UNIVERSIDADE DE LISBOA

FACULDADE DE MOTRICIDADE HUMANA

IMMUNE CELL CHANGES IN SWIMMERS:

RESPONSE TO ACUTE EXERCISE AND TRAINING

Tese elaborada com vista à obtenção do grau de Doutor em
Motricidade Humana na especialidade de Fisiologia do Exercício

Orientador: Professora Doutora Cristina Paula Fidalgo Negreiros Monteiro Bento

Co-orientador: Professor Doutor Francisco José Bessone Ferreira Alves

Júri:
  Presidente
    Professor Doutor António Fernando Rosado
  Vogais
    Professor Doutor José Alberto Ramos Duarte
    Professora Doutora Maria José Cabrita Lucas Laires
    Professor Doutor José Henrique Fuentes Gomes Pereira
    Professor Doutor Luís Manuel Pinto Lopes Rama
    Professora Doutora Cristina Paula Fidalgo Negreiros Monteiro Bento

JOSÉ PEDRO EVANGELISTA DE RESENDES MORGADO

Janeiro 2016

Reprodução da Tese

DECLARAÇÃO

Nome: José Pedro Evangelista de Resendes Morgado

Endereço electrónico: morgado.ze@gmail.com **Telefone:** 965490820

Número do Bilhete de Identidade/Cartão de Cidadão: 11889147 2ZZ7

Título: Immune Cell Changes in Swimmers: Response to Acute Exercise and Training

Orientador: Professora Doutora Cristina Paula Fidalgo Negreiros Monteiro Bento

Co-orientador: Professor Doutor Francisco José Bessone Ferreira Alves

Ano de conclusão: 2016

Designação do ramo de conhecimento do Doutoramento: Fisiologia do Exercício

Nos exemplares das teses de doutoramento entregues para a prestação de provas na Universidade e dos quais é obrigatoriamente enviado um exemplar para depósito legal na Biblioteca Nacional e pelo menos outro para a Biblioteca da FMH/UL deve constar uma das seguintes declarações:

1. É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE/TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

2. É AUTORIZADA A REPRODUÇÃO PARCIAL DESTA TESE/TRABALHO (indicar, caso tal seja necessário, nº máximo de páginas, ilustrações, gráficos, etc.) APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

3. DE ACORDO COM A LEGISLAÇÃO EM VIGOR, (indicar, caso tal seja necessário, nº máximo de páginas, ilustrações, gráficos, etc.) NÃO É PERMITIDA A REPRODUÇÃO DE QUALQUER PARTE DESTA TESE/TRABALHO.

Faculdade de Motricidade Humana – Universidade de Lisboa, ____/ ____ / _____

Assinatura _____

FCT

Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

DEDICATÓRIA:

À minha Mãe Isabel Maria e ao meu Pai Zé Carlos

Acknowledgements/Agradecimentos

Agradeço a todos os que estiveram envolvidos directa ou indirectamente na realização deste trabalho:

Em primeiro lugar, à minha orientadora Professora Doutora Cristina Paula Fidalgo Negreiros Monteiro Bento, apesar de não existirem palavras que traduzam a dimensão do meu apreço e gratidão, bem como o meu sentimento de amizade. É uma referência na minha vida e uma senhora por quem tenho um profundo e enorme respeito. Agradeço pela importante orientação, por tudo o que me ensinou, pelo apoio e acompanhamento efectivos e pela disponibilidade e dedicação em todas as fases do doutoramento. Agradeço a paciência, compreensão, o carinho, a confiança, o incentivo e a motivação. Agradeço por ter ajudado a "encontrar-me" quando tudo parecia não estar a correr bem e o fim estar cada vez mais distante. Sem a Professora o meu percurso académico teria terminado.

À Professora Doutora Maria José Laires, por me ter incentivado a prosseguir na minha carreira académica após a concretização do mestrado, por me ter apoiado e acompanhado no início dos trabalhos do doutoramento, pelas críticas e sugestões aos textos e por todo o entusiasmo e confiança.

Ao Professor Doutor Francisco Alves, pela orientação nos períodos críticos deste estudo e por contribuir para direcionar a acção e objectivos dos trabalhos desenvolvidos.

À Professora Doutora Catarina Matias, a amiga que nunca desistiu de "puxar" por mim, companheira de laboratório e uma valiosa ajuda nos variados assuntos relacionados com a utilização de instrumentos, programas, revisões de textos e publicações. Agradeço a companhia e a possibilidade de poder ter convivido com uma profissional de excelência.

À Professora Doutora Joana Reis, grande amiga de longa data. Não foram apenas as avaliações do doutoramento, a disponibilidade demonstrada, a escrita dos artigos, e a pertinência de todos os comentários e sugestões. Também por estar sempre presente e ser a pessoa com quem pude falar abertamente sem receios acerca de tudo.

À Professora Doutora Isabel Fragoso, pela orientação relacionada com a maturação, as avaliações antropométricas e de composição corporal, pela sinceridade, simpatia e carinho.

À Professora Doutora Júlia Teles, pela orientação relacionada com a análise estatística complexa e pela paciência e disponibilidade reveladas.

Ao Professor Doutor Luis Rama, pelas sugestões e esclarecimentos de dúvidas relativas às diferentes áreas do conhecimento abordadas neste trabalho, e pela orientação e disponibilização de instrumentos e bibliografia relacionados com o treino competitivo e sistema imunitário.

Aos atletas, treinadores e dirigentes desportivos dos clubes de natação que, amavelmente, aceitaram participar neste estudo e disponibilizar as suas instalações, sem os quais o trabalho aqui desenvolvido não teria sido possível, nomeadamente: Sport Lisboa e Benfica (SLB), Sporting Clube de Portugal (SCP), Associação Naval Amorense (ANAM) e Sociedade Filarmónica União Artística Piedense (SFUAP).

Quero especialmente agradecer às profissionais do Instituto Nacional de Saúde Doutor Ricardo Jorge, sem os quais o estudo não se poderia ter realizado, e cuja colaboração foi preciosa no que diz respeito às analises de sangue: à Professora Doutora Mafalda Bourbon, pela coordenação de esforços no sentido de viabilizar e organizar as análises sanguíneas, às Doutoras Fátima Martins, Maria Teresa Seixas e Marta Alvim pelo esforço, disponibilidade e paciência para analisar uma enorme quantidade de colheitas sanguíneas.

À Fátima Raposo, Lara Butes, Ana Mil-Homens, e Joana Simões pelo excelente trabalho na difícil tarefa das recolhas sanguíneas. Um especial agradecimento à Joana Simões que foi inexcedível e fez com que fosse possível ter sempre técnicos especializados para efectuar as recolhas, e ainda o apoio extra de todas nos registos das avaliações.

Aos amigos Luís Madruga, Luís André, Nuno Almeida, que se disponibilizaram para ajudar nas avaliações e registo de resultados.

À minha família, em especial aos meus tios Ana e Zé e às minhas avós Evangelina e Maria dos Anjos, por toda a compreensão e paciência nos momentos difíceis do meu percurso. Obrigado por me terem ajudado a chegar até aqui.

Por considerar fazerem parte da família, agradeço ao Sr. Vitorino e à Sra. Maria de Jesus por serem meus amigos, acreditarem em mim, e por me terem apoiado incondicionalmente nos últimos anos em tudo.

Título

Alterações da Imunidade Celular em Nadadores: Resposta ao Exercício Agudo e ao Treino

Resumo

O treino competitivo envolve exercício intenso e prolongado, capaz de modular o número e actividade das células imunitárias. Quando demasiado exigente poderá induzir fadiga e aumentar a susceptibilidade a doenças.

Esta dissertação apresenta três estudos desenvolvidos no âmbito da Imunologia do Exercício, considerando a análise da resposta celular imunitária sistémica aguda e crónica ao exercício aplicada em situações reais do treino competitivo de natação, controlando factores passíveis de influenciar esta resposta. Pretendeu-se avaliar a resposta imunitária a uma sessão de treino prolongada e intensa, durante as 24h de recuperação (Estudo 1) e a uma época de treino com sete meses (Estudo 2), e estudar a influência de um macrociclo de treino de quatro meses sobre a resposta imunitária à mesma sessão de treino e período de recuperação (Estudo 3), controlando sexo, fases do ciclo menstrual, maturidade, escalão, especialidade, performance, cargas de treino e sintomas respiratórios superiores (URS).

A sessão de treino induziu a diminuição da vigilância imunitária adquirida imediatamente e, pelo menos nas 2h seguintes. Juvenis e seniores recuperaram totalmente 24h depois, mas não os juniores, reforçando a ideia da existência de uma *janela aberta* para a infecção após exercícios prolongados e intensos e sugerindo uma recuperação menos eficiente para os juniores. No período de treino mais intenso da época observou-se uma imunodepressão e maior prevalência de URS. No final da época, a imunidade inata diminuiu aparentando maior sensibilidade aos efeitos cumulativos da carga de treino, enquanto a imunidade adquirida parece ter recuperado após o *taper*. O macrociclo de treino atenuou a resposta imunitária à sessão de treino e aumentou o período de *janela aberta* às infecções (efeitos mais acentuados nos adolescentes).

Os resultados evidenciam a importância de controlar alterações imunitárias durante a época competitiva, especialmente em períodos de treino intenso e quando se realizam sessões de treino intensas consecutivas com recuperações inferiores a 24h.

Palavras-Chave: Imunidade Celular, Nadadores, Sessão de Treino, Época de Treino, Sintomas Respiratórios Superiores.

Title

Immune Cell Changes in Swimmers: Response to Acute Exercise and Training

Abstract

Competitive training demands strenuous prolonged exercise that may modulate the number and activity of circulating immune cells. Over demanding programs can lead to fatigue and increased risk of infection and susceptibility to diseases.

This dissertation presents three studies developed within the Exercise Immunology scope, considering the analysis of acute and chronic systemic immune cell responses to exercise applied in real situations of competitive training, controlling for factors that may affect immune responses. We aimed to evaluate the immune response to a high intensity prolonged swimming training session (Study 1) and to a 7-month swimming training season (Study 2), and the influence of a 4-month training macrocycle on the immune response to a swimming session (Study 3). Subjects characteristics, namely sex, menstrual cycle phase, maturity, swimming age group, and distance specialty were controlled, and training load, performance improvements and Upper Respiratory Symptoms (URS) were monitored.

The swimming session induced an impaired acquired immune surveillance immediately and at least throughout 2h post-exercise, however, 24h after, senior and youth swimmers had totally recovered but not juniors. This supports the idea of an *open window* to infection after prolonged intense exercise, suggesting also a more difficult recovery of juniors. During the season's high intensity training periods immune depression and higher URS prevalence were observed. When the season ended, innate immunity was decreased, appearing to have been more affected by cumulative training loads, while acquired immunity seemed to have adapted and recovered efficiently after the *taper* period. The training season induced an overall attenuation of the immune system's ability to respond to the swimming session, and a subsequent longer *open window* period of susceptibility to infection (more accentuated in adolescents).

These findings enhance the importance of controlling immune alterations throughout the season, especially in heavy training periods and when performing consecutive intense training sessions without 24h of recovery.

Keywords: Cellular Immunity, Swimming, Training session, Training Season, Upper Respiratory Symptoms.

Título

Alterações da Imunidade Celular em Nadadores: Resposta ao Exercício Agudo e ao Treino

Resumo Desenvolvido

O treino competitivo envolve exercício intenso e prolongado, capaz de modular o número e a actividade das células imunitárias. Quando demasiado exigente poderá induzir fadiga e aumentar a susceptibilidade a doenças. A presente dissertação inclui três estudos de investigação desenvolvidos no âmbito da Imunologia do Exercício, considerando a análise da influência do treino de competição sobre a resposta celular imunitária sistémica em nadadores, nomeadamente dos leucócitos totais e das subpopulações neutrófilos, monócitos, eosinófilos e linfócitos, respeitando a distinção clássica entre os efeitos agudos e crónicos do exercício. As subpopulações linfocitárias T (T totais $CD3^+$, T *helper* $CD4^+$ e T *cytotoxic* $CD8^+$), B ($CD19^+$) e *natural killer* (NK, $CD16^+56^+$) foram também avaliadas devido ao papel fundamental que desempenham na resposta imunitária celular, na humoral e na inata, respectivamente. Nesta investigação procurou-se avaliar a resposta imunitária ao exercício no terreno, em situações reais e representativas de processos de treino competitivos, controlando factores passíveis de influenciar esta resposta. No Estudo 1 avaliou-se a resposta imunitária a uma sessão de treino de natação prolongada e intensa, ao longo de um período de recuperação de 24 h. No Estudo 2 avaliou-se a resposta imunitária a uma época de treino de natação com sete meses. No Estudo 3 estudou-se a influência de um macrociclo de treino de 4 meses sobre a resposta imunitária à sessão de natação padronizada, ao longo de um período de recuperação de 24 h. Em todos os estudos controlaram-se variáveis associadas às características dos sujeitos que poderão influenciar a resposta imunitária, nomeadamente: sexo, fases do ciclo menstrual, maturidade, escalão competitivo, e especialidade desportiva com base na distância do principal evento competitivo. Ao longo da época competitiva controlou-se a melhoria da performance e foi quantificada a carga de treino de todas as sessões de treino planeadas da época, no sentido de se caracterizar a dinâmica da carga e perceber como as alterações deste parâmetro poderiam estar associadas à resposta imunitária. A incidência de sintomas respiratórios superiores (URS) foi também monitorizada através de questionários semanais.

Este projeto acompanhou uma época de inverno de natação competitiva com a duração de 30 semanas. A avaliação dos nadadores foi feita em quatro momentos de avaliação (M) denominados M1 (início da época, avaliação de base), M2 (na semana após a competição

principal do 1º macrociclo; a 13ª semana de treino), M3 (na sub fase preparatória específica do 2º macrociclo; a 23ª semana de treino) e M4 (a semana posterior à competição principal do 2º macrociclo; a 30ª semana de treino). Em todos os momentos de avaliação foram recolhidos dados acerca dos índices bioquímicos imunitários em repouso, dados maturacionais e de características físicas. No M2 e M4 foi realizada uma sessão de treino de natação padronizada e depois da sessão avaliaram-se os índices imunitários acima referidos. Estas recolhas foram feitas imediatamente (Post), duas horas (Post 2h) e 24 h após (Post 24h) o término da sessão. A sessão de treino incluída nesta investigação é representativa do típico esforço desenvolvido nas sessões de treino integradas em qualquer processo de treino de natação competitiva.

No Estudo 1, a resposta imunitária aguda à sessão de treino de natação de intensidade elevada, traduziu-se num aumento imediato do número de neutrófilos (neutrofilia), num decréscimo do número de linfócitos (linfopénia) e do número de eosinófilos, que duraram pelo menos duas horas, independentemente do sexo e maturidade. A recuperação da imunidade adquirida, expressa por linfócitos totais e subpopulações T (CD3$^+$) e B (CD19$^+$), parece ter sido mais difícil e lenta nos nadadores do escalão júnior (15 – 17 anos de idade, de acordo com a classificação *Ligue Européene de Natation – LEN*), uma vez que estes parâmetros se mantiveram diminuídos ao longo das 2 h após a sessão de treino e um período de 24 h foi insuficiente para a recuperação completa dos linfócitos totais e subpopulações T. Este facto deverá ser considerado aquando do planeamento de sessões de treino consecutivas. A linfopénia observada no final da sessão sugere uma vigilância imunitária diminuída que poderá aumentar o risco de infecção ou imunidade reduzida dos atletas no período imediatamente após o treino, destacando a necessidade de um cuidado extra quando expostos a agentes ambientais agressivos, como o ambiente das piscinas. No Estudo 2, ao considerar a influência da época de treino de natação sobre a resposta imunitária, observou-se na fase inicial da época (M2) uma diminuição da imunidade adquirida dos nadadores juvenis expressa principalmente pela diminuição dos linfócitos totais e subpopulações CD3$^+$ e CD4$^+$, bem como uma diminuição das subpopulações CD8$^+$ em todos os nadadores. Durante o período de treino mais intenso da época de treino (M3), caracterizado por volumes elevados de treino realizados nas zonas de intensidade aeróbia e de tolerância láctica, verificou-se a manutenção dos valores reduzidos de linfócitos CD8$^+$ e diminuições do número de eosinófilos e linfócitos NK CD16$^+$56$^+$, concomitantes com o maior número de episódios semanais de URS. No final da época de inverno (M4), um período com uma componente de recuperação mais acentuada da carga de treino (período

de *taper*), os nadadores seniores apresentaram uma contagem reduzida de monócitos e todos os nadadores demonstraram decréscimos dos valores de eosinófilos e linfócitos $CD16^+56^+$. No entanto, as subpopulações $CD8^+$ e $CD19^+$ recuperaram com o período de *taper*, e no caso das $CD19^+$, até se verificou o aumento do seu número. Estes resultados sugerem, por um lado, que a imunidade adquirida foi mais influenciada em períodos de carga de treino intensa, enquanto que a imunidade inata parece ter sido mais sensível ao efeito cumulativo do processo de treino de natação de longa duração. No Estudo 3, a investigação acerca da influência de um macrociclo de treino de 4 meses sobre a resposta imunitária aguda a uma sessão de treino de natação revelou que, no final do macrociclo (M4), imediatamente após a sessão de natação, houve uma leucocitose e neutrofilia inferiores e, nas duas horas após, uma recuperação menos eficiente dos linfócitos totais e subpopulação $CD19^+$ (linfócitos B). Vinte e quatro horas após terminar a sessão verificou--se também uma recuperação menos eficiente da subpopulação NK $CD16^+56^+$ nos nadadores adolescentes. Estas alterações dos parâmetros imunitários foram concomitantes com uma frequência mais elevada de URS no final do macrociclo comparativamente ao início (M2). Os resultados sugerem uma resposta imunitária aguda globalmente mais atenuada e um subsequente período de *janela aberta* de susceptibilidade à infecção mais longo. Esta resposta parece ter sido mais acentuada nos nadadores adolescentes. No entanto, é difícil de dizer se estas modificações reflectem mecanismos adaptativos positivos ou negativos, embora seja provável que tenham resultado dos efeitos cumulativos das cargas de treino de natação.

Estes resultados evidenciam a importância de controlar alterações imunitárias durante toda a época de treino, especialmente em períodos de treino intenso e ao realizar sessões de treino de alta intensidade consecutivas que não respeitem um período de recuperação de 24 h entre si, mas também durante os primeiros meses da temporada de treino em particular para os jovens atletas. Desta forma, sugere-se que treinadores e atletas implementem estratégias comportamentais e de intervenção a fim de contribuir para manter as condições de saúde, impedindo o aparecimento da fadiga e a diminuição de desempenho associada, contribuindo assim para evitar doenças, garantir a participação nas sessões de treino e alcançar o máximo desempenho em competições. Os atletas deverão ainda ter precauções especiais durante as primeiras horas após as sessões de treino intensas.

Palavras-Chave: Imunidade Celular, Nadadores, Sessão de Treino, Época de Treino, Sintomas Respiratórios Superiores.

Table of Contents

Figures List

Tables List

Abbreviations

%FM	Fat Mass percentage
A1	Aerobic Threshold
A2	Anaerobic Threshold
APC / APCs	Antigen Presenting Cell (s)
ATS	ANOVA-type statistic
AUL	Arbitrary Units of Load
BIA	Bioelectorical Impedance Analysis
BM	Body Mass
BMI	Body Mass Index
cAMP	Intracellular Cyclic Adenosine Monophosphate
CD	Clusters of Differentiation or Cluster Designators
EDTA	Ethylamindiaminetetraacetic Acid
FFM	Free Fat Mass
Hb	Hemoglobin
HCT	Hematocrit
IAT	Individual Anaerobic Threshold
ICAM /ICAMs	Intracellular Adhesion Molecule (s)
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
INSA	National Health Institute Doutor Ricardo Jorge
LEN	*Ligue Européene de Natation*
LP	Lactate Production
LT	Lactate Tolerance
M1	First moment of evaluation
M2	Second moment of evaluation
M3	Third moment of evaluation
M4	Fourth moment of evaluation
MATS	modified ANOVA-type statistic
MCH	Mean cell hemoglobin
MCHC	Mean cell hemoglobin concentration
MCV	Mean cell volume
MHC	Major Histocompatibility Complex
NK	Natural Killer Cell
OTS	Overtraining Syndrome
Post	Immediately after exercise
Post 24h	Twenty-four hours after exercise
Post 2h	Two hours after exercise
Pre	Before exercise
RBC	Red blood cell
RDW	Red cell distribution width
SD	Standard Deviation
SEM	Standard Error of the Mean
Tc	T *cytotoxic* lymphocyte
Tc1	T *cytotoxic* type 1 lymphocyte
Tc2	T *cytotoxic* type 2 lymphocyte
Th	T *helper* lymphocyte

Th1	T *helper* type 1 lymphocyte
Th2	T *helper* type 2 lymphocyte
TLR / TLRs	Toll-like Receptor (s)
TNF	Tumour Necrosis Factor
Ts	T regulatory or suppressor lymphocytes
URS	Upper Respiratory Symptoms
URTI	Upper Respiratory Tract Infection
VO_2max	Maximal Oxygen Consumption
WBC	White blood cell count or leukocytes
WR	Warm up and Recovery

CHAPTER I

Introduction

Exercise Immunology is an expanding field specially focused on the study of the influence of exercise on immune function. Exercise constitutes a stressful stimulus to the organism and induces immune perturbations. Researches in this field have been exploring the mechanisms related to exercise-induced modulation of the immune system and prevention of diseases by exercise training (Harris, 2011). Regarding competitive training processes, the relationship between the states of fatigue and the risk of infection and susceptibility to diseases has also been studied (Armstrong & VanHeest, 2002). Many exercise immunology researches have focused on the influence of acute exercise on the immune response, and on the immediate and post exercise early recovery effects, referring transient changes of immune cells. Few investigations extended the observation of the post-exercise period to 24 h, reporting the immune recovery to baseline levels. The literature states that these changes do not necessarily imply a clinically enhanced or deficient immune state, and highlight the need for further investigations to help understand their true meaning. The chronic response has been associated to the exercise intensity and duration. Moderate exercise, appears to be beneficial for the recreational, or sedentary populations and also for patients with chronic diseases (Harris, 2011; Sothern, Loftin, Suskind, Udall, & Blecker, 1999). Whereas intense training processes may contribute to an immunodepression state sometimes accompanied by an impaired performance, a combination that, if not reversed, may lead to overtraining (Armstrong & VanHeest, 2002). To date, the mechanisms linking exercise and health are not entirely comprehended, but they possibly underlie either the capability of exercise to both modulate the number of circulating immune cells and their activity. Considering that athletes exercise daily and for several hours performing high-intensity efforts, it seems important to account for the possible detrimental effects of high intensity training sessions, excessive exercise and long-term training on immune system. In this manner, the control of the immune response might be part of the strategy of monitoring and testing in sports training, directed to the acute and chronic exercise adaptations of athletes and also ensure the effectiveness of preparation strategies in sports. In theory, it could contribute to prevent the increase of the susceptibility to infections and illnesses, impaired performances and most of all disease, which may compromise attendance to training sessions, and in the worst case scenario, the onset of overtraining.

The knowledge developed in the exercise immunology field about the mechanisms linking exercise and health may help explain the impact of training in the health of physical active populations involved in training processes and the improvements in the outcomes of clinical disorders such as cancer (Kruijsen-Jaarsma, Revesz, Bierings, Buffart, & Takken, 2013), heart disease, type 2 diabetes, degenerative osteoarticular and reumatological diseases, and other chronic diseases (Gleeson, 2007).

The present dissertation, entitled "Immune Cell Changes in Swimmers: Response to Acute Exercise and Training" aimed to study the influence of acute and chronic exercise effects on the immune cell response of swimmers over the course of a competitive training season, controlling for possible factors that may affect the immune response.

In order to contextualize the investigation that culminated in three research studies, a literature review (*Chapter II*), and an integrated discussion (*Chapter VII*) of the main findings obtained within the three studies (*Chapters IV – VII*) are presented. This dissertation is organized as follows:

Chapter II includes a brief literature review including an introduction to the immune system concerning the main components that constitute the innate and acquired immune response. A detailed review of the current literature about exercise and immune function, including acute and chronic exercise effects, mechanisms and influential factors, is also presented in this chapter. This section finishes by highlighting the main research goals of the dissertation.

A description of the methods used during this project is exposed in *Chapter III*.

Chapters IV to VII correspond to the three studies that were conducted to answer the research goals that were defined in Chapter II. These studies are presented in a format ready to submit to peer reviewed journals.

Chapter VII provides an integrated discussion of the main findings obtained in the three studies over the course of this investigation. Overall conclusions and practical applications were pointed out at the end of this section.

Chapter VIII includes a list of all the references sited in all chapters.

List of articles and conference abstracts as first author

As a result of the complementary work that occurred as a significant part of the doctoral research program, publications in international journals and communications (oral/poster) in international congresses were made as first author:

Peer-reviewed articles published, in press, which are related to the dissertation:

Morgado, JP; Monteiro, CP; Teles, J; Reis, JF; Matias, C; Seixas, MT; Alvim, MG; Bourbon, M; Laires, MJ; Alves, F. (2016) *Immune cell changes in response to a swimming training session during a 24 h recovery period.* Applied Physiology, Nutrition, and Metabolism. DOI: 10.1139/apnm-2015-0488

Morgado, J, Monteiro, C, Matias, C, Alves, F, Pessoa, P, Reis, J, Martins, F, Raposo, M, Seixas, T, Laires, MJ (2014). *Sex-Based Effects on Immune Changes Induced by a Maximal Incremental Exercise Test in Well-Trained Swimmers.* Journal of Sports Science and Medicine, 13: 708-714. URL: http://www.ncbi.nlm.nih.gov/pubmed/25177203

Abstracts that are related to the dissertation:

Morgado, J., Monteiro, C.P., Alves, F., Matias, C., Seixas, M.T., Alvim, M., Bourbon, M., Teles, J., Laires, M.J. (2015) *Immune Response to a Swimming Session during a 24 h Recovery Period.* Accepted for the 20th Annual Congress of the European College of Sports Science. Malmö, Sweden

Morgado, J., Monteiro, C.P., Matias, C., Alves, F., Seixas, M.T., Alvim, M., Bourbon, M., Laires, M.J. (2012) *Immune Response Effects of a Swimming Session Remain for More Than 24 Hours.* Book of Abstracts of the 17th Annual Congress of the European College of Sports Science. Brugges, Belgium (pp. 473)

5

Morgado, J., Alves, F., Monteiro, C.P., Reis J., Laires, M.J. (2012). *One Week of Swimming Training Influences Resting Heart Rate Variability in Young Swimmers.* Book of Abstracts of the 2012 Annual Meeting-World Congress on Exercise is Medicine (San Francisco, California, USA) in Medicine and Science in Sports and Exercise, 44 (5): S240.

Morgado, J., Alves, F., Monteiro, C.P., Reis J., Laires, M.J. (2011) *Effects of one week of swim training on resting Heart Rate Variability in young swimmers.* Book of Abstracts of the 16th Annual Congress of the European College of Sports Science. Liverpool, United Kingdom (pp. 428).

Morgado, J, Monteiro, C, Matias, C, Raposo, M, Seixas, T, Pessoa, P, Reis, J, Martins, F, Laires, MJ, Alves, F. (2011). *Immune Changes Induced by a Maximal Incremental Swimming Test.* Book of Abstracts of the 2011 Annual Meeting-World Congress on Exercise is Medicine (Denver, Colorado, USA) in Medicine and Science in Sports and Exercise, 43 (5): S334.

Morgado, J, Matias, C, Monteiro, C, Raposo, M, Alves, F, Seixas, T, Pessoa, P, Reis, J, Martins, F, Laires, MJ (2008). *Immune changes in elite swimmers over the course of a training cycle.* Book of Abstracts of the 13th Annual Congress of the European College of Sports Science, Estoril, Portugal, (pp. 324-325).

CHAPTER II

Literature Review

2.1. Biological component of the training process

Training is a systematic process that aims to improve physical, psychological and technical qualities or abilities that allows reaching higher levels of performance. This process is based on the principle of biological adaptation. This principle sustains that the existence of stimuli, planned or spontaneous, induces a response that will lead to an adaptation (Alves, 2006; Busso, 2003; Costill et al., 1991; Foss & Keteyian, 1998). Accordingly, the current scientific theory and methodology of sports training highlights the relevance of the different types of stimuli that will induce different physiologic responses, implicating a dose-response relationship between training stimulus and the adaptation of the athlete (Busso, 2003). These stimuli are usually seen as the exercises or tasks, which present structural and load characteristics, and their overall organization is known as training load. The application of physical stimuli affects the psychosomatic, neuroendocrine, and functional (metabolic) responses (Alves, 2006; Foss & Keteyian, 1998; Peake et al., 2015). Generally, physical exercise acts as a mechanical and metabolic stress that disrupts the internal environment balance (homeostasis) of the human body. All systems of the body respond by generating physiological feedback mechanisms that will satisfy the enlarged energy demands, eliminate toxic products and repair the molecular, cellular, and eventually tissue damage that may occur, so as to the body comes back to a new state of homeostasis (Alves, 2006; Menicucci et al., 2013; Peake et al., 2015). This reaction will comprise structural and metabolic changes that will diminish disruptive fluctuations and produce a more efficient and less expensive response to a subsequent stress.

The body's homeostasis can be disrupted also by injury, illness and disease, interfering with the proper functioning of the body. In this case, the immune system contributes to homeostasis by preparing the body to fight off infection and to help the healing process in case harm occurs (Gleeson, 2006, 2007; Harris, 2011; Menicucci et al., 2013). So, an active immune system is required if an athlete aims to produce frequent peak performances. Immune cells respond to growth factors and cytokines, and are involved in muscle growth and repair adaptations to exercise, participating in angiogenesis and tissue

repair (Adams et al., 2011). These functions may be important for the efficiency of muscle repair and muscle fiber hypertrophy induced by heavy resistance exercise (Ihalainen et al., 2014).

In competitive sports, many years of daily training and competition are required to achieve and maintain national and international level performances, which mean that the success of the athlete is determined by the cumulative effect of long-term training resultant from a long-lasting athletic preparation. The cumulative training effect reflects modifications in physiological and biochemical parameters and also in parameters associated to sports specific physical/technical abilities and level of performance (Issurin, 2010). The most noticeable adaptations may be achieved in aerobic abilities, which imply a higher improvement rate in aerobic endurance disciplines (Issurin, 2010; Jones & Carter, 2000).

Endurance sports training processes are seen as promoters of several "beneficial" cardiovascular, metabolic, and respiratory adaptations. Some of those adaptations are: decreased heart rate, and increased levels of stroke volume of the heart, cardiac output, oxygen transportation and distribution efficiency, mitochondrial number and volume in the muscle fibers used in training, aerobic enzymes, glycogen and fat storing and energy source mechanisms, body thermal and pH regulation (Buchheit & Laursen, 2013; Foss & Keteyian, 1998; Jones & Carter, 2000).

The physiological adaptations above mentioned affect positively endurance performance by inducing a rightward shift in the *velocity-time curve*. This change allows athletes to work out for a longer period of time at certain exercise intensity, or to exercise at a higher intensity for a given duration (Issurin, 2010).

The cumulative training effect involves an adequate and competent planning and regulation of training loads throughout long periods. To allow the adaptation to the workloads, an adequate balance between training stimulus and recovery periods is necessary. The conceptual models that study the processes of physical adaptation to training assume that *training load* has, concomitantly, a positive (fitness) and a negative (fatigue) effect on performance (Banister, 1991). The consequences of low training loads and/or excessive recovery or high training loads along with deficient recoveries may impair performance, and in the latter can cause excessive fatigue (Armstrong & VanHeest, 2002; Hackney, 2013; Mackinnon, 2000; Smith, 2004).

