular dystrophy. *EMBO Mol. Med.* **3,** 258–265 (2011).

22. Karolina, D. S. *et al.* MicroRNA 144 Impairs Insulin Signaling by Inhibiting the Expression of Insulin Receptor Substrate 1 in Type 2 Diabetes Mellitus. *PLoS ONE* **6,** e22839 (2011).

23. Baggish, A. L. *et al.* Dynamic regulation of circulating microRNA during acute exhaustive exercise and sustained aerobic exercise training. *J. Physiol.* **589,** 3983–3994 (2011).

24. Bye, A. *et al.* Circulating MicroRNAs and Aerobic Fitness – The HUNT-Study. *PLoS ONE* **8,** e57496 (2013).

25. Aoi, W. *et al.* Muscle-enriched microRNA miR-486 decreases in circulation in response to exercise in young men. *Front. Physiol.* **4,** 80 (2013).

26. Banzet, S. *et al.* Changes in circulating microRNAs levels with exercise modality. *J. Appl. Physiol. Bethesda Md 1985* (2013). doi:10.1152/japplphysiol.00075.2013

27. Baggish, A. L. *et al.* Rapid upregulation and clearance of distinct circulating microRNAs after prolonged aerobic exercise. *J. Appl. Physiol. Bethesda Md 1985* **116,** 522–531 (2014).

28. Gomes, C. P. C. *et al.* Circulating miR-1, miR-133a, and miR-206 levels are increased after a half-marathon run. *Biomark. Biochem. Indic. Expo. Response Susceptibility Chem.* 1–5 (2014). doi:10.3109/1354750X.2014.952663

29. Mooren, F. C., Viereck, J., Krüger, K. & Thum, T. Circulating microRNAs as potential biomarkers of aerobic exercise capacity. *Am. J. Physiol. Heart Circ. Physiol.* **306,** H557–563 (2014).

30. Russell, A. P. *et al.* Regulation of miRNAs in human skeletal muscle following acute endurance exercise and short-term endurance training. *J. Physiol.* **591,** 4637–4653 (2013).

31. Cortez, M. A. *et al.* MicroRNAs in body fluids—the mix of hormones and biomarkers. *Nat. Rev. Clin. Oncol.* **8,** 467–477 (2011).

Capítulo 2

Differential Expression of Circulating miRNAs Following Resistance Exercise and Carbohydrate/Protein Supplementation

Submetido e formatado segundo as normas da revista Applied Physiology, Nutrition, and Metabolism

Resumo

Neste estudo foram investigados os níveis de expressão de 12 microRNAs circulantes (c-miRNAs) envolvidos na proliferação e diferenciação celular, na angiogênese, na inflamação e no controle glicêmico, após uma sessão de exercício resistido (ER) e suplementação nutricional. Doze indivíduos foram submetidos a 10 séries de 10 repetições de exercício de extensão do joelho com o 80% da sua respetiva repetição máxima (RM), e seguido de suplementação com carboidrato ou carboidrato/proteína. Esta suplementação foi feita num delineamento simples cego e aleatório onde os indivíduos foram seus próprios controles. Amostras de sangue foram coletadas antes e depois (3h e 24h) do ER. A expressão relativa dos c-miRNAs foi analisada através da análise de variância com dois factores (ANOVA) com medidas repetidas. A resposta molecular do grupo que recebeu suplementação com proteína foi maior nos c-miRNAs envolvidos em miogênese, em particular o hsa-miR-133a e -503. Ambos os tratamentos produziram uma expressão diferenciada nos c-miRNAs hsa-miR-126 e 16, ambos reguladores de processos de angiogênese. O hsa-miR133a está associado à proliferação de células satélite e é parcialmente responsável pela hipertrofia muscular após o ER. Além disso, a maior e a menor expressão do hsa-miR-126 e do -16, respectivamente, sugerem processos de neovascularização.

Palavras Chave: Proliferação Celular; Angiogênese; Parácrino; Plasma.

Abstract

We investigated the levels of expression of 12 circulating miRNAs (c-miRNAs) involved in cell proliferation, differentiation, angiogenesis, inflammation and glycemic control following resistance exercise (RE) and dietary supplementation. Twelve subjects performed 10 sets of 10 repetitions with 80% of their respective one-repetition maximum (1RM) followed by either carbohydrate or carbohydrate/protein supplementation in a randomized single-blind counter-balanced design. Samples of blood were collected before RE, 03 and 24 hours afterwards. The relative expression data of all of the genes were analyzed using a two-way analysis of variance with repeated measures. The molecular response in the group that supplemented with protein was more pronounced for c-miRNAs involved in myogenesis, particularly hsa-miR-133a and -503. Both treatments revealed a differential expression of the c-miRNAs hsa-miR-126 and -16, which regulate angiogenesis. We argue that hsa-miR-133a is associated with satellite cell proliferation, and might be partially responsible for muscle hypertrophy following RE. Further, both the up- and down-regulation of hsa-miR-126 and -16, respectively, are likely to reflect neovascularization.

Keywords: Muscle Cell Proliferation; Angiogenesis; Paracrine; Plasma.

Introduction

In response to resistance exercise (RE) and dietary protein supplementation, there is an accretion of myofibrillar proteins, which normally results in hypertrophy and gains in strength (Yang et al. 2012; Moore et al. 2014; Witard et al. 2014).

MicroRNAs (miRNAs) are small non-coding molecules of RNA that regulate gene expression at the posttranscriptional level. MiRNAs bind to the 3'UTR sites of target mRNA, which suppresses protein synthesis or triggers mRNA degradation. Consequently, miRNAs occupy critical positions in a broad range of physiological processes (Sita-Lumsden et al. 2013; Ambros 2004). Accumulating evidence suggests that muscle-specific miRNAs, referred to as myomirs, regulate muscle differentiation and growth. For instance, Dicer-knockout mice show substantially reduced muscle mass and abnormal fiber morphology (O'Rourke et al. 2007). Further, the expression of miR-208, -208b and -499 determines the type and content of myosin heavy chain and the type of the muscle fiber (Rooij and Quiat 2009). Following RE and dietary protein supplementation in young men, the expression of the myomir hsa-miR-1 is decreased (Drummond et al. 2008). This is attributed to an enhanced rate of muscle protein synthesis as hsa-miR-1 inhibits the insulin-like growth factor I (IGF-I)- protein-kinase B (AKT) pathway (Elia et al. 2009).

There is evidence to suggest that c-miRNAs are secreted into the bloodstream into exosomes (Hunter et al. 2008) or transported by RNA-binding proteins (Arroyo et al. 2011) and high-density lipoproteins (Vickers et al. 2011). Thus, c-miRNAs are highly stable and protected from degradation. Similar to hormones and circulating growth factors, c-miRNAs bear the potential of regulating intricate

networks of cellular communication that probably involve not only cell-to-cell, but also tissue-to-tissue crosstalk.

To date, only a handful of studies have investigated changes on the levels of c-miRNAs in response to exercise. In a recent study, the expression levels of hsa-mir-181b and -214 increased after uphill walking whereas hsa-miR-1, -133a, -133b and -208b increased following downhill walking. The number, but not the function, of c-miRNAs secreted in response to downhill exercise was attributed to a higher degree of muscle injury (Banzet et al. 2013). A similar pattern of expression was observed in response to exhaustive exercise before and after three months of training. While some c-miRNAs are differentially expressed following a single bout of exhaustive exercise in untrained subjects, others are expressed only in trained individuals (Baggish et al. 2011).

Here, we reasoned that the c-miRNAs expressed in response to RE and dietary protein supplementation would be pivotal for muscle growth and vascularization. Unfortunately, thus far no study has examined the effect of nutritional interventions and RE on the expression of c-miRNAs. This line of inquiry is important because it will shed light on some of the molecular mechanisms underlying the adaptation to exercise. To test this premise, we investigated the response to RE and protein supplementation of 12 c-miRNAs previously associated with cellular proliferation (hsa-miR-133a), differentiation (hsa-miR-503), angiogenesis (hsa-miR-16, -20a, -21, -210, -221 and -222), endothelial injury (hsa-miR-126), inflammation/response to hypoxia (hsa-miR-146), glycemic control (hsa-miR-223), and cancer (hsa-miR-34a).

Materials and Methods

Subjects

The subjects were 12 recreationally active young males (aged 22.02 ± 2.49 years, height 177.10 ± 8.05 cm, BMI 23.60 ± 0.71 kg/m2). All of the subjects declared having participated in ~3 h/wk of structured physical activity for at least two months previous to this study. None of them smoked, had significant medical history or was taking regular or incidental medication during the study. All of the subjects gave their written informed consent before participating in the study. The experimental protocol was in accordance with the Declaration of Helsinki and approved by the respective Institutional Review Board (protocol 332.117).

Design

Two weeks before the collection of the samples, the subjects performed a one-repetition maximum (1RM) strength test on a leg press machine (Cybex Leg Press 16110. Medway, USA). Proper form was explained to all of the subjects. The values obtained from the strength test were used to determine the load of the subsequent RE sessions. The subjects completed two exercise sessions, separated by 10 days in a randomized single-blind counterbalanced design. On each session and after a light warm-up (3 sets of 10 repetitions with 40% of 1RM), the subjects completed 10 sets of 10 repetitions with 80% of 1RM of bilateral leg extensions. The rest interval between sets was two minutes. Similar exercise protocols have been previously shown to promote hypertrophy in humans (Drummond et al. 2008; Dalbo et al. 2013). Verbal feedback and encouragement was constantly provided to ensure proper form and completion of the test. All of the tests were performed between 8:30 and 9:00 AM. Samples of

blood (± 4mL) from the antecubital vein were collected into EDTA-coated tubes from each subject under resting conditions before the RE session, 03 and 24 h afterwards. These points were chosen to examine both the recovery and adaptation to RE (Mahoney et al. 2008). The blood samples were obtained by a qualified phlebotomist using standardized venipuncture techniques. The samples were obtained each from a different puncture to avoid the influence of local inflammation on the levels of c-miRNAs.

Diet and supplementation

The subjects were asked to refrain from exercise and alcohol 48 h prior to each exercise session. Further, the subjects were required to have finished breakfast at least 1.5 h before the beginning of each session. Immediately after completing the RE protocol, the subjects consumed in a randomized single-blind fashion a solution (300 mL) consisting of either carbohydrate [0.5 gr/kg BW of maltodextrin (Sports Supplements Ltd., UK) or protein + carbohydrate [the same proportion of maltodextrin mentioned above + 0.15 gr/kg BW of whey protein (Gold Standard; Optimum Nutrition Inc., USA)]. This amount of protein has previously been shown to stimulate muscle protein synthesis in humans (Drummond et al. 2008; Dalbo et al. 2013; Dreyer et al. 2008). The two solutions were similar in color and taste. The day before the RE sessions, the subjects received three standardized meals (~12Kcal/Kg BW; ~60% of energy as carbohydrate, ~20% of energy as fat and ~20 of energy as protein) (Dreyer et al. 2008; Verdijk et al. 2009) to consume during the days of the RE sessions as breakfast, lunch and dinner, respectively. The subjects were allowed to consume light snacks and water as desired between meals. The subjects were required to eat lunch no less than three hours after the exercise session (only after the collection of the second blood sample). The collection of blood 24 h after exercise (~07:30 AM) took place with the subjects under fasting conditions. All of the subjects reported total compliance with the diet. The design of the study is shown in Figure 1.

RNA extraction

The plasma was obtained by centrifugation at 1.900 x g for 10 min at 4°C shortly after blood collection. The supernatant (upper yellow phase) was transferred to conical microtubes for a second high-speed centrifugation step at 16.000 x g for 10 min at 4°C. All of the samples were processed on ice. Total RNA enriched with small RNAs including miRNAs was extracted from plasma using the miRNeasy Mini Kit (Qiagen, USA). Total RNA was extracted from 200 µL of plasma and mixed with 1mL of Qiazol. Chloroform of an equal volume of the starting sample was added and shaken vigorously for 30 s. The samples were then centrifuged at 12.000 x g for 15 min at 4°C. The upper aqueous phase was transferred to a new collection tube and mixed with 900 µL of 100% ethanol. Using RNeasy MinElute spin columns, the solution was centrifuged at 8.000 x g for 15 s and total RNA eluted in 12 µL of RNase-free water. The concentration of RNA in the samples was quantified by spectrophotometer (Nanodrop ND-100; Nanodrop Technologies, Inc. USA). The concentration of RNA extracted from plasma ranged from 13 to 62 ng/µL. Cel-miR-39 was spiked-in and used as a positive control for PCR. The samples were stored frozen at -80°C until analysis.

RT-qPCR

cDNA was produced using the miScript Reverse Transcription Kit (Qiagen, USA). Briefly, 9 µL of template RNA were mixed with 4µL miScript HiSpec buffer, 2 µL miScript Nucelics Mix, 2 µL miScript RT mix and 3 µL of RNase-free water. The solution was incubated for 60 min at 37°C. The circulating levels of plasma miRNA were quantified by RT-qPCR using the miScript SYBR Green PCR Kit (Qiagen, USA) on a Step One Plus Real-Time PCR System (Applied Biosystems, USA). All primers were ordered from Qiagen (USA). For each 96-well plate, a solution containing 1.375 mL 2x SYBR Green PCR Master Mix, 275 µL 10x

miScript Universal Primer, 1 mL RNAse-free water and 100µL cDNA template was prepared. For each well, 25 µL of the solution were used. Each plate was then incubated at 95 °C for 15 min, followed by 40 amplification cycles of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s. Transcripts of Cel-miR-39 were quantified as the reference gene. The fluorescence data were analyzed with the software Step One Plus (Applied Biosystems, USA) with automated settings for the determination of baselines and quantifications cycles. All of the miR expression data are normalized to the reference gene Cel-miR-39. The miR expression data corresponding to 03 h and 24 h after the RE session are normalized to the levels of miR before each RE session, which were determined at a relative expression of 1.

Statistical Analysis

We tested the effect of treatment (carbohydrate vs. carbohydrate/protein) over time on the levels of expression of c-miRNAs. To accomplish this, the relative expression data of all of the genes were analyzed using a two-way analysis of variance (ANOVA) with repeated measures followed by the Bonferroni post hoc test for multiple comparisons of means when appropriate. For all of the analyses, significant results were defined at the level of $p<0.05$.

Results

Figures 2 and 3 provide the expression pattern of the four c-miRNAs with significant variation following RE. The differentially expressed c-miRNAs are involved in cellular proliferation (hsa-miR-133a), differentiation (hsa-miR-503), and angiogenesis (hsa-miR-16 and -126). Interestingly, acute ingestion of dietary protein elicited increases in the expression of hsa-miR-133 and -503 during the

first 03 h after RE with decreasing levels over the following 24 h. Comparison between the groups revealed a significant effect of time for hsa-miR-503 [F (2, 33) = 4.45, p = 0.019] and both time [F (2, 33) = 20.64, p > 0.0001] and treatment [F (1, 33) = 12.67, p = 0.001] for hsa-miR-133a. On the other hand, we found opposite profiles of expression for the miRNAs involved in angiogenesis. Carbohydrate supplementation triggered increases on the levels of hsa-miR-126 [F (1, 33) = 5.66, p = 0.023] shortly after RE. Supplementation with protein almost reached significance [F (2, 33) = 3.21, p = 0.053]. Contrary to this, protein ingestion led to decreased levels of hsa-miR-16 [F (2, 33) = 6.31, p = 0.004] 24 h after RE with a tendency for significance between treatments [F (1, 33) = 3.89, p = 0.057]. As expected, these findings suggest that dietary protein supplementation induces a greater molecular response following RE than exercise alone (Figures 2 and 3). Lastly, we found no significant effects in other c-miRNAs related to angiogenesis (hsa-miR-20a, -21, -210, -221 and -222), inflammation/response to hypoxia (hsa-miR-146), glycemic control (hsa-miR-223) or cancer (hsa-miR-34a).

Discussion

The main findings of this study are that c-miRNAs associated with cellular proliferation, differentiation and angiogenesis are differentially expressed following RE and dietary protein supplementation. Whereas c-miRNAs required for muscle growth were upregulated shortly after RE, those associated with angiogenesis showed contrasting dynamics with respect to both treatment and time. These findings support our initial hypothesis and we were able to demonstrate that c-miRNAs involved in endothelial cell differentiation and angiogenesis are also critical to the adaptation of the contractile apparatus to exercise.

During the immediate hours after exercise, the skeletal muscle recovers by resynthesizing glycogen and repairing mechanical and free radical induced-damage. On the other hand, adaption occurs over the subsequent days as a result of mitochondrial biogenesis and myofibrillar protein synthesis (Mahoney et al. 2005; Mahoney et al. 2008). Resistance exercise alone or accompanied by dietary protein supplementation has repeatedly been shown to increase the rate of myofibrillar protein synthesis up to 48 h after exercise (Reitelseder et al. 2014; Drummond et al. 2008). Convincing evidence now supports the notion that down-regulation of some of the inhibitors of the AKT-mTOR pathway, such as protein regulated in development and DNA damage responses (REDD1), tuberous sclerosis 1 and 2 (TSC1 and 2) and myostatin, coupled with a higher expression of positive regulators, such as Ras homolog enriched in brain (Rheb) and MyoD are amongst the mechanisms responsible for fiber hypertrophy (Fujita et al. 2007; Drummond et. al. 2009). These observations are in strong agreement with recent findings showing that validated targets of muscle-specific miRNAs influence cell growth and satellite cell proliferation (McCarthy and Esser 2007; Simon et al. 2008).

Our results show that hsa-miR-133a substantially increased 3h after RE. Strikingly, the expression was approximately three times greater in the group that supplemented with protein even though it was significant for both groups. Hsa-miR-133a is specifically expressed in cardiac and skeletal muscle and targets n-polypyrimidine tract-binding protein (nPTB) and uncoupling protein 2 (UCP2). This means that hsa-miR-133a promotes myoblast proliferation while represses differentiation (Chen et al. 2006; Pangukuri et al. 2010). So far, conflicting data have been published in regard to the role of miR-133a during muscle growth. In rodents, miR-133a has been shown to decrease after 7 days of functional overload of the plantaris muscle (McCarthy and Esser 2007). The authors proposed that miR-133a inhibits positive regulators of growth signaling pathways such as IGF-1 and serum response factor (SRF). Further, the administration of a

cocktail of TNF-like weak inducer of apoptosis (TWEAK), a cytokine known to produce muscle atrophy, significantly reduced miR-133a/b (Panguluri et al. 2010). In humans, the levels of expression within the muscle of hsa-miR-133a have been reported to increase after 1 h of cycling (Nielsen et al., 2010), and to remain unaltered following a single bout or 12 weeks of RE (Drummond et al. 2008; Davidsen et al. 2011). However, in agreement with our findings others have found differentially up-regulated levels of circulating hsa-miR-133a/b after RE and eccentric exercise in young males (Banzet et al. 2013; Uhlemann et al. 2012).

Based on these data, it is difficult to interpret the exact role of hsa-miR-133a during skeletal muscle recovery or adaptation. Hsa-miR-133a might have a role in satellite cell proliferation during muscle hypertrophy. Recent evidence indicates that the myomirs 1 and 206 as well as miR 27b regulate the expression of paired-box transcription factor Pax3 and Pax7, proteins required for the survival and migration of satellite cells during myogenesis (Chen et al. 2010; Crist et al. 2009). Particularly, these miRNAs allow the transition from the proliferation to the differentiation stage by down-regulating Pax3 and Pax7. Consequently, in a similar fashion, higher levels of hsa-mir-133a might be associated with satellite cell proliferation, and be partially responsible for muscle hypertrophy following RE.

Somewhat unexpectedly, the levels of expression of hsa-miR-126 were significant only for the group that supplemented with carbohydrate. Similar to miR-133a, miR-126 is also a tissue-specific miRNA highly expressed in endothelial cells (EC). In a very elegant study, Fish and colleagues presented data indicating that miR-126 plays a pivotal role in the maintenance of the vascular structure. By directly targeting sprouty-related protein (SPRED1) and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2), which are endogenous

repressors of vascular endothelial growth factor (VEGF), miR-126 enhances VEGF signaling, angiogenesis and vascular integrity in vivo (Fish et al. 2008). Fascinatingly, in response to tissue injury, apoptotic bodies enriched in miR-126 serve as a paracrine signal by inducing the production of CXC motif chemokine 12 (CXCL12), and the subsequent recruitment of endothelial progenitor cells (Zernecke et al. 2009). This mechanism has been extended into humans with type 2 diabetes mellitus and endothelial dysfunction in whom the loss of circulating hsa-miR-126 was inversely proportional to the level of blood glucose (Zampetaki et al. 2010). Hence, the increase in hsa-miR-126 in our study is most likely associated with angiogenesis and overall vascular adaptation in response to exercise.

Two of the miRNAs differentially expressed in our study have not been previously described in response to exercise, so we are unable to compare our data. Recently, however, important evidence has been provided with respect to the role of hsa-miR-503 in myoblast differentiation. Repression of cell cycle regulators, particularly cell division cycle 25 homolog A (Cdc25a) by hsa-miR-503 is one of the very intricate mechanisms by which C2C12 cells differentiate into myotubes (Sarkar et al. 2010). Similar to hsa-miR-503, no other study appears to have reported variations in the levels of hsa-miR-16 in response to exercise. miR-16 has also been implicated in EC survival and angiogenesis. Increased levels of miR-16 hinder growth of EC by reducing the expression of vascular endothelial growth factor receptor 2 (VEGFR2), and the survival-promoting effects of VEGF while inhibiting PI3k-Akt signaling (Chamorro et al. 2011). In our study, hsa-miR-16 was markedly downregulated 24 h after RE. The expression profile of both hsa-miR-503 and -16 suggests a consistent modulation of muscle differentiation and angiogenesis following RE and dietary protein supplementation. Especially, taking into consideration the similar expression of hsa-miR-133a and -126. However, because a single molecule of miRNA might

modulate several targets, interpretations of the cell differentiation and angiogenic properties of hsa-miR-503 and -16, respectively, should be made with caution.

Some limitations to this study should be acknowledged. First, at this point no statements can be made in regard to the source and release of c-miRNAs. Previous attempts to correlate the expression of c-miRNAs to known markers of muscle injury or circulating growth factors such as creatine kinase or IGF-1, respectively, have failed or shown only weak correlations (Sawada et al. 2013). More importantly, even if strong correlations were found, this would not necessarily demonstrate that c-miRNAs were passively leaked out, rather than being actively released into the circulation. Especially, considering that concentric endurance exercise, which does not induce muscle injury, has been known to elicit a distinctive profile of c-miRNAs (Baggish et al. 2011; Uhlemann et al. 2012). Of further interest will be to investigate if membrane proteins are involved in the release of exosomes enriched in miRNAs following mechanical or nutritional stimuli. Secondly, our subjects had had previous experience with RE and constituted a somewhat modest sample. By including untrained subjects, the c-miRNA response to RE could have probably been more comprehensive. On the other hand, our sample was very homogeneous and the subjects served as their own control. Thus, we believe that the controlled diet and the random counter-balanced design allowed us to observe important differences in the profile of c-miRNAs in response to RE when only dietary supplementation was modified.

Overall, our findings suggest a distinct profile of expression in c-miRNA between dietary carbohydrate and carbohydrate/protein supplementation following RE. As expected, the molecular response in the group that supplemented with protein was more pronounced for c-miRNAs involved in the regulation of myogenesis. Both treatments showed a differential expression of miRNAs involved in angiogenesis. Our findings add hsa-miR-503 and -16 to the list of c-miRNAs differentially expressed following RE.

Acknowledgements

We are grateful to participants for their involvement and understanding towards the instructions giving throughout the study. This study was supported by from a grant from the National Council for Scientific and Technological Development (CNPQ). M.M.D.G. and O.L.B.J. received graduate fellowships from the Coordination for the Improvement of Higher Education- Personnel (CAPES) and the Brazilian Program for Post Graduate Students (PECPG - CAPES), respectively. F.S.E. received the fellowship Research Productivity from CNPQ. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of Interest: None reported.

References

Ambros, V. 2004. The functions of animal microRNAs. Nature. 16;431(7006):350-5.

Arroyo, J.D.;, Chevillet, J.R.; Kroh, E.M.; Ruf, I.K.; Pritchard, C.C.; Gibson, D.F.; Mitchell, P.S.; Bennett, C.F.; Pogosova-Agadjanyan, E.L.; Stirewalt, D.L.; Tait, J.F.; Tewari, M. 2011. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A. ;108(12):5003-8. doi: 10.1073/pnas.1019055108.

Baggish, A.L.; Hale, A.; Weiner, R.B.; Lewis, G.D.; Systrom, D.; Wang, F.; Wang, T.J.; Chan, S.Y. 2011. Dynamic regulation of circulating microRNA during acute exhaustive exercise and sustained aerobic exercise training. J Physiol.; 589(Pt 16):3983-94. doi: 10.1113/jphysiol.2011.213363.

Banzet, S.1.; Chennaoui, M.; Girard, O.; Racinais, S.; Drogou, C.; Chalabi, H.; Koulmann, N. 2013. Changes in circulating microRNAs levels with exercise modality. J Appl Physiol (1985).; 1;115(9):1237-44. doi: 10.1152/japplphysiol.00075.2013.

Boutz, P.L.; Chawla, G.; Stoilov, P.; Black, D.L. 2007. MicroRNAs regulate the expression of the alternative splicing factor nPTB during muscle development. Genes Dev.;21(1):71-84. doi: 10.1101/gad.1500707.

Chamorro-Jorganes, A.; Araldi, E.; Penalva, L.O.; Sandhu, D.; Fernández-Hernando, C.; Suárez, Y. 2011. MicroRNA-16 and microRNA-424 regulate cell-autonomous angiogenic functions in endothelial cells via targeting vascular endothelial growth factor receptor-2 and fibroblast growth factor receptor-1. Arterioscler Thromb Vasc Biol.; 31(11):2595-606. doi: 10.1161/ATVBAHA.111.236521.

Chen, J.F.; Callis, T.E.; Wang, D.Z. 2009. microRNAs and muscle disorders. J Cell Sci.; 122(Pt 1):13-20. doi: 10.1242/jcs.041723.

Chen, J.F.; Mandel, E.M.; Thomson, J.M.; Wu, Q.; Callis, T.E.; Hammond, S.M.; Conlon, F.L.; Wang, D.Z. 2006. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet.; 38(2):228-33. doi: 10.1038/ng1725.

Chen, J.F.; Tao, Y.; Li, J.; Deng, Z.; Yan, Z.; Xiao, X.; Wang, D.Z. 2010. microRNA-1 and microRNA-206 regulate skeletal muscle satellite cell proliferation and differentiation by repressing Pax7. J Cell Biol.; 190(5):867-79. doi: 10.1083/jcb.200911036.

Crist, C.G1.; Montarras, D.; Pallafacchina, G.; Rocancourt, D.; Cumano, A.; Conway, S.J.; Buckingham, M. 2009. Muscle stem cell behavior is modified by microRNA-27 regulation of Pax3 expression. Proc Natl Acad Sci U S A.; 106(32):13383-7. doi: 10.1073/pnas.0900210106.

Dalbo, V.J.; Roberts, M.D.; Hassell, S.; Kerksick, C.M. 2013. Effects of pre-exercise feeding on serum hormone concentrations and biomarkers of myostatin and ubiquitin proteasome pathway activity. Eur J Nutr.; 52(2):477-87. doi: 10.1007/s00394-012-0349-x.

Davidsen, P.K.1.; Gallagher, I.J.; Hartman, J.W.; Tarnopolsky, M.A.; Dela, F.; Helge, J.W.; Timmons, J.A.; Phillips, S.M. 2011. High responders to resistance exercise training demonstrate differential regulation of skeletal muscle microRNA expression. J Appl Physiol (1985).; 110(2):309-17. doi: 10.1152/japplphysiol.00901.2010.

Dreyer, H.C.; Drummond, M.J.; Pennings, B.; Fujita, S.; Glynn, E.L.; Chinkes, D.L.; Dhanani, S.; Volpi, E.; Rasmussen, B.B. 2008. Leucine-enriched essential amino acid and carbohydrate ingestion following resistance exercise enhances mTOR signaling and protein synthesis in human muscle. Am J Physiol Endocrinol Metab.; 294(2):E392-400. doi: 10.1152/ajpendo.00582.2007.

Drummond, M.J.; Dreyer, H.C.; Pennings, B.; Fry, C.S.; Dhanani, S.; Dillon, E.L.; Sheffield-Moore, M.; Volpi, E.; Rasmussen, B.B. 2008. Skeletal muscle protein anabolic response to resistance exercise and essential amino acids is delayed with aging. J Appl Physiol (1985).; 104(5):1452-61. doi: 10.1152/japplphysiol.00021.2008.

Drummond, M.J.; Fujita, S.; Abe, T.; Dreyer, H.C.; Volpi, E.; Rasmussen, B.B. 2008. Human muscle gene expression following resistance exercise and blood flow restriction. Med Sci Sports Exerc.; 40(4):691-8. doi: 10.1249/MSS.0b013e318160ff84.

Drummond, M.J.; McCarthy, J.J.; Fry, C.S.; Esser, K.A.; Rasmussen, B.B. 2008. Aging differentially affects human skeletal muscle microRNA expression at rest and after an anabolic stimulus of resistance exercise and essential amino acids. Am J Physiol Endocrinol Metab; 295(6):E1333-40. doi: 10.1152/ajpendo.90562.2008.

Drummond, M.J.1.; Miyazaki, M.; Dreyer, H.C.; Pennings, B.; Dhanani, S.; Volpi, E.; Esser, K.A.; Rasmussen, B. B. 2009. Expression of growth-related genes in young and older human skeletal muscle following an acute stimulation of protein synthesis. J Appl Physiol (1985).; 106(4):1403-11. doi: 10.1152/japplphysiol.90842.2008.

Elia, L.; Contu, R.; Quintavalle, M.; Varrone, F.; Chimenti, C.; Russo, M.A.; Cimino, V.; De Marinis, L.; Frustaci, A.; Catalucci, D.; Condorelli, G. 2009. Reciprocal regulation of microRNA-1 and insulin-like growth factor-1 signal transduction cascade in cardiac and skeletal muscle in physiological and pathological conditions. Circulation.;120(23):2377-85. doi: 10.1161/CIRCULATIONAHA.109.879429.

Fish, J.E.; Santoro, M.M.; Morton, S.U.; Yu, S.; Yeh, R.F.; Wythe, J.D.; Ivey, K.N.; Bruneau, B.G.; Stainier, D.Y.; Srivastava, D. 2008. miR-126 regulates angiogenic signaling and vascular integrity. Dev Cell.; 15(2):272-84. doi: 10.1016/j.devcel.2008.07.008.

Fujita, S.; Dreyer, H.C.; Drummond, M.J.; Glynn, E.L.; Cadenas, J.G.; Yoshizawa, F.; Volpi, E.; Rasmussen, B.B. 2007. Nutrient signalling in the

regulation of human muscle protein synthesis. J Physiol.; 582(Pt 2):813-23. doi: 10.1113/jphysiol.2007.134593

Hunter, M.P.; Ismail, N.; Zhang, X.; Aguda, B.D.; Lee, E.J.; Yu, L.; Xiao, T.; Schafer, J.; Lee, M.L.; Schmittgen, T.D.; Nana-Sinkam, S.P; Jarjoura, D.; Marsh, C.B. 2008. Detection of microRNA expression in human peripheral blood microvesicles. PLoS One.; 3(11):e3694. doi: 10.1371/journal.pone.0003694.

Mahoney, D.J.; Parise, G.; Melov, S.; Safdar, A.; Tarnopolsky, M.A. 2005. Analysis of global mRNA expression in human skeletal muscle during recovery from endurance exercise. FASEB J.; 19(11):1498-500. doi: 10.1096/fj.04-3149fje.

Mahoney, D.J.; Safdar, A.; Parise, G.; Melov, S.; Fu, M.; MacNeil, L.; Kaczor, J.; Payne, E.T.; Tarnopolsky, M.A. 2008. Gene expression profiling in human skeletal muscle during recovery from eccentric exercise. Am J Physiol Regul Integr Comp Physiol.; 294(6):R1901-10. doi: 10.1152/ajpregu.00847.2007.

McCarthy, J.J.; Esser, K.A. 2007. MicroRNA-1 and microRNA-133a expression are decreased during skeletal muscle hypertrophy. J Appl Physiol (1985).; 102(1):306-13. doi: 10.1152/japplphysiol.00932.2006

Moore, D.R.; Camera, D.M.; Areta, J.L.; Hawley, J.A. 2014. Beyond muscle hypertrophy: why dietary protein is important for endurance athletes. Appl Physiol Nutr Metab. 7:1-11. doi: 10.1139/apnm-2013-0591.

Nielsen, S.; Scheele, C.; Yfanti, C.; Akerström, T.; Nielsen, A.R.; Pedersen, B.K.; Laye, M.J. 2010. Muscle specific microRNAs are regulated by endurance

exercise in human skeletal muscle. J Physiol.; 588(Pt 20):4029-37. doi: 10.1113/jphysiol.2010.189860.