Fatigue is characterized by a reduction of performance probably associated with insufficient muscular recovery, substrate depletion, neuroendocrine alterations, and/or microtrauma. This state is inextricable linked with the training process and also may be

exacerbated in the presence of other external factors (environment, relationship stress, schoolwork, lack of sleep, poor nutrition) and eventually be damaging to certain organs and systems of the body (Alves, 2006; Armstrong & VanHeest, 2002; Mackinnon, 2000). Furthermore, if not timely reversed it can become permanent and evolve to cumulative fatigue leading to overtraining syndrome (OTS) (Armstrong & VanHeest, 2002; Mackinnon, 2000; Smith, 2004).

Overtraining syndrome (OTS) is also referred to as the *unexplained underperformance syndrome*, and is usually characterized by a long-term decreased performance capacity, and chronicle maladaptations in which the restoration of performance capacity and overall health condition may take several weeks or months. These maladaptations are associated to physiological, biochemical, immunological and psychological symptoms, and also inadequate nutrition (Halson & Jeukendrup, 2004; Hellard et al., 2013; Meeusen et al., 2013). The OTS most reported symptoms besides decreased physical performance and general fatigue are: malaise, loss of vigor, insomnia, change in appetite, irritable, restless, excitable, anxious, loss of bodyweight, loss of motivation, lack of mental concentration, and feelings of depression (Armstrong & VanHeest, 2002).

Several biological parameters, mostly blood constituents, regarded as possible markers of OTS, have been assessed in athletes. Among others, there are variables related to the endocrine and immune function that present reduced values in the athletes diagnosed with overtraining, namely hematocrit, hemoglobin, leukocytes and subsets, catecholamines, testosterone, cortisol, growth hormone, adrenocorticotropic hormone, and prolactin (Armstrong & VanHeest, 2002).

As suggested by Hellard et al. (2005) in a study concerning the residual effects of training on the swimming performance in Olympic swimmers, it is essential to maintain the training loads below the overtraining limit in order to obtain an optimal development of physical capacities.

From all endurance sports, swimming stands out for its special characteristics. Swimming is an individual cyclic sport, considerably different from dry land sports since the water environment induces in the body different metabolic and biomechanical displays (Holmer, Stein, Saltin, Ekblom, & Astrand, 1974; Toussaint & Beek, 1992; Zamparo, Capelli, & Pendergast, 2011). Since the majority of swimming events lasts longer than 30s, and similarly to other sports enduring similar range of exercise durations, the majority of training and competition loads induce a high participation of the oxidative system (Bangsbo, Michalsik, & Petersen, 1993; Craig et al., 1995; Spencer & Gastin, 2001). Well

trained swimmers, including short distance specialists, usually present elevated levels of aerobic power and capacity, much similar to those of other traditionally endurance sports. In fact, this predominant aerobic participation even in shorter events, such as 50 m and 100 m, is a singularity of this sport (Toussaint & Beek, 1992; Zamparo et al., 2011).

Apparently, in no other sport is efficiency more important than in swimming and the highest efficiency levels are achieved through a combination of ideal anatomical structure and technical perfection of the stroke (Toussaint & Beek, 1992). So, in swimming it is of common use the implementation of cycles of high training volume and intensity, which include consecutive highly demanding training sessions with little recovery time in between, in order to optimize aerobic and movement economy adaptations (Sargent, Halson, & Roach, 2014). The majority of such training is performed using an interval training format to allow reaching higher levels of the swimmers aerobic and anaerobic capacity. Consequently, the usual levels of energy expenditure are also elevated (Buchheit & Laursen, 2013; Sweetenham & Atkinson, 2003).

Moreover, most of the training sessions are conducted in swimming pools, implicating the repeated exposure to warm humid environment, temperature variations and chlorine-rich atmosphere, thus predisposing swimmers to respiratory illness (Aubry, Hausswirth, Louis, Coutts, & Y, 2014; Bernard, Nickmilder, Voisin, & Sardella, 2009; Bougault et al., 2012; Gleeson, 2000; Gleeson et al., 1995; Gleeson et al., 2000; Mackinnon, 1997; Reid, Gleeson, Williams, & Clancy, 2004; Spence et al., 2007), especially sinusitis, otitis and conjunctivitis (Ahmadinejad, Alijani, Mansori, & Ziaee, 2014). This predisposition seems to be accentuated during the heaviest training periods characterized by high loads imposed continuously over several weeks as the number of upper respiratory symptoms (URS) reported increases (Morgado et al., 2012; Rama et al., 2013).

Although, in some cases the intense stimulation of adaptive mechanisms related to metabolic, hormonal, circulatory and respiratory responses may have some negative influence on performance and health status, in most cases this may be reversed by a tapering or recovering period (Gleeson & Bishop, 2005; Shephard & Shek, 1999; Suzui et al., 2004).

Thus, it becomes essential to know the athlete's skills and limitations and determine the ability of the swimmer to withstand the training loads. In addition, it appears important to analyze, among other variables, the functional state of the organism and health status (via functional tests and / or medical examinations).

Therefore, the preparation of the career of swimmers who pursuit high performances and national and international competitions, which requires daily training and preparation that consumes several hours and involves frequent periods of heavy training, should consider the possible overall detrimental effects of excessive exercise and long-term training on immune system. These evidences support the need for specific research on this sport.

2.2. Introduction to the immune system

The immune system is responsible for the maintenance of a healthy body, defending it against infectious organisms, such as bacteria, protozoa, parasites, fungi, and viruses. It is also able to recognize altered host cells (for example leading to cancer) and even non-infectious substances that can promote the initiation of an immune response, such as proteins, polysaccharides and other macromolecules. The protection given by the immune system can be divided into two related activities: recognition and response (Kindt, Goldsby, & Osborne, 2007). The immune recognition is remarkable for its ability to distinguish between internal molecules of the host and foreign molecules (*self - non self* discrimination). The recognition of a pathogen by the immune system causes an effector response that aims to eliminate or neutralize the attacker. The first contact with pathogens elicits a memory response characterized by a faster and more exuberant immune response in subsequent contacts (Kindt et al., 2007; Parslow, Stites, Terr, & Imboden, 1997).

The immune system contains a great number of components that can be divided into cellular and soluble elements. Cellular components are transported across the blood, lymph and lymphoid organs, and comprise all leukocytes (immune system main cells), which include neutrophils, eosinophils, basophils, mast cells, dendritic cells, monocytes, macrophages, and lymphocytes. Soluble components include acute phase-proteins, complement system, lysozymes, cytokines and immunoglobulins. All these components are associated to the *Innate* or to the *Acquired* immune responses, which work together synergistically, and the adequate and controlled interaction between them dictate an effective immune response. The innate response is activated as a first line of defense, attacking indiscriminately pathogens, eliminating altered host cells, or restricting the entry of microorganisms into the body. It comprises surface barriers (skin, epithelial layers and mucosal secretions), soluble factors, phagocytes (neutrophils, eosinophils, monocytes, and

macrophages) and *natural killer* (NK) lymphocytes (Parslow et al., 1997). The acquired response often succeed the innate response and is generally based on the proliferation of specific lymphocytes that attack the invader directly or through the production of antibodies (immunoglobulins; Ig) that target specific antigens. These lymphocytes have an antigen-specific memory of such pathogens (Smith, 2004). A clinical infection occurs when the pathogen overlaps the innate mechanisms of the immune system.

Lymphocytes are the only body cells with the capacity to specifically recognize and distinguish different antigenic determinants and thus are responsible for the two defining characteristics of the acquired immunity: specificity and memory, operating as mediators of humoral and cellular immunity (Kindt et al., 2007; Parslow et al., 1997). They constitute 20 to 25% of the leukocytes and may be divided into three major populations, based on the function and components of the cell membrane: NK, B and T lymphocytes or cells, which are again subdivided into T *helper* (*Th*1 and *Th*2), T *cytotoxic* (*Tc*1 and *Tc*2), and T *suppressor* (*Ts*) lymphocytes (Gleeson, 2006; Kindt et al., 2007; Parslow et al., 1997).

Morphological differences between the populations of granulocytes and between them and monocytes and lymphocytes allow for the identification and quantification of these cells. For differentiation and quantification of lymphocytes subsets, it is necessary to selectively detect membrane bound molecular markers specific to each group of cells. The different membrane bound molecular markers are called clusters of differentiation or cluster designators (CD) and each has been given a number (e.g. CD3 – present on all T lymphocytes; CD4 – present on T *helper* (*Th*) lymphocytes; CD8 – present on T *cytotoxic* (*Tc*) and T *suppressor* (*Ts*) lymphocytes; CD19 – present on B lymphocytes; CD16 and CD56 – present on NK lymphocytes (NK cells are identified mainly by the presence of one or both markers simultaneously) (Lancaster, 2006; Shephard, 2010). Cell populations are usually defined using a "+" or a "-" symbol to indicate whether a certain cell fraction expresses or lacks a CD molecule. Thus, CD molecules are utilized in cell sorting using various methods including flow cytometry (Biosciences, 2000; Lancaster, 2006; Radbruch, 2000). CD molecules can act in numerous ways, often as receptors or ligands (activate a receptor) usually initiating a signal cascade that alters the behaviour of the cell, or as intracellular cell adhesion molecules (ICAMs) (Dimitrov, Lange, & Born, 2010).

Maturation of B cells (5 to 15% of total lymphocytes) occurs in the bone marrow. These cells subsequently develop in the lymphoid organs, and have immunoglobulins anchored on their membrane surface, which will function as receptors for antigens. B cells are activated when they encounter an antigen and then initiate the production of antibodies,

thereby functioning as a mediator of humoral immunity. The inactive/naïve B lymphocytes do not produce immunoglobulin, however when stimulated by cytokines they undergo clonal expansion, becoming active cell called plasmocyte (Parslow et al., 1997).

T lymphocytes (60 to 75% of total lymphocytes), like B cells, originate in the bone marrow but mature in the thymus. Unlike B cells that recognize the isolated antigen, T cells only recognize antigens when connected to the Major Histocompatibility Complex (MHC) class II. When T cells recognize an antigen combined with a MHC molecule in an antigen presenting cell (APC), it proliferates and differentiates into memory and effector T cells (T *helper* and T *cytotoxic*) (Gleeson, 2006; Kindt et al., 2007). *Th* lymphocytes (CD4$^+$) are regulatory immune cells, because they send messages to all leukocytes in order to fight the "aggression". These cells stimulate growth and proliferation of *Tc* and *Ts*, and B lymphocytes into plasmocytes, to produce antibodies against the antigens. They also regulate macrophages activation and lymphocytes self-stimulation. T *cytotoxic* lymphocytes (CD8$^+$) receptors recognize the MHC-class I expressed by foreign cells attacking them directly, thereby lysing the cell. T *suppressor* cells are lymphocytes which have the function of modulating the immune response by inactivating T *helper* and T *cytotoxic* cells, limiting their action in the body during an immune reaction (Kindt et al., 2007; Parslow et al., 1997). *Th* lymphocytes activate *Ts* and the latter will control the activity of the first, inhibiting their action in a negative feedback. The *Ts* cells are also involved in the immune tolerance mechanism by which the immune system prevents the leukocytes from attack the body's own cells. *Th* cells can be subdivided on the basis of their cytokine production profile in two types: type 1 (*Th*1) produces interferon-γ (IFNγ), interleukin-2 (IL-2), and tumor necrosis factor (TNF), and type 2 (*Th*2) secrete interleukins IL-4, IL-5 and IL-13. Type 1 lymphocytes stimulate the cellular defence against intracellular pathogens whereas type 2 lymphocytes stimulate the humoral and cellular defence against extracellular pathogens (Lancaster et al., 2004). NK cells (10-20% of total lymphocytes) comprise a small population of granular lymphocytes that do not express CD3 cell-surface marker. These cells exert a spontaneous cytolytic activity against a wide variety of tumor and other cells infected with viruses. This kind of response is called nonspecific immune response because their action is independent of the MHC. Also, they are able to produce cytokines that activate other immune cells (Gleeson, 2006; Kindt et al., 2007; Parslow et al., 1997).

The development of an effective immune response involves both lymphocytes and granulocytes. The complex interactions between these cells are mediated by cytokines, a

group of small glycoproteins, which are secreted by various sources like immune cells and other tissues, including muscle, in response to various stimuli, such as infections, trauma, physical and chemical agents, tissue necrosis, and foreign bodies. Through autocrine, paracrine, and in some cases endocrine actions, cytokines either stimulate or inhibit proliferation, differentiation and maturation of leukocytes, and regulate the inflammatory process, the acute phase response, tissue regeneration and management of energy stores (Peake et al., 2015). Cytokines are produced in small quantities and the main producers are T *helper* cells, dendritic cells and macrophages, although B cells, endothelial cells, fibroblasts, and various stromal cells may also produce cytokines (Kindt et al., 2007). Cytokines can be divided into interleukins (IL), interferons (IFN), colony-stimulating factors (CSF), and tumour necrosis factors (TNF).

The basic structure and main components of the immune system and immune response abovementioned are represented in Fig 2.1.

Fig 2.1. The basic structure and main components of the immune system response (adapted from Hackney (2013) and Gleeson (2006)).

The Immune System

Leukocytes Responses to Pathogens

Innate Immune Responses
- First line of defense
- Indiscrimantely attacks pathogens

↔

Acquired Immune Responses
- Follows innate response
- Target specific pathogens
- Produces antigen specifc memory

Mucosal Immunity

Neutrophils
Eosinophils
Basophils
Monocytes
Macrophages
Natural killer cells (NK)

T lymphocytes
B lymphocytes

IgA, IgM

Cell-mediated Immunity

Humoral Immunity

Other components: surface barriers, acute phase proteins, cytokines

Abbreviations: IgA=Immunoglobulin A; IgM=immunoglobulin M

2.3. Exercise and immune function

Exercise Immunology research has been focused on the mechanisms related to exercise-induced modulation of the immune system and prevention of diseases by exercise training (Harris, 2011).

It is important to distinguish acute (e.g. one training session) from chronic effects (training processes) of exercise on the capability to modulate both the number of circulating immune cells and their activity. This distinction takes into account the length, volume, intensity and type of activity that characterizes the physical exercise session or workout. The acute effects of exercise on the immune system reflect changes that arise in immune parameters in response and as a result of that workout. Conversely, the chronic effects of physical exercise refer to the stress caused by exercise activities and/or training sessions imposed cyclically over long-term periods (Gleeson, 2006).

The immune system reacts differently to the acute and chronic stresses of exercise and the alterations may be observed in the number and functions of circulating blood leukocytes, in the concentrations of blood Igs and cytokines, and also in the concentrations of salivary Igs (Gleeson, 2006). However, these changes do not reach all constituents with the same magnitude because they depend on the degree of susceptibility of each type of cell, on the intensity and duration of the physical exercise sessions and on the intervals between sessions.

Many exercise immunology studies have focused on the influence of acute exercise on the immune response, mostly in the adult population. The literature refers a consistent increase in total leukocytes (Gabriel, Schwarz, Steffens, & Kindermann, 1992a; Kargotich, Keast, Goodman, Crawford, & Morton, 1997; McFarlin, Flynn, Stewart, & Timmerman, 2004; Starkie, Rolland, Angus, Anderson, & Febbraio, 2001), but also in its subpopulations, namely: neutrophils (Ibfelt, Petersen, Bruunsgaard, Sandmand, & Pedersen, 2002; Kakanis et al., 2010; Kargotich et al., 1997; Steensberg et al., 2001a), monocytes Gabriel et al. (1992a); (Kakanis et al., 2010; Kargotich et al., 1997), and lymphocytes (Gabriel et al., 1992a; Kakanis et al., 2010; Kargotich et al., 1997; Steensberg et al., 2001a; Steensberg, Toft, Schjerling, Halkjaer-Kristensen, & Pedersen, 2001b), during and immediately after exercise and a transient imbalance that may occur throughout the first hours of recovery reflecting divergent changes of the immune cells. These post exercise changes are proportional to exercise intensity and duration and appear to have recovered to resting

values within 24 h (Gleeson, 2006; Walsh et al., 2011). Although some of the mechanisms underlying these changes have been identified, their true significance remains rather inconclusive, and so it is still not clear whether it means enhanced immune response, or increased risk of infection resulting from supressed immunity.

As for the chronic response, regular moderate primarily aerobic exercise has been pinpointed as a potential enhancer of various immune parameters, strengthening the immune system and improving resistance to disease and infection (Gleeson, 2007; Kakanis et al., 2010; Nehlsen-Cannarella et al., 1991a; Nehlsen-Cannarella et al., 1991b). However, when considering regular and intense training processes, the controversial outcomes in the literature are based in studies that evaluated primarily adult sedentary populations or recreational physical activity subjects, involved in rigorous experimental controls. The characteristics of the above mentioned samples are different from the athletic population, which *per si* present also some diverse inter-subjects characteristics, and may misrepresent the sports training influence on the immune response. Also, instead of the beneficial effects associated with moderate exercise, it is generally acknowledged that high intensity exercise, or physical activities, when accompanied by environmental or competitive stress, may lead to an "open window" for infection throughout the time of the stimulus, probably representing the basis for the progressive reduction of immune system's competence (Armstrong & VanHeest, 2002; Walsh et al., 2011). This immunodepression can be more evident during the intense training periods of the season, particularly when consecutive high intensity training sessions with little recovery in between are performed and an adequate lifestyle and care in the recovery processes are not respected (Aubry et al., 2014; Sargent et al., 2014). Despite this trend for lowered immunity, most athletes cannot be considered clinically immune deficient. It is possible that the combined effects of small changes in several immune parameters, either in response to prolonged intense acute exercise or to high intensity training periods, may compromise resistance to minor illnesses such as the Upper Respiratory Symptoms (URS) and Upper Respiratory Tract infections (URTI). Moreover, the concomitant occurrence of cell-mediated immunosuppression and higher URS-URTI risk converges with the *Open Window* and *J-Curve Response* concepts about exercise training and illness developmental state (Gleeson, 2006; Hackney, 2013; Mackinnon, 2000; Nieman, 2000b).

Fig 2.2. The *Open Window* concept associated with acute immune responses to exercise (adapted from Gleeson (2006), Hackney (2013), Mackinnon (2000), and Nieman (2000b))
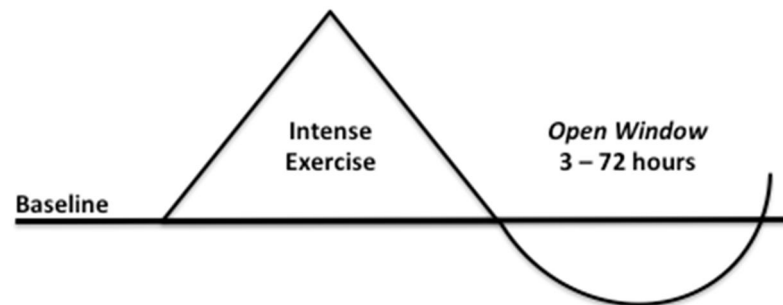
The *Open Window* theory (Fig. 2.2.) highlights the increase in the susceptibility to illnesses, such as URTI, due to a post exercise decline in the host defence mechanisms, in the 3 h - 72 h period following intense exercise, during which viruses and pathogens may enter the host. There is the possibility of this "window" to be extended for a longer period if daily rest is insufficient throughout consecutive days of intensive training.

The *J-Curve Response* model (Fig. 2.3.) suggests that sedentary persons are considered to be at normal risk of URTI, individuals who are engaged in a regular and moderate physical activity programs are associated with reduced risk of URTI (resulting in lower incidence and duration of symptoms), while high-intensity exercise is associated with an increased risk of infection.

Fig 2.3. The *J-Curve* model about the relationship between exercise-dose and infection risk based on upper respiratory tract infections prevalence (adapted from Gleeson (2006), Hackney (2013), Mackinnon (2000), and Nieman (2000b))
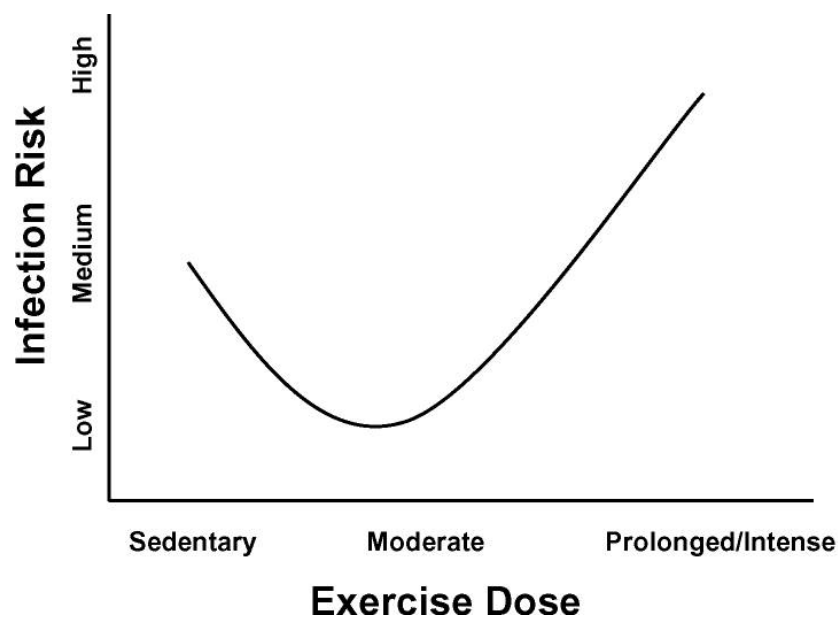
However, in most studies, a limited number of common symptoms and signs were reported and the information provided lacked confirmation by a physician and/or laboratory analyses for the detection of pathogens (Cox et al., 2008; Spence et al., 2007). Consequently, it is possible that the outcomes could have misled the conclusions, probably tending to overestimate the frequency of URTI in athletes (Gleeson, Pyne, & Callister, 2004; Hellard, Avalos, Guimaraes, Toussaint, & Pyne, 2015). Hence, it appears difficult to assess the difference between inflammation and infection, even for medical personnel, once lab confirmation of the presence of a pathogen is required for accurate results (Cox et al., 2008; Spence et al., 2007). However, medical diagnosis and laboratory confirmation are not always possible and since the symptoms are often very similar in self reported upper airway infection or inflammation, lately the literature suggested the use of the term URS to classify signs and symptoms that affect the upper airways (Spence et al., 2007).

2.3.1. Acute cellular immune response to exercise

The number of leukocytes increases in the blood (leucocytosis) during and immediately after acute exercise (McCarthy et al., 1991; McCarthy et al., 1992; Natale et al., 2003; Yamada et al., 2000). This pattern of mobilization is usually observed for all leukocytes subsets after exercise, and has been reported in the literature: rises in the number of neutrophils (neutrophilia) (Ferrer, Tauler, Sureda, Tur, & Pons, 2009; Gabriel et al., 1992a; Kargotich et al., 1997; Yamada et al., 2000), monocytes (monocytosis) (Gabriel et al., 1992a; Kargotich et al., 1997), and total lymphocytes (lymphocytosis) (Gabriel et al., 1992a; Ibfelt et al., 2002; Kakanis et al., 2010; Starkie et al., 2001; Yamada et al., 2000) and lymphocytes subsets (Natale et al., 2003), whereas eosinophils remained unchanged (Kakanis et al., 2010). The magnitude of the increases depends on the intensity, duration and type of exercise (Blannin et al., 1998). Yet, although some authors have mentioned that the immune changes are more dependent on exercise intensity (Gabriel, Urhausen, & Kindermann, 1992b), others point out a more meaningful influence of the duration of exercise as indicated by a larger leucocytosis magnitude observed in prolonged exercises compared to short-term high intensity exercises (Nieman et al., 1998a; Robson, Blannin, Walsh, Castell, & Gleeson, 1999; Suzuki et al., 2003).

During the recovery period following acute exercise, a delayed leucocytosis and neutrophilia has been reported at 1h (Gabriel et al., 1992a; Green, Rowbottom, &

Mackinnon, 2003) and at 2 h after accomplishing the exercise (Gabriel et al., 1992a; Yamada et al., 2000), also observed 3 h into recovery after different types of cycling tasks (McCarthy et al., 1991; McCarthy et al., 1992; Natale et al., 2003) and after a resistance exercise circuit (Natale et al., 2003). No alterations in monocytes (Kakanis et al., 2010; Kargotich et al., 1997) and eosinophils (Kakanis et al., 2010) have been reported in this early recovery of exercise.

However, Gabriel *et al.* (1992a) reported a fall in lymphocytes (lymphocytopenia), reflecting essentially the decline in $CD3^+$ lymphocytes below rest levels. Other studies also reported declines of $CD4^+$ and $CD8^+$ cells (Ibfelt et al., 2002; McFarlin et al., 2004) and of type 1 $CD4^+$ and $CD8^+$ cells (Steensberg et al., 2001a). As for other lymphocyte subpopulations, several investigations showed decreases of $CD16^+$ (Gabriel et al., 1992a), $CD56^+$ (McFarlin et al., 2004), and $CD16^+56^+$ NK cell counts (Gabriel et al., 1992b) and others increased values of $CD19^+$ lymphocytes (Natale et al., 2003) during recovery. According to Natale *et al.* (2003) the levels of $CD3^+$, $CD4^+$ and $CD8^+$ cells return to baseline by 3 h after exercise and in the same period $CD19^+$ cells can remain elevated. McFarlin *et al.* (2004) added that the total numbers of $CD4^+$ and $CD8^+$, and $CD56^+$ NK subsets were not different from pre-exercise levels at 4h following exercise.

Investigations about the acute response of leukocytes and subpopulations (including lymphocytes subsets) to exercise throughout the post exercise 24 h recovery are scarce, and essentially reported the return to baseline levels of leukocytes (Gabriel et al., 1992a; Zhang et al., 2006), neutrophils (Kakanis et al., 2010), and monocytes (Gabriel et al., 1992a). As for lymphocytes, the recovery to pre-exercise values of lymphocytes total (Gabriel et al., 1992a; Steensberg et al., 2001a) and subsets $CD3^+$ (Gabriel et al., 1992a), $CD4^+$ (Gabriel et al., 1992a; Steensberg et al., 2001a), and $CD8^+$ (Gabriel et al., 1992a) has been observed, although the latter subset has also been reported to stay below baseline values (Steensberg et al., 2001a).

2.3.1.1. Innate immunity cell functions response to exercise: chemotaxis, adherence, phagocytosis, degranulation, oxidative burst

Neutrophils affinity to chemical receptors that allow *chemotaxis* (and consequent facilitate the movement of these granulocytes to the desired location) may be elevated (Giraldo, Garcia, Hinchado, & Ortega, 2009; Ortega, Collazos, Maynar, Barriga, & De la Fuente,

1993) after the completion of both moderate and intense exercise. Moreover, neutrophils *adherence* to the endothelium, which may facilitate diapedesis, has shown not to be affected by acute exercise (Lewicki, Tchorzewski, Denys, Kowalska, & Golinska, 1987). As for neutrophils *phagocytosis* activity, an elevation (Blannin, Chatwin, Cave, & Gleeson, 1996) and unaltered responses to exercise (Lewicki et al., 1987) were observed. Phagocytosis efficiency depends on the number of neutrophils, on the percentage of active neutrophils (those that effectively promote phagocytosis, usually this percentage decreases after exercise), and on individual phagocytic ability of neutrophils (Blannin et al., 1996). The digestion of microorganisms by neutrophils during phagocytosis implicates the release of granular lytic enzymes (degranulation) and generation of reactive oxygen species (respiratory burst) and these combined effects generate an hostile environment for the destruction of pathogens (Kindt et al., 2007). Exercise has been indicated as inducer of degranulation (Suzuki et al., 2003), essentially through means of their increased number and not so through their ability to degranulate, which seems to be lowered facing stimulation (Robson et al., 1999). Neutrophils oxidative burst activity in response to acute exercise has shown to be dependent on exercise intensity. So, moderate exercise is associated to an enhanced oxidative burst and inversely high intensity exercise to a reduction in respiratory burst activity. The reduction of neutrophils functions is associated with recurrent infections (Parslow et al., 1997) and it has suggested that this condition could help to an augmented susceptibility to infection in athletes (Pyne, 1994).

Regarding monocytes, their phagocytic function has shown to be increased after prolonged exercise while the oxidative burst activity appears to be unaltered (Nieman et al., 1998b). Also, macrophages functions such as adherence, chemotaxis, and phagocytosis seem to be positively affected by moderate exercise and remain unaltered facing intense exercise. Furthermore, monocytes act as APCs and the Toll Like Receptors (TLRs) enable the APCs recognition, phagocytosis, digestion, and presentation of pathogens, and also stimulate the acquired immune response, especially by activating naïve T cells (Kindt et al., 2007). With exercise an immediate reduction in the expression of TLRs on monocytes surface may occur (Simpson et al., 2009) and remain below baseline levels for 2 h (Lancaster et al., 2005), thus suggesting a compromised recognition of the pathogens associated molecular patterns and the succeeding initiation of innate and acquired inflammatory responses.

NK cells are important in both innate and acquired immune response, and its functions of proliferation, differentiation, cytotoxicity, and degranulation are regulated by a balance in the activation and inhibition of cytokines such as interleukins. Recently, researches about

exercise-induced phenotypic and functional changes in circulating lymphocytes have been based on the distinctive surface cell markers within each lymphocytes subset. Two NK cell subsets CD56^{dim} and CD56^{bright} have been distinguished, and their functionality appears to be different: whereas CD56^{bright} NK cells produce cytokines such as IFN-γ and have lower cytotoxicity, CD56^{dim} NK cells participate in natural and antibody-dependent cell-mediated cytotoxicity (Campbell et al., 2009; Millard et al., 2013).

Investigations about NK cell function response to exercise have been controversial. High intensity exercise appears to induce an immediate post exercise elevation (Nieman et al., 1993) followed by a diminution in the CD56^{+} NK cytotoxic activity (McFarlin et al., 2004). However, Suzui et al. (2004) observed that intense training was related to a diminished CD56^{+} NK cytotoxicity per cell. Furthermore, higher increases of CD56^{dim} comparing to CD56^{bright} NK cells after exercise were also reported, suggesting that CD56^{dim} NK cells are more responsive to exercise (Campbell et al., 2009; Millard et al., 2013). The preferential mobilization of CD56^{dim} NK cells, as well of specific CD8^{+} lymphocytes subsets, suggests that exercise induces a selective mobilization of cells that present certain functional and phenotypic characteristics, such as high cytotoxicity, low proliferative ability, and high tissue-migrating potential (Campbell et al., 2009; Millard et al., 2013).

2.3.1.2. Acquired immunity cell functions response to exercise: activation, cytokine production, proliferation, and cytotoxicity

T cell activation is indicated by the expression of protein markers of activation on the surface of the cell, such as CD69 (early activation), CD25 (IL-2 receptor), CD45RO (memory/effector) and the HLA-DR antigen (MHC class II determinant) (Kindt et al., 2007). Investigations about the responsiveness of these markers of CD4^{+} and CD8^{+} cells to acute exercise have revealed inconsistent findings, although they seem to be, as with many other aspects of immune function, proportional to exercise intensity and duration. Still, increases in the T cell activation can indicate either a selective recruitment of subsets of active cells into circulation (Fry, Morton, & Keast, 1992b), or a state of activation due to hormonal influence (Gabriel et al., 1993), or even both concurrently (Gleeson, 2006).