O'Rourke, J.R.; Georges, S.A.; Seay, H.R.; Tapscott, S.J.; McManus, M.T.; Goldhamer D.J.; Swanson, M.S.; Harfe, B.D. 2007. Essential role for Dicer during skeletal muscle development. Dev Biol ;311(2):359-68.

Panguluri, S.K.; Bhatnagar, S.; Kumar, A.; McCarthy, J.J.; Srivastava, A.K.; Cooper, N.G.; Lundy, R.F.; Kumar, A. 2010. Genomic profiling of messenger RNAs and microRNAs reveals potential mechanisms of TWEAK-induced skeletal muscle wasting in mice. PLoS One.; 5(1):e8760. doi: 10.1371/journal.pone.0008760.

Reitelseder, S.; Agergaard, J.; Doessing, S.; Helmark, I.C.; Schjerling, P.; van Hall, G.; Kjaer, M.; Holm, L. 2014. Positive muscle protein net balance and differential regulation of atrogene expression after resistance exercise and milk protein supplementation. Eur J Nutr.;53(1):321-33. doi: 10.1007/s00394-013-0530-x.

Rosenberg, M.I.; Georges, S.A.; Asawachaicharn, A.; Analau, E.; Tapscott, S.J. 2006. MyoD inhibits Fstl1 and Utrn expression by inducing transcription of miR-206. J Cell Biol.; 175(1):77-85. doi: 10.1083/jcb.200603039

Sarkar, S.; Dey, B.K.; Dutta, A. 2010. MiR-322/424 and -503 are induced during muscle differentiation and promote cell cycle quiescence and differentiation by

down-regulation of Cdc25A. Mol Biol Cell.; 21(13):2138-49. doi: 10.1091/mbc.E10-01-0062.

Sawada, S.; Kon, M.; Wada, S.; Ushida, T.; Suzuki, K.; Akimoto, T. 2013. Profiling of circulating microRNAs after a bout of acute resistance exercise in humans. PLoS One.; 8(7):e70823. doi: 10.1371/journal.pone.0070823.

Simon, D.J.; Madison, J.M.; Conery, A.L.; Thompson-Peer, K.L.; Soskis, M.; Ruvkun, G.B.; Kaplan, J.M.; Kim, J.K. 2008. The microRNA miR-1 regulates a MEF-2-dependent retrograde signal at neuromuscular junctions. Cell.; 133(5):903-15. doi: 10.1016/j.cell.2008.04.035.

Sita-Lumsden, A.; Dart, D.A.; Waxman, J.; Bevan, C.L. 2013. Circulating microRNAs as potential new biomarkers for prostate cancer. Br J Cancer. 28; 108(10):1925-30. doi: 10.1038/bjc.2013.192

Uhlemann, M.; Möbius-Winkler, S.; Fikenzer, S.; Adam, J.; Redlich, M.; Möhlenkamp, S.; Hilberg, T.; Schuler, G.C.; Adams, V. 2014. Circulating microRNA-126 increases after different forms of endurance exercise in healthy adults. Eur J Prev Cardiol.; 21(4):484-91. doi: 10.1177/2047487312467902.

van Rooij, E.; Quiat, D.; Johnson, B.A.; Sutherland, L.B.; Qi, X.; Richardson, J.A.; Kelm, R.J. Jr.; Olson, E.N. 2009. A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance. Dev Cell. 2009 Nov;17(5):662-73. doi: 10.1016/j.devcel.2009.10.013.

Verdijk, L.B1.; Jonkers, R.A.; Gleeson, B.G.; Beelen, M.; Meijer, K.; Savelberg, H.H.; Wodzig, W.K.; Dendale, P.; van Loon, L.J. 2009. Protein supplementation before and after exercise does not further augment skeletal muscle hypertrophy after resistance training in elderly men. Am J Clin Nutr.; 89(2):608-16. doi: 10.3945/ajcn.2008.26626.

Vickers, K.C.; Palmisano, B.T.; Shoucri, B.M.; Shamburek, R.D.; Remaley, A.T. 2011. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol.; 13(4):423-33. doi: 10.1038/ncb2210.

Zampetaki, A.; Kiechl, S.; Drozdov, I.; Willeit, P.; Mayr, U.; Prokopi, M.; Mayr, A.; Weger, S.; Oberhollenzer, F.; Bonora, E.; Shah, A.; Willeit, J.; Mayr, M. 2010. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. Circ Res.; 107(6):810-7. doi: 10.1161/CIRCRESAHA.110.226357.

Witard, O.C.; Cocke, T.L.; Ferrando, A.A.; Wolfe, R.R.; Tipton, K.D. 2014. Increased net muscle protein balance in response to simultaneous and separate ingestion of carbohydrate and essential amino acids following resistance exercise. Appl Physiol Nutr Metab. 39(3):329-39. doi: 10.1139/apnm-2013-0264.

Yang, Y.; Churchward-Venne, T.A.; Burd, N.A., Breen, L.; Tarnopolsky, M.A.; Phillips, S.M. 2012. Myofibrillar protein synthesis following ingestion of soy protein isolate at rest and after resistance exercise in elderly men. Nutr Metab (Lond) 14;9(1):57. doi: 10.1186/1743-7075-9-57.

Zernecke, A.; Bidzhekov, K.; Noels, H.; Shagdarsuren, E.; Gan, L.; Denecke, B.; Hristov, M.; Köppel, T.; Jahantigh, M.N.; Lutgens, E.; Wang, S.; Olson, E.N.; Schober, A.; Weber, C. 2009. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. Sci Signal.; 2(100):ra81. doi: 10.1126/scisignal.2000610.

Figure Legends

Figure 1. Timeline of the RE sessions and collection of samples. The subjects attended the RE sessions in a randomized single-blind counter-balanced design. The time at which the subjects had dinner during the days of the RE sessions is not shown.

Figure 2. Changes in c-miRNAs following RE and carbohydrate or carbohydrate/ protein supplementation. Values shown (n = 12 subjects per group; means ± SD) are the fold regulation relative to baseline, which was determined at a relative expression of 1.0. Significant results were defined at $p < 0.05$. α = Significant effects over time. β = Significant effects between treatments.

Figure 3. Scatter plots of significantly expressed c-miRNAs following RE and carbohydrate or carbohydrate/ protein supplementation. Values shown (n = 12 subjects per group) are the fold regulation relative to baseline, which was determined at a relative expression of 1.0.

Figure 1

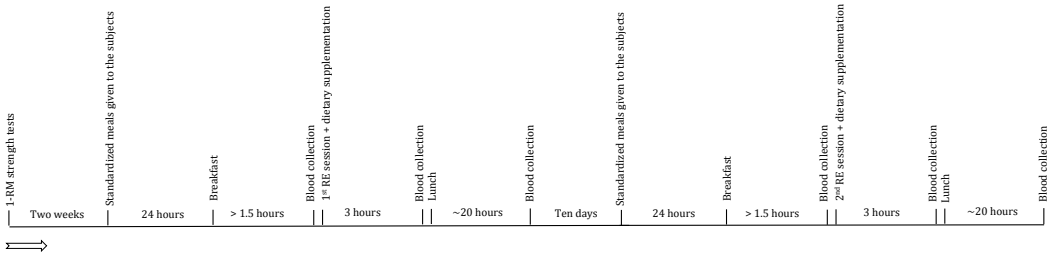

1-RM strength tests
Two weeks
Standardized meals given to the subjects
24 hours
Breakfast
> 1.5 hours
Blood collection
1st RE session + dietary supplementation
3 hours
Blood collection
Lunch
~20 hours
Blood collection
Ten days
Standardized meals given to the subjects
24 hours
Breakfast
> 1.5 hours
Blood collection
2nd RE session + dietary supplementation
3 hours
Blood collection
Lunch
~20 hours
Blood collection

Figure 2

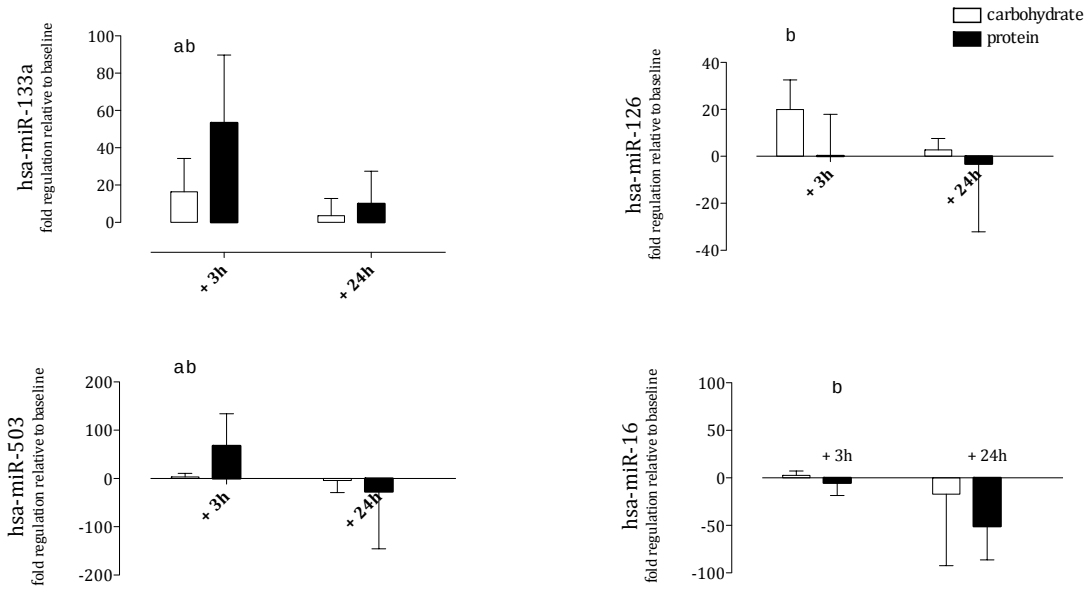
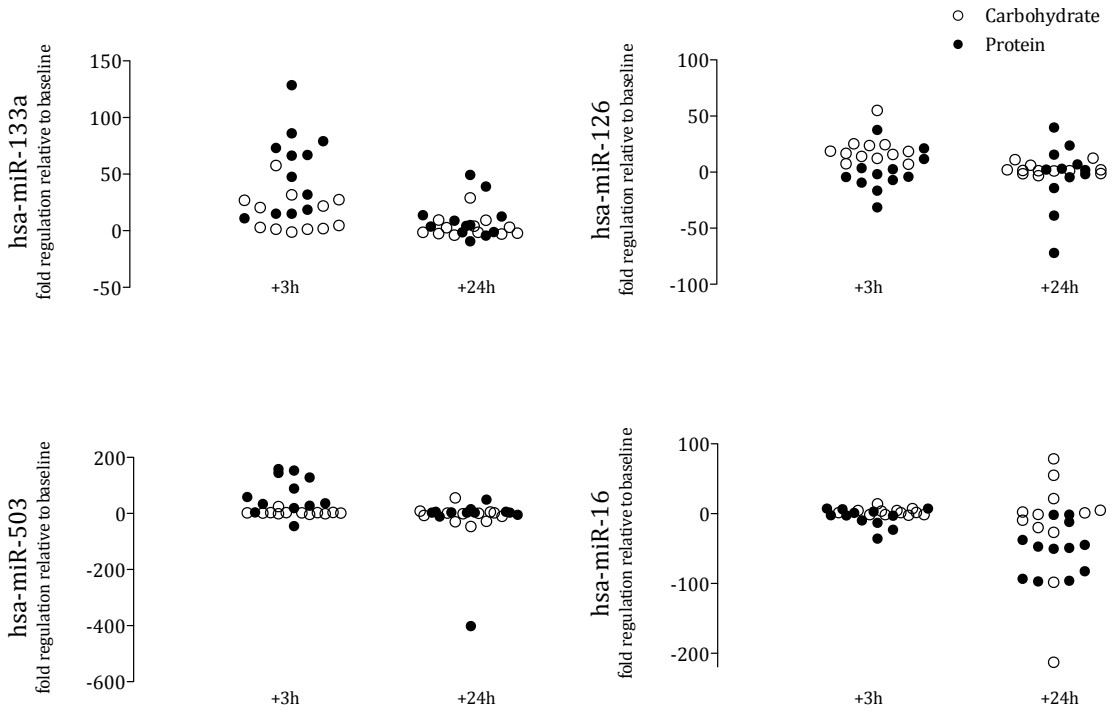

carbohydrate
protein
hsa-miR-133a
fold regulation relative to baseline
100
80
60
40
20
0
a b
+ 3h
+ 24h
hsa-miR-126
fold regulation relative to baseline
40
20
0
-20
-40
b
+ 3h
+ 24h
hsa-miR-503
fold regulation relative to baseline
200
100
0
-100
-200
a b
+ 3h
+ 24h
hsa-miR-16
fold regulation relative to baseline
100
50
0
-50
-100
b
+ 3h
+ 24h

Figure 3


Carbohydrate
Protein
hsa-miR-133a
fold regulation relative to baseline
150
100
50
0
-50
+3h
+24h
hsa-miR-126
fold regulation relative to baseline
100
50
0
-50
-100
+3h
+24h
hsa-miR-503
fold regulation relative to baseline
200
0
-200
-400
-600
+3h
+24h
hsa-miR-16
fold regulation relative to baseline
100
0
-100
-200
+3h
+24h

Capítulo 3

Salivating over Biological Markers; A Call for a Closer Collaboration between Coaches and Sports Scientists

A ser submetido à revista British Journal of Sports Medicine

Resumo

Durante a última década, a medicina esportiva tem dado um maior interesse ao uso da saliva como matriz substituta do sangue. A saliva oferece claras vantagens sobre o sangue, no que diz respeito não só à sua coleta, mas também ao seu manuseamento e armazenamento. Ainda assim, embora muitos sejam já os métodos analíticos aplicados para estudar a resposta de uma vasta gama de moléculas na saliva à prática esportiva, na nossa opinião, a informação daí retirada é ainda pouco útil. Parece haver um desacordo entre a abordagem da procura de biomarcadores e da informação relevante para o treinador e, por isso, é crítico discriminar entre os efeitos que o exercício promove nos parâmetros salivares e a informação que o marcador, utilizado para avaliar a resposta fisiológica ao treinamento, deve oferecer. Nenhuma molécula por si só deve ser considerada um marcador biológico devido à diferença estatística na sua concentração em resposta ao exercício. Por outras palavras, na medicina esportiva, os marcadores biológicos devem estar associados à variação da carga de treinamento; devem identificar um estado de recuperação ou inadaptação; ou devem ter o potencial de prever o sucesso atlético. Por outro lado, alguns estudos têm de fato revelado um enorme potencial dos marcadores salivares no monitoramento do treinamento esportivo. No presente trabalho, são discutidos os estudos que identificaram as moléculas salivares que, quando usadas juntamente com os parâmetros clássicos de desempenho na fisiologia do exercício, podem oferecer uma compreensão mais aprofundada de como os atletas lidam com o treinamento e, portanto, podem auxiliar no planeamento e prescrição do mesmo. Esta revisão é uma tentativa de, em primeiro lugar, enfatizar a forma como os marcadores biológicos salivares devem ser utilizados e interpretados e, em segundo lugar, demonstrar o potencial dos marcadores salivares na medicina desportiva.

Palavras Chave: Carga de treino; Recuperação; Fadiga; Desidratação; Lactato; Saliva; Esporte; Desempenho.

Abstract

During the last decade considerable interest has been directed towards using saliva as a surrogate matrix for blood in sports medicine. Saliva offers clear advantages over blood in regards to collection, handling and storage. Event though many analytical methods have been applied to study the response of a broad range of salivary analytes to sports and exercise, in our opinion little useful information has been accumulated thus far. There seems to be a disagreement in our approach to look for biomarkers and the information that is relevant for coaches. Thus, it is critical that we be able to discriminate between the effects that exercise evokes in salivary parameters and the information that a marker used to evaluate the physiological response to training should offer. Any molecule should not be considered a biological marker solely because of a statistical difference in their concentration in response to exercise. Phrased differently, biological markers in sports medicine must be associated with the variation of training load, identify a state of recovery or maladaptation or bear the potential to predict athletic success. On the other hand, some studies have in fact revealed great potential of salivary markers to monitor sports training. Here, we discuss the studies that identified salivary molecules that when used together with classical parameters of performance in exercise physiology might offer a deeper understanding on how athletes cope with training and hence, assist in the planning and prescription of training. In order to produce data that can be translated into the field, both scientists and coaches should participate in the aim and design of the studies as well as the interpretation and application of the findings. This review is an attempt to 1) emphasize how salivary biological markers should be employed and interpreted and 2) demonstrate the translational potential of salivary markers in sports medicine.