The production of cytokines by T cells is also affected by acute exercise, and the type of cytokines produced will dictate if the immune response is cell mediated (IL-2 and IFN-γ)

or humoral (IL-4, IL-5, IL-6, and IL-13). So far, investigations have reported mostly a decreased in the cytokine production after accomplishing high intensity workouts. Specifically, this reduction was observed for the production of Interleukin - 2 (IL-2) by *Th* type 1 cells (Steensberg et al., 2001a), and of IL-2 and IFN-γ by *Th* type 1 cells (Ibfelt et al., 2002) by CD4^{+} and CD8^{+} type 1 cells, and appears to be associated to the exercise induced rises in adrenaline (Steensberg et al., 2001a). As for cortisol no correlations with type 1 or type 2 cells were observed (Ibfelt et al., 2002; Steensberg et al., 2001a).

Usually, the proliferation of CD3^{+} T cells declines during and after prolonged exercise (Fry, Morton, Crawford, & Keast, 1992a; Henson et al., 1999), appears to reflect decreases in the number of responsive cells, instead of a diminished responsiveness of each cell.

The functional capacity of B cells has essentially been evaluated through the production of mucosal and serum antibodies in vitro and in vivo studies. The most common used immunoglobulins in exercise immunology research have been the IgM, IgG and IgA, which slightly increased (Nehlsen-Cannarella et al., 1991a) or maintained their blood concentrations in response to exercise (Gleeson, 2006).

2.3.1.3. Immune mechanisms of the acute exercise response

The alterations in the number of leukocytes counts and their populations in response to acute exercise may be explained by three different processes: cell traffic, cell proliferation or cell death (Kruger, Lechtermann, Fobker, Volker, & Mooren, 2008; Kruger & Mooren, 2014). It is thought that these processes are concurrent and their relative magnitude probably depends on the mode of exercise (Kruger & Mooren, 2014). Cell traffic depends upon the adherence of cells to the endothelium and on their redistribution amongst organs or compartments, especially between the circulation and the lung, spleen and muscle (Adams et al., 2011). Leukocyte trafficking and function can be influenced, during exercise and immediately after its ending, by increases of cardiac output, shear stress, and blood flow to working muscle, and by changes in pH and temperature (Adams et al., 2011). This physiological response reflects an increase of sympathetic activity and an activation of the hypothalamic-pituitary axis inducing the secretion of circulating catecholamines (Ottaviani & Franceschi, 1996). Catecholamines also contribute, directly and indirectly, to the decrease of the adherence of leukocytes to the endothelium (demargination) and consequently increase the number of circulating leukocytes (Gabriel et al., 1992a). Directly

by reducing the number of cell adhesion molecules on the cells' surface, and indirectly by accelerating heart rate, increasing blood flow and shear stress. Moreover, it is believed that catecholamines exert a direct action on lymphocytes, particularly increasing the density of β2 adrenergic receptors. In addition, exercise enhances the exposure of these receptors to catecholamines and their activation increases intracellular cyclic adenosine monophosphate (cAMP) concentration. This molecule will induce decreases in lymphocytes ICAM expression and/or affinity for the ligands expressed by the vascular endothelial cells, thus, resulting in mobilization of marginated lymphocytes (Shephard, 2003). The use of β-blockers during exercise attenuates the elevation of lymphocytes, supporting the idea that these hormones constitute one of the possible mechanisms involved in the lymphocytosis during and immediately after exercise, before the lymphocytopenia (Nemet, Mills, & Cooper, 2004). The lymphocytosis observed immediately after exercise as shown to be different among subsets, with higher increases (listed in order of magnitude), in $CD56^+$ NK cells followed by $CD8^+$ and $CD4^+$, possibly related to higher β_2-adrenergic receptor density on the cell surface (Zhang et al., 2006). Furthermore, it has been suggested that to promote significant changes in the number of B cells, exercise until exhaustion, regardless of duration, must be performed (Fry et al., 1992a; Miles et al., 2003).

According to McCarthy et al. (1991; 1992), and Mignini et al. (2008) catecholamines appear to be in the basis of the acute effects of exercise, particularly lymphocytosis, participating in the regulation of lymphocyte subset redistribution, and cortisol seems to contribute, during the recovery period, to generate and uphold both the lymphopenia and neutrophilia (the last may be produced by release of neutrophils from the bone marrow).

The proliferation of T cells seems to decrease during and after exercise, and this function may be affected by the number of activated cells (Fry et al., 1992a; Henson et al., 1999). However, exercise has shown to induce a reduction of the expansion of cell populations by increasing the rate of apoptosis of both $CD4^+$ and $CD8^+$ T cells, rather than a decrease in the rate of proliferation (Rowbottom & Green, 2000).

Cell death or apoptosis plays an important role in the maintenance of the balance between the generation of new cells and removal of damaged or aged cells. Findings about apoptosis regulation of immune cells after exercise in athletes have essentially focused neutrophils and lymphocytes. It is commonly accepted that the transient lymphopenia after exercise occurs in part due to enhanced apoptosis (Mars, Govender, Weston, Naicker, & Chuturgoon, 1998; Mooren, Bloming, Lechtermann, Lerch, & Volker, 2002), although no influence was mentioned by others (Simpson et al., 2007), while for other cells, such as

neutrophils, the post-exercise apoptosis regulation remains controversial (Kruger & Mooren, 2014).

Despite the contradictory results, these authors worked on the assumption that this mechanism would involve the lymphocyte recruitment from the marginated pool, therefore, bringing to circulation cells that eventually had different functional abilities to those already in the circulation thus enlarging the naïve T-cell repertoire (Kruger & Mooren, 2014). However, it also should be taken into account that most of lymphocytes are not on the blood stream, which lead us to argue whether the changes observed in these peripheral blood cells after acute exercise reflect changes in the reservoirs.

Another mechanism that is not yet fully understood has to do with apoptosis and whether the deletion of autoreactive cells can be seen as beneficial due to the generation of "free space" for new lymphocytes, thus enlarging the naïve T-cell repertoire. Or instead, if it should be considered harmful, once a suppressed immune response occurs.

Additionally strenuous exercise induces increased levels in a number of pro-and anti-inflammatory cytokines, especially IL-6, which is predominantly produced within the contracting skeletal muscle. The net release from the muscle can account for the exercise-induced increase in arterial concentration, which can reach up to 100 fold after a marathon race (Pedersen, Steensberg, & Schjerling, 2001). Suwa et al. (2000) suggested that IL-6 causes a biphasic neutrophilia where the first peak (2 – 6 h) results from the mobilization of neutrophils into the circulating pool from the marginated pool and the second peak (12 – 24 h) results from an accelerated bone marrow release of polimorphonuclear cells. Epinephrine may only partly influence the plasma levels of IL-6 during exercise (Steensberg et al., 2001b).

2.3.2. Chronic cellular immune response to exercise

The literature about the effects of chronic exercise on immune cell system, is consensual regarding the moderate exercise beneficial effects on physical and mental health especially if performed regularly and with aerobic characteristics (Laires & Monteiro, 2008).

Another commonly accepted notion is that during periods of heavy training, the stimulation of adaptive mechanisms related to metabolic, hormonal, circulatory and respiratory responses may compromise performance and negatively influence health status,

although this situation may be reversible by a tapering or recovering period (Gleeson & Bishop, 2005; Shephard & Shek, 1999; Suzui et al., 2004).

Researches that have assessed the chronic response of leukocytes and subpopulations (including lymphocytes subsets) in athletes of different sports such as running (Denguezli et al., 2008), basketball (Brunelli et al., 2014), volleyball (Dias et al., 2011) and soccer (Bury, Marechal, Mahieu, & Pirnay, 1998; Del Giacco, Scorcu, Argiolas, Firinu, & Del Giacco, 2014; Rebelo et al., 1998; Suda et al., 2013) to long-term training periods or competitive training seasons showed controversial outcomes, somehow suggesting that, besides training load, particular characteristics of the sports and of the athletes may also affect the immune response.

Neutropenia (decreased neutrophils) and monocytopenia (decreased monocytes) were observed along a 10-year retrospective study in elite athletes of 14 different sports, including athletics, swimming, cycling, triathlon, basketball, volleyball, rugby, soccer, among others (Horn, Pyne, Hopkins, & Barnes, 2010). Prolonged cycling training induced a reduction in neutrophils phagocytic ability at rest. The decrease in the phagocytic function at rest seems to occur in response to prolonged periods of intense training (Lewicki et al., 1987; Mackinnon, 2000), especially in endurance sports training (Gleeson, 2006). Studies that followed up a 7-month swimming training season reported a reduction in neutrophils and monocytes resting values (Morgado et al., 2012) and decreased $CD56^+$ NK cells (Gleeson et al., 1995; Rama et al., 2013). After a 3-month swimming training program $CD56^+$ NK cells were also diminished (Gleeson et al., 2000). T and B lymphocytes functions have also shown to be sensitive to increases in the training load in well-trained athletes, with falls in circulating type 1 *Th* cells counts, decreased T cell proliferative responses and reductions in stimulated B cell Ig synthesis (Baj et al., 1994; Lancaster et al., 2004; Verde, Thomas, & Shephard, 1992).

The long term negative changes in neutrophils counts and functions predispose athletes to bacterial infection. The exercise-induced neutrophil apoptosis and consequent lower neutrophil lifespan may be one of the mechanisms that contribute to these cells alterations (Kruger & Mooren, 2014). The declines in T, B and NK cells numbers, activity, and proliferation appear to compromise the humoral immune response, thus lowering the host protection level against intracellular pathogens such as viruses and "opening the window" for infections and illnesses. These lymphocytes subsets decreases may be caused by elevations of the circulating stress hormones, particularly cortisol, and anti-inflammatory cytokines, induced by consecutive bouts of exercise (e.g. IL-6, IL-10, IL-Ira) (Giraldo et

al., 2009; Gleeson, 2007; Suzuki et al., 2003). Over the course of a swimming training season, resting cortisol concentrations also increased (Morgado et al., 2012; Rama et al., 2013), whereas monocytes, dendritic cells, and neutrophils capacity to produce inflammatory cytokines in response to an external stimulation was decreased (Morgado et al., 2012), especially during the observation periods with elevated training volumes. So, in response to excessive exercise, it seems that these anti-inflammatory factors (cortisol and cytokines) produced by the immune system, may persist for long periods and possibly cause immune suppression (Dinarello, 1997).

2.4. Factors affecting immune function response to exercise

It is acknowledged that genetic and environmental factors can interfere with the way the immune system works. Regarding exercise and sports training, the type of sport/activity, training load, subject fitness level, age, sex, and maturity have been pointed as potential influential factors of the immune response (Vleck, Millet, & Alves, 2014). Additionally, the divergent findings of the immune response to exercise have often been explained by a possible influence of these factors, along with the variety of exercise protocols, methods of data collection, and differences in the length and seasonality of observation periods.

2.4.1. Subjects characteristics

In general, at rest, athletes seem to have leukocyte and lymphocyte subsets counts and functions similar to those of non-athletes (Nieman, 2000a), and trained individuals seem to have lower neutrophils adherence to the endothelium than controls (Lewicki et al., 1987). When considering specific sports, runners have shown total leukocyte and lymphocyte subsets similar to controls (Nieman et al., 1995a; Nieman et al., 1995b), as well as cyclists (Baj et al., 1994). Tennis players' number of leukocytes, total lymphocytes, $CD3^+$ and $CD19^+$ subsets, eosinophils, and basophils were not different, but neutrophils were lower and $CD16^+56^+$ NK cells were higher than in non-athletes controls (Nieman, Kernodle, Henson, Sonnenfeld, & Morton, 2000). Leukocytes and neutrophil counts were lower in

endurance sports athletes, namely cycling and triathlon compared with team or skill-based sports such as water polo, cricket and volleyball (Horn et al., 2010).

As for swimmers, they have presented similar lymphocytes counts to controls but lower numbers of circulating total leukocytes (Gleeson et al., 1995). Moreover, no differences were observed between non-swimmers and swimmers in the response of total leukocytes, total lymphocytes, lymphocytes subsets $CD8^+$, $CD19^+$, $CD16^+56^+$ and monocytes to the Wingate anaerobic test, except for $CD3^+$, immediately after exercise and for $CD4^+$ at 1 h post-exercise (Boas et al., 1996).

Evidences of maturity differential effects on the immune response to acute physical exercise between adolescents and adults have been highlighted by Timmons et al. (2004). Generally, chronological age may not reflect biological age, especially during puberty. This is a period where each individual presents different timings of growth and maturation, and where the growth patterns of lymphoid, neural, general and genital domains of the body's are not similar. Consequently, it is commonly observed a diversity in anthropometric characteristics and body composition of subjects during this development phase (Boggin, 1999). When considering the normal development of the immune system, the typical curve of lymphoid development shows an increasing line until puberty which afterwards decreases, independently at what time puberty happens (Molinari & Gasser, 2004). This variety stands out when contemplating also the sex-related differences in the physiological levels of some hormones (e.g. catecholamines, cortisol, estrogen, and testosterone) in association with a differential effect of these hormones and cytokines on lymphocyte subsets (Fragala et al., 2011).

Regarding the effect of the menstrual cycle phases over the immune response in females, controversial data has been found. No differences were observed between luteal and follicular phases by Morgado et al. (2014), contrasting with the higher responsiveness during the luteal phase comparing to the follicular phase observed by Timmons et al. (2005).

The few studies that have assessed the influence of sex on the immune response to exercise have revealed controversial results as well. No differences between the sexes were observed, at rest, in total blood leukocyte, neutrophil, monocyte and lymphocyte counts of endurance training athletes, however males had higher B and $CD56^+$ NK cells than females (Gleeson, Bishop, Oliveira, McCauley, & Tauler, 2011). Contrarily, females have shown weaker immune responsiveness than males to a swimming test, represented by lower neutrophils (Ferrer et al., 2009; Tauler et al., 2008) and lymphocytes count and subsets

responses (Morgado et al., 2014). Nonetheless, the CD56^{+} NK cells counts elevation have shown to be higher in females than in males after running up and down 150 stair-steps for about 1 min (Millard et al., 2013), and higher in girls than in boys after 60 min of cycling at 70% of maximal oxygen uptake (Timmons, Tarnopolsky, & Bar-Or, 2006b). Likewise, Timmons et al. (2006c) observed that post exercise elevations in total lymphocytes and CD16^{+}56^{+} NK cells were superior in older girls versus older boys, without any dissimilarity between young girls and young boys. Furthermore, the abovementioned authors described bigger rises in total leukocytes, lymphocytes, and subset CD16^{+}56^{+} NK counts in girls than in boys of similar pubertal status (an indicator of maturity).

Puberty effects on the immune response to exercise have also been noted. Post pubertal boys have shown less acute changes in the immune parameters than the prepubertal and peripubertal boys in response to a Wingate anaerobic test (Boas et al., 1996). Leucocytosis and lymphocytosis, and elevation of CD3^{+}, CD4^{+}, and CD8^{+} lymphocytes was observed in prebubertal and peripubertal boys but not in postpubertal boys. Despite this, 1 h after exercise, this effect was not evident over total leukocytes, and, in the prepubertal group, nor over total lymphocytes, having all returned to baseline values (Boas et al., 1996). In the peripubertal group, lymphocytes total and subsets CD3^{+}, CD4^{+}, and CD8^{+}, decreased below resting values. CD16^{+}56^{+} NK cells and CD19^{+} cells responses were different within puberty groups. In postpubertal boys, CD16^{+}56^{+} was the only parameter that changed immediately post-exercise.

All these evidences pinpoint the need to take age, pubertal stage, and sex into account when interpreting the immune responses to exercise, as referred by Timmons et al. (2007). Furthermore, in the particular case of swimming, the competitive teams include males and females of different ages and maturational states, involving diversity in the characteristics of subjects. In order to deal with this diversity, swimmers are divided into sex and age groups that compete within each group. Therefore, seldom training programs are individualized; instead they are applied to each sex and age group according to a set of fundamental capacities that is expected to have been developed, and respect the biological maturation/growth expected for that age. So, it appears also pertinent to contemplate a possible differential immune response considering the different swimming age groups.

Little is also known or assumed about the effects of ability level, and/or event distance specialization (distance specialty) on the immune response to training either in swimming or others sports. Actually, regarding endurance sports, since short- and long-distance specialists normally differ in the proportion of the time that is spent on training at higher

intensities, it would be interesting to know if the immune status would be affected differentially.

2.4.2. Training load

The influence of training load on sports performance is essential for training science and the practice of sport. The training load is characterized by two main factors that will determine the degree of adaptation to training: volume and intensity. The volume is directly related to the frequency (number of repetitions and the number of sets) and duration of the stimulus or stimuli, whereas the intensity refers to the effort level required by the stimulus (Costill et al., 1991). In order to assess the training loads impact and to perceive the load dynamic throughout the training processes it is necessary to quantify this variable.

In competitive swimming, the immediate physiological demands of swimming events dictate the particular abilities (aerobic endurance, aerobic power, lactate tolerance, and sprint) that will need to be developed and trained under specific training regimens in order to maximize the performance in those competitive events. Each of these characteristics implies training sets that have different intensities, duration and rest intervals and that are frequently adopted in training methodologies, thus promoting the specific desired adaptions (Maglischo, 2003; Sweetenham & Atkinson, 2003). The adjustment of one or more of these parameters may effectively target one of the abovementioned training intensities. These evidences raise the question of the use of the total distance swam as not clearly reflecting the physiological stress produced at different levels of intensity of the various training sets (Sharp, 1993). In fact, coaches need to quantify the work accomplished in the various levels of intensity associated to each training set, to have an estimation of the physiological stress of training sets, and consequently of the training sessions, training mycrocycles, mesocycles and macrocycles and eventually of the whole training season.

In order to estimate the values of intensity of training Mujika et al. (1995) proposed the quantification of the magnitude of the training load expressed by dimensionless units of load, named arbitrary units of load (AUL). AUL determination is based on a stress index scale and is a procedure that allows adjustment to the exponential function determined by the curve of lactate accumulation in relation to the intensity of effort determined by the

average swimming speed on each training zone. The description of the zones of intensity and correspondent swimming speeds percentage in relation to race time, theoretical blood lactate accumulation levels, and the associated stress index scale values, are represented in Table 2.1. The AUL can then be obtained from the ratio between the sum of the volumes swam in each zone of intensity multiplied by the respective index and the total volume effectively completed.

Table 2.1. Characterization of the intensity levels of swimming training based on the average swimming speed on tasks, theoretical lactatemia, and stress index values (adapted from the works by Mujika et al. (1995), Maglischo (2003), and Sweetenham & Atkinson (2003))

Zones of Intensity (Intensity levels)	Description (Training Purpose)	Average swimming speed on tasks (relative to race time; %)	Lactatemia (mmol.l^{-1})	Stress Index
I – WR	Warm up and Recovery	until 60%	0 - 2	1
II – A1	Aerobic Threshold	until 75%	2 - 3	2
III – A2	Anaerobic Threshold	≈ 80%	3 - 4	3
IV – VO$_2$max	VO$_2$max	≈ 85%	6 - 9	4
V – LT	Lactate Tolerance	≈ 90%	>8	6
VI – LP	Lactate Production	≈ 95%	>8	8
VII - Sprint	Power Training (Alactic)	maximal	-	10

The literature review revealed that several cross-sectional and longitudinal studies have investigated the systemic exercise induced changes of leukocytes and subpopulations (including lymphocytes subsets), however some limitations upsurge when discussing the emerged findings. Few cross-sectional studies included observations of these immune cell parameters and up to 24 h after acute exercise, and time-course response patterns are difficult to define until complete recovery. There seem to be convergent findings about the response of leukocytes, neutrophils, total lymphocytes, and monocytes to exercise. However, lymphocyte subsets behaviour has shown inconsistent results. Most of the cross sectional studies primarily evaluated male adults and majorly adopted an exercise stimulus based on cycle ergometer and treadmill protocols in laboratories, or competition events, and not representative training sessions (Gabriel et al., 1992a; Kakanis et al., 2010; Steensberg et al., 2001a). Longitudinal studies that evaluated these immune cells at rest

over the course of long-term training periods or competitive training seasons of different sports (Brunelli et al., 2014; Del Giacco et al., 2014; Denguezli et al., 2008; Dias et al., 2011; Suda et al., 2013) presented dissimilarities in the length and seasonality of the observation periods that difficult the comparison of the outcomes. Additionally, to our best knowledge no longitudinal study was conducted with the intent of exploring a long-term training process influence on the immediate immune cell response to an acute exercise, or training session, and the subsequent recovery along the 24 h after.

The somewhat divergent findings have often been explained by the diversity of exercise protocols, training loads, length and seasonality of the observational periods, methods of data collection, sample sizes and inter-subjects characteristics, such as subject fitness level, sex, maturity, ability level, and/or event distance specialization. Another factor associated to this heterogeneity may be the type of activities or sports and the specific periodization and training characteristics. This evidence seems to be more relevant when considering endurance sports, and, as above mentioned, competitive swimming. Although some researches have been conducted about this subject, in laboratory or in the field, we intended to apply the analysis of immune cell systemic basic parameters, namely, leukocytes and subpopulations (including lymphocytes subsets) directly in the field with the follow-up of real and representative competitive training situations, in a particular sport such as competitive swimming, where few investigations have been made.

With this in mind, specific research on this subject appears to be pertinent to help understanding how to handle these apparently harmful effects of high intensity training sessions and long-term training on immune system and how to take advantage of the immune system participation on the adaptation to exercise, while minimizing the deleterious effect of intense exercise.

In fact, we believe this comprehension and the control of the immune response may improve the strategies of monitoring and testing used in the preparation of the swimmers' career, in preventing the increase of the susceptibility to potential infections and illnesses, impaired performances and most of all health, which may compromise attendance to training sessions, and in the worst case scenario, the onset of overtraining. Furthermore, knowing better the particular responses to exercise of the immune system according with the subject's characteristics may be useful regarding the individualization of the training process.

2.5. Aim of the investigation

The present dissertation includes three research studies conducted under the scope of the effects of training on swimmers immune system, considering the classic distinction of acute and chronic exercise effects:

Study 1 (*Chapter IV*) aimed to evaluate the acute systemic immune cell response to a representative high intensity swimming training session integrated in a normal training process during a competitive swimming season, during a 24 h recovery period, in well trained swimmers, taking into account, sex, menstrual cycle phases, maturity and swimming age groups effects in the interpretation of these immune responses.

Study 2 (*Chapter VI*) aimed to investigate the variation of resting systemic immune cell parameters over the course of a 7-month swimming training season, in a large cohort of well-trained swimmers involved in their regular training environment, taking into account sex, maturity, swimming age groups, performance improvements, and distance specialty effects in the interpretation of these immune variations.

Study 3 (*Chapter VII*) intended to explore the influence of a 4-month training macrocycle of a swimming training season over the immune cell response to a standardized high intensity swimming training session integrated in the normal training process, during a 24 h recovery period, whilst controlling systematically and simultaneously for the effects of sex, maturity, age group, performance improvements, and distance specialty.

CHAPTER III

Methodology

A brief description of the sample will be provided in this chapter, followed by a description of the methods and inherent procedures used throughout the present investigation.

3.1. Study design and sampling

This project used an observational design with a follow-up over a swimming competitive training season lasting 30 weeks. The evaluation of the swimmers was made at four moments of evaluation, named M1 (at the beginning of the season; baseline evaluation), M2 (the week after the main competition of the 1st macrocycle; 13th week of training), M3 (at the specific preparatory sub phase of the preparatory phase of the 2nd macrocycle; 23rd week of training) and M4 (the week after the main competition of the 2nd macrocycle; 30th week of training). At M2 and M4 a standardized real high intensity swimming training session was performed in order to understand the influence of training over the acute response of biochemical immune indices to exercise.

According to this organization, each study was performed considering certain moments of evaluation. *Study 1* was based on M2, *Study 2* on M1, M2, M3 and M4, and *Study 3* on M2 and M4. In the latter, although analysing only two evaluation moments, which could be referred as moment 1 and moment 2, the designation M2 and M4 was preserved in order to respect the logical sequence of the study design and facilitate the integration of the results in the general discussion (*Chapter VII*).

At each moment of evaluation data collected for all subjects included subjects' physical characteristics and maturity, and biochemical immune indices.

Throughout the follow up season the incidence of URS and the menstrual cycle phases for females were monitored weekly and training load and mean intensity of all scheduled swimming sessions were quantified. In general, the characteristics of the training regimens and competition schedules were not modified by the present study in anyway nor any swimmer suffered from major injury or sickness preventing them from training for more than one day.

3.1.1. Subjects

The subjects of this study were members of four different Portuguese swimming teams belonging to the *Lisbon Swimming Association.*

3.1.1.1. Swimming age groups

Swimmers were classified according to the different age group they belonged to and thus had different background in competitive swimming amongst the age groups considered (average of 5.3 ± 1.9 yrs. and range $\simeq$ 3 to 11 yrs. of practice).

The classification of the swimming age groups according to the *Portuguese Swimming Federation* and the *Ligue Européene de Natation* (*LEN*) is described in Table 3.1.:

Table 3.1. Swimming age groups description in both sexes according to the *Portuguese Swimming Federation* and the *Ligue Européene de Natation* (*LEN*)

Swimming Age Groups	Age range (years)	
	Females	Males
Youth	13 - 14	14 – 16
Junior	14 – 16	16 – 18
Adult	≥ 17	≥ 18

3.1.1.2. Distance specialty

Swimmers were also grouped regarding their swim specialty on a distance type basis: short or long-distance. This division was made according to the coaches' classifications of their swimmers, and was primarily based on the swimmers' main event distance: 50 m, 100 m and 200 m swimmers were classified as short distance and 400 m, 800 m and 1500 m swimmers as long distance swimmers.

All participants and their parents or legal guardians were informed about the possible risks of the investigation before giving their written informed consent to participate (Appendix A). All procedures were approved by the Ethic Committee of the Faculty of Human

Kinetics, University of Lisbon and conducted in accordance to the declaration of Helsinki for human studies of the World Medical Association (World Medical Association, 2008).

This study started with 103 participants at M1. Over the course of the training season not all subjects had a continuous participation in the study especially due to dropping out swimming competition training, or being ill or injured at the times of the evaluation moments.

Table 3.2. presents the number of female and male swimmers, chronological age range and study design for the three studies included in this dissertation. Specific and individualized details of the sample and the methods will be provided in each study (*Chapters IV* to *VI*).

Table 3.2. Number of female and male swimmers, chronological age range and study design of each study of the dissertation

Study	Females (N)	Males (N)	Chronological age range (yrs)	Study design
1	30	35	13 - 21	Cross-sectional (in season)
2	25	29	13 - 21	Longitudinal (7-month winter training season)
3	16	27	13 - 21	Longitudinal (4-month training macrocycle; in season)

3.1.2. Swimming training season

The follow up training program started after detraining from the previous swimming year (transition phase) and lasted throughout a competitive season lasting 30 weeks (Fig. 3.1.). Swimmers followed the training program set by the coaches of each different team.

The evaluation of the swimmers was made at four moments of evaluation (M) named M1 (at the beginning of the season; baseline evaluation), M2 (the week after the main competition of the 1st macrocycle; 13th week of training), M3 (at the specific preparatory sub phase of the preparatory phase of the 2nd macrocycle; 23rd week of training) and M4 (the week after the main competition of the 2nd macrocycle; 30th week of training).

The study was divided into three main periods that represented distinctive training phases: M1 to M2 (three months) corresponded to the 1st macrocycle of the training season, which began with the general preparatory sub phase and lasted until the main competitive sub phase. This first macrocycle aimed to prepare the athletes to the National Youth Long-distance Championship, to the winter National Championships and to the National

Interclubs Championship. This period was characterized by an aerobic training predominance and the progressive increase of training volumes and intensities in the first two months and for the maintenance of high intensities and progressive decrease of volumes in the last month. At the National Championships all swimmers have accomplished at least one personal best time in the races they were enrolled in.

M2 to M3 (two months) coincided with the entire preparatory phase of the 2nd macrocycle of the season. This development period was characterized by a progressive increase in training volume; intensity and frequency that lasted until the end of the specific preparatory sub phase, where the higher peak of training load of the season was reached and the swimmers were evaluated (M3). In this period there was also a more frequent participation in competitions (including international meetings);

M3 to M4 (one month) was a period of training that occurred during the competitive phase of the 2nd macrocycle of the season and included a specific preparatory period followed by a competitive period that lead to important competitions for which training load was progressively reduced: National Youth Championship and National Junior and Senior Championships.

Fig. 3.1. Periodization of the 7-month winter swimming training competitive season and schedule of the moments of evaluation: M1, M2, M3 and M4

DATES	MONTHS	SEPTEMBER			OCTOBER					NOVEMBER				DECEMBER				JANUARY					FEBRUARY				MARCH				APRIL
	1st DAY OF WEEK	12	19	26	3	10	17	24	31	7	14	21	28	5	12	19	26	2	9	16	23	30	6	13	20	27	5	12	19	26	2
COMPETITION CALENDAR — TEAMS										X					X																
COMPETITION CALENDAR — LOCAL							X				X									X	X						X				
COMPETITION CALENDAR — NATIONAL												X			X														X		X
COMPETITION CALENDAR — INTERNATIONAL																	X			X	X										

PERIODIZATION																															
TRAINING PHASES	PREPARATORY 1							COMPETITIVE 1						TRANS.		PREPARATORY 2									COMPETITIVE 2						
TRAINING SUBPHASES	GENERAL PREP.				SPECIFIC PREP.			PRE COMP.		MAIN COMPETITIVE				TRANS.		GENERAL PREP.				SPECIFIC PREP.		PRE COMP.		MAIN COMPETITIVE							
MACROCYCLES	1															2															
MESOCYCLES	1				2			3				4				5				6			7				8				
MYCROCYCLES	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
PEAKING	4			3			2			1				5		4			3			2				1					
CONTROL TESTS		X		X	X		X	X		X					X		X		X			X		X			X				

Legend: Dryland Training, Technique Training, VOLUME, INTENSITY, PEAKING, MOMENTS OF EVALUATION; PEAKING scale 1–5; vertical axis 10%–100%; M1, M2, M3, M4.

41

3.1.3. Swimming training session

At M2 and M4 a standardized high intensity swimming training session was performed in order to explore the acute response of biochemical immune indices to exercise. The pre-selected swim training session started with a 1500 m standardized warm-up lasting 30 to 35 min followed by a high intensity main task that lasted 50 min and a 500 m recovery task (8 min of duration). The main task was designed to induce maximal lactate accumulation and had a total distance of 1000 to 1200 m, depending on the age group considered. For the youth group the main task consisted of two sets of four repetitions of 75 m front crawl on a five min cycle, with 10 min of active recovery between sets (400 m freestyle). Each repetition had to be accomplished at 90 - 95% of 100 m Freestyle personal best race time. The task organization was identical for juniors and seniors but with repetitions of 100 m. Swimming times were registered in each repetition and the mean time was used to determine the mean effort intensity percentage (%), in relation to the personal best time at the 100 m freestyle race.

The organization of the session tasks is described below:

Warm up (1500 m, 30 – 35 min):
500 m (200 m Freestyle/ 150 m Breaststroke / 100 m Backstroke/ 50 m Butterfly)
8 x 75 m Medley 2x(Freestyle/ Breaststroke / Backstroke/ Butterfly) on 1:25
12 x 25 m (Freestyle / 1≠Freestyle) on :30
100 m Freestyle, catch-up, bilateral

Main Set: Maximal Lactate Accumulation, 90 – 95% of 100 m Freestyle of personal best time (1000 – 1200 m Freestyle, 50 min):
≥16 yrs: 2 x (4 x 100 m, on 5:00) with 10:00 active recovery (400 m)
13-15 yrs: 2 x (4 x 75 m, on 5:00) with 10:00 active recovery (400 m)

Recovery (500 m, 8m20s)
10 x 50 m Freestyle on :50, bilateral

3.2. Methods

3.2.1. Subjects characteristics

Chronological age, anthropometric characteristics (stature, weight, body mass index, fat mass percentage, and free fat mass), and an indicator of biological maturation (pubertal Tanner stages) were used to characterize swimmers enrolled in this study.

3.2.1.1. Chronological age

Decimal chronological age was calculated as the difference between date of birth (in years, months, and days) and date on which the evaluations were made.

3.2.2.2. Anthropometric characteristics

Stature and body mass were measured following procedures of Martin & Saller (1957) always after wakening in the fasted state. Stature was measured to the nearest 0.1 cm with *Siber-Hegner* anthropometric kit (DKSH Ltd., Zurich, SW), as the distance from the standing surface (the anthropometer's flat platform) to the top (vertex) of the head (the anthropometer's head piece). The examiner assisted with positioning the anthropometer and correcting swimmers posture. Subjects were in standard erect posture with weight evenly distributed between both feet, heels together, arms hanging relaxed at the sides and the head in the Frankfurt horizontal plane. The head was in the Frankfurt plane when the horizontal line from the ear canal to the lower border of the orbit of the eye was parallel to the floor and perpendicular to the vertical line of the anthropometer. The anthropometer headpiece was lowered so that it rested firmly on top of the participant's head, with sufficient pressure to compress the hair. Subjects were instructed to stand as tall as possible, to take a deep breath, and hold this position. Intra-observer technical errors of measurement were 0.29 units.

Participants were weighed to the nearest 0.1 kg wearing a bathing suit without shoes on an electronic scale (TANITA BC-601 body composition scale monitor). This electronic scale

also calculated swimmers Fat Mass percentage (%FM) using Bioelectrical Impedance Analysis (BIA) with a measuring current of 50 kHz, 100 μA. Body mass index (BMI) was calculated as body mass (BM, kg) divided by the square of the stature (m). Free fat mass (FFM) was calculated according to the formula:

$$FFM \ = \ BM - (BM \ x \ \%FM)$$

Where FFM is the Fat Free Mass expressed in kilograms, BM is the Body Mass expressed in kilograms, and %FM is the Fat Mass percentage expressed in percentage.

3.2.2.3. Indicators of biological maturation

In adolescence, chronological age is not a reliable parameter for biological characterization of individuals. During this period, individuals with the same age are frequently in different stages of puberty considering that its onset and progression are highly variable. So, in the present study a non-invasive method was utilized to assess biological maturity of participants: sexual maturation.

Sexual maturation is a continuous process that extends from sexual differentiation in the period of the embryo through puberty to full sexual maturity and fertility (Malina, Bouchard, & Bar-Or, 2004). The assessment of sexual maturation allows for the evaluation of the adolescents pubertal developmental stage.

Stages of puberty provide an indication of maturity status at the time of observation. Swimmer's sexual maturity was assessed by questionnaire and classified according to the pubertal Tanner's stages based on the secondary sex characteristics (1962). A self-evaluation method, with figures, was used to identify the degree of development of pubic hair in each gender, breast development and age at menarche in girls and genital and voice changes development in boys. Breasts and genitals are examined according to size, shape, and characteristics and pubic hair according to quantity and distribution (Appendix B). Stage 1 (Tanner 1) corresponds to the pre-pubertal phase and stage 5 (Tanner 5) corresponds to late-pubertal (adult) phase. In this sense, stages 2, 3, and 4, or the mid-pubertal stages, represent puberty. Stages 2 to 4 are conventionally called sexual maturation stages, or Tanner stages.

3.2.3. Quantification of the training load

Training load was determined through the total amount of meters swam (volume) and also by the balance of the distance completed at each level of intensity based on the work of Mujika et al. (1996a; 1995).

The use of a stress index scale of difficulty has been established in reference to the theoretical values of blood lactate accumulation usually associated with the different swimming training zones of intensity. In the present dissertation, the training zones adopted considered the works by Mujika et al. (1995), Maglischo (2003), and Sweetenham & Atkinson (2003) (described in Table 2.1.), and were: I - warm up and recovery, II - aerobic 1, III – aerobic 2, IV - VO$_2$max, V - lactate tolerance, VI - lactate production and VII - sprint. In order to evaluate the swimming sessions training load the volume accomplished in each zone of intensity was quantified (mI, mII, mIII, mIV, mV, mVI and mVII). The magnitude of the load was then expressed in dimensionless units of load, or arbitrary units of load (AUL), obtained from the ratio between the sum of the volumes swam in each zone of intensity multiplied by the respective index (1, 2, 3, 4, 6, 8, 10) and the total volume effectively completed, according to the formula:

$$AUL = \frac{1\,mI + 2\,mII + 3\,mIII + 4\,mIV + 6\,mV + 8\,mVI + 10\,mVII}{\text{swimming training session volume}}$$

Where AUL is the arbitrary units of load expressed in dimensionless units of load, the numerator is the weighed volume expressed in meters, mI, mII, mIII, mIV, mV, mVI and mVII are the meters accomplished at the following swimming training zones of intensity: I- warm up and recovery, II - aerobic 1, III – aerobic 2, IV - VO$_2$max, V - lactate tolerance, VI - lactate production and VII – sprint, the numbers 1, 2, 3, 4, 6, 8, 10 are the indexes associated to each zone of intensity, the denominator is the swimming training session volume expressed in meters.

This was performed for all season sessions considering each age group and within all swimming teams.

The mycrocycle or weekly load was quantified by the volume (total of meters swam), by the weighed volume (sum of the multiplications of the volume accomplished in each zone of intensity by the respective stress index values) and by the intensity (determined through the sum of the resulting dimensionless unit of load of each session of training).

3.2.4. Performance improvements

The effect of training on performance was evaluated by magnitude of the change of the race time at competitive events. It was expressed as a relative difference, which represented a percentage of change, comparing the race time accomplished at M4 with that at the end of the previous season for study 2, and with that at M2 for study 3. In this manner, two groups were created according to the level of improvement: the less efficient group, which presented under 2% changes in performance, and the efficient group, which presented changes of 2% and above (Table 3.1.). This 2% level of improvement was adopted based on the consistently mean improvements of around 3% mentioned in the literature (Mujika et al., 1996a).

3.2.5. Upper Respiratory Symptoms

Subjects were asked to answer to a weekly questionnaire that was sent every Monday to their email address. This questionnaire (Appendix C) consisted of a daily logbook in which they noted their symptoms associated with illnesses related to Upper Respiratory Symptoms (URS) such as: headache, fever, ear pain, chills, runny or blocked nose, pharyngitis/tonsillitis, bronquitis, asthma, phlegm, cough, conjunctivitis; itchy, watery eyes, nausea/vomiting, and diarrhoea. All swimmers were asked to indicate the medication they were on and female subjects to point out the days of menstruation. If subjects had no symptoms they simply recorded that and reply to the email.

If fever or at least two concomitant symptoms persisted for at least 48 hours, separated from previous symptoms by at least one week, they were considered an episode of URS (Bishop, 2006). Symptoms separated by less than one week were regarded as a recurrence or continuation of the initial episode and were regarded as part of the same episode. The counting of the URS episodes was expressed and displayed graphically as the weekly number of episodes of URS over the course of the training season.

3.2.6. Blood samples collection and analysis

Peripheral venous blood was obtained early in the morning (resting samples; (6:00 – 6:30 a.m.), in the fasted state (after a period of 8 – 10 h without food ingestion) by standard procedures and collected into tubes containing EDTA for assessment of haemogram and leukogram and lymphocytes subpopulations. At M2 and M4 blood samples were also collected immediately after (Post), 2 h after (Post 2h) and 24 h after (Post 24h) the selected swimming session. Post, Post 2h and Post 24h exercise values were corrected for plasma volume variations with hemoglobin concentration and hematocrit values according to Dill & Costill (1974).

Haemogram and leukogram was performed in an automated hematology analyzer *Coulter LH 750* (*Beckman Coulter*) which produced the following parameters (abbreviation and SI units): hematocrit (HCT; % $L.L^{-1}$), hemoglobin concentration (Hb; $g.dL^{-1}$), red blood cell count, (RBC; $*10^{12}.L^{-1}$), mean cell volume (MCV; fL), mean cell hemoglobin (MCH; pg), mean cell hemoglobin concentration (MCHC; $g.dL^{-1}$), red cell distribution width (RDW; %), white blood cell count or leukocytes (WBC), neutrophils, monocytes, eosinophils, basophils, platelets (the leukocytes and subsets were expressed as $*10^{9}.L^{-1}$) and also the percent (%) of neutrophils, lymphocytes, monocytes, eosinophils and basophils.

Total lymphocytes and subsets were counted by flow cytometry, using the FACS Calibur Becton, Dickinson and Company cytometer equipped with two laser beams at 635 e 488 nm. This cytometer was linked to a *Macintosh* computer that uses the software *Multiset* to analyse the cytometry results (Burtis & Ashwood, 1999; Goldsby, Kindt, Osborne, & Kuby, 2003; Radbruch, 2000; Rose, 2002).

The lymphocytes subsets analysed were $CD3^{+}$ (total T lymphocytes; T cells), $CD4^{+}$ (T *helper*; *Th*), $CD8^{+}$ (T *cytotoxic*; *Tc*), $CD16^{+}56^{+}$ (NK cells) and $CD19^{+}$ (B cells) and results were expressed as $cells.\mu L^{-1}$.

Flow cytometry is a technology that concurrently measures and then analyses multiple physical characteristics of single particles, usually cells, as they flow, one by one, in a fluid stream through a beam of light. The properties measured involve a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. The determination of these characteristics is made by an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence. Flow cytometry integrates the use of multiple fluorochromes, which cause the emission of

secondary light with a different wavelength; to identify and isolate subset populations from a single sample, thus used as cellular markers (Biosciences, 2000).

3.2.7. Statistical analysis

The statistical analyses were performed with the software IBM SPSS Statistics (SPSS Inc., an IBM Company, Chicago, Illinois, USA) version 21, and the R software (R Core Team, 2012), version 2.15.1, and a significance level of 5% was considered.

The statistical procedures common to all studies are presented in this section as follows:

- Descriptive statistics, including means and standard deviation (mean ± SD) were performed for participants' characteristics and training sessions intensity and training load quantification measurements and also for biochemical indices relative difference values in study 3.

- Descriptive statistics, including means and standard error of the mean (mean ± SEM), were performed for biochemical indices in studies 1 and 2.

- Normality of the outcome variables was analysed using the *Shapiro-Wilk* test.

- One sample T-test (*t*) was used to compare group means with the reference range provided by the *National Health Institute Doutor Ricardo Jorge* (*INSA*) (Lewis, Bain, & Bates, 2006) and indicate if participants were within the "clinical normal" values associated with each variable.

- The effects of menstrual cycle phases (follicular, luteal, and not menstruated), sex, Tanner's stages (adolescent and adult), swimming age-groups (youth, juniors and seniors), distance specialty (short and long distance swimmers), performance (efficient and less efficient), and the interaction effect of each one of these factors with the moment of evaluation on the response of the variables of interest was analysed using nonparametric mixed-design ANOVAs. The within-subjects factor was the moment of evaluation (four levels: M1, M2, M3 and M4, or Pre, Post, Post2h and Post 24, and also two levels: M2 and M4), which is referred to as the effect of acute exercise or training, and the subjects' factors were the aforementioned influential variables. The nonparametric mixed-design ANOVA has an ANOVA-type statistic (ATS) for each effect, and also a modified ANOVA-type statistic (MATS) for the between subjects factor. The option for the nonparametric approach was due to the violation of the

assumptions of parametric mixed ANOVA in some groups, namely the normality of the dependent variables in each factor's level, the homogeneity of variances and the sphericity. This nonparametric analysis was performed with the *nparLD* package (Noguchi, Gel, Brunner, & Konietschke, 2012) from the *R* software.

- If an interaction term between the factors mentioned and the variables of interest was non-significant, subsequent analysis of the effects of exercise and training were performed using the whole sample. Otherwise, exercise effects were analysed separately considering each group of effects that influenced the variables of interest.

- When an effect of a factor with three levels occurred, *Kruskal-Wallis* test with the *Dunn-Bonferroni* post hoc tests were executed to assess between which levels the differences existed. If the interaction effect between each one of the factors aforementioned and the moment of evaluation on the variables of interest was non-significant, subsequent analysis of the effects of exercise were performed not distinguishing participants by the levels of each between-subjects factor. Otherwise, exercise effects on the variables of interest were analyzed separately considering each between-subjects factor's level. Repeated measures ANOVA was used for the assessment of training effects on immune parameters. Normality and sphericity assumptions were evaluated with the *Shapiro-Wilk* and *Mauchly's* test, respectively. Post hoc tests with *Bonferroni* correction were performed to determine between which moments a significant difference was observed. If the repeated measures ANOVA assumptions were not met, the exercise effect was assessed by *Friedman* test. Post hoc analyses were performed using *Dunn-Bonferroni* test (Dunn, 1964) or, if necessary, due to the conservative characteristic of the *Bonferroni* procedure, according to Conover et al. (1999).

- *Wilcoxon* signed ranks test was used for comparisons between the relative difference values of the acute response at M2 and M4. Statistical significance was set at $p < .05$ in all cases.

CHAPTER IV

Study 1 – Acquired immunity impairment in response to a high intensity swimming session

4.1. Abstract

Exercise immunology studies have shown convergent findings about total leukocytes, neutrophils, total lymphocytes, and monocytes response to exercise but lymphocyte subsets behavior has shown inconsistent results. Also, seldom representative team training sessions have been studied. This study aimed to evaluate the acute systemic immune cell response to a real swimming session during a 24 h recovery period, taking into account, sex, menstrual cycle phases, maturity and swimming age groups. Competitive swimmers (30 females; 15 ± 1.3 yrs., and 35 males; 16.5 ± 2.1 yrs.) performed a high intensity swimming training session (main set at 91.4 ± 4.7 % of personal best time) at the end of the first macrocycle of the season. Blood samples were collected before (Pre), immediately after (Post), 2 h after (Post 2h) and 24 h after (Post 24h) exercise, by standard procedures for assessment of leukogram by automated counting (Coulter LH 750, Beckman) and lymphocytes subsets by flow cytometry (FACS Calibur BD Biosciences). Subjects were grouped according to the Portuguese Swimming Federation age groups, and monitored for pubertal Tanner stage, and girls for menstrual cycle phases. Statistical significance was set at $p < .05$.

At rest, immune system mean baseline values were within the reference interval. At Post, it was observed an increase in neutrophils in youth and junior swimmers, and a decrease in monocytes, eosinophils and lymphocytes counts, reflecting lymphocytes subsets $CD3^+$, $CD8^+$, $CD19^+$, $CD16^+56^+$ in all subjects, and in juniors and seniors also $CD4^+$. At Post 2h, leukocytes and neutrophils increased in all groups, with neutrophils increasing above the normal range, however eosinophils, and lymphocytes, except for $CD4^+$ in female seniors and male juniors, and $CD19^+$ in youth and seniors, persisted low, and monocytes recovered to pre exercise values. At Post 24h, the total lymphocytes and $CD3^+$ subsets in the junior group remained below the pre-exercise levels, while all other cells counts returned to

baseline values. No effects of menstrual cycle phases or maturity were observed, throughout the 24 h period. A demanding real swimming training session, representative of the typical effort developed in any training process, induced a significant acute neutrophilia, lymphopenia and low eosinophils count lasting for at least two hours, independently of sex and maturity. Furthermore, when considering the junior swimming age group (15 – 17 yrs. of age), a 24 h period revealed to be insufficient to attain total recovery of the acquired immunity, i.e. total lymphocytes and total T lymphocytes ($CD3^+$). This fact must be taking into account when planning consecutive training sessions. The observed lymphopenia suggests a lower immune surveillance at the end of the session that may increase the risk of infection or suppressed immunity of athletes in the period just after training, highlighting for the need of extra care when exposed to aggressive environmental agents, such as swimming pools.

Keywords: Cellular Immunity, Swimmers, Training Session.

4.2. Introduction

In adults, considerable evidence suggests that immunocompetence is compromised after intensive exercise, especially when the latter is accompanied by environmental or competitive stress (Walsh et al., 2011), particularly in highly trained athletes (Lopes, Osiecki, & Rama, 2011; Pyne & Gleeson, 1998).

Competitive swimmers are usually under high-intensity training processes that frequently include highly demanding training sessions with little recovery time in between (Sargent et al., 2014). Additionally, they are repeatedly exposed to warm humid environment and chlorine-rich atmosphere (Sa, Boaventura, & Pereira, 2011). These training conditions may contribute to compromise immunocompetence of the swimmers and support the need for specific research on this sport.

The understanding of the impact of the training sessions on the immune system, especially during heavy training periods, is crucial for the adequate periodization of training, in order to prevent the negative influence on health and performance status of the athlete. In fact, if the organization of consecutive acute bouts of intensive exercise is not well managed throughout high-load training periods, a "prolonged maladaptation" of the athlete and of

several biochemical, neurochemical, and hormonal regulation mechanisms may occur and lead to the settling of overtraining syndrome (Meeusen et al., 2013).

Moreover, traditionally, swimming teams include males and females and athletes of different ages and maturational states, implicating diversity in the characteristics of subjects. This diversity is somewhat associated to the different timings of growth and maturation of each individual, involving different types of growth (lymphoid, neural, general and genital) (Boggin, 1999). Thus, chronological age may not reflect biological age, especially during puberty. Considering a normal development of the immune system, the typical curve of lymphoid development shows an increasing line until puberty which afterwards decreases, independently at what time puberty happens (Molinari & Gasser, 2004). This variety stands out when contemplating also the sex-related differences in the physiological levels of some hormones (e.g. catecholamines, cortisol, estrogen, and testosterone) in association with a differential effect of these hormones and cytokines on lymphocyte subsets (Fragala et al., 2011).

In order to deal with this diversity, in sports, athletes are divided into sex and age groups that compete within each group. Therefore, seldom training programs are individualized; instead they are applied to each sex and age group according to a set of fundamental capacities that is expected to have been developed, and respect the biological maturation/growth expected for that age.

So, when exploring the immune response to exercise, and taking into account that in the past years the studies about the acute immune response to exercise and sports activities have created a pool of data that has some heterogeneity, where the divergent findings have often been explained by the variety of exercise protocols, methods of data collection, and subjects' different levels of conditioning, age, and sex, it appears pertinent to consider the particularities of the response of males versus females, pre-pubertal versus pubertal versus post-pubertal, and youth versus juniors versus seniors.

Regarding previous studies of the acute immune cells response to exercise, several Authors have reported consistent patterns, namely a rise in the number of total leukocytes (leukocytosis) (McCarthy et al., 1991; McCarthy et al., 1992; Natale et al., 2003; Yamada et al., 2000), neutrophils (neutrophilia) (Ferrer et al., 2009; Gabriel et al., 1992a; Kargotich et al., 1997; Yamada et al., 2000), and total lymphocytes (lymphocytosis) (Gabriel et al., 1992a; Ibfelt et al., 2002; Kakanis et al., 2010; Starkie et al., 2001; Yamada et al., 2000) just after high intensity exercise. Throughout the recovery period, leukocytosis and neutrophilia have been reported at 1h (Gabriel et al., 1992a; Green, Croaker, &

Rowbottom, 2003), 2 h (Gabriel et al., 1992a; Yamada et al., 2000), and 3 h (McCarthy et al., 1991; McCarthy et al., 1992; Natale et al., 2003) after accomplishing the exercise. However, 2 h after intense exercise a fall in total lymphocytes (lymphocytopenia) (Gabriel et al., 1992a; Kakanis et al., 2010; Steensberg et al., 2001a; Steensberg et al., 2001b) was observed, reflecting essentially the decline in $CD3^+$ lymphocytes below rest levels (Gabriel et al., 1992a; McFarlin et al., 2004; Steensberg et al., 2001a) although decreases for $CD16^+$ (Gabriel et al., 1992a), and $CD56^+$ (McFarlin et al., 2004) NK cells and $CD19^+$ (Gabriel et al., 1992a) were also observed. Twenty four hours after exercise, several studies reported the return to pre-exercise values (Kakanis et al., 2010; Nielsen, Secher, Christensen, & Pedersen, 1996; Nieman et al., 1989; Nieman et al., 1991; Zhang et al., 2006) while others reported still altered values which included increased total leukocytes and lymphocytes, and lymphocytes subsets $CD3^+$, $CD4^+$, $CD8^+$ (Gabriel et al., 1992a) or decreased $CD8^+$ subset (Steensberg et al., 2001a).

When considering competitive swimmers, research about the acute immune response to swimming sessions or exercise protocols is scarce, specifically as regards changes in circulating leukocytes and subpopulations (Ferrer et al., 2009; Kargotich et al., 1997; Morgado et al., 2014; Tauler et al., 2008). These studies referred the immediate post-exercise leukocytosis, lymphocytosis, reflecting the increase of all subsets, and a $CD4^+/CD8^+$ ratio decline after high intensity swimming (Kargotich et al., 1997; Morgado et al., 2014). Moreover, throughout the recovery period subsequent to high intensity swimming exercises, leucocytosis was observed at 2 h and 2.5 h post exercise (Kargotich et al., 1997) and neutrophilia at 1 h (Tauler et al., 2008), 2 h (Ferrer et al., 2009; Kargotich et al., 1997) and 2.5 h post exercise (Kargotich et al., 1997). As for lymphocytes subsets, a fall in lymphocytes total and subsets $CD4^+$, $CD8^+$ and $CD19^+$ at 1 h post swimming was reported, which maintained below pre-exercise levels at 2 h and 2.5 h post swimming; $CD16^+$ NK cells fully recovered at 1 h post and $CD4^+/CD8^+$ ratio increased 1 h post and recovered at 2 h post (Kargotich et al., 1997). On the contrary, no changes occurred in the number of lymphocytes at 2 h post swimming in the study of Ferrer et al. (2009).

When considering sex, maturity or age differences in the immune response to acute exercise the number of studies designed to monitor these characteristics is scarce and the results are not consistent. When studying sex-based differences, the effect of menstrual cycle phase must be considered. Previous studies have reported either no differences between phases (Morgado et al., 2014), or a higher responsiveness during the luteal comparing to the follicular phase (Timmons et al., 2005). In general, females have shown

either weaker immune responsiveness than males to a swimming test, represented by lower neutrophils (Ferrer et al., 2009; Tauler et al., 2008) and lymphocytes count responses (Morgado et al., 2014), or higher increases of CD56^{+} NK cells counts after 60 min of cycling at 70% of maximal oxygen uptake (Timmons et al., 2006b). Regarding maturity postpubertal boys had less responsiveness than prepubertal and peripubertal boys in response to the Wingate anaerobic test (Boas et al., 1996). These facts emphasizes the need to take sex, pubertal stage, and age into account when interpreting the immunological responses to exercise, as referred by Timmons et al. (2006c).

In most of exercise immunology investigations, the immune response was studied using exercise protocols or competition events and not real training sessions. To our knowledge, this is the first study addressing the immune cell response to a representative training session whilst systematically and simultaneously examining the influence of sex, menstrual cycle phase, maturity, and swimming age groups throughout the recovery period.

Considering all the above evidences it is pertinent to ask if a usual high intensity and prolonged swimming training session is too demanding in ways that athletes become at risk of infection, if recovery is complete until the next training session, or if the risk for infection or for recovery capacity differs between athletes of different sexes, maturity states or swimming age groups.

This study aimed to evaluate the acute systemic immune cell response to a representative high intensity swimming training session integrated in a normal training process during a competitive swimming season, during a 24 h recovery period, in well trained swimmers, taking into account, sex, menstrual cycle phase, maturity and swimming age groups effects in the interpretation of these immunological responses.

4.3. Methods

4.3.1. Participants

Sixty-five swimmers (30 females, 35 males) members of four different Portuguese swimming teams, undertaking 13 - 15 h of pool training and 4 h of dry-land training per week, were evaluated in this study.

The swimmers were included into different swimming age groups according to the regulation of *Portuguese Swimming Federation* and the *Ligue Européene de Natation* (*LEN*) (described in Table 4.1.) and had different competitive swimming backgrounds (5.3 ± 1.9 yrs. ranging from $\simeq 3$ to 11 yrs. of practice).

Six girls had not reached menarche at the time of the evaluations and the other female participants reported regular menstrual cycles for at least the 4 months prior to participation in this study. No one was undergoing oral contraceptive therapy. The testing coincided with the beginning of the follicular phase of the menstrual cycle in 14 girls and with the luteal phase in nine girls.

After receiving detailed information about the aim of the study and the possible risks of the investigation, either the subjects or their parents, as appropriate, provided their written informed consent to participate. All procedures were approved by the Ethics Committee of the Faculty of Human Kinetics of the University of Lisbon and were conducted in accordance with the Declaration of Helsinki for human studies (World Medical Association, 2008).

4.3.2. Study design

Swimmers performed a representative high intensity swimming training session designed by experienced coaches. Data collected included subjects' chronological age and body composition measurements, menstrual cycle phases for girls and an indicator of biological maturity (pubertal Tanner stages). Biochemical immune indices were evaluated before (Pre), immediately after (Post), 2 h after (Post 2h) and 24 h after (Post 24h) in order to examine the acute immune response to exercise. Athletes were instructed not to consume anything but water after 10:00 p.m. of the preceding day and to have a minimum of 8 h rest before testing. To standardize pre-exercise food intake and to avoid extending the duration of their fasted state, participants consumed a sandwich with butter and a juice after the body composition measurements and the resting blood sample collection. The experimental session took place between 6:30 and 10:00 a.m.. All swimmers performed the evaluation at the same period of the season. The evaluation took place immediately after the correspondent main competition of the first macrocycle of the season (short course). The month prior to the competition was characterized by the maintenance of high intensities and progressive decrease of training volumes.

4.3.3. Body composition measurements

Stature and body mass were measured always after wakening in the fasted state. Stature was measured to the nearest 0.1 cm with *Siber-Hegner* anthropometric kit (DKSH Ltd., Zurich, SW). Participants were weighed to the nearest 0.1 kg wearing a bathing suit without shoes on an electronic scale (TANITA BC-601 body composition scale monitor). This electronic scale was also used to calculate swimmers Fat Mass percentage (%FM) using Bioelectrical Impedance Analysis with a measuring current of 50 kHz, 100 μA. Body Mass Index (BMI) was calculated as body mass (BM; kg) divided by the square of the stature (m). Free Fat Mass (FFM) was calculated according to the formula:

$$FFM = BM - (BM \ x \ \%FM)$$

Where FFM is the Fat Free Mass expressed in kilograms, BM is the Body Mass expressed in kilograms, and %FM is the Fat Mass percentage expressed in percentage.

4.3.4. Maturity - Tanner stages

After receiving detailed instructions, the participants self-assessed their degree of genital organ, breast, and pubic hair development using a questionnaire (Tanner, 1962) accompanied by figures and were then grouped according to pubertal stage.

4.3.5. Swimming training session

The swimming session started with a 1500 m standardized warm-up lasting 30 to 35 min followed by a high intensity main task that lasted 50 min and a 500 m recovery task (8 min of duration). The main task was designed to induce maximal lactate accumulation and had a total distance of 1000 to 1200 m, depending on the age group considered. For the youth group the main task consisted of two sets of four repetitions of 75 m front crawl on a five min cycle, with 10 min of active recovery between sets (400 m freestyle). Each repetition had to be accomplished at 90 - 95% of 100 m Freestyle personal best race time. The task organization was identical for juniors and seniors but with repetitions of 100 m. Swimming times were registered in each repetition and the mean time was used to determine the mean effort intensity percentage (%), in relation to the personal best time at the 100 m freestyle race.

4.3.6. Immune system parameters

Peripheral venous blood samples were collected via standard procedures before (Pre, between 6:00 – 6:30 a.m. in the fasted state), immediately after (Post), 2 h after (Post 2h) and 24 h after (Post 24h) the swimming training session. Venous blood was collected into tubes containing EDTA for assessment of hemogram and leukogram and for counting of total and subpopulations of lymphocytes. Hemogram and leukogram was performed in an automated hematology analyzer (Coulter LH 750, Beckman) which produced information about the following parameters: hemoglobin concentration ($g.dL^{-1}$), hematocrit (%) and counts of white blood cells namely: leukocytes, neutrophils, monocytes, and eosinophils. Total and subpopulations of lymphocytes were counted by flow cytometry (FACS Calibur, BD Biosciences). The lymphocytes subpopulations analyzed were $CD3^+$ (total T lymphocytes; T cells), $CD4^+$ (T *helper*; *Th* cells), $CD8^+$ (T *cytotoxic*; *Tc* cells), $CD16^+56^+$ (NK cells) and $CD19^+$ (B cells). Results were expressed as number of cells.$10^9.L^{-1}$ for leukogram parameters and as number of cells.μL^{-1} for total and subpopulations of lymphocytes counts. Post, Post 2h and Post 24h exercise values were corrected for plasma volume variation (Dill & Costill, 1974).

4.3.7. Statistical analysis

The statistical analyses were performed with the software IBM SPSS Statistics, version 21, and the *R* software (R Core Team, 2012), version 2.15.1, and a significance level of 5% was considered.

Descriptive statistics, including means and standard deviation (mean ± SD) were performed for participant's characteristics measurements and training sessions intensity, and including means and standard error of the mean (mean ± SEM) for biochemical indices. Normality of the outcome variables was analysed using the *Shapiro-Wilk* test.

One sample *t* test was used to compare group means with the upper or lower limits of the reference interval provided by the *National Health Institute Doutor Ricardo Jorge* (*INSA*) to verify if participants were within the "clinically normal" values associated with each variable.

The effects of the menstrual cycle phases (follicular, luteal, and not menstruated), sex, pubertal Tanner's stages (1st and 2nd stages of adolescence, and adult stage), swimming

age-groups (youth, juniors and seniors), and the interaction effect of each one of these factors with the moment of evaluation on the response of the variables of interest was analysed using nonparametric mixed-design ANOVAs. The within-subjects factor was the moment of evaluation (four levels: Pre, Post, Post 2h and Post 24h), which is referred as the effect of exercise, and the subjects' factors were the aforementioned influential variables. The nonparametric mixed-design ANOVA has an ANOVA-type statistic (ATS) for each effect, and also a modified ANOVA-type statistic (MATS) for the subject's factor. The option for the nonparametric approach was due to the violation of the assumptions of parametric mixed ANOVA, namely the normality of the dependent variables in each factor's level, the homogeneity of variances and the sphericity. This nonparametric analysis was performed with the *nparLD* package (Noguchi et al., 2012) from the *R* software.

When an effect of a factor with three levels occurred, *Kruskal-Wallis* test with the *Dunn-Bonferroni* post hoc tests were executed to assess between which levels the differences existed. If the effect of interaction between menstrual cycle phase and the moment of evaluation on the variables of interest was non-significant, subsequent analyses were performed not distinguishing girls by menstrual cycle phase. After that, if the interaction effect between each one of the other factors aforementioned and the moment of evaluation on the variables of interest was non-significant, subsequent analysis of the effects of exercise were performed not distinguishing participants by the levels of each between-subjects factor. Otherwise, exercise effects on the variables of interest were analyzed separately considering each between-subjects factor's level.

Repeated measures ANOVA was used for the assessment of exercise effects on immune parameters. Normality and sphericity assumptions were evaluated with the *Shapiro-Wilk* and *Mauchly's* test, respectively. Post hoc tests with *Bonferroni* correction were performed to determine between which moments a significant difference was observed. If the repeated measures ANOVA assumptions were not met, the exercise effect was assessed by *Friedman* test. Post hoc analyses were performed using *Dunn-Bonferroni* test (Dunn, 1964) or, if necessary, due to the conservative characteristic of the *Bonferroni* procedure, according to Conover et al. (1999).