Keywords: Training load; Recovery; Fatigue; Dehydration; Lactate; Performance; Saliva; Exercise; Sports.

Introduction

During the last decade considerable interest has been directed towards using saliva as a surrogate matrix for blood in sports medicine. Saliva offers clear advantages over blood in regards to collection, handling and storage. Furthermore, considering some of the limitations imposed by the International Olympic Committee to obtain blood samples from professional athletes, monitoring the physiological adaptation to training by means salivary markers would be ideal for both coaches and athletes.

To date, many analytical methods have been applied to study the response of a broad range of salivary analytes to sports and exercise. However, in our opinion little useful information has been accumulated thus far because a large number of these findings are not strictly relevant to sports medicine. To be useful, any marker should offer information on the physiological status of the athlete. As such, markers are used to diagnose how efficient training is and the impact it has on the athletes. For instance, the use of power output and heart rate is critical to determine the training load (TL) in cycling. The combination of the two allows coaches to estimate the physiological cost of training at precise speeds. Similarly, maximal oxygen consumption (VO_{2max}) and the percentage at which the lactate threshold (LT) is reached might be used as predictors of performance in long-distance runners[1]. In addition, significant reductions in cortisol and blood counts are effective markers to determine excessive training loads and/or ineffective recovery. Thus, biological markers in sports medicine are not different from any other marker used to differentiate healthy from diseased conditions such as blood pressure for cardiovascular disease, blood cholesterol for hyperlipidemia or glycated hemoglobin for diabetes. These markers offer precise and clear information about the physiological state of the individual. Similarly, in

sports training markers allow coaches to identify adaptation to training (improved performance) or inappropriate recovery.

In general, most studies on salivary markers have focused on either proteins, such as salivary alpha-amylase (sAA) or salivary immunoglobulin A (sIgA), hormones such as cortisol (sC) and testosterone (sT), or metabolites such as lactate (sLac) and nitrite (sNO_2). Whereas most proteins are actively secreted into saliva from the salivary glands after sympathetic innervation, other substances such as steroid hormones and sLac diffuse passively from blood. The pioneer work of Chatterton et al, [2,3] on the reactivity of the sympathetic nervous system reported increases in sAA after exercise and parachute jumping. They reasoned that because salivary glands received sympathetic stimulation, proteins released into saliva in response to certain stimuli, such as exercise, might be used as a proxy for catecholamines. Because catecholamines, to a certain extent, mediate the fight-or-flight response, they suggested sAA as a marker of stress. Since then, several studies in sports science adopted a similar experimental design and have considered as a marker of "physical stress" any salivary protein or molecule that increases in response to exercise[4–6]. However, in this context increases in heart rate, respiration rate, skin conductance, rate of perceive exertion or even transpiration rate could be considered as markers of "physical stress". More importantly, "physical stress" as used in this context is an ambiguous term with little, if any value in sports medicine. Fatigue and overtraining are other categories into which salivary parameters, mainly metabolites, have been classified mostly because of statistical differences in their concentration have been observed in response to exercise[7]. Physical effort, even at moderate intensities might elicit major rearrangements in the metabolic balance to lead to statistical differences in certain molecules when compared to resting values. However, the changes that occur during exercise to maintain homeostasis are not necessarily an indication of physiological adaptations to sports training or inappropriate recovery from it.

Thus, there seems to be a disagreement in our approach to look for biomarkers and the information that is relevant for coaches. It is critical that we be able to discriminate between the effects that exercise evokes in salivary parameters and the information that a marker used to evaluate the physiological response to training should offer. Any molecule should not be considered a biological marker solely because of a statistical difference in their concentration in response to exercise. Phrased differently, biological markers in sports medicine must be associated with the variation of TL, identify a state of recovery or maladaptation or bear the potential to predict athletic success.

On the other hand, some studies have in fact revealed great potential of salivary markers to monitor sports training. Here, we discuss the studies that identified salivary molecules in a way that, when used together with classical parameters of performance in exercise physiology might offer a deeper understanding on how athletes cope with training and hence, assist in the planning and prescription of TL. A summary of these data is shown in Table 1. A closer collaboration between us, as sports scientists, and coaches is essential in the search for (salivary) biological markers. In order to produce data that can be translated into the field, both scientists and coaches should participate in the aim and design of the studies as well as the interpretation and application of the findings[8]. This review is an attempt to 1) emphasize how salivary biological markers should be employed and interpreted and 2) demonstrate the translational potential of salivary markers in sports medicine.

Training load

The methodological oscillation in the volume and intensity of training allows athletes to reach peaks of performance at the most important competitions[9]. Biological and psychological markers of the impact of training must exhibit a comparable pattern to the oscillations in training load. Abnormal behavior in

these markers might relate to inappropriate recovery and adaptation. Catecholamines occupy critical positions in the regulation of physiological processes during exercise[10]. On the other hand, it has been shown that circulating nitrite predicts exercise capacity in trained subjects[11]. With this mind, we investigated the response of salivary proteins and nitrite to training in elite swimmers and compared it against plasma catecholamines and nitrite, respectively[9,10]. We found strong correlations between sPT (r=0.59), sAA (r=0.89) and sCgA (r=0.52) and plasma adrenaline and between all of the above against the intensity and load of training. Event though no significant correlations were found between salivary and plasma nitrite, the former also predicted training intensity (r=0.33) and load (r=0.68) as has been previously reported[9]. Other salivary proteins such as immunoglobulin A (sIgA) and lysozyme (sLys) have been found to decrease in response to elite training[12]. In this study, 31 professional rugby players were monitored for eleven months. Even though no correlation analysis was performed between salivary parameters and training load, it was found that the athletes were more disposed to infections of the upper respiratory tract (URTI) when training intensity was higher and this was associated with a decreased in salivary proteins and elevations in salivary cortisol (sC)[12].

In addition to salivary proteins, the concentration of hormones in saliva, mainly testosterone (sT) (or its metabolic intermediates) and sC have been used to track the physiological impact of elite training as markers of anabolic and catabolic status, respectively. In this regard, 36 female professional handball players participated in a 16-week training program that consisted of 8 weeks of strength training at ~80% of the one-repetition maximum (1RM) and endurance training at the LT and 8 weeks of interval and high intensity training. The training program lead to an increase in the concentration of dehydroepiandrosterone (sDHEA)[13]. Interestingly, the sDHEA/sC ratio showed a negative linear relationship (r = -0.73) with TL at the end of the 16 weeks[13]. Similar findings were observed in adolescent tennis players during four weeks of tennis-specific training for

endurance, power and agility and one subsequent week of tapering[14]. Whereas sC correlated positively with TL (r=0.64), the sT/sC ratio correlated negatively (r=-0.77). The response of salivary hormones to training was also strongly associated with negative mood states[14]. In 18 Naval Special Warfare operators submitted to four months of physical training aimed to promote power and strength endurance sT, sC, sDHEA as well as the sDHEA/sC ratio increased during the months with higher volume and intensity and decreased concomitantly with TL. However, no changes were observed for the sT/sC ratio[15].

The cortisol awakening response (CAR) is a steep increase in the circulating levels of cortisol approximately 30 minutes after awakening. The CAR is thought to reflect the reactivity of the hypothalamic-pituitary-adrenal (HPA) axis to lasting and strenuous physiological and psychological demands[16]. After 16 weeks of endurance, strength and power training in 12 professional female tennis players, the diurnal rhythm and the levels upon awakening and 30 min afterwards of sC decreased when compared to pre-training levels. This was accompanied by concomitant increases in sAA upon awakening and as such, it was suggested that progressive increases in training load induce asymmetry in the activation of the HPA axis and the sympathetic nervous system, respectively[17]. Blunted rhythms of cortisol in response to excessive training have been suggested to represent a subclinical form of hypocortisolism[17]. However, the clinical meaning of such variation during elite training remains inconclusive.

Finally, conflicting data still exists. In a group of 18 football players during two weeks of football-specific drills, strength and power training, sC failed to monitor changes in either TL or performance whereas submaximal exercise heart rate (r=-0.8 and r=0.2, respectively) and mood states (r=0.2 and r=0.6, respectively) were significantly correlated[18].

Fatigue

Only a couple of studies have been able to diagnose fatigue by means of salivary markers in trained subjects. Ideally, the appropriate monitoring of TL would allow the necessary strategies for recovery to avoid overtraining. Yet, in ultra-endurance sports or especially in military training where sleep deprivation is common, the exceptionally high volume of exercise might lead to fatigue. The ratio between two peptides found in acidic and basic proline-rich salivary proteins was shown to increase in line with the rate of perceived exertion when nine amateur cyclists performed sets of 19-minute walking and 20-minute cycling at 70% of their maximum ventilator threshold with 10-minute rest between sets for ten consecutive hours[19]. These findings were later replicated by the same group in a larger cohort of subjects in an experimental model of mental fatigue[20]. Cognitive performance and mood states were assessed in 19 subjects (+16 controls) during 48 hours of sleep deprivation. The Fatigue Biomarker Index, as coined by the authors, could identify poor performance and mental fatigue with both selectivity and specificty[20]. On the other hand, sC showed a similar pattern than the work/rest cycles in 11 subjects taking part in the United States Air Force Special Tactics Officer selection. This process consists of five days of exercise such as running, swimming and ruck marching, several-hour missions that include swimming, land navigation, and skill tests as well as leadership reaction courses[21]. However, no correlation analyses were performed to confirm the association between salivary markers and fatigue in these studies.

Lactate

The LT and the maximum lactate steady state (MLSS) are indices of aerobic endurance commonly used to adjust the intensity of training in swimmers and rowers[22,23]. Lactate diffuses passively into saliva from blood and its accumulation in saliva closely reflects the one in blood during exercise. Exercise physiologists

have studied the accumulation of lactate in blood for decades and significant understanding in regard to its dynamics and application to sports medicine have been gathered. Unfortunately, very few studies have been directed towards investigating the suitability of surrogates of blood lactate (bLac) to predict training intensity in elite athletes. Based on the principle that, in general, sympathetic stimulation to the salivary glands leads to the secretion of electrolytes and proteins Chicharro and colleagues had already demonstrated twenty years ago that the salivary concentration of Na^+ and Cl^- show a similar behavior than bLac (r = 0.82) and catecholamines (r=0.75) in healthy males submitted to incremental exercise[24]. Similar results by their group were reported in ten-year-old children a year later[25]. Other studies have followed this rationale and have identified the viability of salivary proteins to predict the salivary LT in active subjects and elite athletes during sub-maximal or maximal exercise. Strong correlations have been reported between bLac and both the activity (r=0.95)[26] and the concentration of sAA (r=0.84 and r=0.81)[27,28], sPT (r=0.90; r=0.78 and r=0.93)[27–29] and sCgA (r=0.82)[28]. Others studies instead tackled the issue directly and determined the potential of salivary lactate (sLac) to predict bLac accumulation. Clearly, directly quantifying salivary lactate (sLac) would provide more useful information. In this regard, it has been demonstrated that sLac is a viable and reliable index to predict MLSS in amateur cyclists with strong correlations between bLac and sLac when the intensity of exercise was expressed in terms of VO_2 (r=0.89) and power (r=0.92)[30]. Using a graded cycle ergometer test with 25-W increments every three minutes until volitional exhaustion in amateur athletes, Segura and colleagues reported a high degree of concordance (r=0.81) between bLac and sLac, with the latter being highly accurate to predict the LT[31]. Similar associations in the pattern of accumulation between bLac and sLac (r=0.71) have been described in experienced marathon runners after a 30-km race[32]. Due to the passive diffusion of lactate from blood into saliva, it was suggested that more rapid increases in workload might complicate the determination of LT[31]. However, we demonstrated that even during short-duration incremental tests (<12min) sLac is a reliable predictor of the LT in elite swimmers[28]. In general, the

concentration of sLac is between 15 and 50% that the one found in blood during exercise[28,31,32]. Stronger correlations have been found in athletes (r=0.511) than in non-athletes (r=0.385) after maximal exercise with the latter group showing higher concentration of sLac at the workloads than the athletes[33]. Finally, equivalent to bLac measurements, sLac is higher in sprinters than long distance runners after a 400-m sprint and a 3000-m run, respectively[34]. Many of the studies mentioned above used common electro enzymatic methods to quantify lactate. Hence, from a methodological standpoint, portable lactate analyzers with lower detection limits could be employed to measure sLac in the field. On the other hand, in the laboratory, sLac has been proven to remain stable for 40 days when stored at 4°C[31]. The absence of red blood cells in saliva eliminates the need of pre-treating the samples with sodium fluoride to preserve glucose and avoid further production of lactate[28].

Hydration status

Euhydration is critical to maintain muscle strength and endurance[35,36]. However, beyond the clear importance of maintaining an appropriate hydration status for athletic performance, monitoring water loss, for instance, is pivotal in fighting and equestrian sports in which fast weight loss practices in the form of dehydration are employed prior to a competition in order to meet a required weight limit. Interesting findings have been accumulated regarding the potential of salivary parameters to track acute dehydration. Some studies have reported strong correlations between saliva osmolality (sOSM; r=0.94), salivary total protein (sPT; r=0.97) and salivary flow rate (r=-0.88) with the percentage of body mass loss (BML) up to 2.9% when subjects exercised at 60% of their VO_{2max} for approximately two hours[37]. When compared to plasma osmolality, saliva (r=0.87) and urine osmolality (r=0.83), and sPT (r=0.91) also correlated strongly[38]. A similar association between sOSM and percentages in BML (r=0.80) was observed when subjects performed two hours of ten-minute work/rest cycles of

treadmill walking at 4.0–4.8 $km \cdot h^{-1}$ while wearing the heavy personal protective equipment used in firefighting[39]. Similar dynamics in sOSM and 3% BML were observed after 48 hours of fluid and caloric restriction (11346±197 kJ and 40 ml/kg*BM per day) followed by exercise-induced dehydration (1.5 hours of walking at 50% VO_{2max} each day)[40]. However, no statistical analysis to correlate these parameters was performed in this report. In a more carefully designed study, strong correlations were observed when subjects were exposed to exercise and heat exposure to induce dehydration (7% BML; r=0.81), partial rehydration (3% BML; r=0.50) and re-dehydration (7% BML; r=0.70)[41]. According to the authors, the high inter-subject variability found in their study makes it difficult for sOSM to be employed in a wider population. Fortunately, this would not be an issue for elite athletes since coaches and medical personnel can easily gain reference values from their athletes throughout the training season.

One important factor to consider is whether dehydration is caused by exercise or heat exposure. A recent study provided evidence that sOSM is more sensible to track BML due to dehydration when subjects exercise (five hours of cycling at a self-selected intensity) rather than when passively exposed to hot and moderate humid environments (36°C and 50% relative humidity for five hours)[42]. They found that mild changes in body mass (~1%) were more closely associated with urine, rather than serum or saliva osmolality when dehydration was achieved through heat exposure. However, the opposite was found during exercise once serum and saliva osmolality distinguished moderate BML (>2%). The authors attributed the increased in sOSM during exercise to a parasympathetic impulse removal to the salivary glands, increasing the concentration of Na^+ and K^+ while reducing the secretion of water[42].

Some limitations of saliva to track mild to moderate dehydration should be acknowledged. First, fluid consumption shortly before the collection of samples would dilute solutes in saliva creating false results. It has been demonstrated that in subjects with 4% of BML due to exercise and heat exposure, an oral rinse

with 50mL of water decreases to the value of sOSM to euhydrated levels. sOSM only returned to the pre-dehydrated levels 15 minutes after the rinse[43]. Second, as mentioned above sOSM poorly discerned BML of 1% when dehydration was caused only by heat exposure[42]. It is a common practice for fighters to dehydrate using a combination of exercise and heat exposure (sauna) to make weight before a fight. Since the magnitude of BML is between 2-10%[44], it remains to be elucidated whereas sOSM tracks BML when dehydration is moderate to severe.