4.4. Results

The participant's characteristics, including body composition related variables, are presented in Table 4.1.

Table 4.1. Mean and SD of the demographics and body composition of female (n=30) and male (n=35) swimmers and number of participants in each maturity group and swimming age group

Swimmers characteristics		Females	Males
	Age (years)	15.0 ± 1.33	16.5 ± 2.07
	Stature (cm)	165 ± 7.03	174 ± 6.87
	Body Mass (kg)	56.0 ± 6.99	64.8 ± 8.35
	BMI (kg.m^{-2})	20.8 ± 1.72	21.3 ± 2.02
	FM (%)	24.7 ± 3.89	15.8 ± 3.09
	FFM (kg)	42.5 ± 4.71	54.5 ± 7.43
Years of swimming practice		5.01 ± 1.33	6.47 ± 2.07
Maturity (Tanner's stages)	1st state of adolescence	n = 5	n = 2
	2nd state of adolescence	n = 18	n = 19
	Adult	n = 17	n = 14
Swimming age group	Youth	13 – 14 yrs; n = 9	14 – 16 yrs; n = 20
	Juniors	14 – 16 yrs; n = 16	16 – 18 yrs; n = 5
	Seniors	≥ 17 yrs; n = 5	≥ 18 yrs; n = 10

Abbreviations: BMI, body mass index; FM, fat mass percentage; FFM, fat free mass; Note: Tanner's stages classification of the maturational state according to Tanner (1962); Swimming age groups classification in both sexes according to the regulation of the *Portuguese Swimming Federation* and *Ligue Européene de Natation* (*LEN*).

Swimmers accomplished the main set at $91.4 \pm 4.7 \%$ of intensity in relation to their personal best time at the 100 m Freestyle race.

Immune system mean baseline values indicated that the participants were within the reference interval associated with each variable (Lewis et al., 2006). At Post 2h, neutrophils were above the upper limit reference interval. During the 24 h recovery period the response of the immune parameters to the swimming session returned to the reference interval.

4.4.1. Effects of sex, maturity and swimming age group on the immune response to the swimming training session

Although no influence was observed for menstrual cycle phase or for maturity Tanner stages, sex influenced the response of CD4$^+$ lymphocytes ($F_{(2.454, \infty)} = 3.285$, $p = .028$); and swimming age group influenced the response of neutrophils ($F_{(3.910, \infty)} = 2.576$,

$p = .037$), total lymphocytes (F(4.750, ∞) = 2.515, $p = .030$), and lymphocytes subpopulations CD3$^+$ (F(4.851, ∞) = 2.801, $p = 0.017$), CD4$^+$ (F(4.455, ∞) = 3.326, $p = .008$), and CD19$^+$ (F(4.561, ∞) = 3.056, $p = .012$) to exercise. CD16$^+$56$^+$ mean values throughout the 24 h post exercise were higher in males than in females (F(1, 60.121 = 4.591, $p = .036$).

The sex (F(1, 61.473) = 10.433, $p = .002$) and swimming age groups effects (F(1.843, 40.353) = 4.271, $p = .023$) over monocytes revealed higher values for males than for females at rest and in response to the swimming session and greater values for youth compared to juniors at Pre, Post 2h and Post 24h, and higher values for seniors compared to juniors at Pre, but lower values for juniors compared to seniors at Post 24h.

The assessment of the effect of a high intensity swimming training session over leukocytes, monocytes, eosinophils, lymphocytes subpopulations CD8$^+$, CD16$^+$56$^+$ and CD4$^+$/CD8$^+$ ratio was done not distinguishing participants by the levels of each between-subjects factor as the interactions were non-significant.

4.4.2. Immune response to the swimming training session

At Post, significantly lower values for monocytes, eosinophils, total lymphocytes, and subpopulations of lymphocytes CD3$^+$, CD8$^+$, CD19$^+$ and CD16$^+$56$^+$ were observed, whereas the CD4$^+$/CD8$^+$ ratio was higher than baseline. Neutrophils for the youth and junior groups were also elevated and CD4$^+$ lymphocytes for the male and female junior and senior groups decreased (Fig. 4.1.).

At Post 2h, leukocytes increased; monocytes returned to baseline values; eosinophils, total lymphocytes and subpopulations of lymphocytes CD3$^+$, CD8$^+$, and CD16$^+$56$^+$ maintained lower values while CD4$^+$/CD8$^+$ ratio maintained higher values; CD19$^+$ lymphocytes in the groups of youth and seniors returned to baseline whereas in the juniors group this parameter increased but without reaching baseline values.

Neutrophils increased for the senior group and maintained higher values for the youth and junior groups. CD4$^+$ lymphocytes returned to Pre values for the male junior group and the female senior group and remained lower for the male senior and female junior groups (Fig. 4.1.).

At Post 24h, leukocytes, neutrophils, eosinophils, subpopulations of lymphocytes CD8$^+$ and CD16$^+$56$^+$, and CD4$^+$/CD8$^+$ ratio returned to Pre levels. As for total lymphocytes and

subpopulation $CD3^+$ lymphocytes there was a recovery to baseline values in the youth and senior groups but for the junior group these variables stayed below Pre levels although they had augmented comparing to Post and Post 2h. $CD19^+$ lymphocytes in the junior group and $CD4^+$ lymphocytes in the female junior and male senior groups recovered to baseline levels which had already been observed at Post 2h for the youth and senior groups in $CD19^+$ and female senior and male junior groups for $CD4^+$.

$CD4^+$ lymphocytes for the youth group remained similar to baseline in response to the swimming session throughout the 24 h evaluation period.

Fig 4.1. Mean and SEM values of the acute response of leukocytes, neutrophils, eosinophils, total lymphocytes and subsets CD3+, CD4+, CD8+, CD16+56+, CD19+ counts and CD4+/CD8+ ratio to a representative high intensity swimming training session. Pre = before exercise, Post = immediately after exercise, Post 2h = two hours after exercise, and Post 24h = 24 hours after exercise.
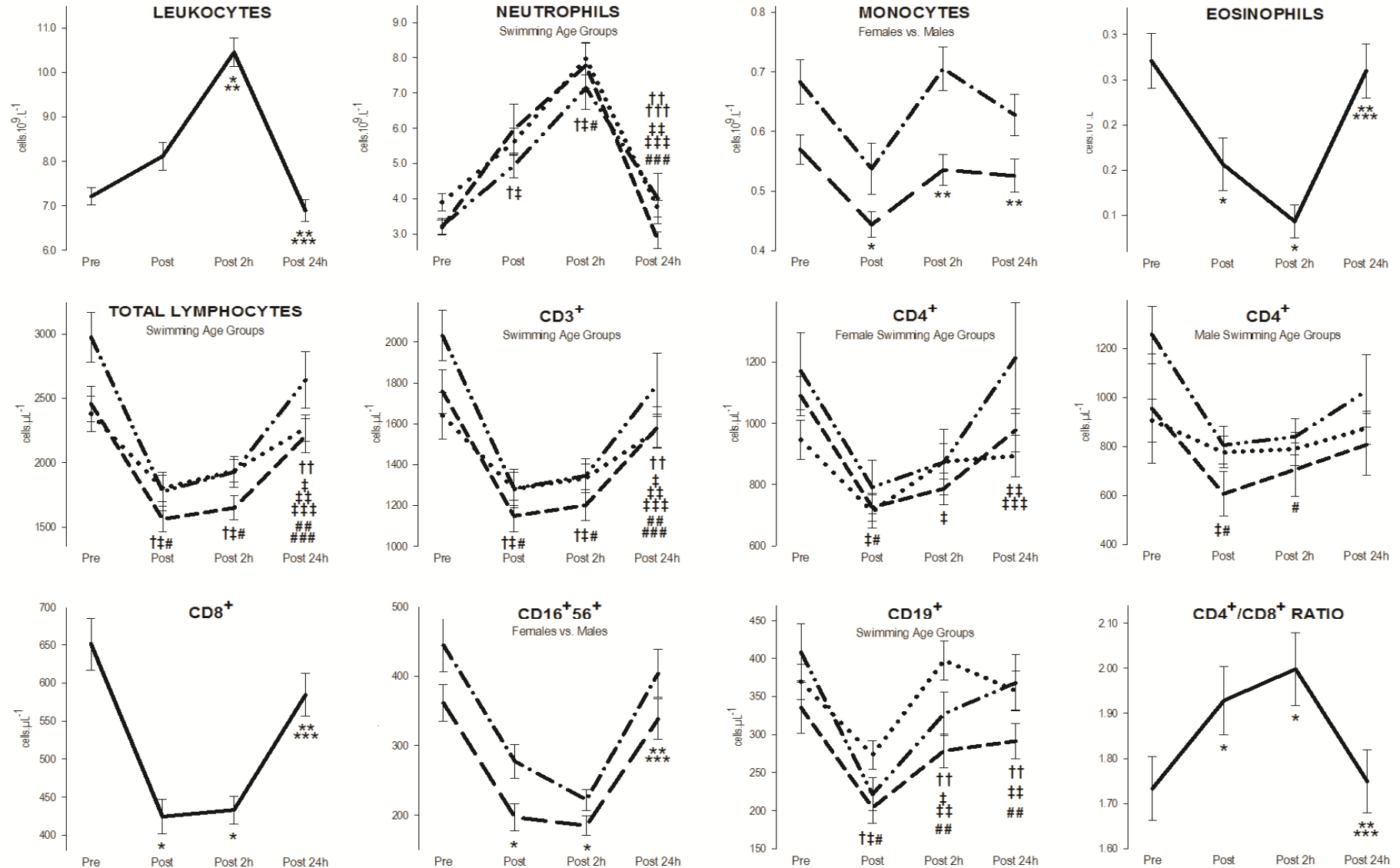
Legend: ——, * Whole Group; •••, † Youth; – –, ‡ Juniors; —··—, # Seniors; — — Females; —·— Males;
*, †, ‡, # different from Pre;   **, ††, ‡‡, ## different from Post;   ***, †††, ‡‡‡, ### different from Post 2h (p<.05)

4.5. Discussion

Studies that have assessed the acute response of leukocytes and subpopulations (including lymphocytes subsets) to exercise immediately after the accomplishment of exercise, and 2 h after, are scarce (Ibfelt et al., 2002; Kargotich et al., 1997; McFarlin et al., 2004; Starkie et al., 2001; Steensberg et al., 2001b; Yamada et al., 2000), and if we consider adding the evaluation 24 h after, they are even more scarce (Gabriel et al., 1992a; Kakanis et al., 2010; Steensberg et al., 2001a). Furthermore, most of these studies were performed using cycle ergometers and treadmills at laboratories or in swimming pools using specific exercise protocol tests but not real training sessions and mainly evaluating adult male subjects. So, to date, research on the comprehensive analysis of exercise-induced immune cell counts changes in athletes respecting the effects of sex, menstrual cycle phase, maturity and sports age groups are clearly lacking. In this way, our study has a marked descriptive component given the great and varied amount of information collected.

Regarding total leukocytes we did not observe any change immediately after the swimming session, despite a marked elevation trend has occurred. This finding is divergent from the ones that refer a stereotypical post-exercise leukocytosis either after swimming tests (Kargotich et al., 1997; Morgado et al., 2014), or after cycling continuously either for short or long period at several intensities (e.g. approximately 90 min at 85% and 100% of individual anaerobic threshold (IAT) (Gabriel et al., 1992a); 20 min at 80% of VO_2 max (Starkie et al., 2001); 60 min at 75 – 80% of VO_2 max (McFarlin et al., 2004)), or even after treadmill running to exhaustion (Yamada et al., 2000). This may result from the balance between the rise of neutrophils that opposes the decrease of monocytes, eosinophils and lymphocytes. Nonetheless, leukocytosis happened at Post 2h, which is in accordance with some of the abovementioned studies (Gabriel et al., 1992a; Kargotich et al., 1997; McFarlin et al., 2004; Starkie et al., 2001) although it does not necessarily imply increased immune defences. As described before (Gabriel et al., 1992a; Zhang et al., 2006), at 24 h leukocytes had return to baseline values.

When analysing leukocyte subsets, in particular the innate immune cells studied, the neutrophilia, observed in this study, at Post, in youth and juniors groups, and at Post 2h, in all swimming age groups, is in accordance with other studies where it has been described in adults after intense prolonged exercise (Ibfelt et al., 2002; Kakanis et al., 2010; Kargotich et al., 1997; Steensberg et al., 2001a) lasting at least for 2 h and having returned

to pre-exercise values at 24 h (Kakanis et al., 2010). However, the decrease in monocytes and eosinophils observed at Post, and maintenance of eosinophils below pre-exercise values at Post 2h oppose the studies that reported post exercise monocyte rises (Gabriel et al., 1992a; Kargotich et al., 1997) or absence of eosinophils changes in response to exercise, either immediately or at Post 2h, although as in our study the authors observed the recovery of monocytes at Post 2h (Kakanis et al., 2010; Kargotich et al., 1997).

Considering the cells of the adaptive immune response, the lymphopenia observed just after the swimming training session, and at Post 2h, appears to be due to a decrease of $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+56^+$ and $CD19^+$ subsets below baseline values at Post, plus their maintenance below pre-exercise levels, with the exceptions of $CD4^+$ in female seniors and male juniors, and of $CD19^+$ in youth and senior groups that had returned to baseline values, at Post 2h. In the youth group, although $CD4^+$ lymphocytes remained similar to baseline throughout the 24 h, there was a trend to decrease immediately after the swimming training session. These results contrast those of other investigations where a lymphocytosis reflecting an increase of lymphocytes subsets was observed just after swimming (Kargotich et al., 1997; Morgado et al., 2014), running (Ibfelt et al., 2002), and cycling (Gabriel et al., 1992a), or where no alterations in total lymphocytes (Steensberg et al., 2001a; Steensberg et al., 2001b), and in $CD4^+$ (Kakanis et al., 2010) and $CD8^+$ subsets (Kakanis et al., 2010; Steensberg et al., 2001a) were observed. However, regarding the recovery period the results from previous studies are heterogeneous. Two hours after the exercise tasks declines in total lymphocytes (Gabriel et al., 1992a; Kakanis et al., 2010; Kargotich et al., 1997; Steensberg et al., 2001a; Steensberg et al., 2001b), $CD3^+$ (Gabriel et al., 1992a; McFarlin et al., 2004), $CD4^+$ (Gabriel et al., 1992a; Kargotich et al., 1997; McFarlin et al., 2004; Steensberg et al., 2001a), $CD8^+$ (Gabriel et al., 1992a; Kargotich et al., 1997; Steensberg et al., 2001a), $CD16^+56^+$ (Ibfelt et al., 2002), and $CD19^+$ subsets (Kargotich et al., 1997), or cell counts similar to pre-exercise values for total lymphocytes (Ferrer et al., 2009; Ibfelt et al., 2002), $CD3^+$ (Ibfelt et al., 2002), $CD4^+$, $CD8^+$ (Ibfelt et al., 2002; Kakanis et al., 2010), $CD16^+$ (Gabriel et al., 1992a; Kargotich et al., 1997), and $CD19^+$ (Gabriel et al., 1992a) have been observed. At Post 24h, the recovery to pre-exercise values of lymphocytes total and subsets $CD3^+$, $CD4^+$, and $CD8^+$ was observed by Gabriel et al. (1992a) after 90 min cycling at 85% of IAT, and Steensberg et al. (2001a) observed the recovery of lymphocytes total and $CD4^+$ cells but not of $CD8^+$ cells that, contrastingly, stayed below baseline values.

The increased $CD4^+/CD8^+$ ratio observed at Post and maintenance of higher levels at Post 2h diverges from the reduction observed post-swimming (Kargotich et al., 1997; Morgado et al., 2014) and cycling (Gabriel et al., 1992a) and from the absence of changes at Post 2h (Gabriel et al., 1992a; Kargotich et al., 1997) reported in the literature, suggesting a more accentuated reduction of $CD8^+$ comparing to $CD4^+$ in the present study.

Regarding all the aforementioned discussed results, the authors suggest that the changes in the number of leukocytes and their populations in response to acute exercise may be explained by three different processes: cell traffic, cell proliferation or cell death (Kruger et al., 2008). It is thought that these processes occur concomitantly and their relative magnitude probably depends on the mode of exercise (Kruger & Mooren, 2014). Cell traffic depends upon the adherence of cells to the endothelium and on their redistribution amongst organs or compartments, especially between the circulation and the lung, spleen and muscle (Adams et al., 2011). Leukocyte trafficking and function can be influenced, during exercise and immediately after its ending, by increases of cardiac output, shear stress, and blood flow to working muscle, and by changes in pH and temperature (Adams et al., 2011). This physiological response reflects an increase of sympathetic activity and an activation of the hypothalamic-pituitary axis inducing the secretion of circulating catecholamines (Ottaviani & Franceschi, 1996).

This well-known rising of catecholamines levels during exercise protocols and just after their ending (Gabriel et al., 1992a; Timmons & Bar-Or, 2007; Timmons et al., 2006b) and of cortisol concentrations immediately post-exercise (Gabriel et al., 1992a; Timmons, Hamadeh, & Tarnopolsky, 2006a; Timmons et al., 2006b) may influence the number and activity of leukocytes and its subpopulations. According to McCarthy et al. (1991; 1992), and Mignini et al. (2008) catecholamines appear to be in the basis of the acute effects of exercise, particularly lymphocytosis, participating in the regulation of lymphocyte subset redistribution, and cortisol seems to contribute, during the recovery period, to generate and uphold both the lymphopenia and neutrophilia (the last may be produced by release of neutrophils from the bone marrow). Catecholamines also contribute, directly and indirectly, to the decrease of the adherence of leukocytes to the endothelium (demargination) and consequently increase the number of circulating leukocytes (Gabriel et al., 1992a). Directly by reducing the number of cell adhesion molecules on the cells' surface, and indirectly by accelerating heart rate, increasing blood flow and shear stress.

Cell death or apoptosis plays an important role in the maintenance of the balance between the generation of new cells and removal of damaged or aged cells. It is commonly

accepted that the transient lymphopenia after exercise occurs in part due to enhanced apoptosis, while for other cells, such as neutrophils, the post-exercise apoptosis regulation remains controversial (Kruger & Mooren, 2014). Moreover, these authors referred that exercise-induced lymphocyte apoptosis may generate "free space" for new lymphocytes thus enlarging the naïve T cell repertoire.

The long duration and high intensity of the swimming training session, may explain the declines of monocytes, eosinophils and lymphocytes total and subsets observed in this study immediately after the swimming training session, that oppose the rising of these cells counts mentioned in the literature (Gabriel et al., 1992a; Ibfelt et al., 2002; Kakanis et al., 2010; Kargotich et al., 1997). These results may reflect a negative balance generated by proliferation, demargination, and mobilization of cells from reservoirs, and by entry of cells into tissues along with apoptosis.

Additionally, strenuous exercise induces increased levels in a number of pro-and anti-inflammatory cytokines, especially IL-6, which is predominantly produced within the contracting skeletal muscle. The net release from the muscle can account for the exercise-induced increase in arterial concentration (Pedersen et al., 2001). Suwa et al. (2000) suggested that IL-6 causes a biphasic neutrophilia where the first peak (2 – 6 h) results from the mobilization of cells into the circulating pool from the marginated pool and the second peak (12 – 24 h) results from an accelerated bone marrow release. Epinephrine may only partly influence the plasma levels of IL-6 during exercise (Steensberg et al., 2001b).

The similarity in the immune response to the swimming session regardless of menstrual cycle phase is in accordance with the immediate post-exercise results obtained by Morgado et al. (2014) for a 7 x 200 m swimming maximal test, but contrasts with those obtained by Timmons et al. (2005) for 90 min cycling at 65% maximal oxygen uptake which reported a lower lymphocyte response to exercise during the follicular phase than during the luteal phase.

When considering sex-based differences, monocytes and CD16^{+}56^{+} values were higher for males than for females at rest and throughout the recovery period. Nonetheless, the immune response to the swimming training session was similar for these two variables regardless of sex. Regarding monocytes, our results are in accordance with Timmons et al. (Timmons et al., 2005; 2006c) who observed no differences between sexes in response to exercise. The results obtained for the CD16^{+}56^{+} response to exercise diverge from the conflicting few results mentioned in the literature e.g. higher responsiveness in males than in females just after a swimming test (Morgado et al., 2014) and a more elevated

$CD16^+56^+$ counts in girls than in boys after cycling (Timmons et al., 2006c). For the other studied variables the mean values were not different between sexes along this investigation and the response to exercise was similar regardless of sex with the exception of $CD4^+$, where females at Post tended to decrease more and at Post 24h recovered more efficiently than males. The absence of differences between sexes is in conformity with the findings for total lymphocytes of Ferrer et al. (2009), but do not comply with the sex differences reported by the same authors for neutrophils (Ferrer et al., 2009). The similar sex response immediately after exercise has been reported for leukocytes (Timmons et al., 2005), neutrophils, monocytes (Timmons et al., 2005; Timmons et al., 2006c), total lymphocytes (Timmons et al., 2005), $CD3^+$, $CD4^+$ and $CD8^+$ (Timmons et al., 2005; Timmons et al., 2006c), and for $CD19^+$ lymphocytes (Morgado et al., 2014; Timmons et al., 2006c). However, in a previous study we observed that a swim set induced an increase in leukocytes, total lymphocytes, $CD3^+$, $CD4^+$, $CD8^+$, and $CD16^+56^+$ counts in the male group while in the female group only leukocytosis, but of a lower magnitude, occurred (Morgado et al., 2014). Conversely, Timmons et al. (2006c) observed a strong response of leukocytes in females, and of $CD56^+$ as referred before. To our knowledge few studies followed the recovery period. Timmons et al. (2006c) observed no sex differences for leukocytes and subsets, including lymphocytes subsets, except for $CD3^+$ where young girls had higher values than young boys, 1 h after exercise.

Regarding maturity, our findings showed similar responses of the immune cell counts to the swimming training session which is consistent with the similar post-exercise immediate leukocytosis and neutrophilia across pubertal stages in boys and girls (Timmons et al., 2006c), but opposes the outcomes reported by Boas et al. (1996) in which postpubertal boys had less immune responsiveness to cycling than the prepubertal and peripubertal boys.

As in real training sessions, the youth group swimmers performed a main set adapted to their competitive level in order to reach an effort percentage similar to that of juniors and seniors. However, the immune response observed for the youth group was consistent with a blunter immune reaction than that of the other groups. This might be the result of a reduced immune responsiveness of these younger swimmers.

Conversely, the exercise effect over the immune response along the 24 h recovery period was more noticeable over the junior group for total lymphocytes and $CD3^+$ subsets. This was a particular finding of the present study which was not observed elsewhere when considering a prolonged and intense exercise performed either by female athletes between

14 and 16 yrs. or by male athletes between 16 and 18 yrs.. Generally, if we consider all other immune parameters, their recovery at Post 24h has been mentioned in previous investigations that studied male adults (Gabriel et al., 1992a; Kakanis et al., 2010; Steensberg et al., 2001a; Zhang et al., 2006).

The physiological response of sex hormones such as cortisol, oestrogen, and testosterone in association with a differential effect of these hormones and cytokines on leukocyte subsets (Fragala et al., 2011) may be implicated in the differential effects of sex and swimming age group we observed in the acute response and recovery of systemic cell immunity to the swimming training session.

In general, just after the training session, the swimmers showed a decrease of the adaptive immune response, but also of some parameters of the innate immune response as NK cells, monocytes and eosinophils also decreased. This was only counteracted by the increase of neutrophils. As we did not assess the cells functions we could not evaluate whether their reduction in number was compensated by their activation. However, previous studies suggested a diminished activity of some leukocytes subsets, namely neutrophils (Robson et al., 1999), monocytes (Nieman et al., 1998b; Simpson et al., 2010) and CD56^{+} NK lymphocytes (Suzui et al., 2004). So we can argue that at least in the first 2 h after intense training sessions such as the one performed by our swimmers, immune defenses of athletes may be compromised.

4.6. Conclusions

The swimming training session performed in this study represents the real and typical effort developed in training sessions involved in any training process. This swimming session provoked a significant acute neutrophilia; lymphopenia and low eosinophils count lasting for at least two hours, independently of sex and maturity.

Furthermore, the recovery of acquired immunity reflected by total lymphocytes and total T (CD3^{+}) and B (CD19^{+}) cells seems to have been more affected in the junior swimming age group (15 – 17 yrs. of age, according to the *LEN* classification). Both parameters persisted low along the 2 h after swimming and a 24 h period revealed to be insufficient to attain total recovery total lymphocytes and T cells.

The observed lymphopenia suggests a lower immune surveillance at the end of the session that may increase the risk of infection or suppressed immunity of athletes in the period just after training, highlighting for the need of extra care when exposed to aggressive environmental agents. Concerning juniors, the insufficient 24 h period for recovery of the immunological levels must be considered when planning consecutive training sessions.

So, athletes and coaches should consider taking actions in order to avoid exposure of the athletes to potential infections, which may compromise attendance to training sessions, performance and most of all health.

CHAPTER V

Study 2 – Innate and acquired immunity are affected by long term swimming training

5.1. Abstract

In endurance sports such as swimming, many years of daily training and competition are required to progressively improve performances, and usually the cycles of high training volume and intensity that include consecutive training sessions with little recovery time in between may lead to transient imbalances between training loads and recovery contributing to the onset of fatigue and eventually illness in athletes.

This study aimed to investigate resting cellular immune changes over a 7-month swimming season, controlling for sex, maturity, age groups, distance specialty, and performance. Blood resting samples were taken from 54 swimmers (29 males, 16 ± 2.0 yrs., and 25 females, 15 ± 1.5 yrs.) at 4 moments of evaluation: M1 (beginning of the season), M2 (after the main competition of the 1st macrocycle, 13th wk.), M3 (preparatory phase of the 2nd macrocycle, 23rd wk.) and M4 (after the main competition of the 2nd macrocycle, 30th wk.). Samples were collected by standard procedures for assessment of leukogram by automated counting (Coulter LH 750, Beckman) and lymphocytes subsets by flow cytometry (FACS Calibur, BD Biosciences). Upper Respiratory Symptoms (URS) episodes were monitored using daily logbooks. Training load was quantified. Statistical significance was considered at $p<.05$.

Only sex influenced the response of monocytes to training, and swimming age group influenced the response of monocytes, total lymphocytes and subsets $CD3^+$ and $CD4^+$, while $CD19^+$ lymphocytes values were higher for males than females despite similar response to training. $CD8^+$ lymphocytes decreased at M2, remained below baseline values at M3 and recovered at M4. $CD16^+56^+$ lymphocytes and eosinophils decreased at M3, and remained diminished at M4. At M4, $CD19^+$ lymphocytes were elevated. Along the 4 moments of evaluation, no alterations in leukocytes and neutrophils were observed. In

juveniles, total lymphocytes, lymphocytes subpopulations CD3$^+$ and CD4$^+$, decreased at M2 and recovered at M3, but remained unchanged in juniors and seniors. Monocytes were unchanged, except for the senior males, which were elevated at M4. The heaviest training load, both in volume and intensity, and the higher frequency of URS episodes happened at M3.

The swimming training season had a cumulative effect towards a decrease of the innate immunity, while the acquired immunity appeared to be more affected at the most intense training period, recovering after a taper period. Younger swimmers presented acquired immune depression earlier in the training season. At the heaviest training period both innate and acquired immunity impairments contributed to a more pronounced immune depression alongside with higher prevalence of upper respiratory symptoms.

Key Words: Cellular Immunity, Swimmers, Training Season.

5.2. Introduction

It is generally acknowledged that the immune system may experience a functional reduction when exposed to successive psychological and physical stressful stimulus, such as the competitive training process (Walsh et al., 2011).

In endurance sports such as swimming, many years of daily training and competition are required to achieve and maintain national and international level performances. It is of common use the implementation of cycles of high training volume and intensity that include consecutive training sessions with little recovery time in between in order to optimize aerobic and movement economy adaptations (Sargent et al., 2014). This can lead to transient imbalances between training loads and recovery contributing to the onset of fatigue and eventually illness in athletes (Aubry et al., 2014). When this transitory impaired performance settles, an extra pressure on immune function can be generated, and an immunodepression state characterized by substrate depletion, hormonal and immune functions disturbances and infectious episodes, which are normally reported by athletes, is usually the response to these hard training periods (Cordova, Sureda, Tur, & Pons, 2010; Dias et al., 2011; Gleeson, 2007; Gleeson & Williams, 2013; Morgado et al., 2012; Rama et al., 2013). Additionally, swimmers are repeatedly exposed to warm humid environment,

temperature variations and chlorine-rich atmosphere, thus being more predisposed to respiratory illness (Aubry et al., 2014; Bernard et al., 2009; Bougault et al., 2012; Gleeson, 2000; Gleeson et al., 1995; Gleeson et al., 2000; Mackinnon, 1997; Reid et al., 2004; Spence et al., 2007). In fact, there is a shared belief that the frequency of upper respiratory symptoms (URS) is bigger in elite endurance athletes after single bouts of ultra-endurance exercise and through periods of intensified training (Nieman, 1994; Spence et al., 2007), with the common cold being the most reported common infection episode. Altogether, these training conditions can lead to the stimulation of adaptive mechanisms related to metabolic, hormonal, circulatory and respiratory responses that may compromise performance and negatively influence health status, although this situation may be reversible by a tapering or recovering period (Gleeson & Bishop, 2005; Shephard & Shek, 1999; Suzui et al., 2004).

Moreover, it has been argued that the immunological response to training may depend on factors such as training load, subject fitness level, performance, sex, maturity, age group, ability level, and/or event distance specialization (Vleck et al., 2014).

So, it is reasonable to ask which variables and factors are possible to monitor and control in ways that help coaches and athletes to handle with the related transitory impaired performance and immunosuppression state that is usually generated in periods of heavy training, preventing the onset of fatigue and upsurge of related infections and illnesses. The answer to this question may be crucial for the adequate periodization of training, and eventually to the individualization of the training process, in order to prevent the negative influence on health and performance status of the athlete.

Studies that followed up a 7-month swimming training season reported a reduction in neutrophils and monocytes resting values (Morgado et al., 2012) and decreased $CD56^+$ NK cells (Gleeson et al., 1995; Rama et al., 2013). After a 3-month swimming training program $CD56^+$ NK cells were also diminished (Gleeson et al., 2000). So, it seems that long-term intensified training can affect the number of innate immune cells, possibly contributing to an elevated risk of infection. Nevertheless, T and B cell functionality has shown signs of hampering in athletes engaging long-term periods of intense training (Walsh et al., 2011).

In general, at rest, athletes seem to have leukocyte and lymphocyte subsets counts and functions similar to those of non-athletes (Baj et al., 1994; Nieman, 2000a; Nieman et al., 1995a; Nieman et al., 1995b). Gleeson et al. (2011), reported no differences between the sexes, at rest, in total blood leukocyte, neutrophil, monocyte and lymphocyte counts of

endurance training athletes, however males had higher B and CD56^{+} NK cells (Gleeson et al., 2011). Additionally, Timmons et al. (2004) referred that adolescents and adults may have slightly different immunological responses to physical exercise, although on an acute response basis. As for swimmers, they have presented lymphocytes counts similar to controls but lower numbers of circulating total leukocytes (Gleeson et al., 1995).

When comparing the investigations about the behaviour of the immune system that made a follow-up of the training load or periodization and the occurrence of upper respiratory symptoms, several limitations can be found in what refers to differences in the length and seasonality of the observation periods, and control for subjects characteristics.