Performance

It has been reported that the resting levels of sT and sC are related to power and strength in elite rugby players. The improvement in performance in both the squat jump (SJ) and the box squat (BS) throughout four weeks of power and strength training in 18 rugby players correlated, though weakly, with the pre-exercise levels of sT (vs. SJ r=0.20 and vs. BS r=0.44) and sC (vs. SJ r=0.30 and vs. BS r=0.36)[45]. On the other hand, stronger correlations between performance in the 10-m sprint (r=-0.87) and 1RM squat (r=0.92) and sT were observed in five trained weightlifters during 40 days of training[46]. Although the original cohort included 10 subjects, the authors found only significant correlations in the data from the subjects able to squat more than twice their body weight before the beginning of training program. These findings are in agreement with the variation in performance and salivary hormones in wrestlers. In the course of 15 weeks of training, 15 elite junior wrestlers experienced gains on lean body mass (~1.5Kg), maximum mechanical power output (+12.8%), bench press (+5.7%), squat (+23.1%), power clean (+6.1%), and time to 3,000- and 30-m sprints (-3.6, -1.3%, respectively). These improvements in performance was associated with changes in sC, sT and the sT/sC ratio[47]. Further associations between neuromuscular performance and the pattern of hormone secretion were observed in 34 professional rugby players assessed for running speed, strength and power. sT correlated modestly with the 10 and 20-mt sprint (r=-0.48; -0.56, respectively), sC

correlated with the SJ (r=0.41), and the BS (r=0.39) while the sT/sC ratio correlated with the 10-20-mt sprint SJ (r=-0.39) and bench press throw (r=0.41)[48]. Of note, these data suggest that the concentration of both sT and sC prior to exercise might determine the adaptation to training. Furthermore, it indicates that the hormonal response to training depends on training status and thus it could be used to adjust TL on an individual basis throughout yearlong training programs[45,46,48]. Similarly, significant increases in sT (30.9%) were observed prior to winning games in 13 rugby players. The variation in sC was not related to either winning or losing games nor was it significant across the 7-week training period that the team was monitored[49].

We have demonstrated positive correlations between negative mood states and peak sC levels after awakening (r=0.71)[16] and between mood states and the concentration levels of sTP (r=0.67), sAA (r= 0.59) and CgA (r=0.61) in 12 professional swimmers during a national competition[50]. In a group of 15 football players submitted to a 9-day intensified training block, the percentage of change in the performance of the counter-movement jump (CMJ) was positively associated with increases in CAR after training (r=0.79)[51]. However, in this study the subjects were arbitrarily divided into responders and non-responders and only six of them showed a training-induced increase in CAR. Finally, sDHEA (r=0.38), but not sT, together with classic anthropometric and pubertal development characteristics have been shown to predict explosive leg power in 51 teenage football players and as such could be incorporated as an objective parameter for talent identification in sports[52]. Thus, the levels of steroid hormones might not only partially modulate the adaptation to training but also predict short and long-term performance in elite athletes.

Other applications

The other applications of the published data on salivary markers in sports medicine include the monitoring of recovery and the impact of long-term elite training on the profile of hormonal secretion. The effects of cryotherapy to assist in recovery have been confirmed by means of salivary hormones. Recently, Grasso and colleagues showed that in professional rugby players submitted to four hours of daily training, exposure for three minutes to -140°C in a temperature-controlled cryochamber twice a day for seven days reduced the resting concentration of sC, sDHEA and salivary estradiol while increased the levels of sT and the sT/sC ratio[53]. Hence, this study indicates that the benefits in recovery after cryotherapy might be modulated, at least in part, by variation in steroid hormones. Finally, saliva has also been used to study the effect that elite training bears on hormonal circadian rhythms. In a group of 239 (142 females) teenage elite gymnasts, the concentration of sC was higher in females during the morning (8-10 AM) and late in the afternoon (6-8 PM) when compared with 81 (40 females) age-matched controls not engaged in sports. This was associated with higher levels of perceived psychological stress[54]. Even though only two sampling points are not comprehensive enough to detect variations in diurnal biological rhythms, these findings suggest that long-term elite training might lead to over-activation of the HPA axis[54].

Conclusions

Considering the advantages of collection, handling and storage that saliva offers over blood, there is significant promise in using salivary markers in sports medicine. Thus far, compelling evidence has been accumulated in relation to TL, fatigue, recovery, lactate, hydration status and performance. However, these studies are still limited in number. From a biological standpoint, it is interesting to understand the response of a myriad of salivary molecules to single bouts of exercise. However, a more purposeful approach to identifying markers under scenarios of elite training is needed. The information gathered should be clear

and precise in detecting the physiological effect of training. Consequently, in our opinion, the potential of saliva to assist in the preparation of professional athletes would be fully explored as the result of a closer collaboration between sports scientists and coaches.

References

1. Hausswirth, C. & Lehénaff, D. Physiological demands of running during long distance runs and triathlons. *Sports Med. Auckl. NZ* **31,** 679–689 (2001).
2. Chatterton, R. T., Jr, Vogelsong, K. M., Lu, Y. C., Ellman, A. B. & Hudgens, G. A. Salivary alpha-amylase as a measure of endogenous adrenergic activity. *Clin. Physiol. Oxf. Engl.* **16,** 433–448 (1996).
3. Chatterton, R. T., Jr, Vogelsong, K. M., Lu, Y. C. & Hudgens, G. A. Hormonal responses to psychological stress in men preparing for skydiving. *J. Clin. Endocrinol. Metab.* **82,** 2503–2509 (1997).
4. Toda, M., Den, R., Hasegawa-Ohira, M. & Morimoto, K. Effects of woodland walking on salivary stress markers cortisol and chromogranin A. *Complement. Ther. Med.* **21,** 29–34 (2013).
5. Capranica, L. *et al.* Salivary cortisol and alpha-amylase reactivity to taekwondo competition in children. *Eur. J. Appl. Physiol.* **112,** 647–652 (2012).
6. Gallina, S. *et al.* Salivary chromogranin A, but not α-amylase, correlates with cardiovascular parameters during high-intensity exercise. *Clin. Endocrinol. (Oxf.)* **75,** 747–752 (2011).
7. Ra, S.-G., Maeda, S., Higashino, R., Imai, T. & Miyakawa, S. Metabolomics of salivary fatigue markers in soccer players after consecutive games. *Appl. Physiol. Nutr. Metab. Physiol. Appl. Nutr. Metab.* 1–7 (2014). doi:10.1139/apnm-2013-0546
8. Roos, L., Taube, W., Brandt, M., Heyer, L. & Wyss, T. Monitoring of Daily Training Load and Training Load Responses in Endurance Sports: What Do Coaches Want? *Schweiz. Z. Für Sportmed. Sporttraumatologie* **61,** 30–36 (2013).
9. Diaz, M. M., Bocanegra, O. L., Teixeira, R. R., Soares, S. S. & Espindola, F. S. Salivary Nitric Oxide and Alpha-Amylase as Indexes of Training Intensity and Load. *Int. J. Sports Med.* (2012). doi:10.1055/s-0032-1316318

10. Díaz Gómez, M. M., Bocanegra Jaramillo, O. L., Teixeira, R. R. & Espindola, F. S. Salivary Surrogates of Plasma Nitrite and Catecholamines during a 21-Week Training Season in Swimmers. *PLoS ONE* **8,** e64043 (2013).

11. Rassaf, T. *et al.* Nitric oxide synthase-derived plasma nitrite predicts exercise capacity. *Br. J. Sports Med.* **41,** 669–673; discussion 673 (2007).

12. Cunniffe, B. *et al.* Time course of changes in immuneoendocrine markers following an international rugby game. *Eur. J. Appl. Physiol.* **108,** 113–122 (2010).

13. Filaire, E., Duché, P. & Lac, G. Effects of amount of training on the saliva concentrations of cortisol, dehydroepiandrosterone and on the dehydroepiandrosterone: cortisol concentration ratio in women over 16 weeks of training. *Eur. J. Appl. Physiol.* **78,** 466–471 (1998).

14. Gomes, R. V. *et al.* MONITORING TRAINING LOADS, STRESS, IMMUNE-ENDOCRINE RESPONSES AND PERFORMANCE IN TENNIS PLAYERS. *Biol. Sport* **30,** 173–180 (2013).

15. Oliver, J. M. *et al.* SALIVARY HORMONE RESPONSE TO 12-WEEK BLOCK- PERIODIZED TRAINING IN NAVAL SPECIAL WARFARE OPERATORS. *J. Strength Cond. Res. Natl. Strength Cond. Assoc.* (2014). doi:10.1519/JSC.0000000000000628

16. Díaz, M. M. *et al.* The relationship between the cortisol awakening response, mood States, and performance. *J. Strength Cond. Res. Natl. Strength Cond. Assoc.* **27,** 1340–1348 (2013).

17. Filaire, E., Ferreira, J. P., Oliveira, M. & Massart, A. Diurnal patterns of salivary alpha-amylase and cortisol secretion in female adolescent tennis players after 16 weeks of training. *Psychoneuroendocrinology* **38,** 1122–1132 (2013).

18. Buchheit, M. *et al.* Monitoring fitness, fatigue and running performance during a pre-season training camp in elite football players. *J. Sci. Med. Sport Sports Med. Aust.* **16,** 550–555 (2013).

19. Michael, D. J., Daugherty, S., Santos, A., Ruby, B. C. & Kalns, J. E. Fatigue biomarker index: an objective salivary measure of fatigue level. *Accid. Anal. Prev.* **45 Suppl,** 68–73 (2012).

20. Michael, D. J., Valle, B., Cox, J., Kalns, J. E. & Fogt, D. L. Salivary biomarkers of physical fatigue as markers of sleep deprivation. *J. Clin. Sleep Med. JCSM Off. Publ. Am. Acad. Sleep Med.* **9,** 1325–1331 (2013).

21. Cuddy, J. S., Reinert, A. R., Hailes, W. S., Slivka, D. R. & Ruby, B. C. Accelerometry and salivary cortisol response during Air Force Special Tactics Officer selection. *Extreme Physiol. Med.* **2,** 28 (2013).

22. Toubekis, A. G. & Tokmakidis, S. P. Metabolic Responses at Various Intensities Relative to Critical Swimming Velocity. *J. Strength* **27,** 1731–1741 (2013).

23. Smith, T. B. & Hopkins, W. G. Measures of rowing performance. *Sports Med. Auckl. NZ* **42,** 343–358 (2012).

24. Chicharro, J. L. *et al.* Saliva electrolytes as a useful tool for anaerobic threshold determination. *Eur. J. Appl. Physiol.* **68,** 214–218 (1994).

25. Chicharro, J. L. *et al.* Anaerobic threshold in children: determination from saliva analysis in field tests. *Eur. J. Appl. Physiol.* **70,** 541–544 (1995).

26. Calvo, F. *et al.* Anaerobic threshold determination with analysis of salivary amylase. *Can. J. Appl. Physiol. Rev. Can. Physiol. Appliquée* **22,** 553–561 (1997).

27. De Oliveira, V. N. *et al.* Changes in the salivary biomarkers induced by an effort test. *Int. J. Sports Med.* **31,** 377–381 (2010).

28. Bocanegra, O. L., Diaz, M. M., Teixeira, R. R., Soares, S. S. & Espindola, F. S. Determination of the lactate threshold by means of salivary biomarkers: chromogranin A as novel marker of exercise intensity. *Eur. J. Appl. Physiol.* (2012). doi:10.1007/s00421-011-2294-4

29. Bortolini, M. J. S. *et al.* Total protein of whole saliva as a biomarker of anaerobic threshold. *Res. Q. Exerc. Sport* **80,** 604–610 (2009).

30. Pérez, M., Lucía, A., Carvajal, A., Pardo, J. & Chicharro, J. L. Determination of the maximum steady state of lactate (MLSS) in saliva: an alternative to blood lactate determination. *Jpn. J. Physiol.* **49,** 395–400 (1999).

31. Segura, R. *et al.* A new approach to the assessment of anaerobic metabolism: measurement of lactate in saliva. *Br. J. Sports Med.* **30,** 305–309 (1996).

32. Santos, R. V. T., Almeida, A. L. R., Caperuto, E. C., Martins, E., Jr & Costa Rosa, L. F. B. P. Effects of a 30-km race upon salivary lactate correlation with blood lactate. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **145,** 114–117 (2006).

33. Tékus, E. *et al.* Comparison of blood and saliva lactate level after maximum intensity exercise. *Acta Biol. Hung.* **63 Suppl 1,** 89–98 (2012).

34. Ohkuwa, T., Itoh, H., Yamazaki, Y. & Sato, Y. Salivary and blood lactate after supramaximal exercise in sprinters and long-distance runners. *Scand. J. Med. Sci. Sports* **5,** 285–290 (1995).

35. Eric D. B. Goulet, M. L.-L. Pre-Exercise Hypohydration Effects On Jumping Ability And Muscle Strength, Endurance And Anaerobic Capacity: A Meta-Analysis: 1681. *Med. Sci. Sports Exerc. - MED SCI SPORT Exerc.* **42,** (2010).

36. Pierre-Yves Gigou, M. L.-L. Meta-Analysis Of The Effects Of Pre-Exercise Hypohydration On Endurance Performance, Lactate Threshold And Vo2max: 1679. *Med. Sci. Sports Exerc. - MED SCI SPORT Exerc.* **42,** 361–362 (2010).

37. Walsh, N. P., Montague, J. C., Callow, N. & Rowlands, A. V. Saliva flow rate, total protein concentration and osmolality as potential markers of whole body hydration status during progressive acute dehydration in humans. *Arch. Oral Biol.* **49,** 149–154 (2004).

38. Walsh, N. P. *et al.* Saliva parameters as potential indices of hydration status during acute dehydration. *Med. Sci. Sports Exerc.* **36,** 1535–1542 (2004).

39. Smith, D. L., Shalmiyeva, I., Deblois, J. & Winke, M. Use of salivary osmolality to assess dehydration. *Prehospital Emerg. Care Off. J. Natl. Assoc. EMS Physicians Natl. Assoc. State EMS Dir.* **16,** 128–135 (2012).

40. Oliver, S. J., Laing, S. J., Wilson, S., Bilzon, J. L. J. & Walsh, N. P. Saliva indices track hypohydration during 48h of fluid restriction or combined fluid and energy restriction. *Arch. Oral Biol.* **53,** 975–980 (2008).

41. Taylor, N. A. S. *et al.* Observations on saliva osmolality during progressive dehydration and partial rehydration. *Eur. J. Appl. Physiol.* **112,** 3227–3237 (2012).

42. Muñoz, C. X. *et al.* Assessment of hydration biomarkers including salivary osmolality during passive and active dehydration. *Eur. J. Clin. Nutr.* **67,** 1257–1263 (2013).

43. Ely, B. R., Cheuvront, S. N., Kenefick, R. W. & Sawka, M. N. Limitations of salivary osmolality as a marker of hydration status. *Med. Sci. Sports Exerc.* **43,** 1080–1084 (2011).

44. Franchini, E., Brito, C. J. & Artioli, G. G. Weight loss in combat sports: physiological, psychological and performance effects. *J. Int. Soc. Sports Nutr.* **9,** 52 (2012).

45. Crewther, B. T., Lowe, T., Weatherby, R. P. & Gill, N. Prior sprint cycling did not enhance training adaptation, but resting salivary hormones were related to workout power and strength. *Eur. J. Appl. Physiol.* **105,** 919–927 (2009).

46. Crewther, B. T., Cook, C. J., Gaviglio, C. M., Kilduff, L. P. & Drawer, S. Baseline strength can influence the ability of salivary free testosterone to predict squat and sprinting performance. *J. Strength Cond. Res. Natl. Strength Cond. Assoc.* **26,** 261–268 (2012).

47. Passelergue, P. A. & Lac, G. Salivary hormonal responses and performance changes during 15 weeks of mixed aerobic and weight training in elite junior wrestlers. *J. Strength Cond. Res. Natl. Strength Cond. Assoc.* **26,** 3049–3058 (2012).

48. Crewther, B. T., Lowe, T., Weatherby, R. P., Gill, N. & Keogh, J. Neuromuscular performance of elite rugby union players and relationships with salivary hormones. *J. Strength Cond. Res. Natl. Strength Cond. Assoc.* **23,** 2046–2053 (2009).

49. Crewther, B. T. *et al.* The workout responses of salivary-free testosterone and cortisol concentrations and their association with the subsequent competition outcomes in professional rugby league. *J. Strength Cond. Res. Natl. Strength Cond. Assoc.* **27,** 471–476 (2013).

50. Diaz, M. M., Bocanegra, O. L., Teixeira, R. R., Soares, S. S. & Espindola, F. S. Response of Salivary Markers of Autonomic Activity to Elite Competition. *Int. J. Sports Med.* (2012). doi:10.1055/s-0032-1304638

51. Minetto, M. A. *et al.* Changes in awakening cortisol response and midnight salivary cortisol are sensitive markers of strenuous training-induced fatigue. *J. Endocrinol. Invest.* **31,** 16–24 (2008).

52. Baldari, C. *et al.* Is explosive performance influenced by androgen concentrations in young male soccer players? *Br. J. Sports Med.* **43,** 191–194 (2009).

53. Grasso, D. *et al.* Salivary steroid hormone response to whole-body cryotherapy in elite rugby players. *J. Biol. Regul. Homeost. Agents* **28,** 291–300 (2014).

54. Georgopoulos, N. A. *et al.* Abolished circadian rhythm of salivary cortisol in elite artistic gymnasts. *Steroids* **76,** 353–357 (2011).

Table 1 Salivary Markers in Sports Medicine

Parameter	Marker	Findings	Sport / Model	Reference
TL	sPT, sAA, sCgA & sNO_2	Strong correlation between salivary markers, TL and plasma catecholamines during 21 week of training	Swimming	[9, 10]
	sIgA, sLys & sC	Vulnerability to during periods of intense training associated with a reduction in salivary proteins and an increase in sC	Rugby	[12]
	sC & sDHEA	Negative strong correlation between TL and the sDHEA/sC ratio	Handball & Volleyball	[13]
	sT & sC	Strong positive and negative correlations between TL and sC and the sT/sC ratio, respectively	Tennis	[14]
	sT, sC, & sDHEA	Similar patterns between the variation of volume and intensity of exercise and salivary hormones	Power & Strength Training	[15]
	sAA & sC	Opposite awakening levels between sAA and sC after training	Tennis	[17]
	sC	No correlation with TL	Football	[18]
Fatigue	Proline-rich salivary	Similar patterns between the ratio of two salivary proteins and physical fatigue	Long-duration	[19]

	proteins		Exercise	
	sC	Similar patterns between work/rest cycles and sC	Military Training	[21]
Lactate	Na^+ & Cl^-	Strong correlations between ions in saliva with bLac and catecholamines	Incremental Exercise	[24, 25]
	sPT, sAA, sCgA & sLac	Strong correlations between salivary proteins, bLac and sLac	Swimming / Incremental Exercise	[26-29]
	sLac	Strong correlations with bLac	Running	[30-34]
Hydration Status	sPT , sOSM, & flow rate	Strong correlations with BML	Moderate Exercise / Heat Exposure	[37-43]
Performance	sT & sC	Weak correlations between salivary hormones and improvements in the SJ and BS	Rugby	[45]
	sT	Strong correlations between with improvements in 1RM and 10-m sprint	Weightlifting	[46]
	sT & sC	Correlations between salivary hormones and body lean mass gain as well as improvements in power and strength	Wrestling	[47]
	sT & sC	Correlations between salivary hormones and improvements	Rugby	[48]

		in power and strength		
	sT	Increases in sT prior to winning games	Rugby	[49]
	sPT, sAA, sCgA & sC	Moderate correlations between negative mood states and salivary parameters prior to a national competition	Swimming	[16, 50]
	sC	Strong correlation between a training-induced increases in the awakening levels of sC and improvements in CMJ	Football	[51]
	sDHEA	Moderate correlations between sDHEA and explosive leg power	Football	[52]
Recovery	sT, sC, sDHEA & salivary estradiol	Cryotherapy reduced the resting concentration of sC, sDHEA and salivary estradiol while increased the levels of sT and the sT/sC ratio.	Rugby	[53]
Circadian Rhythms	sC	Elevated diurnal concentration of sC associated with higher levels of perceived psychological stress	Gymnastics	[54]

TL: Training Load; sPT: salivary total protein; sAA: salivary alpha-amylase; sCgA: salivary chromogranin A; sNO_2: salivary nitrite; sIgA; salivary immunoglobulin A; sLys: salivary lysozyme; sC: salivary cortisol; URTI: upper respiratory tract infections; sDHEA: salivary dehydroepiandrosterone; sT; salivary testosterone; bLac: blood lactate; sLac: salivary lactate; sOSM: saliva osmolality; BML: body mass loss; SJ: squat jump; BS; box squat; 1RM; one-maximum repetition; CMJ: counter-movement jump.

Capítulo 4

Salivary Surrogates of Plasma Nitrite and Catecholamines During a 21-week Training Season in Swimmers

Manuscrito formatado como publicado na revista Plos One no dia 23 de maio de 2013

DOI: 10.1371/journal.pone.0064043

Resumo

A coleta de amostras de saliva é simples e não invasiva, o que a torna um fluido ideal para o monitoramento da resposta adaptativa ao treinamento. Neste estudo foi investigada a resposta das proteínas salivares alfa-amilase (sAA), da cromogranina A (sCgA) e a concentração de proteína total (sPT) e de nitrito salivar (sNO_2) em relação à concentração de catecolaminas e de nitrito plasmático (pNO_2), respectivamente. A variação ocorrida nestes marcadores foi comparada com a intensidade e carga de treinamento ao longo de 21 semanas de treinamento, em 12 nadadores profissionais. Em geral, as proteínas salivares apresentaram uma dinâmica similar à adrenalina plasmática e correlações negativas relativamente aos parâmetros de treinamento. Não houve correlação entre o nitrito salivar e o sanguíneo. No entanto, o sNO_2 demonstrou uma correlação positiva com a intensidade e carga de treinamento. Uma menor atividade simpática é responsável pela diminuição da concentração de proteínas salivares ao longo da sessão de treinamento. Além disso, o aumento da concentração de nitrito estará muito provavelmente relacionado com as variações hemodinâmicas e com a regulação do tono vascular. A relação entre os marcadores salivares e os parâmetros de treinamento ressalta o potencial destes marcadores como marcadores não invasivos de adaptação ao treinamento, em atletas profissionais.

Palavras Chave: Treinamento; Biomarcador; Saliva; Exercício; Adaptação

ABSTRACT

The collection of samples of saliva is noninvasive and straightforward, which turns saliva into an ideal fluid for monitoring the adaptive response to training. Here, we investigated the response of the salivary proteins alpha-amylase (sAA), chromogranin A (sCgA), and the concentration of total protein (sTP) as well as salivary nitrite (sNO_2) in relation to plasma catecholamines and plasma nitrite (pNO_2), respectively. The variation in these markers was compared to the intensity and load of training during a 21-week training season in 12 elite swimmers. Overall, the salivary proteins tracked the concentration of plasma adrenaline and were inversely correlated with the training outcomes. No correlations were observed between sNO_2 and pNO_2. However, sNO_2 correlated positively with the intensity and load of training. We argue that the decrease in sympathetic activity is responsible for the decrease in the concentration of proteins throughout the training season. Furthermore, the increase in nitrite is likely to reflect changes in hemodynamics and regulation of vascular tone. The association of the salivary markers with the training outcomes underlines their potential as noninvasive markers of training status in professional athletes.

Keywords: Training; Biomarker; Saliva; Exercise; Adaptation.

INTRODUCTION

Periodization is a structured approach based mainly upon the variation of the volume and the intensity of training. Periodization allows athletes to reach maximal performance at appropriate times by providing the necessary physiological adaptation and recovery [1,2]. Intense and continuous training can induce changes in a broad series of biochemical parameters such as the release of muscle proteins into the blood and variations in cortisol, urea, iron, catecholamines, and blood counts [3,4]. These parameters are often used to monitor the physiological response to training. Abnormal levels of skeletal muscle proteins in the blood, for instance, can be interpreted as a signal of muscle damage [5]. High concentrations of cortisol and urea are widely regarded as markers of increased protein turnover [6] whereas decreased levels of iron might compromise performance due to its critical role in the delivery and utilization of oxygen by the active muscle [7]. Finally, variations in catecholamines and leukocytes regularly suggest inadequate recovery from training [5,6]. However, the quantification of these parameters requires blood sampling and it can be inconvenient for the athletes or pose safety risks. Furthermore, for some people venipuncture is painful and stressful. Consequently, the collection of blood might increase the levels of catecholamines and cortisol, thus invalidating the assay. By contrast, the collection of saliva is noninvasive and straightforward. Therefore, analyzing salivary components is clearly appealing in sports medicine.

Catecholamines occupy critical positions in the regulation of physiological processes during exercise. The concentration of plasma catecholamines rises rapidly during exercise, especially at high intensities. This results in increased cardiac output, vasoconstriction in the non-contracting muscles, stimulation of the sweat glands, transportation of oxygen and energetic substrates to the active muscles, and increased contractility of the skeletal muscles [8]. On the other hand, plasma nitrite (pNO_2) is the product of the oxidation of nitric oxide (NO) and is essential for vasodilation in the systemic and renal vasculature. Further, it has been demonstrated that the concentration of pNO_2 at rest predicts exercise capacity and is correlated with flow-mediated vasodilation in healthy subjects [9].

Recently, we demonstrated that salivary alpha-amylase (sAA) and salivary nitrite (sNO_2) show a proportional response to the variation of the intensity and load of training[10]. Salivary alpha-amylase is the most abundant enzyme in saliva and has digestive and anti-microbial properties [11]. The reasoning behind the use of sAA to monitor training is that sAA is released into the saliva mainly after sympathetic stimulation and thus, is considered a surrogate marker for catecholamines [12]. Considering the role of nitrite in vasodilation, we proposed that sNO_2 would show an equivalent response to the intensity of training [10]. However, the levels of nitrite in saliva are substantially higher than in blood due to the reduction of nitrate by oral bacteria [13]. Therefore, the argument that sNO_2 is correlated to pNO_2 warrants confirmation.

A series of studies by Chatterton and colleagues in the late 1990s stimulated considerable interest in sAA as a marker of sympathetic activity [12,14]. In these studies, it was reported that the levels of sAA increased significantly before parachute jumping [14] and were correlated with plasma noradrenaline (r=.64) and adrenaline (r=.49) after a single bout of exercise [12]. Ever since, a great deal of research has been devoted to investigating changes in the activity of sAA to a broad series of acute exercise protocols. However, none of them investigated further relations between sAA and catecholamines. On the other hand, less attention has been given to sNO_2. Few articles have reported changes in sNO_2 after single bouts of exercise [15,16] but only our previous study has shown the response of sNO_2, and sAA, to long-term training under resting conditions. In that study, both sAA and sNO_2 behaved proportionally, although in opposite directions, to the intensity and load of training. While sAA correlated negatively with the parameters of training, sNO_2 correlated positively. Because they reflect the activity of biological systems pivotal to the adaptation to training, sAA and sNO_2 could prove to be appealing markers in sports medicine. Further, to be useful, the variation of salivary parameters in response to training should be assessed under resting conditions, as it is done with most of the traditional markers in blood, and that is why longitudinal designs are particularly important

The current study aimed to extend the previous findings on sAA and sNO_2 as markers of the intensity and load of training . We assessed 12 professional swimmers throughout 21 weeks of training. In addition to sAA and sNO_2, salivary chromogranin A (sCgA), and salivary total protein (sTP) were quantified in saliva. These parameters were compared to plasma catecholamines and $pNO_{2,}$. We hypothesized that there would be a strong correlation between salivary proteins and plasma catecholamines and between sNO_2 and pNO_2. We expected that the variations in these markers would be equivalent to the oscillation of the intensity and load of training.

METHODS

Ethics Statement

The subjects were 12 professional swimmers (8 men and 3 women aged 19.3±1.4 years; BMI 24.3±1.7 kg/m^2; competition experience; 4.6±2.8 years) different from those who participated in our previous study [10]. None of them smoked, had significant medical or oral health history or was taking regular or incidental medication during the study. One week before the beginning of the collection of the samples, the subjects gave their written informed consent. The experimental protocol was approved by the Institutional Review Board of the Federal University of Uberlandia (Protocol CEP/UFU 483/10).

Design

The subjects were evaluated during their regular training season. The experimental design has been described elsewhere [10]. In sum, the subjects completed nine training sessions per week that included predominantly swimming. The volume, intensity and load during the swimming sessions throughout the 5-month study are shown in Figures 1A and 1B. The intensity of training was established by means of blood lactate measures, with an intensity of 100% corresponding to a swimming velocity at the anaerobic threshold for each individual. Every four weeks during the 5-month season, the subjects attended the laboratory under fasting conditions for collection of saliva and blood. All collection procedures took place at 8 am. On each visit, heart rate (HR) and blood pressure

(BP) were recorded. The subjects completed the Profile of Mood States Questionnaire (POMS) immediately before the collection of saliva. The POMS is a 65-item questionnaire measuring tension, depression, anger, confusion, vigor and fatigue on a 5-point Likert scale. Diet logs were kept to ensure consistent caloric and nitrate intake for the 48 hours before each sample collection (Table 1).

Sample collection

Whole saliva was collected with no exogenous stimulation. The saliva was allowed to pool in the mouth and then drooled into pre-weighted collection vials after two minutes. The subjects were asked to refrain from drinking, eating or tooth brushing during the hour prior to the collection of the samples. Immediately after collecting saliva, blood from the antecubital vein (± 10 mL) was withdrawn into two EDTA-coated tubes. The samples were obtained by a qualified phlebotomist using standardized venipuncture techniques. The analysis of the blood samples was performed immediately after the collection. The subjects had had their blood routinely sampled prior to this study and none of them reported the procedure as stressful. After the collection of saliva, the samples were stored frozen at -20°C.

Catecholamines

The blood samples were centrifuged at 4°C for 5 min at 5000 rpm and the plasma was separated. The concentration of plasma catecholamines was determined by high performance liquid chromatography with electrochemical detection. The catecholamines were extracted by alumina and eluted by acetic acid. The separation was performed using a Supelcosil LC-18-DB column (Sigma. St. Louis, MO, USA). The mobile phase consisted of 13.8 g of monosodium phosphate, 100 mg of EDTA, 0.2 g of sodium octanesulfonate, and 30 mL of acetonitrile, pH 3.0, delivered at flow rate of 1mL/min. The electrochemical detector consisted of a triple-electrode system (Electrochemical Detection, Coulochem, ESA, Chelmsford, MA, USA). The concentration of catecholamines in each sample was corrected using 3,4-dihydroxybenzylamine as the internal standard [17]. The assay sensitivity was approximately 10pg. The intra-assay coefficients of variation were below 10%.

Nitrite

To avoid the reaction of nitrite with hemoglobin, the blood samples were immediately centrifuged after collection to separate the plasma. Nitrite was determined by acid diazotation [18]. Fifty µL of saliva or plasma were incubated with 50 µL of Griess reagent (1% sulfanilamide in 2.5% H_3PO_4 and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride) at room temperature for 10 min. The absorbance was measured at 570 nm using a microplate reader (Molecular Devices, Menlo Park, CA, USA). The content of nitrite was calculated based on a standard curve constructed with $NaNO_2$ at the concentrations of 400, 200, 100, 50, 25, 12.5, 6.25 and 3.12 µM. The assay sensitivity was approximately 125 pmoL. The intra and inter-assay coefficients of variation for duplicate samples was 4.8% and 6.3% and 12.3% and 14.2% for sNO_2 and pNO_2, respectively.

Salivary proteins

On the day of analysis, the samples of saliva were thawed and centrifuged at 3000 rpm for 15 minutes. The concentration of the total protein in the samples was determined by the Bradford method [19] using Coomassie Brilliant Blue G-250 (Fisher BioReagents, Fair Lawn, NJ, USA) and bovine serum albumin (Sigma. St. Louis, MO, USA) as the standard protein. The limit of detection of the Bradford method is 1 µg of protein. The intra and inter-assay coefficients of variation for the duplicate samples was 3.2% and 6.1%, respectively. The concentration of the total protein in the samples was used as loading control in western blots. All of the samples from each subject were assayed on the same plate in duplicate. To avoid the possible effects of salivary flow rate on the concentration of proteins, ten micrograms of the total protein from each sample were denatured under reducing conditions and applied on 5–20% SDS–polyacrylamide gradient gels, as previously suggested [20,21]. The proteins were separated and then transferred onto nitrocellulose membranes in transfer buffer (25 mM Tris, 190 mM glycine, 20% MeOH, pH 7.8-8.4) for two hours at 200 mA and 4°C. The protein transfer was confirmed by visualization with Ponceau. The membranes were blocked for 4 hours at 4°C in blocking buffer (5% non-fat dry milk in PBS w/v). The membranes were then incubated overnight at 4°C with purified polyclonal rabbit anti-human sAA (dilution 1:5000) (produced in our laboratory) and mouse monoclonal anti-

human CgA (dilution 1:1000) (Millipore, Temecula, CA), respectively. The membranes were subsequently incubated with secondary antibodies for two hours. After the incubations with specific primary and then secondary antibodies, the labeled proteins were detected with ECL reagents and by exposing the developed blots to GE Healthcare films**.** The densitometrical analysis of the spots was performed using ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA) by a researcher who was blinded to the experimental design. The area in pixels of each spot was determined in triplicate, and the means were used for statistical analyses.