To our knowledge, no one has addressed the effect on the circulating leukocytes and subpopulations (including lymphocyte subset populations) of a long-term training process of any physical activity or sports controlling for subjects characteristics. Thus, this study aimed to investigate the variation of resting systemic immunological cell parameters over the course of a 7-month swimming training season, in a large cohort of well-trained swimmers involved in their regular training environment, taking into account sex, maturity, swimming age groups, performance, and event distance specialization effects in the interpretation of these immunological variations.

Consequently, we hypothesised that the most intense periods of training over the competitive training season would lead to some immunological depression as compared to the beginning of the season, and that the upper respiratory symptoms occurrence would be greater during the intense periods. Conversely we hypothesized that at the end of the training period, where recovery was provided to swimmers and peak performance was expected, the immunological condition of the athlete would have recovered from the intensive periods thus conferring a healthy condition necessary for achieving best performances in competition (Aubry et al., 2014).

5.3. Methods

5.3.1. Participants

Fifty-four swimmers (25 females, 29 males) members of four different Portuguese swimming teams, undertaking 13 – 15 h of pool training and 4 h of dry-land training per

week, were evaluated in this study. The swimmers were included into different swimming age groups according to the regulation of *Portuguese Swimming Federation* and the *Ligue Européene de Natation* (*LEN*) (described in Table 5.1.) and had different competitive swimming backgrounds (4.6 ± 1.6 yrs. in females and of 5.9 ± 2.0 yrs. in males, ranging from ≃ 3 to 11 yrs. of practice).

After receiving detailed information about the aim of the study and the possible risks of the investigation, either the subjects or their parents, as appropriate, provided their written informed consent to participate. All procedures were approved by the Ethics Committee of the Faculty of Human Kinetics of the University of Lisbon and were conducted in accordance with the Declaration of Helsinki for human studies (World Medical Association, 2008).

5.3.2. Study design

This study used an observational design with a follow-up over a swimming competitive training season lasting 30 weeks. Swimmers followed the training program set by the coaches of each different team.

The evaluation of the swimmers was made at four moments of evaluation (M) named M1 (at the beginning of the season; baseline evaluation), M2 (the week after the main competition of the 1st macrocycle; 13th week of training), M3 (at the specific preparatory sub phase of the preparatory phase of the 2nd macrocycle; 23rd week of training) and M4 (the week after the main competition of the 2nd macrocycle; 30th week of training). At each moment of evaluation, data collected for all subjects included subjects' chronological age and body composition measurements, an indicator of biological maturity (pubertal Tanner stages) and biochemical immune indices. Athletes were instructed not to consume anything but water after 10 p.m. of the preceding day and to have a minimum of 8 h rest before testing. The body composition measurements and the resting blood sample collection were performed in a fasted state (between 6:30 and 9 a.m.). Throughout the follow up season the incidence of URS and the menstrual cycle phases for girls were monitored weekly and training load and mean intensity of all scheduled swimming sessions were quantified. The characteristics of the training regimens and competition schedules were not modified by the present study in anyway nor any swimmer suffered from major injury or sickness preventing them from training for more than one day.

5.3.3. Swimmers characteristics

5.3.3.1. Body composition measurements

Trained and experienced staff measured swimmers stature and body mass always after wakening in the fasted state wearing a bathing suit without shoes. Stature was measured to the nearest 0.1 cm with *Siber-Hegner* anthropometric kit (DKSH Ltd., Zurich, SW). Participants were weighed to the nearest 0.1 kg on an electronic scale (TANITA BC-601 body composition scale monitor) that also calculated Fat Mass percentage (%FM) using Bioelectorical Impedance Analysis with a measuring current of 50 kHz, 100 μA . Body Mass Index (BMI) was calculated as body mass (BM; kg) divided by the square of the stature (m) and Free Fat Mass (FFM) according to the formula:

$$FFM \ = \ BM - (BM \ x \ \%FM)$$

Where FFM is the Fat Free Mass expressed in kilograms, BM is the Body Mass expressed in kilograms, and %FM is the Fat Mass percentage expressed in percentage.

5.3.3.2. Maturity - Tanner stages

Participants received detailed instructions after which they made a self-assessment of their degree of genital organ, breast, and pubic hair development using a questionnaire (Tanner, 1962) accompanied by figures and were then grouped according to pubertal stage (1st and 2nd stages of adolescence, and adult stage). Considering the heterogeneity and mean chronological age of the swimmers, it was possible that some might develop from one stage to the succeeding along the 7-month training process. In fact, at M2, some swimmers classified themselves as having developed into the subsequent stage in comparison to M1. Yet, all swimmers maintained the classification they had at M2 throughout the rest of the training season. So, in this study, the maturity stage at M2 (which was equal at M3 and M4) was considered for the differentiation in two maturity groups (adolescents and adults) (Table 5.1.).

5.3.3.3. Distance specialty

Swimmers were grouped regarding their swim specialty on a distance type basis: sprint or long-distance. This division was made according to the coaches' classifications of their swimmers, and was primarily based on the swimmers' main event distance: 50m, 100m and 200m swimmers were classified as short distance and 400m, 800m and 1500m swimmers as long distance swimmers (Table 5.1.).

5.3.4. Swimming training season

The study was divided into three main periods that represented distinctive training phases (Fig. 5.1.):

M1 to M2 (three months) corresponded to the 1st macrocycle of the training season, which began with the general preparatory sub phase and lasted until the main competitive sub phase. This first macrocycle aimed to prepare the athletes to the National Youth Long-distance Championship, to the winter National Championships and to the National Interclubs Championship. This period was characterized by an aerobic training predominance and the progressive increase of training volumes and intensities in the first two months and for the maintenance of high intensities and progressive decrease of volumes in the last month. At the National Championships all swimmers have accomplished at least one personal best time in the races they were enrolled in.

M2 to M3 (two months) coincided with the entire preparatory phase of the 2nd macrocycle of the season. This development period was characterized by a progressive increase in training volume, intensity and frequency that lasted until the end of the specific preparatory sub phase, where the higher peak of training load of the season was reached and the swimmers were evaluated (M3). In this period there was also a more frequent participation in competitions (including international meetings).

M3 to M4 (one month) was a period of training that occurred during the competitive phase of the 2nd macrocycle of the season and included a specific preparatory period followed by a competitive period that lead to important competitions for which training load was progressively reduced: National Youth Championship and National Junior and Senior Championships.

Fig. 5.1. Periodization of the 7-month winter swimming training competitive season and schedule of the four moments of evaluation: M1, M2, M3 and M4

DATES	MONTHS	SEPTEMBER			OCTOBER					NOVEMBER				DECEMBER				JANUARY					FEBRUARY				MARCH				APRIL
	1st DAY OF WEEK	12	19	26	3	10	17	24	31	7	14	21	28	5	12	19	26	2	9	16	23	30	6	13	20	27	5	12	19	26	2
COMPETITION CALENDAR	TEAMS									X				X																	
	LOCAL						X				X									X	X					X					
	NATIONAL												X		X														X		X
	INTERNATIONAL																			X	X		X								
PERIODIZATION	TRAINING PHASES	PREPARATORY 1								COMPETITIVE 1				TRANS.				PREPARATORY 2									COMPETITIVE 2				
	TRAINING SUBPHASES	GENERAL PREP.					SPECIFIC PREP.			PRE COMP.		MAIN COMPETITIVE		TRANS.				GENERAL PREP.					SPECIFIC PREP.			PRE COMP.	MAIN COMPETITIVE				
	MACROCYCLES	1																2													
	MESOCYCLES	1					2				3			4				5			6			7				8			
	MYCROCYCLES	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
PEAKING		4					3			2			1				5			4			3			2			1	
CONTROL TESTS			X		X	X			X	X		X					X		X		X			X		X			X		

Legend (lower chart):
- Dryland Training
- Technique Training
- VOLUME ——
- INTENSITY ·······
- PEAKING – – – –
- MOMENTS OF EVALUATION
- Scale: 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%
- PEAKING scale: 1, 2, 3, 4, 5 — P E A K I N G
- Moments of evaluation: M1, M2, M3, M4

5.3.5. Performance improvements

The effect of training on performance was evaluated by magnitude of the change of the race time at competitive events. It was expressed as a relative difference, which represented a percentage of change, comparing the race time accomplished at M4 with that at the end of the previous season. In this manner, two groups were created according to the level of improvement: the less efficient group, which presented under 2% changes in performance, and the efficient group, which presented changes of 2% and above (Table 5.1.). This 2% level of improvement was adopted based on the consistently mean improvements of around 3% mentioned in the literature (Mujika et al., 1996a).

5.3.6. Quantification of the training load

Training load was determined through the total amount of meters swam (volume) and also by the balance of the distance completed at each level of intensity based on the work of Mujika et al. (1996a; 1995) (Table 5.3).

The use of a stress index scale of difficulty has been established in reference to the theoretical values of blood lactate accumulation usually associated with the different swimming training zones of intensity.

The training zones adopted considered the works by Mujika et al. (1995), Maglischo (2003), and Sweetenham & Atkinson (2003), and were: I - warm up and recovery, II - aerobic 1, III – aerobic 2, IV - VO_2max, V - lactate tolerance, VI - lactate production and VII - sprint. In order to evaluate the swimming sessions training load the volume accomplished in each zone of intensity was quantified (mI, mII, mIII, mIV, mV, mVI and mVII). The magnitude of the load was then expressed in dimensionless units of load, or arbitrary units of load (AUL), obtained from the ratio between the sum of the volumes swam in each zone of intensity multiplied by the respective index (1, 2, 3, 4, 6, 8, 10) and the total volume effectively completed, according to the formula:

$$AUL = \frac{1\,mI + 2\,mII + 3\,mIII + 4\,mIV + 6\,mV + 8\,mVI + 10\,mVII}{\text{swimming training session volume}}$$

Where AUL is the arbitrary units of load expressed in dimensionless units of load, the numerator is the weighed volume expressed in meters, mI, mII, mIII, mIV, mV, mVI and mVII are the meters accomplished at the following swimming training zones of intensity: I- warm up and recovery, II - aerobic 1, III – aerobic 2, IV - VO_2max, V - lactate tolerance, VI - lactate production and VII – sprint, the numbers 1, 2, 3, 4, 6, 8, 10 are the indexes associated to each zone of intensity, the denominator is the swimming training session volume expressed in meters.

This was performed for all season sessions considering each age group and within all swimming teams.

The mycrocycle or weekly load was quantified and expressed by the volume (total of meters swam), by the weighed volume (sum of the multiplications of the volume accomplished in each zone of intensity by the respective stress index values) and by the intensity (determined through the sum of the resulting dimensionless unit of load of each session of training).

5.3.7. Immune system parameters

Peripheral venous blood samples were collected via standard procedures between 6:00 and 6:30 a.m., in the fasted state, at the four moments of evaluation (M1, M2, M3 and M4). Venous blood was collected into tubes containing EDTA for assessment of hemogram and leukogram and for counting of total and subpopulations of lymphocytes. Hemogram and leukogram was performed in an automated hematology analyzer (Coulter LH 750, Beckman) which produced information about the following parameters: hemoglobin concentration ($g.dL^{-1}$), hematocrit (%) and counts of white blood cells namely: leukocytes, neutrophils, monocytes, eosinophils. Total and subpopulations of lymphocytes were counted by flow cytometry (FACS Calibur, BD Biosciences). The lymphocytes subpopulations analyzed were $CD3^+$ (total T lymphocytes; T cells), $CD4^+$ (T *helper*; *Th* cells), $CD8^+$ (T *cytotoxic*; *Tc* cells), $CD16^+56^+$ (NK cells) and $CD19^+$ (B cells). Results were expressed as number of $cells.10^9.L^{-1}$ for leukogram parameters and as number of $cells.\mu L^{-1}$ for total and subpopulations of lymphocytes counts.

5.3.8. Upper Respiratory Symptoms

Subjects were asked to answer to a weekly questionnaire that was sent every Monday to their email address. This questionnaire consisted of a daily logbook in which they noted their symptoms associated with illnesses related to URS such as: headache, fever, ear pain, chills, runny or blocked nose, pharyngitis/tonsillitis, bronquitis, asthma, phlegm, cough, conjunctivitis; itchy, watery eyes, nausea/vomiting, and diarrhoea. All swimmers were asked to indicate the medication they were on and female subjects to point out the days of menstruation. If subjects had no symptoms they simply recorded that and reply to the email.

If fever or at least two concomitant symptoms persisted for at least 48 hours, separated from previous symptoms by at least one week, they were considered an episode of URS (Bishop, 2006). Symptoms separated by less than one week were regarded as a recurrence or continuation of the initial episode and were regarded as part of the same episode. The counting of the URS episodes was expressed as the weekly number of episodes of URS over the course of the training season.

5.3.9. Statistical analysis

The statistical analyses were performed with the software IBM SPSS Statistics, version 21, and the *R* software (R Core Team, 2012), version 2.15.1, and a significance level of 5% was considered. Descriptive statistics, including means and standard deviation (mean ± SD) were performed for participants' characteristics and training load quantification measurements and including means and standard error of the mean (mean ± SEM) for biochemical indices. Normality of the outcome variables was analysed using the *Shapiro-Wilk* test. One sample *t* test was used to compare group means with the upper or lower limits of the reference interval, provided by the *National Health Institute Doutor Ricardo Jorge* (*INSA*) (Lewis et al., 2006), to verify if participants were within the "clinically normal" values associated with each variable.

The effects of sex, pubertal Tanner's stages (adolescence and adult stages), swimming age-groups (youth, juniors and seniors), distance specialty (short and long distance swimmers), performance (efficient and less efficient), and the interaction effect of each one of these factors with the moment of evaluation on the response of the variables of interest was analysed using nonparametric mixed-design ANOVAs. The within-subjects factor was the moment of evaluation (four levels: M1, M2, M3 and M4), which is referred to as the effect of training, and the subjects' factors were the aforementioned influential variables. The nonparametric mixed-design ANOVA has an ANOVA-type statistic (ATS) for each effect, and also a modified ANOVA-type statistic (MATS) for the subject's factor. The option for the nonparametric approach was due to the violation of the assumptions of parametric mixed ANOVA in some groups, namely the normality of the dependent variables in each factor's level, the homogeneity of variances and the sphericity. This nonparametric analysis was performed with the *nparLD* package (Noguchi et al., 2012) from the *R* software.

When an effect of a factor with three levels occurred, *Kruskal-Wallis* test with the *Dunn-Bonferroni* post hoc tests were executed to assess between which levels the differences existed. If the interaction effect between each one of the factors aforementioned and the moment of evaluation on the variables of interest was non-significant, subsequent analysis of the effects of exercise were performed not distinguishing participants by the levels of each between-subjects factor. Otherwise, exercise effects on the variables of interest were analyzed separately considering each between-subjects factor's level. Repeated measures ANOVA was used for the assessment of training effects on immune parameters. Normality

and sphericity assumptions were evaluated with the *Shapiro-Wilk* and *Mauchly's* test, respectively. Post hoc tests with *Bonferroni* correction were performed to determine between which moments a significant difference was observed. If the repeated measures ANOVA assumptions were not met, the exercise effect was assessed by *Friedman* test. Post hoc analyses were performed using *Dunn-Bonferroni* test (Dunn, 1964) or, if necessary, due to the conservative characteristic of the *Bonferroni* procedure, according to Conover et al. (1999).

5.4. Results

The number of participants in each group of maturity, swimming age group, distance specialty, and performance is presented in Table 5.1.

Table 5.1. Number of female (n=25) and male (n=29) participants in each group of maturity, swimming age-group, distance specialty, and performance

		Females	Males
Maturity (Tanner's stages)	Adolescent	12	15
	Adult	14	9
Swimmimng age-group	Youth (female: 13 – 14 yrs, male:14 – 16 yrs)	10	19
	Juniors (female: 14 – 16 yrs, male:16 – 18 yrs)	10	3
	Seniors (female: ≥ 17 yrs, male: ≥ 18 yrs)	5	7
Distance specialty	Short distance (sprinters and individual medley)	20	13
	Long distance (middle to long distance)	5	16
Performance	Efficient (improved performance at the end of the season)	13	15
	Less efficient (maintained performance at the end of the season)	12	14

Note: Tanner's stage classification of the maturational state according to Tanner (1962); Swimming age-group classification in both sexes according to the regulation of the *Portuguese Swimming Federation* and *Ligue Européene de Natation* (*LEN*); Distance specialty groups were based on the participant's main event distance: 50m, 100m and 200m swimmers were classified as Short distance and 400m, 800m and 1500m swimmers as Long distance; Performance groups based on the percent change difference between the personal race best time between at the beginning and at the end of the season: <2% changes = Less efficient (maintained performance), and ≥ 2% changes = Efficient (improved performance)

The participant's characteristics, including demographics and body composition related variables, are presented in Table 5.2.

Table 5.2. Demographics and body composition of swimmers at the four moments of evaluation (M1, M2, M3 and M4)

		Moments of evaluation			
		M1	M2	M3	M4
Females	Age (years)	14.6 ± 1.50	14.9 ± 1.49	15.1 ± 1.50	15.2 ± 1.51
	Stature (cm)	163.0 ± 6.24	163.5 ± 6.22	164.2 ± 6.20 *; **	164.5 ± 6.06 *; **
	Body Mass (kg)	54.9 ± 7.40	55.5 ± 7.21	56.2 ± 7.19 *; **	56.3 ± 6.86 *; **
	BMI (kg.m^{-2})	20.6 ± 1.78	20.7 ± 1.89	20.8 ± 1.83	20.8 ± 1.84
	FM (%)	23.9 ± 3.59	24.9 ± 3.64 *	24.2 ± 3.47	24.2 ± 3.56
	FFM (kg)	41.7 ± 4.99	41.6 ± 4.95	42.5 ± 5.10 **	42.6 ± 4.76 **
Males	Age (years)	15.9 ± 2.04	16.2 ± 2.04	16.4 ± 2.03	16.5 ± 2.04
	Stature (cm)	172.1 ± 7.47	173.0 ± 7.00 *	173.4 ± 6.88 *; **	173.7 ± 6.69 *; **
	Body Mass (kg)	64.0 ± 8.03	64.2 ± 7.96	65.4 ± 7.71 *; **	65.4 ± 7.21 **
	BMI (kg.m^{-2})	21.6 ± 2.05	21.4 ± 2.04	21.8 ± 1.98 **	21.6 ± 1.77
	FM (%)	16.1 ± 3.11	16.5 ± 3.17	16.4 ± 3.01 *; **	16.4 ± 3.11 *
	FFM (kg)	53.6 ± 6.75	53.6 ± 7.11	54.7 ± 7.02	54.7 ± 6.34

Abbreviations: BMI, body mass index; FM, fat mass percentage; FFM, fat free mass; * different from M1; ** different from M2; *** different from M3 (p<.05)

Swimmers physical characteristics changed over the season, especially between M1 and M3. These alterations reflect stature growth between M1 and M2 in males and between M2 and M3 in both groups, and also increases in body mass and FFM.

Immune system values throughout the four moments of evaluation were within the reference interval associated with each variable.

5.4.1. Effects of sex, maturity, swimming age group, distance specialty and performance on the immune response to the swimming training season

No influence was observed for maturity Tanner stages, distance specialty, and performance on the response of the variables of interest to the training season. However, sex influenced the response of monocytes ($F(2.931, \infty) = 3.598$; $p = .014$), and swimming age group influenced the response of monocytes ($F(5.271, \infty) = 2.574$; $p = .022$), total lymphocytes ($F(4.967, \infty) = 3.043$; $p = .010$), and lymphocytes subsets CD3$^+$ ($F(4.678, \infty) = 2.857$; $p = .016$), and CD4$^+$ ($F(4.550, \infty) = 2.493$; $p = .034$). CD19$^+$ lymphocytes revealed higher values for males than females throughout the season ($F(1, 51.314) = 4.635$; $p = .036$) although they presented similar responses of the variables of interest to the training season.

5.4.2. Immune system response to swimming training

At M2, CD8$^+$ subsets decreased. Total lymphocytes and subsets CD3$^+$ and CD4$^+$ decreased in the youth group.

At M3, CD8$^+$ subsets remained below baseline values, eosinophils and CD16$^+$56$^+$ subsets decreased. Total lymphocytes and subsets CD3$^+$ and CD4$^+$ recovered to baseline values in the youth group.

At M4, CD19$^+$ lymphocytes were elevated, CD16$^+$56$^+$ lymphocytes continued to decrease, eosinophils remained below baseline levels and CD8$^+$ lymphocytes recovered to baseline levels. Monocytes were also decreased in the male senior group (Fig. 5.2.).

Fig. 5.2. Mean and SEM values of leukocytes, neutrophils, eosinophils, total lymphocytes and subsets CD3+, CD4+, CD8+, CD16+56+, CD19+ counts, and CD4+/CD8+ ratio, at the four moments of evaluation of the 7-month winter swimming training season (30 wks.). M1 = beginning of the season (1st wk.), M2 = after the main competition of the 1st macrocycle (13th wk.), M3 = preparatory phase of the 2nd macrocycle (23rd wk.) and M4 = after the main competition of the 2nd macrocycle (30th wk.).
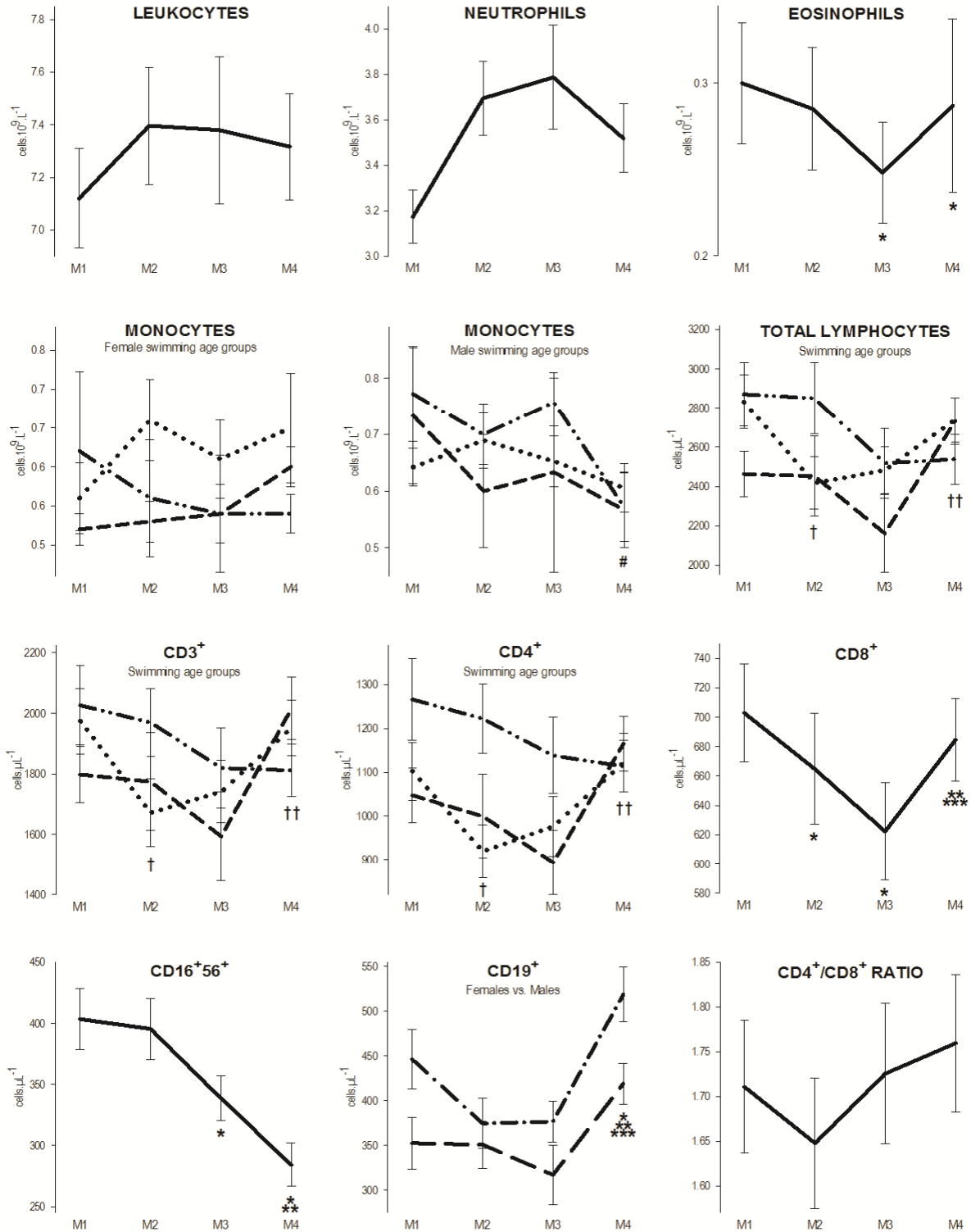
Legend: ____ , * Whole Group; •••, † Youth; _ _ _ , ‡ Juniors; _ . . _ , # Seniors; _ _ _ Females; _ . _ Males;
*, †, # different from M1; **, †† different from M2; *** different from M3 (p<.05)

5.4.3. Seasonal training workload

Training load characterization of the four weeks before the last three moments of evaluation are presented in Table 5.3.

Table 5.3. Mean and SD values of the weekly and total training volume (m), weighed volume (m), load score (AUL) and partial training volumes accomplished at each intensity training zone (m), performed every four weeks before the last three moments of evaluation (M2, M3, and M4) of the 7-month swimming winter training season

Training load parameters and training zones of intensity (weekly value at each zone; m)	M2	M3	M4	Statistic
Volume (m)	30979 ± 4120	47251 ± 12819 **	30110 ± 7519 ***	$F_{(1,\ 112.668)}$ = **.000**
Weighed volume (m)	73956 ± 10456	112344 ± 28542 **	73121 ± 16586 ***	$F_{(1,\ 144.335)}$ = **.000**
Load score (AUL)	12,09 ± 0,63	13,88 ± 0,21 **	11,47 ± 0,73 **; ***	$F_{(1.035,\ 214.309)}$ = **.000**
Warm-up/Recovery (m)	7819 ± 1295	10818 ± 2659 **	8460 ± 1517 **; ***	$F_{(1.036,\ 98.312)}$ = **.000**
Aerobic 1 (m)	12462 ± 1248	18252 ± 6686 **	10883 ± 3372 **; ***	$F_{(1.006,\ 52.469)}$ = **.000**
Aerobic 2 (m)	7325 ± 1272	13113 ± 2861 **	6923 ± 1989 ***	$F_{(1.169,\ 353.347)}$ = **.000**
VO_2 (m)	1983 ± 698	3238 ± 796 **	2091 ± 754 **; ***	$F_{(1.043,\ 186.395)}$ = **.000**
Lactacte Tolerance (m)	404 ± 207	1223 ± 66 **	683 ± 182 **; ***	$F_{(1,\ 416.830)}$ = **.021**
Lactacte Power (m)	374 ± 39	336 ± 133	387 ± 74 ***	$F_{(1.070,\ 5.504)}$ = **.000**
Sprint (m)	555 ± 330	271 ± 70 **	617 ± 189 **; ***	$F_{(1.003,\ 71.318)}$ = **.000**
Training load parameters (total training values) and corresponding RV (%) of the training season	from M1 to M2	from M2 to M3	from M3 to M4	from M1 to M4
Volume (m)	449039 (39,7%)	382953 (33,8%)	300024 (26,5%)	1132016
Weighed volume (m)	1052320 (39,4%)	891645 (33,4%)	724608 (27,2%)	2668573
Load score (AUL)	161.22 (43,6%)	112.93 (30,6%)	95.41 (25,8%)	369.6

Abbreviations: AUL, arbitrary units of load; RV (%); relative value in percentage; ** different from M2; *** different from M3 (p<.05)

In the whole group, as when considering the separated swimming training groups, at M3, training volume, weighed volume, load score (AUL), warm-up/recovery, aerobic 1, aerobic 2, VO_2, and lactate tolerance were higher, and sprint volume was lower than at M2 and M4. At M4, training load score (AUL), aerobic 1, lactate tolerance were lower, whereas warm-up/recovery, and VO_2 were higher, than at M2. At M4, lactate power was higher than at M3, and sprint was greater than at M2. No differences were observed for training volume, weighed volume, and aerobic 2, between M2 and M4, or for lactate power, between M2 and M3. When considering the different age groups of swimmers (youth, juniors and seniors) there was a similar training load pattern.

These results indicate that M3 was the moment of evaluation preceded by the 4 week period with the heaviest training load, both in volume and intensity, as planned by the coaches.

5.4.4. Upper Respiratory Symptoms

The number of episodes of URS was monitored weekly throughout the 7-month swimming winter training season (Fig. 5.3).

Fig. 5.3. Weekly number of episodes of Upper Respiratory Symptoms (URS) over the course of a 7-month swimming winter training season and schedule of the four moments of evaluation: M1, M2, M3 and M4
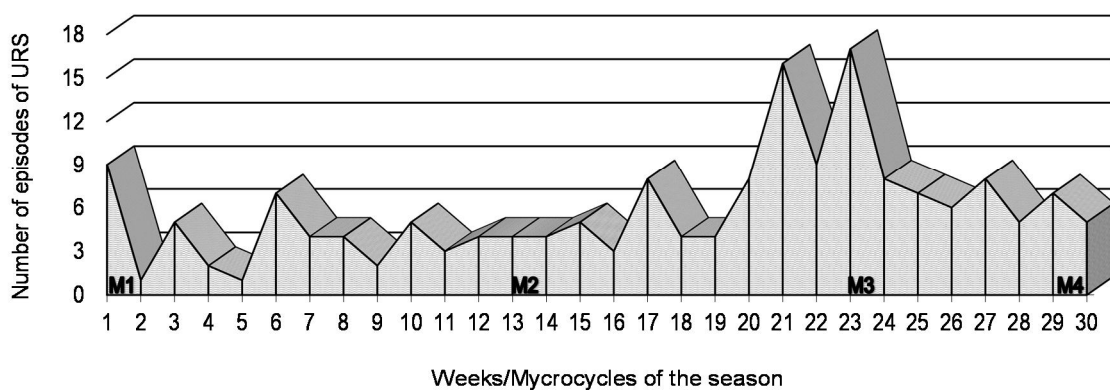
The greater amount of URS episodes happened during the four weeks/mycrocycles prior to M3, and in the two weeks after M3.

5.5. Discussion

Our study shows that training periods with higher load volume and intensity induce a more pronounced immune depression that seemed to be coincident to a greater incidence of URS episodes. Researches that have assessed the chronic response of leukocytes and subpopulations (including lymphocytes subsets) in athletes of different sports such as running (Denguezli et al., 2008), basketball (Brunelli et al., 2014), volleyball (Dias et al.,

2011) and soccer (Del Giacco et al., 2014; Suda et al., 2013) to long-term training periods or competitive training seasons are scarce. If we consider swimming training they are also rare, but rather consistent in what concerns the length and seasonality of the observation periods (Gleeson et al., 1995; Morgado et al., 2012; Mujika, Chatard, & Geyssant, 1996b; Rama et al., 2013; Teixeira et al., 2014). Therefore, since the swimming training season is particularly different from team sports, we chose to focus our discussion on previous studies that monitored immunological alterations along 7-month swimming winter training seasons.