Determination of sAA activity

The samples of saliva were centrifuged at 3000 rpm for 15 min to remove mucins. Ten µL of saliva were diluted (1:200) in MES buffer (MES 50mM, NaCl 300mM, $CaCl_2$ 5mM, KSCN 140mM, pH 6.3) followed by the addition of 300 µL of pre-heated (37 °C) substrate solution (2-chloro-4-nitrophenyl-galactopyranoside maltoside). The optical density was read at 405 nm at one-min intervals during three min at 37 °C using a microplate reader (Molecular Devices, Menlo Park, CA, USA). The enzyme activity was determined using the formula: [Absorbance difference per minute × total assay volume (308 ml) × dilution factor (200)]/ [millimolar absorptivity of 2-chloro-4-nitrophenyl (12.9) × sample volume (.008 ml) × light path (.97) [10]. The enzyme activity (U/mL) was then multiplied by flow rate (mL/min) to estimate the sAA secretion rate (U/min). The assay sensitivity was approximately 0.4 U/mL. The intra-assay coefficient of variation for duplicate samples was 7.6%.

STATISTICAL ANALYSIS

The data were tested for normality using the Shapiro-Wilk test prior to the analyses. All of the variables were compared by one-way analysis of variance (ANOVA) with repeated measures followed by the Tukey test for multiple comparisons. The relationships between biochemical markers and training outcomes were analyzed using a two-tailed Pearson correlation coefficient. For all

of the analyses, significant results were defined at the level of $p < 0.05$. The results shown are means (SD).

RESULTS

Markers of autonomic activity

Figures 2A and 2B show the response to training of the markers of autonomic activity . We noted significant differences in adrenaline [F (4, 9) = 5.26, p = 0.019], noradrenaline [F (4, 9) = 7.47, p = 0.002] and dopamine [F (4, 9) = 6.76, p = 0.004]. The concentration of adrenaline and dopamine decreased up to the middle of the training season and subsequently increased towards the baseline levels. On the other hand, noradrenaline oscillated with each month of training. Similar results to those of adrenaline were observed for the salivary surrogate markers. The activity of sAA [F (4, 12) = 12.43, p = 0.019], as well as its concentration [F (4, 11) = 11.15 $p < 0.0001$], sCgA [F (4, 10) = 25.03, $p < 0.0001$], and sTP [F (4,9) = 18.24, $p < 0.001$] declined up to the middle of the season with following increases by the end (Figures 2 and 3). No differences in salivary flow rate, HR or BP were observed in response to training (Figure 4 and Table 2).

Nitrite

As expected, the concentration of nitrite in saliva was greater than in plasma. The levels of both sNO_2 [F (4, 11) = 3.59 $p < 0.05$] and pNO_2 [F (4, 12) = 6,42 p = 0.027] varied significantly in response to training. However, no significant correlation was found between the two (Figure 5).

Correlation between biochemical markers and training outcomes

The Pearson correlation coefficients between the biochemical markers and the training outcomes are shown in Table 3. Throughout the training season, the variation in sAA (both the activity and the concentration) showed a strong positive correlation with adrenaline whereas sCgA and sPT correlated modestly. Only sTP correlated positively with dopamine. We found no significant correlations between the salivary proteins and noradrenaline. Interestingly, we observed a strong positive correlation between the activity and the concentration of sAA [r(12) = 0.75,

$p < 0.05$]. Furthermore, adrenaline, dopamine and the salivary proteins correlated negatively with the intensity and load of training. On the other hand, sNO_2 but not pNO_2 correlated positively with the training outcomes.

DISCUSSION

Consistent with our original hypothesis, we found strong and modest correlations between the salivary proteins and adrenaline. Further, the salivary proteins and sNO_2 showed a proportional response to the intensity and load of training. However, contrary to our preliminary hypothesis, sNO_2 was not associated to pNO_2 and the latter did not predict the training outcomes.

It is well documented that training modulates autonomic activity. The adaptations associated with long-term training include a decrease in sympathetic activity and an increase in parasympathetic drive [22]. Several reports have found lower resting levels of adrenaline after long-term training in humans [23–26]. In these studies, the decrease in adrenaline appears to be more related to the intensity of exercise than to the duration. In our study, catecholamines varied significantly in response to training, and adrenaline was strongly correlated with the load of training.

Although it would be expected that as direct markers of autonomic activity catecholamines, HR and BP displayed similar patterns during training, it is worth mentioning that the autonomic regulation is a tissue-specific and intricate process. Most of the mechanisms linked to the exercise-induced bradycardia, for instance, are thought to be a consequence of increases in vagal tone and a reduction in intrinsic HR. However, a reduction in sympathetic tone is considered to have little effect on the lower HR observed in trained subjects [26,27]. We failed to observe significant changes in HR and BP. This may be attributed to a “ceiling” effect of training considering the experience of the subjects as competitive athletes, or to the little effect of catecholamines in decreasing sympathetic activity in the heart and blood vessel.

It is interesting to note that the salivary proteins displayed a similar pattern to adrenaline and significantly correlated with the intensity and load of training. As mentioned above, salivary proteins are released into saliva mainly after sympathetic stimulation [28]. Therefore, a decline in the levels of proteins in response to training is expected. Chatterton et al., reported correlations of .64 and .49 between sAA, adrenaline and noradrenaline, respectively, after 10-min intervals of walking, jogging and running [29]. None of the salivary proteins assessed in our study correlated with noradrenaline. Furthermore, although significantly different throughout the training season, noradrenaline failed to correlate with the training outcomes. Under resting conditions, most of the noradrenaline found in plasma is the result of spillover from the sympathetic nerve terminals with a small proportion coming from the adrenal gland [30]. We attribute the lack of correlation between the salivary proteins and noradrenaline in our study to the fact that the subjects were assessed under resting conditions and not after acute submaximal exercise.

Salivary alpha-amylase is the most abundant enzyme in saliva. It is released mainly from the parotid and the submandibular glands and has digestive and anti-fungal properties [11]. Thus far, most of the studies on the sAA response to exercise have incorporated kinetic assays with only a handful of them assessing the concentration of the enzyme [20,21]. It has been suggested that post-translational modifications such as glycosylations, or the formation of protein complexes between sAA and mucins might affect amylolytic function [31]. Therefore, the concentration of sAA rather than its activity would be a more appropriate marker of autonomic drive. No other study has investigated this association in response to training. Here, we observed a strong correlation between the two ($r = 0.75$). Additionally, both the concentration and the activity of sAA correlated significantly with the intensity and load of training. It remains to be seen whether other populations, such as type II diabetics or immunocompromised patients show a corresponding response between the activity and the concentration of sAA during exercise.

Salivary chromogranin A is secreted into saliva from the submandibular gland. As a surrogate marker of autonomic activity, sCgA shares the same rationale that sAA. Both are secreted into saliva mainly after sympathetic innervation to the salivary glands. Most studies agree on increments in sCgA in response to exercise. Salivary chromogranin A has been shown to increase proportionally to the intensity of exercise [20,32] and to correlate with the double product of HR and BP (r = 0.89) as well as the rate of perceived exertion (r = 0.82) during a maximal exercise test [33]. In our study sCgA correlated positively with adrenaline and these two correlated negatively with the intensity and load of training. Our data are in agreement with previous studies showing equivalent responses of sAA and sCgA to acute maximal exercise [20,21,32], exposure to microgravity [34] and adverse psychological stimuli [35]. The study that observed a significant increase in sCgA but not sAA in response to exercise did show that both peaked at the end of the exercise and returned to baseline levels 30 min thereafter [33].

Only recently has research identified sTP as a prospective marker in sports medicine. A previous report, for instance, indicates that sTP could predict dehydration after exercise [36]. We have previously proposed that sTP is an ideal marker of autonomic activity [21] and some studies have shown parallel responses between sTP and other salivary proteins during exercise [20,37]. Here, sTP correlated with adrenaline and the training outcomes. As with sAA and sCgA, the concentration of sTP in saliva rises after innervation to the glands. However, unlike the former proteins, the quantification of sTP is straightforward and inexpensive.

The two major pathways for the production of NO in the body are the arginine/NOS and the nitrate-nitrite-NO pathway. Thus, nitrate and nitrite are important alternative sources of NO and are involved in vascular processes. Nitric oxide is an essential regulator of metabolism and vascular tone. In the striated muscle, NO increases insulin sensitivity, stimulates lipid oxidation and reduces contractility. In the blood vessel, it is known to inhibit contraction, platelet aggregation and promote vasodilation [38]. Here, we have shown that the resting levels of both sNO_2 and pNO_2 increase in response to long-term training. However, the variation in one of them does not reflect changes in the other.

Dietary nitrate is absorbed in the small intestine and concentrated in the salivary glands. Roughly 5% of the ingested nitrate is reduced to nitrite in the oral cavity by commensal bacteria. Consequently, the levels of nitrate in saliva are approximately 10-fold higher than in plasma [39]. On the other hand, only a fraction of plasma nitrate originates from the NOS pathway [40]. Other groups have presented evidence suggesting that nitrite can be transformed to NO either spontaneously in the blood, or enzymatically in the endothelial cells[41,42]. Under hypoxic and acidic conditions, such as exercise, nitrite is converted to NO increasing blood flow [43]. This would constitute a NO-scavenging mechanism whereby erythrocytes are unable to form nitrate and methemoglobin. Thus, the levels of sNO_2 may be more related to plasma nitrate than nitrite. Despite the lack of association between sNO_2 and pNO_2, the former did correlate with the training outcomes and constitutes an attractive marker of the load of training in sports medicine.

For a better understanding of the physiological impact of training, we included analysis of glucose, triglycerides, cholesterol, cortisol, urea, iron, lactate dehydrogenase, creatine kinase and complete blood counts (data not shown). These markers were evaluated at the same points than nitrite, catecholamines and the salivary proteins. Additionally, the subjects answered the POMS immediately before the collection of the samples. Glucose, triglycerides, and cholesterol were selected to monitor substantial changes and/or compliance with the diet before each collection point. Because there were no differential changes in any of these parameters and the diet was standardized 48 hours before collecting the samples, we are certain that the variation in catecholamines, salivary proteins and nitrite was due to the oscillation of the training variables. The lack of any significant difference in these parameters during the training season is broadly consistent with several studies in professional athletes which have reported no effect of training in metabolic, hormonal or immunological markers when the periodization of training is appropriate [44–47]. It is important to point out that the parameters above have been shown to vary mainly in stages of overreaching and overtraining, which frequently imply grueling loads of training. However, there is no reason to expect any change in these markers when the load of training is properly tolerated.

Regarding the female athletes in our study, no difference has been observed in the levels of salivary proteins between men and women under resting conditions [48,49] or in response to adverse stimuli [50]. Moreover, although we did not control for the phase of the menstrual cycle, it is well documented that the variation of sex hormones during the menstrual cycle does not influence such parameters [51–54].

In conclusion, sAA (both the activity and the concentration), sCgA and sTP track the concentration of plasma adrenaline in response to training. Given the simplicity for the quantification of sTP, we propose that sTP be used instead of sAA and sCgA as a marker of autonomic activity. Salivary NO_2 does not reflect changes in pNO_2. However, sNO_2 does predict training outcomes and along with the salivary proteins represent an attractive marker of training status in professional athletes.

REFERENCES

1. Stone MH (1990) Muscle conditioning and muscle injuries. Med Sci Sports Exerc 22: 457–462.
2. Gabbett TJ (2004) Reductions in pre-season training loads reduce training injury rates in rugby league players. Br J Sports Med 38: 743–749. doi:10.1136/bjsm.2003.008391.
3. Banfi G, Colombini A, Lombardi G, Lubkowska A (2012) Metabolic markers in sports medicine. Adv Clin Chem 56: 1–54.
4. Rønsen O, Børsheim E, Bahr R, Klarlund pedersen B, Haug E, et al. (2004) Immuno-endocrine and metabolic responses to long distance ski racing in world-class male and female cross-country skiers. Scandinavian Journal of Medicine & Science in Sports 14: 39–48. doi:10.1111/j.1600-0838.2003.00333.x.
5. Millet GY, Tomazin K, Verges S, Vincent C, Bonnefoy R, et al. (2011) Neuromuscular consequences of an extreme mountain ultra-marathon. PLoS ONE 6: e17059. doi:10.1371/journal.pone.0017059.
6. Hug M, Mullis PE, Vogt M, Ventura N, Hoppeler H (2003) Training modalities: over-reaching and over-training in athletes, including a study of the role of hormones. Best Pract Res Clin Endocrinol Metab 17: 191–209.
7. Peeling P, Dawson B, Goodman C, Landers G, Trinder D (2008) Athletic induced iron deficiency: new insights into the role of inflammation, cytokines and hormones. European Journal of Applied Physiology 103: 381–391. doi:10.1007/s00421-008-0726-6.
8. Zouhal H, Jacob C, Delamarche P, Gratas-Delamarche A (2008) Catecholamines and the effects of exercise, training and gender. Sports Med 38: 401–423.
9. Rassaf T, Lauer T, Heiss C, Balzer J, Mangold S, et al. (2007) Nitric oxide synthase-derived plasma nitrite predicts exercise capacity. Br J Sports Med 41: 669–673; discussion 673. doi:10.1136/bjsm.2007.035758.
10. Diaz MM, Bocanegra OL, Teixeira RR, Soares SS, Espindola FS (2012) Salivary Nitric Oxide and Alpha-Amylase as Indexes of Training Intensity and Load. Int J Sports Med. Available: http://www.ncbi.nlm.nih.gov/pubmed/22960992. Accessed 19 September 2012.

11. Scannapieco FA, Torres G, Levine MJ (1993) Salivary alpha-amylase: role in dental plaque and caries formation. Crit Rev Oral Biol Med 4: 301–307.
12. Chatterton RT Jr, Vogelsong KM, Lu YC, Ellman AB, Hudgens GA (1996) Salivary alpha-amylase as a measure of endogenous adrenergic activity. Clin Physiol 16: 433–448.
13. Archer DL (2002) Evidence that ingested nitrate and nitrite are beneficial to health. J Food Prot 65: 872–875.
14. Chatterton RT Jr, Vogelsong KM, Lu YC, Hudgens GA (1997) Hormonal responses to psychological stress in men preparing for skydiving. J Clin Endocrinol Metab 82: 2503–2509.
15. Rahman ZA, Abdullah N, Singh R, Sosroseno W (2010) Effect of acute exercise on the levels of salivary cortisol, tumor necrosis factor-alpha and nitric oxide. J Oral Sci 52: 133–136.
16. Panossian AG, Oganessian AS, Ambartsumian M, Gabrielian ES, Wagner H, et al. (1999) Effects of heavy physical exercise and adaptogens on nitric oxide content in human saliva. Phytomedicine 6: 17–26.
17. Eisenhofer G, Goldstein DS, Stull R, Keiser HR, Sunderland T, et al. (1986) Simultaneous liquid-chromatographic determination of 3,4-dihydroxyphenylglycol, catecholamines, and 3,4-dihydroxyphenylalanine in plasma, and their responses to inhibition of monoamine oxidase. Clinical Chemistry 32: 2030–2033.
18. Tsikas D (2007) Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: appraisal of the Griess reaction in the L-arginine/nitric oxide area of research. J Chromatogr B Analyt Technol Biomed Life Sci 851: 51–70. doi:10.1016/j.jchromb.2006.07.054.
19. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.
20. Bocanegra OL, Diaz MM, Teixeira RR, Soares SS, Espindola FS (2012) Determination of the lactate threshold by means of salivary biomarkers: chromogranin A as novel marker of exercise intensity. European Journal of Applied Physiology. Available: http://www.ncbi.nlm.nih.gov/pubmed/22227853. Accessed 30 January 2012.

21. Diaz MM, Bocanegra OL, Teixeira RR, Soares SS, Espindola FS (2012) Response of Salivary Markers of Autonomic Activity to Elite Competition. International journal of sports medicine. Available: http://www.ncbi.nlm.nih.gov/pubmed/22581684. Accessed 2 July 2012.

22. Mueller PJ (2007) Exercise training and sympathetic nervous system activity: evidence for physical activity dependent neural plasticity. Clin Exp Pharmacol Physiol 34: 377–384. doi:10.1111/j.1440-1681.2007.04590.x.

23. Winder WW, Hagberg JM, Hickson RC, Ehsani AA, McLane JA (1978) Time course of sympathoadrenal adaptation to endurance exercise training in man. J Appl Physiol 45: 370–374.

24. Winder WW, Hickson RC, Hagberg JM, Ehsani AA, McLane JA (1979) Training-induced changes in hormonal and metabolic responses to submaximal exercise. J Appl Physiol 46: 766–771.

25. Lacour JR, Pequignot JM, Geyssant A, Coudert J, Peyrin L (1982) [Effect of training on plasma levels of catecholamines on the course of submaximal exercise]. J Physiol (Paris) 78: 838–842.

26. Mazon J, Gastaldi A, Di Sacco T, Cozza I, Dutra S, et al. (2011) Effects of training periodization on cardiac autonomic modulation and endogenous stress markers in volleyball players. Scandinavian Journal of Medicine & Science in Sports: no–no. doi:10.1111/j.1600-0838.2011.01357.x.

27. Smith ML, Hudson DL, Graitzer HM, Raven PB (1989) Exercise training bradycardia: the role of autonomic balance. Med Sci Sports Exerc 21: 40–44.

28. Proctor GB, Carpenter GH (2007) Regulation of salivary gland function by autonomic nerves. Auton Neurosci 133: 3–18. doi:10.1016/j.autneu.2006.10.006.

29. Chatterton RT Jr, Vogelsong KM, Lu YC, Ellman AB, Hudgens GA (1996) Salivary alpha-amylase as a measure of endogenous adrenergic activity. Clin Physiol 16: 433–448.

30. Goldstein DS, Eisenhofer G, Kopin IJ (2003) Sources and significance of plasma levels of catechols and their metabolites in humans. J Pharmacol Exp Ther 305: 800–811. doi:10.1124/jpet.103.049270.

31. Mandel AL, Peyrot des Gachons C, Plank KL, Alarcon S, Breslin PAS (2010) Individual differences in AMY1 gene copy number, salivary α-amylase

levels, and the perception of oral starch. PLoS ONE 5: e13352. doi:10.1371/journal.pone.0013352.

32. Allgrove JE, Gomes E, Hough J, Gleeson M (2008) Effects of exercise intensity on salivary antimicrobial proteins and markers of stress in active men. J Sports Sci 26: 653–661. doi:10.1080/02640410701716790.

33. Gallina S, Di Mauro M, D'Amico MA, D'Angelo E, Sablone A, et al. (2011) Salivary chromogranin A, but not α-amylase, correlates with cardiovascular parameters during high-intensity exercise. Clin Endocrinol (Oxf) 75: 747–752. doi:10.1111/j.1365-2265.2011.04143.x.

34. Rai B, Kaur J (2011) Salivary stress markers and psychological stress in simulated microgravity: 21 days in 6° head-down tilt. J Oral Sci 53: 103–107.

35. Filaire E, Dreux B, Massart A, Nourrit B, Rama LM, et al. (2009) Salivary alpha-amylase, cortisol and chromogranin A responses to a lecture: impact of sex. Eur J Appl Physiol 106: 71–77. doi:10.1007/s00421-009-0991-z.

36. Walsh NP, Montague JC, Callow N, Rowlands AV (2004) Saliva flow rate, total protein concentration and osmolality as potential markers of whole body hydration status during progressive acute dehydration in humans. Arch Oral Biol 49: 149–154.

37. Bortolini MJS, De Agostini GG, Reis IT, Lamounier RPMS, Blumberg JB, et al. (2009) Total protein of whole saliva as a biomarker of anaerobic threshold. Res Q Exerc Sport 80: 604–610.

38. Levine AB, Punihaole D, Levine TB (2012) Characterization of the role of nitric oxide and its clinical applications. Cardiology 122: 55–68. doi:10.1159/000338150.

39. McKnight GM, Smith LM, Drummond RS, Duncan CW, Golden M, et al. (1997) Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. Gut 40: 211–214.

40. Dejam A, Hunter CJ, Pelletier MM, Hsu LL, Machado RF, et al. (2005) Erythrocytes are the major intravascular storage sites of nitrite in human blood. Blood 106: 734–739. doi:10.1182/blood-2005-02-0567.

41. Millar TM, Stevens CR, Benjamin N, Eisenthal R, Harrison R, et al. (1998) Xanthine oxidoreductase catalyses the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions. FEBS Lett 427: 225–228.

42. Hunt JF, Fang K, Malik R, Snyder A, Malhotra N, et al. (2000) Endogenous airway acidification. Implications for asthma pathophysiology. Am J Respir Crit Care Med 161: 694–699.

43. Gladwin MT, Shelhamer JH, Schechter AN, Pease-Fye ME, Waclawiw MA, et al. (2000) Role of circulating nitrite and S-nitrosohemoglobin in the regulation of regional blood flow in humans. Proc Natl Acad Sci USA 97: 11482–11487. doi:10.1073/pnas.97.21.11482.

44. Meyer T, Meister S (2011) Routine Blood Parameters in Elite Soccer Players. International Journal of Sports Medicine 32: 875–881. doi:10.1055/s-0031-1280776.

45. Banfi G, Del Fabbro M, Mauri C, Corsi MM, Melegati G (2006) Haematological parameters in elite rugby players during a competitive season. Clin Lab Haematol 28: 183–188. doi:10.1111/j.1365-2257.2006.00771.x.

46. Mujika I, Chatard JC, Geyssant A (1996) Effects of training and taper on blood leucocyte populations in competitive swimmers: relationships with cortisol and performance. Int J Sports Med 17: 213–217. doi:10.1055/s-2007-972834.

47. Ostojic SM, Ahmetovic Z (2009) Indicators of iron status in elite soccer players during the sports season. International Journal of Laboratory Hematology 31: 447–452. doi:10.1111/j.1751-553X.2008.01064.x.

48. Rohleder N, Nater UM, Wolf JM, Ehlert U, Kirschbaum C (2004) Psychosocial stress-induced activation of salivary alpha-amylase: an indicator of sympathetic activity? Ann N Y Acad Sci 1032: 258–263. doi:10.1196/annals.1314.033.

49. Rantonen PJ, Meurman JH (2000) Correlations between total protein, lysozyme, immunoglobulins, amylase, and albumin in stimulated whole saliva during daytime. Acta Odontol Scand 58: 160–165.

50. Harm DL, Schlegel TT (2002) Predicting motion sickness during parabolic flight. Auton Neurosci 97: 116–121.

51. Tenovuo J, Laine M, Söderling E, Irjala K (1981) Evaluation of salivary markers during the menstrual cycle: peroxidase, protein, and electrolytes. Biochem Med 25: 337–345.

52. Bhoola KD, Matthews RW, Roberts F (1978) A survey of salivary kallikrein and amylase in a population of schoolgirls, throughout the menstrual cycle. Clin Sci Mol Med Suppl 55: 561–566.
53. D'Alessandro S, Curbelo HM, Tumilasci OR, Tessler JA, Houssay AB (1989) Changes in human parotid salivary protein and sialic acid levels during pregnancy. Arch Oral Biol 34: 829–831.
54. Laine M, Pienihäkkinen K, Ojanotko-Harri A, Tenovuo J (1991) Effects of low-dose oral contraceptives on female whole saliva. Arch Oral Biol 36: 549–552.

Table 1 Daily dietary intake for the subjects during the 48 hours prior to each collection of the samples. Values are Means (SD).

Total energy (Kcal)	3254 (758.71)
Carbohydrates (g)	486.3 (110.12)
Proteins (g)	143.2 (42.62)
Total fats (g)	84.36 (22.73)
Saturated fats (g)	42.21 (2.97)
Monounsaturated fats (g)	34.53 (2.88)
Polyunsaturated fats (g)	34.78 (5.01)
Nitrate (mg)	84.62 (4.61)

Table 2 Heart rate and blood pressure throughout the 21-week training season. Values are Means (SD).

	Week 1	Week 6	Week 11	Week 16	Week 21
Heart rate (bpm)	64.57 (12.12)	58.20 (6.68)	57.67 (6.32)	57.90 (6.24)	62.83 (9.49)
Systolic pressure (mm Hg)	121.0 (13.70)	109.0 (11.01)	110.0 (9.48)	114.0 (13.50)	116.0 (8.43)
Diastolic pressure (mm Hg)	71.40 (5.70)	67.00 (8.23)	66.50 (6.25)	69.00 (7.37)	67.00 (8.53)

Table 3 Pearson correlation coefficients between the biochemical markers and the training outcomes at $p < 0.05$.

	sAA	[sAA]	sCgA	sTP	Adr	Nor	Dop	sNO_2	pNO_2
sAA	-	-	-	-	-	-	-	-	-
[sAA]	.75	-	-	-	-	-	-	-	-
sCgA	-	-	-	-	-	-	-	-	-
sTP	-	-	-	-	.78	-	-	-	-
Adr	.83	.89	.52	.59	-	-	-	-	-
Nor	ns	ns	ns	ns	-	-	-	-	-
Dop	ns	ns	ns	.96	-	-	-	-	-
sNO_2	-	-	-	-	-	-	-	-	-
pNO_2	-	-	-	-	-	-	-	ns	-
Volume	ns	-.87	ns	ns	ns	ns	ns	ns	ns
Intensity	-.53	-.30	-.85	-.90	-.49	ns	-.88	.33	ns
Load	-.80	-.83	-.46	-.59	-.84	ns	-.57	.68	ns

sAA = salivary alpha-amylase (activity); [sAA] = salivary alpha-amylase (concentration); Adr = adrenaline; Nor = noradrenaline; Dop = dopamine.

Figures

Figure 1 Training outcomes.

Figure 1A shows the variation in the intensity and volume of training. Figure 1B illustrates the variation in the load of training (expressed as a function of volume x intensity).

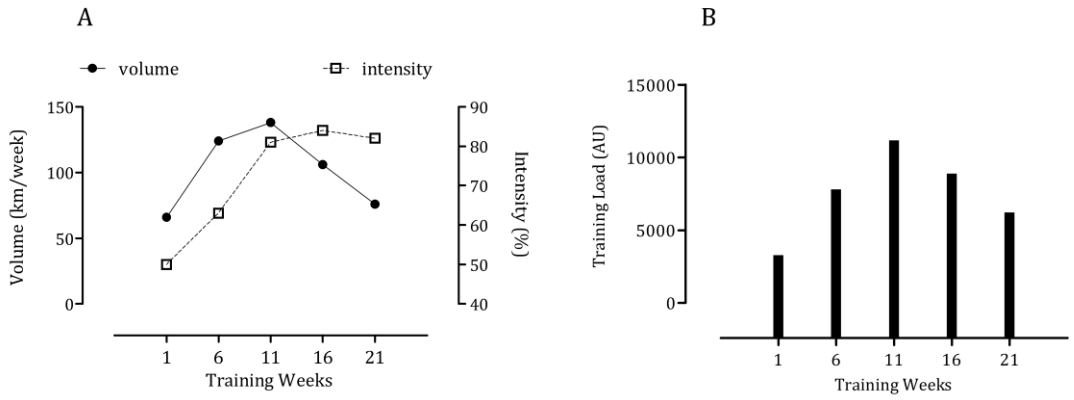


Figure 2 Plasma catecholamines and salivary proteins.

Figure 2A shows the variation in adrenaline and noradrenaline in response to training. Adrenaline: • Different from Training week 16. * Different from Training Week 21. Noradrenaline: δ Different from Training Week 11. σ Different from Training Week 21. Figure 2B shows the variation in dopamine. * Different from Training Week 16. Figure 2C shows the response of sAA activity vs. concentration. sAA activity: * Different from Training Week 11. sAA concentration: • Different from Training Week 21. Figure 2D shows the variation in sCgA and sTP in response to training. sTP: * Different from Training Week 11. sCgA: • Different from training week 6. δ Different from training week 11. σ Different from Training Week 16.

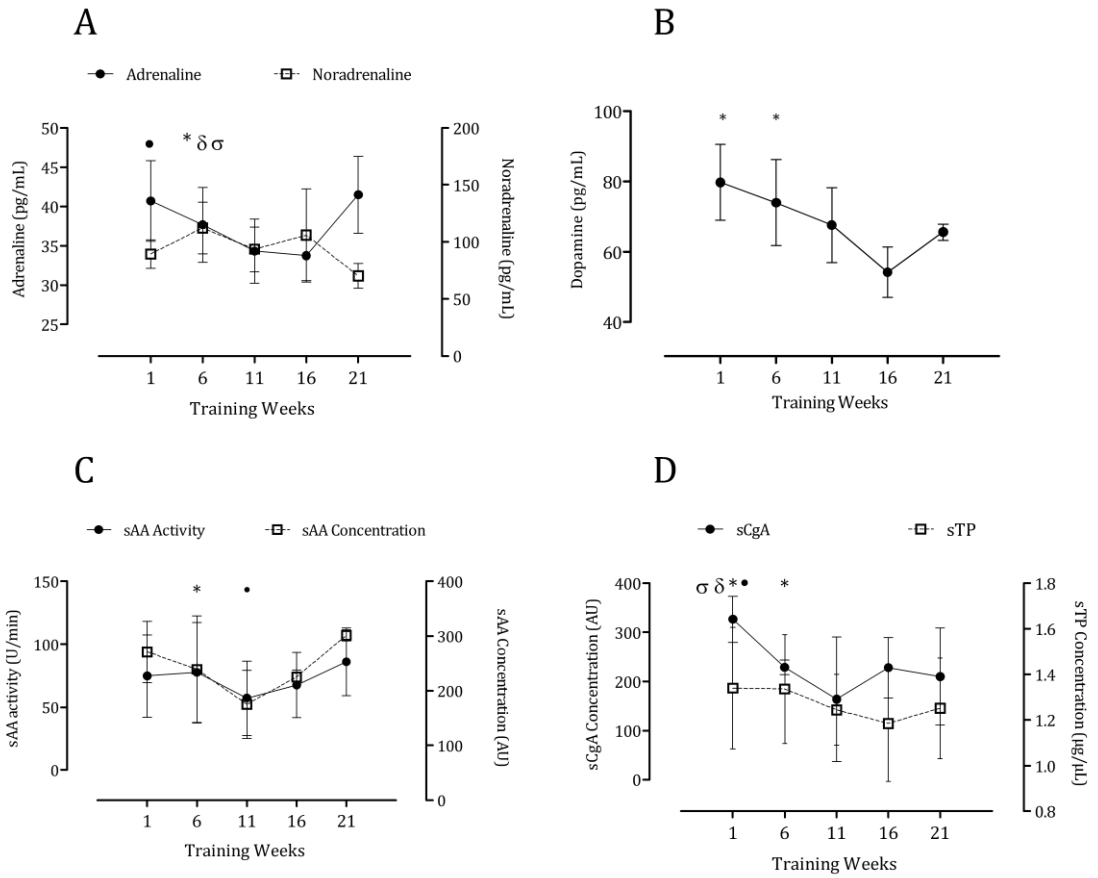


Figure 3 Representative blots of sAA and sCgA in response to training.

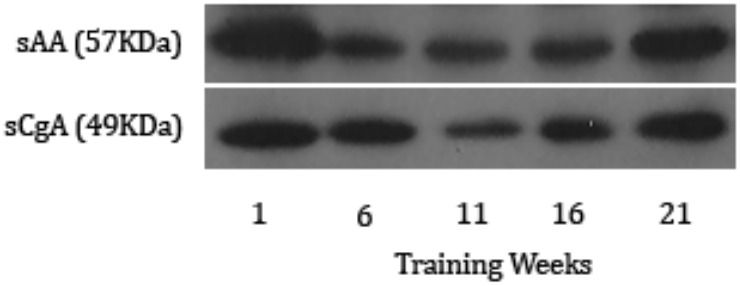


Figure 4 Salivary flow.

No difference in salivary flow was observed throughout the training season.

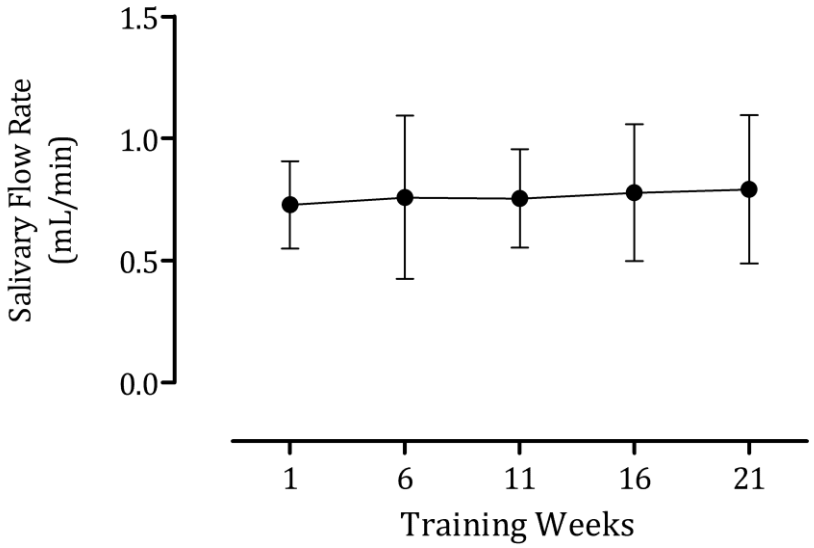


Figure 5 Plasma and salivary nitrite.

* Different from Training Week 21 (pNO_2).