The diminished $CD16^+56^+$ cells observed in the present study were also reported by Rama et al. (2013) at the heaviest training period (M3, 23rd wk. of training). However, the lower values observed for $CD8^+$ subsets at M2 and M3 contradict the absence of changes observed for these cells throughout the several evaluation moments in a previous study (Teixeira et al., 2014). The decreases in eosinophils, $CD16^+56^+$ and $CD8^+$ subsets suggest an impairment of the cellular immunity, which increases the susceptibility for viral infections. The elevated number of $CD19^+$ subsets observed at the end of the season is also contrary to the unaffected response to training of these cells reported by Gleeson et al. (1995). $CD19^+$ subsets were also higher in males than in females. Theoretically, these higher $CD19^+$ levels confer the capacity to produce more antibodies/Ig's, thus increasing humoral immunity. As in our study other authors reported stable values of leukocytes (Gleeson et al., 1995; Mujika et al., 1996b) and neutrophils (Morgado et al., 2012; Mujika et al., 1996b).

Immunological values throughout the training season were similar between adolescents and adults as classified by Tanner (1962). According to Table 5.2., it appears that the whole group of swimmers was under a maturational development over the season reflected essentially by stature growth. The immune system is highly influenced by the physiological levels of some hormones (e.g. growth hormone, cortisol, estrogen, and testosterone) that are permanently changing during puberty. The fact that no influence of maturity was observed on the immune response may be due to the somewhat subjective self-reported methodology used to assess the maturity stage. This does not allow for the positioning of subjects in a continuous process. Although not supported by any evaluation of biological maturity, swimmers are in practice classified according with swimming age groups using chronological age ranges that differ between sexes. This difference aims to take into consideration the classical earlier maturational development of girls compared to boys that occurs throughout adolescence (Boggin, 1999). In fact, in this study, the

swimming age group classification of subjects revealed more differences in the response of the immune cell parameters to the training season than Tanner's stages of maturity, maybe suggesting an alternative and very practical tool to differentiate recommendations for preventing episodes of immune depression or URS.

We also explored the relation between the evolution of immune cells during the training season and the outcome of the training process, evaluated through the impact on performance, and the distance specialty. We chose to explore distance specialty because swimmers tend to explore their particular physical and physiological characteristics for the achievement of the best performance possible. Yet, no influences were observed.

Thus, in the youth group total lymphocytes and subsets $CD3^+$ and $CD4^+$ decreased at M2 and recovered to baseline values afterwards. This suggests that the initial training load of the season affected the acquired immune response, in particular $CD4^+$ (T *helper*), with reflections on $CD3^+$ (total T) and even on total lymphocytes specifically in the youth group. This behavior was not expected hence the most intense period of training was M3. In fact, juniors and seniors showed total lymphocytes and subsets $CD3^+$ and $CD4^+$ values similar to baseline throughout the season, which is in accordance with previous studies that evaluated primarily junior and senior swimmers (Gleeson et al., 1995; Mujika et al., 1996b; Teixeira et al., 2014).

Furthermore, males presented higher monocytes values than females throughout the season, and the response to training was different between males and females with male seniors having diminished monocytes count at M4 compared to M1 and male juniors showing a similar trend profile. Although this last evaluation moment was preceded by a taper period, both seniors and juniors had the lower monocytes count values, suggesting a cumulative effect of the training load, from which swimmers could not efficiently recover with the taper period. This cumulative effect was also noticed for $CD16^+56^+$ subset and eosinophils in the whole group but not for $CD8^+$ or $CD19^+$ subsets, which recovered at the end of the season, with $CD19^+$ even increasing.

The heaviest training period preceded M3 and was characterized by greater volumes in aerobic and lactate tolerance training zones. Increases in training load in well trained athletes undertaking a period of intensified training such as M3 have been described as causing immune depression that may lead to opportunistic infections. In our study, the immune depression was more evident for the innate immunity that decreased during the heaviest training period and persisted below baseline levels until the end of the season although the training load decreased. Immune depression was also noticed for the acquired

immunity but earlier in the training season, suggesting a higher susceptibility to the cumulative training load of the innate immunity while acquired immunity seems to be able to adapt and recover more efficiently when the subject is allowed a period of taper. Indeed, T and B lymphocytes functions have shown to be sensitive to increases in the training load in well-trained athletes, with falls in circulating type 1 T cells counts, decreased T cell proliferative responses and reductions in stimulated B cell Ig synthesis (Baj et al., 1994; Lancaster et al., 2004; Verde et al., 1992). The cause of this depression in acquired immunity may be related to the cumulative effects of repeated bouts of intense exercise which can cause elevations of the circulating stress hormones, particularly cortisol, and anti-inflammatory cytokines (e.g. IL-6, IL-10, IL-Ira) (Gleeson & Bishop, 2005). Overall, the result appears to be a temporary inhibition of Type 1 T cell cytokine production, with a relative diminution of the Type 1 (cell-mediated) response (Gleeson & Bishop, 2005). The literature refers cortisol as a potential conditioner of the entry of lymphocytes into the circulation after intense and prolonged exercise contributing to their return to lymphoid compartments (Nieman, 1994). However, the overall long-term training effects of cortisol over lymphocytes remain unclear. An augmented incidence of viral infections can be the consequence of a defect in T cell number and function (Fabbri, Smart, & Pardi, 2003) either associated or not with cortisol action.

At the heaviest training period both innate and acquired immunity impairments contributed to a more pronounced immune depression. In fact, the higher frequency of URS episodes seems to have happened along this training phase, reinforcing the idea of a disturbed immune resilience of the swimmers. Our results are in agreement with other studies that have also reported an increase in URS symptoms during the heaviest training periods characterized by high loads imposed continuously over several weeks (Morgado et al., 2012; Rama et al., 2013).

The results of the present investigation enhance the importance of controlling immunological alterations during in-season training, especially in heavy training periods but also in the first months of training for young athletes, as a result of the diminished innate and acquired immunity along with higher incidence of URS. This difference in the youth group may be related with the traditional stepper increase in the training load that characterizes the transition for this age group. Furthermore the accumulated effect of years of training can be responsible for the less responsive behaviour of the junior and senior groups in the first macrocycle of the season.

5.6. Conclusions

The long term swimming training process had a cumulative effect towards a decrease of the innate immunity, while the acquired immunity appeared to be more affected at the most intense training period, recovering after a taper period. Younger swimmers presented acquired immune depression earlier in the training season. At the heaviest training period both innate and acquired immunity impairments contributed to a more pronounced immune depression alongside with higher prevalence of upper respiratory symptoms.

CHAPTER VI

Study 3 – Long term swimming training modifies the immune response to high intensity swimming sessions

6.1. Abstract

Long-term training influence on the acute immune cell response to exercise in athletes has been poorly studied, despite the complexity of both chronic and acute adaptations induced by training programs performed throughout the years of the athlete's career.

This study aimed to investigate the influence of a 4-month swimming training macrocycle on the immune cell response to a representative high intensity swimming training session, during a 24 h recovery period, controlling for sex, maturity, age group, performance, and distance specialty effects.

Forty-three swimmers (16 females; 14.4 ± 1.05 yrs., and 27 males; 16.2 ± 2.01 yrs.) performed a standardized training session, at the beginning (M2) and at the end (M4) of a 4-month training macrocycle. Blood samples were collected before (Pre), immediately after (Post), 2 h after (Post 2h) and 24 h after (Post 24h) the training sessions, by standard procedures for assessment of leukogram by automated counting (Coulter LH 750, Beckman) and lymphocytes subsets by flow cytometry (FACS Calibur, BD Biosciences). Throughout the season Upper Respiratory Symptoms (URS) episodes were monitored and training load was quantified. Statistical significance was considered at $p < .05$.

At the end of the training macrocycle, immediately after the swimming session, there was a lower leukocytosis and neutrophilia. From Post 2h to Pre, total lymphocytes and CD19$^+$ subset had a less efficient recovery in the whole group. CD4$^+$/CD8$^+$ ratio, in the youth group continued to increase at M2 but not at M4, while in the senior group continued to increase at M4 and at M2 was returning to baseline. CD16$^+$56$^+$ cells' recovery from Post 24h to Pre values was less efficient in adolescents than in adults.

At the end of the training macrocycle, there seems to be a general attenuated acute immune response, more accentuated in the younger athletes. The higher URS frequency at this period reinforces the idea of a potential immune depression and a longer interval of immune susceptibility to infection. Nonetheless, it is difficult to say if this response reflects positive or negative adaptive mechanisms. However, it appears that the overall changes resulted from the cumulative effects of the swimming training loads.

Key Words: Cellular Immunity, Swimmers, Training Session, Training Season.

6.2. Introduction

The acute immune response to various types of exercises (Gabriel et al., 1992a; Mignini et al., 2008; Natale et al., 2003), and the chronic response of immune cells resting values to long-term training periods or competitive training seasons in different sports (Brunelli et al., 2014; Del Giacco et al., 2014; Dias et al., 2011; Suda et al., 2013) have been investigated. However, there is little evidence about the long-term training effects upon the acute immune systemic and mucosal response to exercise. In fact, to our knowledge the literature refers only one investigation about this topic, which has specifically overviewed the impact of a 3-month training program on mucosal immunity response to swimming training sessions and the incidence of respiratory illness in swimmers (Gleeson et al., 2000). Nonetheless, the influence of the long-term training effects on the acute immune cell response to exercise remains unclear and has not yet been studied, despite the complexity of both chronic and acute adaptations induced by training processes that are extended through many years with daily training and frequent competitions.

At the time of important competitions, a healthy immunological, metabolic, hormonal, circulatory and respiratory condition along with an optimized functional capacity is needed (Hellard et al., 2013). This optimal functionality allows the athlete to achieve the best performance in competition, and is usually the result of an adequate balance between training loads and recovery throughout the different phases of the periodization of a training season (Mujika et al., 1995). However, in endurance sports, such as swimming, during the cycles of high training volume and intensity that include consecutive training sessions with little recovery time in between, athletes may experience a temporary

diminished performance concomitant with an immunodepression state (Dias et al., 2011; Gleeson, 2007; Gleeson & Williams, 2013; Morgado et al., 2012; Rama et al., 2013). Moreover, swimmers are frequently exposed to warm humid environment, temperature variations and chlorine-rich atmosphere, thus being more predisposed to respiratory illness (Aubry et al., 2014; Bernard et al., 2009; Bougault et al., 2012; Gleeson et al., 1995; Gleeson et al., 2000; Mackinnon, 1997; Spence et al., 2007). These combined factors influence negatively the health status must be seriously considered and well managed especially during taper periods (Gleeson & Bishop, 2005) so that the overall functional levels of the athlete may recover in time for competition.

Studies concerning the immune systemic response to swimming, both acute (Ferrer et al., 2009; Kargotich et al., 1997; Morgado et al., 2014; Tauler et al., 2008) and chronic (Gleeson et al., 1995; Morgado et al., 2012; Mujika et al., 1996b; Rama et al., 2013; Teixeira et al., 2014), have also been addressed separately. Like most of the exercise immunology literature, the consistent immediate post-exercise rise in the number of leukocytes (leukocytosis), neutrophils (neutrophilia), monocytes (monocytosis), and lymphocytes (lymphocytosis), reflecting the increase of all lymphocytes subsets, and a $CD4^+/CD8^+$ ratio decline was also observed after high intensity swimming (Kargotich et al., 1997; Morgado et al., 2014). Furthermore, during the first hours of recovery following high intensity swimming exercises, leucocytosis and neutrophilia (Ferrer et al., 2009; Kargotich et al., 1997; Tanner, 1978), monocytes recovery to baseline levels (Kargotich et al., 1997), and declines in lymphocytes total and subsets $CD4^+$, $CD8^+$, $CD16^+$ and $CD19^+$ have been reported (Kargotich et al., 1997), although others noticed no alterations on lymphocytes (Ferrer et al., 2009). When considering the effects of long-term swimming training seasons, reductions in neutrophils and monocytes resting values (Morgado et al., 2012) and decreased $CD56^+$ NK cells (Gleeson et al., 1995; Rama et al., 2013) were observed after a 7-month competitive season, and after a 3-month swimming training program, $CD56^+$ NK cells were also reduced (Gleeson et al., 2000). Summarizing, intense training over long periods appears to affect the number and function of innate and acquired immune cells, possibly contributing to upraise the risk of infection (Walsh et al., 2011).

Besides all the above mentioned factors concerning the immune response to exercise and sports activities, there are still divergent findings in the literature for both acute and chronic immune responses that have been explained by the diversity of exercise protocols, training loads, length and seasonality of the observational periods, methods of data

collection, sample sizes and inter-subjects characteristics, such as subject fitness level, performance, sex, maturity, age group, ability level, and/or event distance specialization.

This study aimed to investigate the influence of a 4-month training macrocycle of a swimming season over the immune cell response to a representative high intensity swimming training session integrated in the normal training process, during a 24 h recovery period, whilst controlling systematically and simultaneously the effects of sex, maturity, age group, performance, and distance specialty.

6.3. Methods

6.3.1. Participants

Forty-three swimmers (16 females, 27 males) members of four different Portuguese swimming teams, undertaking 13 – 15 h of pool training and 4 h of dry-land training per week, were evaluated in this study.

The swimmers were included into different swimming age groups according to the regulation of *Portuguese Swimming Federation* and the *Ligue Européene de Natation (LEN)* (described in Table 6.1.) and had different competitive swimming backgrounds (5.5 ± 0.3 yrs. ranging from $\simeq 4$ to 11 yrs. of practice).

After receiving detailed information about the aim of the study and the possible risks of the investigation, either the subjects or their parents, as appropriate, provided their written informed consent to participate. All procedures were approved by the Ethics Committee of the Faculty of Human Kinetics of the University of Lisbon and were conducted in accordance with the Declaration of Helsinki for human studies (World Medical Association, 2008).

6.3.2. Study Design

This study used an observational design with a follow-up of the second macrocycle of a swimming winter training season lasting 17 weeks. Swimmers followed the training program set by the coaches of each different team.

The evaluation of the swimmers was made at two moments of evaluation named M2 (the week after the main competition of the 1st macrocycle, the beginning of the 2nd macrocycle; 13th week of the training season) and M4 (the week after the main competition of the 2nd macrocycle; 30th week of the training season).

At each moment of evaluation, swimmers performed a representative high intensity swimming training session designed by experienced coaches, and data collected for all subjects included subjects' chronological age and body composition measurements, an indicator of biological maturity (pubertal Tanner stages (Tanner, 1962)) and biochemical immune indices that were evaluated before (Pre), immediately after (Post), 2 h after (Post 2h) and 24 h after (Post 24h) in order to examine the acute response of biochemical immune indices to exercise. Athletes were instructed not to consume anything but water after 10:00 p.m. of the preceding day and to have a minimum of 8 h rest before testing. To standardize pre-exercise food intake and to avoid extending the duration of their fasted state, participants consumed a sandwich with butter and a juice after the body composition measurements and the resting blood sample collection, which were performed in a fasted state. The experimental session took place between 6:30 and 10:00 a.m..

Throughout the follow up season the incidence of Upper Respiratory Symptoms (URS) was monitored weekly and training load and mean intensity of all scheduled swimming sessions were quantified. The characteristics of the training regimens and competition schedules were not modified by the present study in anyway nor any swimmer suffered from major injury or sickness preventing them from training for more than one day.

6.3.3. Swimmers characteristics

6.3.3.1. Body composition measurements

Stature and body mass were measured always after wakening in the fasted state. Stature was measured to the nearest 0.1 cm with *Siber-Hegner* anthropometric kit (DKSH Ltd., Zurich, SW). Participants were weighed to the nearest 0.1 kg wearing a bathing suit without shoes on an electronic scale (TANITA BC-601 body composition scale monitor). This electronic scale was also used to calculate swimmers Fat Mass percentage (%FM) using Bioelectrical Impedance Analysis with a measuring current of 50 kHz, 100 μA. Body

Mass Index (BMI) was calculated as body mass (BM; kg) divided by the square of the stature (m). Free Fat Mass (FFM) was calculated according to the formula:

$$FFM \ = \ BM - (BM \ x \ \%FM)$$

Where FFM is the Fat Free Mass expressed in kilograms, BM is the Body Mass expressed in kilograms, and %FM is the Fat Mass percentage expressed in percentage.

6.3.3.2. Maturity - Tanner stages

Participants received detailed instructions after which they made a self-assessment of their degree of genital organ, breast, and pubic hair development using a questionnaire (Tanner, 1962) accompanied by figures and were grouped according to pubertal stage. In this study, the maturity stage at M2 was equal at M4 and so swimmers were divided in two groups: adolescents and adults (Table 6.1.).
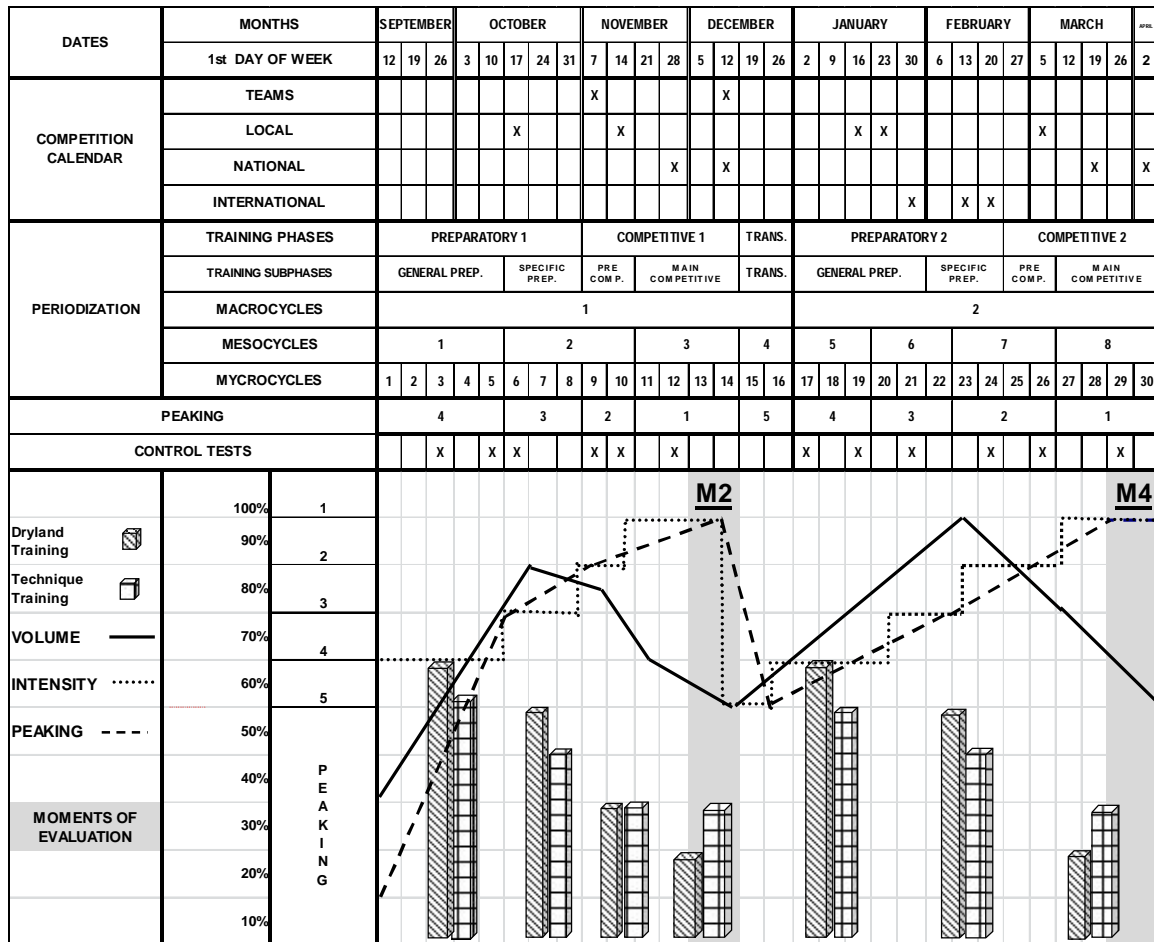
6.3.3.3. Distance specialty

This division was made according to the coaches' classifications of their swimmers, and was primarily based on the swimmers' main event distance: 50m, 100m and 200m swimmers were classified as short distance and 400m, 800m and 1500m swimmers as long distance swimmers (Table 6.1.).

6.3.4. Swimming training season

The observed 4-month training macrocycle was the second macrocycle of a swimming winter training competitive season (Fig. 6.1.). This macrocycle started with a development period characterized by an increasing training volume, intensity and frequency that lasted until the end of the specific preparatory sub phase, where the higher peak of training load of the season was reached. In this period there was also a more frequent participation in competitions (including international meetings). Afterwards, the competitive phase of the 2nd macrocycle of the season occurred, and included a specific preparatory period followed

by a competitive period that lead to important competitions for which training load was progressively reduced: National Youth Championship and National Junior and Senior Championships.

Fig. 6.1. Periodization of the swimming winter training competitive season in which the 4-month training macrocycle is incorporated and schedule of the two moments of evaluation: M2 and M4

DATES	MONTHS	SEP	OCTOBER	NOVEMBER	DECEMBER	JANUARY	FEBRUARY	MARCH	APR
	1st DAY OF WEEK	12 19 26	3 10 17 24 31	7 14 21 28	5 12 19 26	2 9 16 23 30	6 13 20 27	5 12 19 26	2
COMPETITION CALENDAR	TEAMS			X	X				
	LOCAL		X	X		X X	X		
	NATIONAL			X	X			X	X
	INTERNATIONAL					X	X X		

PERIODIZATION			
TRAINING PHASES	PREPARATORY 1 / COMPETITIVE 1 / TRANS.	PREPARATORY 2 / COMPETITIVE 2	
TRAINING SUBPHASES	GENERAL PREP. / SPECIFIC PREP. / PRE COMP. / MAIN COMPETITIVE / TRANS.	GENERAL PREP. / SPECIFIC PREP. / PRE COMP. / MAIN COMPETITIVE	
MACROCYCLES	1	2	
MESOCYCLES	1 2 3 4	5 6 7 8	
MYCROCYCLES	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16	17 18 19 20 21 22 23 24 25 26 27 28 29 30	
PEAKING	4 3 2 1 5	4 3 2 1	
CONTROL TESTS	X X X X X X	X X X X X X	

Legend (graph):
- Dryland Training
- Technique Training
- VOLUME (solid line)
- INTENSITY (dotted line)
- PEAKING (dashed line)
- MOMENTS OF EVALUATION

Graph y-axis: 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%; PEAKING scale 1–5. Moments of evaluation marked M2 and M4.

6.3.5. Quantification of training load

Training load was determined through the total amount of meters swam (volume) and also by the balance of the distance completed at each level of intensity based on the work of Mujika et al. (1996a; 1995) (Table 6.3.).

The use of a stress index scale of difficulty has been established in reference to the theoretical values of blood lactate accumulation usually associated with the different swimming training zones of intensity. The training zones adopted considered the works by

Mujika et al. (1995), Maglischo (2003), and Sweetenham &Atkinson (2003)), and were: I - warm up and recovery, II - aerobic 1, III – aerobic 2, IV - VO$_2$max, V - lactate tolerance, VI - lactate production and VII - sprint. In order to evaluate the swimming sessions training load the volume accomplished in each zone of intensity was quantified (mI, mII, mIII, mIV, mV, mVI and mVII). The magnitude of the load was then expressed in dimensionless units of load, or arbitrary units of load (AUL), obtained from the ratio between the sum of the volumes swam in each zone of intensity multiplied by the respective index (1, 2, 3, 4, 6, 8, 10) and the total volume effectively completed, according to the formula:

$$AUL = \frac{1\,mI + 2\,mII + 3\,mIII + 4\,mIV + 6\,mV + 8\,mVI + 10\,mVII}{\text{swimming training session volume}}$$

Where AUL is the arbitrary units of load expressed in dimensionless units of load, the numerator is the weighed volume expressed in meters, mI, mII, mIII, mIV, mV, mVI and mVII are the meters accomplished at the following swimming training zones of intensity: I- warm up and recovery, II - aerobic 1, III – aerobic 2, IV - VO$_2$max, V - lactate tolerance, VI - lactate production and VII – sprint, the numbers 1, 2, 3, 4, 6, 8, 10 are the indexes associated to each zone of intensity, the denominator is the swimming training session volume expressed in meters.

This was performed for all season sessions considering each age group and within all swimming teams.

The mycrocycle or weekly load was quantified and expressed by the volume (total of meters swam), by the weighed volume (sum of the multiplications of the volume accomplished in each zone of intensity by the respective stress index values) and by the intensity (determined through the sum of the resulting dimensionless unit of load of each session of training).

6.3.6. Swimming training session

The swimming session started with a 1500 m standardized warm-up lasting 30 to 35 min followed by a high intensity main task that lasted 50 min and a 500 m recovery task (8 min of duration). The main task was designed to induce maximal lactate accumulation and had a total distance of 1000 to 1200 m, depending on the age group considered. For the youth group the main task consisted of two sets of four repetitions of 75 m front crawl on a five min cycle, with 10 min of active recovery between sets (400 m freestyle). Each repetition

had to be accomplished at 90 – 95 % of 100 m Freestyle personal best race time. The task organization was identical for juniors and seniors but with repetitions of 100 m. Swimming times were registered in each repetition and the mean time was used to determine the mean effort intensity percentage (%), in relation to the personal best time at the 100 m freestyle race.

6.3.7. Performance improvements

The effect of training on performance was evaluated by magnitude of the change of the race time at competitive events. It was expressed as a relative difference, which represented a percentage of change, comparing the race time accomplished at M4 with that at M2. In this manner, two groups were created according to the level of improvement: the less efficient group, which presented under 2% changes in performance, and the efficient group, which presented changes of 2% and above (Table 6.1.). This 2% level of improvement was adopted based on the consistently mean improvements of around 3% mentioned in the literature (Mujika et al., 1996a).

6.3.8. Immune system parameters

Peripheral venous blood samples were collected via standard procedures before (Pre, between 6:00 – 6:30 a.m. in the fasted state), immediately after (Post), 2 h after (Post 2h) and 24 h after (Post 24h) the swimming training sessions. Venous blood was collected into tubes containing EDTA for assessment of hemogram and leukogram and for counting of total and subpopulations of lymphocytes. Hemogram and leukogram was performed in an automated hematology analyzer (Coulter LH 750, Beckman) which produced information about the following parameters: hemoglobin concentration ($g.dL^{-1}$), hematocrit (%) and counts of white blood cells namely: leukocytes, neutrophils, monocytes, and eosinophils. Total and subpopulations of lymphocytes were counted by flow cytometry (FACS Calibur, BD Biosciences). The lymphocytes subpopulations analyzed were $CD3^+$ (total T lymphocytes; T cells), $CD4^+$ (T *helper*; *Th* cells), $CD8^+$ (T *cytotoxic*; *Tc* cells), $CD16^+56^+$ (NK cells) and $CD19^+$ (B cells). Results were expressed as number of $cells.10^9.L^{-1}$ for leukogram parameters and as number of $cells.\mu L^{-1}$ for total and subpopulations of

lymphocytes counts. Post, Post 2h and Post 24h exercise values were corrected for plasma volume variation (Dill & Costill, 1974).

6.3.9. Upper Respiratory Symptoms

Subjects were asked to answer to a weekly questionnaire that was sent every Monday to their email address. This questionnaire consisted of a daily logbook in which they noted their symptoms associated with illnesses related to URS such as: headache, fever, ear pain, chills, runny or blocked nose, pharyngitis/tonsillitis, bronquitis, asthma, phlegm, cough, conjunctivitis; itchy, watery eyes, nausea/vomiting, and diarrhoea. All swimmers were asked to indicate the medication they were on and female subjects to point out the days of menstruation. If subjects had no symptoms they simply recorded that and reply to the email. If fever or at least two concomitant symptoms persisted for at least 48 hours, separated from previous symptoms by at least one week, they were considered an episode of URS (Bishop, 2006). Symptoms separated by less than one week were regarded as a recurrence or continuation of the initial episode and were regarded as part of the same episode. The counting of the URS episodes was displayed graphically as the weekly number of episodes of URS over the course of the training season.

6.3.10. Statistical analysis

The statistical analyses were performed with the software IBM SPSS Statistics, version 21, and the *R* software (R Core Team, 2012), version 2.15.1, and a significance level of 5% was considered.

In order to have a single value that indicated the change between the moments of observation of the acute immune response (Pre, Post, Post 2h, and Post 24h), the relative differences in percentage from Pre to Post, Pre to Post 2h, Pre to Post 24h, Post to Post 24h, and from Post 2h to Post 24h were calculated according to the formula:

$$RV = \frac{X-Y}{Y} \times 100$$

Where RV is the Relative Difference in percentage, X is the final value, Y is the starting value, the ratio is multiplied by 100 so RV can be expressed as percentage.

Descriptive statistics, including means and standard deviation (mean ± SD) were performed for all quantitative outcome measurements. Normality of the outcome variables was analysed using the *Shapiro-Wilk* test.

The effects of the sex, pubertal Tanner's stages (adolescents and adults), swimming age-groups (youth, juniors and seniors), distance specialty (short and long distance swimmers), and performance (efficient and less efficient), and the interaction effect of each one of these factors with the moment of evaluation on the response of the variables of interest was analysed using nonparametric mixed-design ANOVAs.

The within-subjects factor was the moment of evaluation (two levels: M2 and M4), which is referred as the effect of training, and the subjects' factors were the aforementioned influential variables. The nonparametric mixed-design ANOVA has an ANOVA-type statistic (ATS) for each effect, and also a modified ANOVA-type statistic (MATS) for the subject's factor. The option for the nonparametric approach was due to the violation of the assumptions of parametric mixed ANOVA, namely the normality of the dependent variables in each factor's level, the homogeneity of variances and the sphericity. This nonparametric analysis was performed with the *nparLD* package (Noguchi et al., 2012) from the *R* software.

It was determined if sex, maturity, swimming age group, distance specialty, and performance interactions contributed to the relationships between the immune response to a swimming session (from Pre to Post, Pre to Post 2h, and Pre to Post 24h in percent changes values in relation to pre-exercise values) and the moments of evaluation (M2 and M4). When an interaction occurred exercise effects on the variables of interest were analyzed separately considering each between-subjects factor's level.

The majority of the collected data did not follow the normal distribution and so nonparametric *Wilcoxon* test was used to analyze the influence of training over the acute immune response to exercise.

6.4. Results

The number of participants in each group of maturity, swimming age group, distance specialty, and performance is presented in Table 6.1.

Table 6.1. Number of participants in each group of maturity, swimming age group, swim specialty, and performance

		Number of Participants
Maturity (Tanner's stages)	Adolescent	28
	Adult	15
Swimmimng age group	Youth (female: 13 – 14 yrs, male:14 – 16 yrs)	26
	Juniors (female: 14 – 16 yrs, male:16 – 18 yrs)	10
	Seniors (female: ≥ 17 yrs, male: ≥ 18 yrs)	7
Distance specialty	Sprinters (sprinters and individual medley)	22
	Long distance (middle to long distance)	21
Performance	Improved performance at the end of the season	25
	Maintained performance at the end of the season	18

Note: Tanner's stage classification of the maturational state according to Tanner (1962); Swimming age-group classification in both sexes according to the regulation of the *Portuguese Swimming Federation* and *Ligue Européene de Natation* (*LEN*); Distance specialty groups were based on the participant's main event distance: 50m, 100m and 200m swimmers were classified as Short distance and 400m, 800m and 1500m swimmers as Long distance; Performance groups based on the percent change difference between the personal race best time between at the beginning and at the end of the season: <2% changes = Less efficient (maintained performance), and ≥ 2% changes = Efficient (improved performance)

The participant's characteristics, including demographics and body composition related variables, are presented in Table 6.2.

Table 6.2. Demographics and body composition of swimmers at the moments of evaluation M2 and M4

	Females		Males	
	M2	M4	M2	M4
Age (years)	14.4 ± 1.05	14.7 ± 1.06	16.2 ± 2.01	16.5 ± 2.05
Stature (cm)	162.2 ± 6.13	163.5 ± 6.23	173.6 ± 6.42	174.3 ± 6.18
Body Mass (kg)	54.3 ± 8.87	55.4 ± 6.60	64.7 ± 7.85	66,0 ± 7.31
BMI (kg.m^{-2})	20.6 ± 1.96	20.5 ± 1.82	21.5 ± 3,0	21.8 ± 1.82
FM (%)	25.0 ± 3.33	24.8 ± 2,91	16.6 ± 3.05	16.6 ± 2.90
FFM (kg)	40.7 ± 5.02	41.6 ± 4.69	54.0 ± 7,05	55.0 ± 6.29

Abbreviations: BMI, body mass index; FM, fat mass percentage; FFM, fat free mass

Swimmers physical characteristics slightly changed over the training macrocycle, reflecting little increases in height, body mass and FFM. The main sets of the swimming training sessions were accomplished at the requested high intensity in relation to their personal best time at the 100 m Freestyle race: 92.3 ± 4.7 % at M1, and 93.4 ± 7.2 % at

M4. Immune system mean baseline values indicated that the participants were within the reference interval associated with each variable (Lewis et al., 2006).

6.4.1. Influence of training on the acute exercise immune response

Regarding the effects of the training macrocycle on the response to the training session, maturity influenced $CD16^+56^+$ subsets (from Pre to Post 24h: $F(1, \infty) = 4.470$, $p = .035$), and swimming age group influenced $CD4^+/CD8^+$ ratio (from Pre to Post 2h: $F(1.881, \infty) = 10.847$, $p = .000$) (Fig 6.2. E. and F.). Sex, distance specialty, and performance had no influence over the effect of the training macrocycle on the response to the training session.

At M4, from Pre to Post, leukocytes and neutrophils ascendant response was lower. From Pre to Post 2h, total lymphocytes and $CD19^+$ subset absolute relative differences were higher. For seniors, $CD4^+/CD8^+$ ratio relative ascendant response was more accentuated at M4, while in the youth group it was more accentuated at M2. From Pre to Post 24h, for adolescents $CD16^+56^+$ subset absolute relative difference was more accentuated at M4 (Fig. 6.2.).

Fig. 6.2. Mean and SD of the relative difference (%) values of leukocytes (A.), neutrophils (B.), total lymphocytes (C.) and subsets CD19+ (D.), CD16+56+ (E.), and CD4+/CD8+ ratio (F.) in response to a representative high intensity swimming training session performed at the beginning (M2) and at the end (M4) of a 4-month swimming training macrocycle (corresponding to the 2nd macrocycle of a 7-month swimming season)
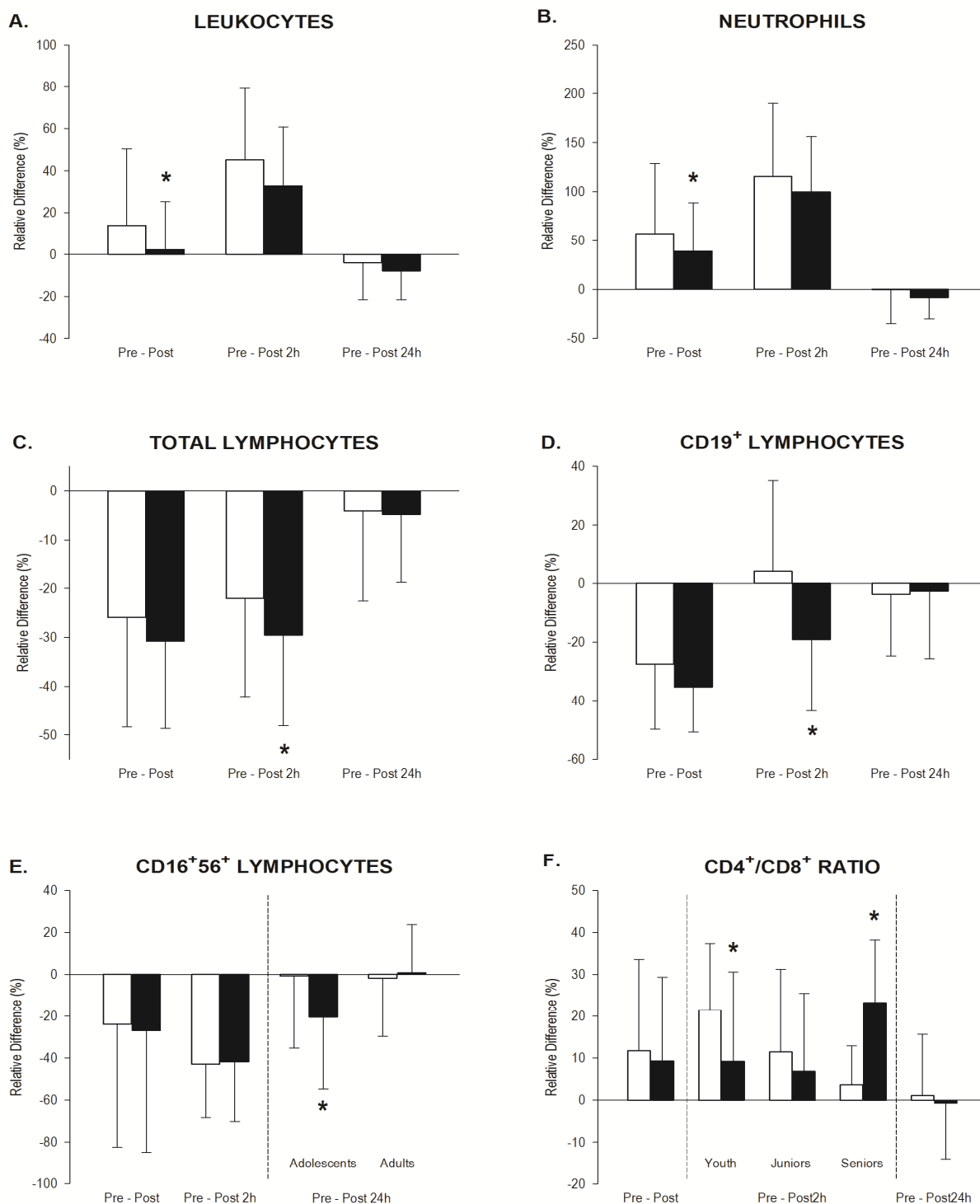
6.4.2. Seasonal training workload

Training load characterization of the four weeks before each moment of evaluation is presented in Table 6.3. The mean weekly training load score (AUL) throughout the 30th weeks of the swimming winter training season in which the 4-month macrocycle is incorporated is presented in Fig. 6.3.

Table 6.3. Mean and SD values of the weekly and total training volume (m), weighed volume (m), load score (AUL) and partial training volumes accomplished at each intensity training zone (m), performed every four weeks before the two moments of evaluation that defined the beginning (M2) and the end (M4) of the 4-month swimming training macrocycle

Training load parameters and training zones of intensity (weekly value at each zone; m)	M2	M4	Macrocycle mean weekly value (m)	Macrocycle total (m) and RV (%) values
Volume (m)	30696 ± 3991	29157 ± 7397	40175	682977
Weighed volume (m)	72951 ± 11005	70868 ± 18177	95074	1616254
Load score (AUL)	12.0 ± 0.8	11.4 ± 0.8 *	12.3	208.3
Warm-up/Recovery (m)	7564 ± 1131	8110 ± 1417 *	9967	24.8
Aerobic 1 (m)	12625 ± 1782	10586 ± 3666 *	15435	38.4
Aerobic 2 (m)	7310 ± 1331	6920 ± 2306	10464	26.0
VO_2 (m)	1893 ± 708	1843 ± 971	2722	6.8
Lactacte Tolerance (m)	416 ± 228	674 ± 281 *	807	2.0
Lactacte Power (m)	368 ± 162	403 ± 167	345	0.9
Sprint (m)	518 ± 346	615 ± 219	436	1.1

Abbreviations: AUL, arbitrary units of load; RV (%); relative value of the macrocycle total values in percentage;
* different from M2 (p<.05)

At M4, the lower weekly AUL and Aerobic 1 volume, along with a higher volume accomplished at the warm-up/recovery training zone suggests that M4 had a more accentuated recovery component than M2.

Fig. 6.3. Mean weekly training load score (AUL) values over the course of the training season in which the 4-month swimming training macrocycle was included (between M2 and M4; corresponding to the 2nd macrocycle of the season)
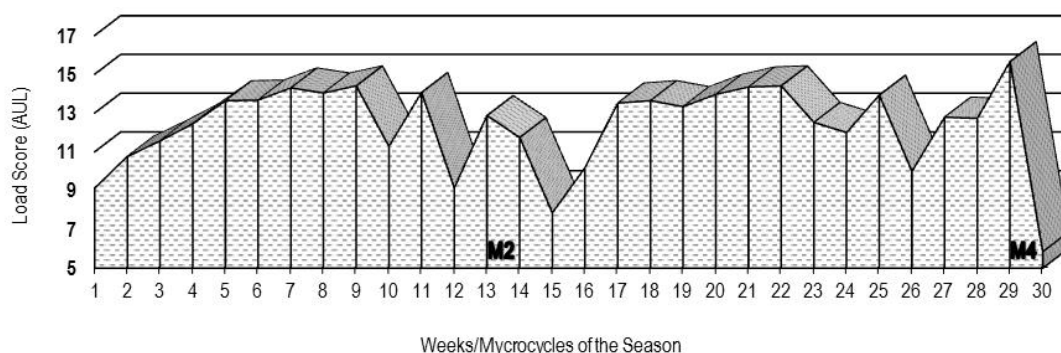
6.4.3. Upper Respiratory Symptoms

The number of episodes of URS was monitored weekly throughout the 7-month swimming winter training season in which the 4-month macrocycle (between M2 and M4) was included (Fig. 6.4.).

Fig. 6.4. Weekly number of episodes of Upper Respiratory Symptoms (URS) over the course of the training season in which the 4-month swimming training macrocycle was included (between M2 and M4; corresponding to the 2nd macrocycle of the season)
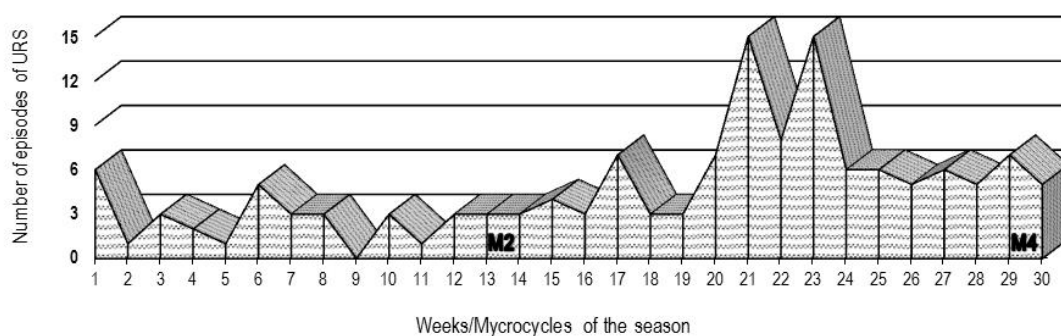
The higher number and weekly frequency of URS episodes of the training season occurred between the 20th and the 24^{h} weeks of training. When comparing the two moments of evaluation, during the four weeks before M4 there was a superior frequency of URS episodes than along the four weeks prior to M2.

114

6.5. Discussion

In the present investigation, to understand the influence of training over the acute response to intense prolonged exercise, a representative high intensity swimming training session was performed at the beginning (M2) and at the end (M4) of a 4-month swimming training macrocycle.

At the end of the training macrocycle, immediately after the swimming session, there was a lower leukocytosis and neutrophilia, suggesting an attenuated acute response to the swimming session. It was also observed a less efficient recovery of total lymphocytes and $CD19^+$ subset (B cells) from Post 2h to Pre values in the whole group. As for $CD4^+/CD8^+$ ratio, in the youth group their ratio continued to increase at M2 but not at M4, while in the senior group their number continued to increase at M4 and at M2 was returning to baseline, from Post to Post 2h. $CD16^+56^+$ NK cells' recovery from Post 24h to Pre values was less efficient in adolescents than in adults.

At the end of the swimming training macrocycle, the immediate response to the swimming training session seems to involve a smaller recruitment of cells from the reservoirs or marginated pool of cells, as shown by the variations of neutrophils reflected into the variation of leukocytes. In addition, the acquired immune response, in particular $CD19^+$ lymphocytes, appears to have a less efficient recovery in the first 2 h after the intense training session in all swimmers, suggesting a longer interval of immune susceptibility to infection than at the beginning of the macrocycle. Likewise, but only in the adolescent swimmers, a less efficient recovery of the innate immunity, namely $CD16^+56^+$, to the swimming session was observed even at 24h post, indicating that the general acute immune response was more attenuated than adults, and also that the innate immune response of adolescents to acute exercise was apparently more sensitive to the influence of long term training. Throughout adolescence, especially during puberty, the physiological levels of some hormones (e.g. catecholamines, cortisol, growth hormone, estrogen, and testosterone) in association with a differential effect of these hormones and cytokines on lymphocyte subsets (Nemet & Eliakim, 2010; Steensberg et al., 2001b) may influence the exercise-induced immune response.

The training process might have contributed to the reduced leukocytes acute response to exercise, through a diminished cell traffic and cell proliferation and/or increased cell death responses (Kruger et al., 2008). A reduction in cell trafficking could have relied upon the

long term adaptations of the adherence of cells to the endothelium and their redistribution amongst organs or compartments, and of the physiological responses to acute exercise, namely, cardiac output, shear stress, and blood flow to working muscle, and improved ability to counteract pH and temperature changes (Adams et al., 2011). Moreover, training might have influenced catecholamines, and cortisol concentrations and their regulation of lymphocyte subset redistribution (McCarthy et al., 1991; Mignini et al., 2008). During the post exercise recovery period cortisol acts as conditioner of the entry of lymphocytes into the circulation contributing to their return to lymphoid compartments (Nieman, 1994), and regulates both lymphopenia and neutrophilia (McCarthy et al., 1991; Mignini et al., 2008). Although the overall long-term training effects of cortisol over resting lymphocytes values remains unclear, and herein was not evaluated, we may argue that cortisol's levels may partially explain the less efficient recovery response of total lymphocytes and $CD19^+$ subset to the swimming session at the end of the macrocycle.

It is commonly accepted that training load increments in well-trained athletes undertaking periods of elevated training volume and intensity can lead to the stimulation of adaptive mechanisms related to metabolic and hormonal circulatory and respiratory responses that can compromise performance and induce an impaired immune status, including falls in the number and activity of T and B cells (Baj et al., 1994; Lancaster et al., 2004; Verde et al., 1992). This conjuncture can contribute to elevate the risk of infection, despite this situation may be reversible by a tapering or recovering period (Gleeson & Bishop, 2005; Walsh et al., 2011).

In our investigation, although the training load intensity decreased from M2 to M4, the number of URS tended to be higher at M4 than at M2. This evidence suggests a potential immune depression, and we can argue that this may result from the cumulative effects of the swimming training loads. We still have to consider seasonal variations of infectious agents, although M2 occurred in winter and M4 in spring.

We did not observe any interaction between the improvement of performance during the macrocycle and the immune response to the swimming session. However, swimming performance depends on a multiplicity of factors, including biomechanical, energetic, psychological, that can mask the role that the immune system has on the outcome (Costill et al., 1985; Reis, Alves, Bruno, Vleck, & Millet, 2012; Toussaint & Beek, 1992). Thus, we may argue that the changes observed in the immune ability to respond to exercise during this period were not associated to the efficiency of the physical, psychological and technical qualities that allow reaching the desired performance.

Finally, coaches and athletes ought to implement intervention and behavioural strategies during taper periods in order to contribute to maintain health conditions, preventing the onset of fatigue and associated diminished performance, thus helping to avoid illness and reaching the peak performance at competitions. Also, athletes should take special precautions during the first hours after intense training sessions.

6.6. Conclusions

In the present investigation, at the end of the training macrocycle, the lower magnitude of the immediate leukocytosis and neutrophilia followed by the more prolonged recovery of total lymphocytes and B cells in response to the swimming session appears to dictate a general attenuated acute immune response. This response seemed even more attenuated in the younger athletes, reflected by the difficulty of $CD16^+56^+$ to recover in 24 h in adolescents, and by the lower magnitude of the $CD4^+/CD8^+$ ratio response during the early recovery in the youth group. Concurrently, there was a higher URS frequency, which reinforces the idea of a potential immune depression and a longer interval of immune susceptibility to infection. Nonetheless, it is difficult to say if this response reflects positive or negative adaptive mechanisms. However, it appears that the overall changes resulted from the cumulative effects of the swimming training loads.

CHAPTER VII

General Discussion

7.1. Main findings

In order to address and enrich some of the above mentioned issues and limitations, which are scrutinized in the literature review section, three research studies were conducted. A detailed discussion of each study and the respective main findings were included in the corresponding chapters (*Chapters IV* to *VI*). This current section aims to gather and integrate the contributions of the three studies, by summarizing the main results and globally reflecting on the implications and practical applications. Limitations of these studies and future research avenues are also disclosed.

In the present dissertation we investigated the acute immune cell response to a representative high intensity swimming training session, during a 24 h recovery period (*Study 1*), and to the chronic effects of the 7-month swimming winter training season, in which the training session was integrated, at rest (*Study 2*). We also investigated the influence of a 4-month training macrocycle over the acute immune response to exercise (*Study 3*). It was also an objective to control systematically and simultaneously inter-subjects diversity, namely sex, menstrual cycle phases for females, maturity, swimming age group, and distance specialty, and also performance improvements and training load along the training season. The review of the state of the art revealed evidences about the possible influence of the aforementioned variables on the immune response to exercise. Thus, since the interpretation of results could be affected by these variables, and there was the possibility of evaluating a large sample of subjects with diverse characteristics, it has been found interesting to control the effects of these variables on the immune response to exercise.

The immune response to the high intensity and prolonged swimming training sessions, which represent a typical effort developed in training sessions included in competitive swimming training processes, was similar throughout the season as it was observed in *Study 1* and *Study 3*. In general, there was a rise of neutrophils, opposed by a decrease of monocytes, eosinophils and total lymphocytes and subsets CD3$^+$ (total T), CD4$^+$ (*Th*),

CD8$^+$ (*Tc*), CD16$^+$56$^+$ (NK), and CD19$^+$ (B), with the neutrophilia and lymphopenia lasting for approximately 2 h after exercise ended. The neutrophilia and lymphopenia suggest a change in the type of circulatory surveillance with increased innate immunity (neutrophils) and decreased acquired immunity (lymphocytes). We can argue that this may result in a broader immune response but also slower and less efficient, which may have advantages in case of infections by agents to which the athlete has never previously been exposed but is disadvantageous for reinfection situations. As the immune cells function was not evaluated, it is not possible to evaluate whether any elevation or diminution in cells counts observed in *Study 1* and *Study 3*, were counteracted by the reduction or enhancement of their ability to respond. Still, previous investigations suggested a diminished activity of some leukocytes subsets, namely neutrophils (Robson et al., 1999), monocytes (Nieman et al., 1998b; Simpson et al., 2010) and CD56$^+$ NK lymphocytes (Suzui et al., 2004). So, it is possible that at least in the first 2 h after intense training sessions such as the ones performed by our swimmers, athletes may be more susceptible to infection, highlighting the need for extra care when exposed to aggressive environmental agents. Except for total lymphocytes and subsets CD3$^+$ in the junior group in *Study 1* and for CD16$^+$56$^+$ NK subsets in adolescents in *Study 3*, all immune parameters recovered to baseline values at 24 h after the swimming session, pointing out the importance of the resting period after intense exercise sessions when planning consecutive training sessions. Three different processes that probably occur concomitantly may explain the variations of the number of these immune cells: cell traffic, cell proliferation or cell death (Kruger et al., 2008; Kruger & Mooren, 2014). These changes and processes are also affected by the release of the exercise-induced hormones catecholamines and cortisol (Gabriel et al., 1992a; Timmons et al., 2006b), and cytokines (Giraldo et al., 2009; Gleeson, 2007). In *Study 1* and *Study 3*, the long duration of the swimming sessions may have revealed a scenario where the influence of cortisol was more evident, with the increase of neutrophils, and the reduction of lymphocytes probably mobilized into tissues such as lungs, spleen and muscles (Adams et al., 2011). These processes and mechanisms associated to the acute immune cell changes induced by exercise are explained in detail in *Chapter II*, *Study 1*, and in *Study 3*.

When considering the influence of *long term training on the immune cells* at rest (*Study 2*), and on the acute response to the swimming session (*Study 3*), our results suggest that the innate and acquired immune cell responses to swimming training vary throughout the competitive season possibly affected by the dynamic of the training load.

Both the innate and acquired immune responses decreased was similar at the highest intensity training period of the season (M3, *Study 2*), reflecting the decreases in $CD16^+56^+$ NK (innate) and $CD8^+$ subsets (acquired) and contributing to an overall impaired immunity, concomitant with the highest weekly number of URS episodes. These evidences support the idea of a disturbed immune resilience of the swimmers and an increased susceptibility to infections at periods of high training load, which is in accordance with the literature (Morgado et al., 2012; Rama et al., 2013). Also, earlier in the training season (M2, *Study 2*), the youth swimmers presented a diminished acquired immunity compared to the beginning of the season, expressed mostly by decreased total lymphocytes and subsets $CD3^+$ and $CD4^+$, whereas $CD8^+$ subsets were diminished in the whole group. This different and more accentuated acquired immune response to training in the youth group may be related with the traditional stepper increase in the training load that characterizes the transition for this age group. The decreased number of the immune cells at M2 and M3 is probably associated to the increased resting cortisol concentrations (Morgado et al., 2012; Rama et al., 2013) and decreased cytokines production by immune cells (Morgado et al., 2012), which occur in response to exhaustive training.

The end of the season (M4, *Study 2* and *Study 3*) was preceded by a *taper* period (recovery period). At M4, in *Study 2*, we observed that the *taper* might have enabled an efficient recovery to baseline values of the resting acquired immunity ($CD8^+$ and $CD19^+$ subsets). Contrarily, resting innate immunity ($CD16^+56^+$ cells and eosinophils) persisted below baseline levels suggesting a higher susceptibility to the cumulative effect of the long term swimming training. At M4, in *Study 3*, there was an overall attenuated acute immune response to the swimming training session reflected by smaller leukocytosis and neutrophilia, and a subsequent longer *open window* period of susceptibility to infection indicated by the less efficient recovery of total lymphocytes and $CD19^+$ (B cells) subset. The $CD16^+56^+$ NK subset recovery from Post 24h to Pre values was less efficient in adolescents than in adults. The leukocytosis seems to have involved a smaller recruitment of cells from the reservoirs or marginated pool of cells, probably through diminished cell traffic and cell proliferation and/or increased cell death responses (Kruger & Mooren, 2014), as a consequence of the long term physiological and biochemical adaptations induced by the predominant endurance type of training included in swimming training (Bangsbo et al., 1993; Craig et al., 1995; Spencer & Gastin, 2001). Also, as previously explained in *Study 2* and *Study 3*, and although herein was not evaluated, the elevated circulating levels of cortisol and cytokines as consequence of exhaustive exercise can

persist for long periods (Dinarello, 1997), and may partially explain the less efficient recovery response of total lymphocytes and subsets $CD16^+56^+$ and $CD19^+$ to the swimming session. Whether this altered acute response reflects positive or negative adaptive mechanisms it is difficult to say, however, it is likely that the overall changes resulted from the cumulative effects of the swimming training loads.

7.2. Intervention strategies

The outcomes of the present dissertation suggest that athletes and coaches should consider taking several actions, mostly preventive, in order to avoid exposure of the athletes to potential infections, which may compromise attendance to training sessions, performance and most of all health.

In general, coaches should consider immune susceptibility when planning training loads at the level of the whole squad, sub-groups (e.g., internationals, nationals, juniors, youth) and individual swimmers. Strategies for surveillance of health status and to detect individuals more susceptible to infection, in particular in period of high training load should be implemented. Furthermore, these training periods should be applied with discretion and the coach should be aware of the repercussions that it implies. Additionally, a careful planning of the general conditioning undertaken in the early stages of the season, during intensive and post-competition periods is needed (Hellard et al., 2015). Besides, during the heavy training phases, several intervention strategies should be adopted to prevent health issues and the onset of fatigue and associated diminished performance thus helping to avoid illness and absence from training sessions. In this manner, coaches should carefully monitor recuperation by providing the adequate rest and recovery periods and the balance between training volume and intensity. A psychological approach, in ways of developing the self-management and coping skills of the athletes and at the same time monitor their responses to the psychological and psychosocial stresses of training and competition, should also be considered. Moreover, according to several authors (Ahmadinejad et al., 2014; Gleeson, 2006; Hackney, 2013; Nieman, 2001; Walsh et al., 2011) preventive behavioural plans for athletes should be contemplated as well, in what refers adopting some actions, such as paying attention to the exposure to common infections and infected or sick persons, and keep vaccine(s) administration updated; protect airways from very

cold or dry air when performing intense exercise; follow a well-balanced diet with adequate nutritional intake; sleep for at least seven hours a night; and try to minimize other life stressors (e.g. management of academic, personal and professional areas).

Nonetheless, in case of illness, a quick and complete recovery is essential to allow the athlete to return to the training activities. Yet, it must be a "safe" recovery, conferring the healthy and functional condition required for adequate participation in the demanding training processes. The timing for return to training should be adapted to the case and circumstance and the specific illness. In these cases, after returning from illness, the application of the behavioural strategies abovementioned appears to assume a greater importance.

7.3. Future directions

Regarding the long-term prospective swimming studies and the outcomes about the relationship between infection, training variables and immune parameters, it would be interesting trying to establish a "health friendly" range of the weekly training load, particularly, for the heavy training periods of the training seasons. Additionally, long term studies conducted in the same athletes over consecutive training seasons can probably help to understand if innate immunity is actually more susceptible to the cumulative training load than acquired immunity.

As the understanding of the immune response to exercise appears to be associated to the influence of hormones, it is of particular importance to evaluate biological maturity when considering youth and adolescent populations. In our study we assessed sexual maturation based on the Tanner puberty stages classification. This somewhat subjective self-reported methodology turned out to be limitative and it did not allow for the positioning of subjects in a continuous process. Other methods of estimating the stage of maturity should be considered, especially those that have shown agreement with estimates based on skeletal age such as relative distance from adult stature and hand-wrist X-ray.

Furthermore, knowing better the subjects characteristics effects upon the immune response to exercise, may be useful regarding the individualization of the training process.

7.4. Conclusions

In the present research dissertation, we observed an impaired acquired immune surveillance immediately and at least throughout a 2 h period after the end of a representative high intensity prolonged swimming training session that may have increased the risk of infection in the period just after training, highlighting the need for extra care when exposed to aggressive environmental agents. This extra care is particularly important for the junior swimming age group that apparently takes longer to recover to baseline immune levels.

During the heavier training periods of the winter season an immune depression was also observed, in particular of the acquired immunity. This was accompanied by higher prevalence of upper respiratory symptoms, reinforcing the idea of a disturbed immune resilience of the swimmers and an increased susceptibility for infections. There were also evidences for a higher susceptibility of the innate immunity to the cumulative effects of the training load while acquired immunity seems to be able to adapt and recover more efficiently when the subject is allowed a period of taper.

The long term training appear to have induced an overall attenuated acute immune response to the swimming session and a subsequent longer *open window* period of susceptibility to infection, apparently more accentuated in the adolescent swimmers. Whether this altered acute response reflects positive or negative adaptive mechanisms it is difficult to say, however, it is likely that the overall changes resulted from the cumulative effects of the swimming training loads.

These findings enhance the importance of controlling immune alterations throughout the season, especially in heavy training periods and when performing consecutive high intensity training sessions without a 24 h recovery period in between, but also during the first months of the training season particularly for young athletes. Accordingly, coaches and athletes ought to implement intervention and behavioural strategies in order to contribute to maintain health conditions, preventing the onset of fatigue and associated diminished performance, thus helping to avoid illness and reaching the peak performance at competitions. Also, athletes should take special precautions during the first hours after intense training sessions.

CHAPTER VIII

References

Adams, G. R., Zaldivar, F. P., Nance, D. M., Kodesh, E., Radom-Aizik, S.Cooper, D. M. (2011). Exercise and leukocyte interchange among central circulation, lung, spleen, and muscle. *Brain Behav Immun.*

Ahmadinejad, Z., Alijani, N., Mansori, S.Ziaee, V. (2014). Common sports-related infections: a review on clinical pictures, management and time to return to sports. *Asian J Sports Med, 5*(1), 1-9.

Alves, F. (2006). A síndrome do sobretreino *Fadiga e Desempenho* (pp. 55-67): Faculdade de Motricidade Humana – Universidade Técnica de Lisboa.

Armstrong, L. E.VanHeest, J. L. (2002). The unknown mechanism of the overtraining syndrome: clues from depression and psychoneuroimmunology. *Sports Med, 32*(3), 185-209.

Aubry, A., Hausswirth, C., Louis, J., Coutts, A. J.Y, L. E. M. (2014). Functional overreaching: the key to peak performance during the taper? *Med Sci Sports Exerc, 46*(9), 1769-1777.

Baj, Z., Kantorski, J., Majewska, E., Zeman, K., Pokoca, L., Fornalczyk, E., Tchorzewski, H., Sulowska, Z.Lewicki, R. (1994). Immunological status of competitive cyclists before and after the training season. *Int J Sports Med, 15*(6), 319-324.

Bangsbo, J., Michalsik, L.Petersen, A. (1993). Accumulated O2 deficit during intense exercise and muscle characteristics of elite athletes. *Int J Sports Med, 14*(4), 207-213.

Banister, E. W. (1991). Modeling Elite Athletic Performance. In J. D. Macdougall, Wenger, H. A. & Green, H. J. (Ed.), *Physiological Testing of Elite Athletes*. Champaign, Illinois: Human Kinetics.

Bernard, A., Nickmilder, M., Voisin, C.Sardella, A. (2009). Impact of chlorinated swimming pool attendance on the respiratory health of adolescents. *Pediatrics, 124*(4), 1110-1118.

Biosciences, B. (2000). Introduction to Flow Cytometry: A Learning Guide (Vol. Manual Part Number: 11-11032-01). San Jose, CA: Becton, Dickinson and Company.

Bishop, N. C. (2006). Exercise and Infection Risk. In M. Gleeson (Ed.), *Immune function in sports and exercise* (pp. 1-14). Edinburgh: Churchill Livingstone Elsevier.

Blannin, A. K., Chatwin, L. J., Cave, R.Gleeson, M. (1996). Effects of submaximal cycling and long-term endurance training on neutrophil phagocytic activity in middle aged men. *Br J Sports Med, 30*(2), 125-129.

Blannin, A. K., Robson, P. J., Walsh, N. P., Clark, A. M., Glennon, L.Gleeson, M. (1998). The effect of exercising to exhaustion at different intensities on saliva immunoglobulin A, protein and electrolyte secretion. *Int J Sports Med, 19*(8), 547-552.

Boas, S. R., Joswiak, M. L., Nixon, P. A., Kurland, G., O'Connor, M. J., Bufalino, K., Orenstein, D. M.Whiteside, T. L. (1996). Effects of anaerobic exercise on the immune system in eight- to seventeen-year-old trained and untrained boys. *J Pediatr, 129*(6), 846-855.

Boggin, B. (1999). *Patterns of Human Growth* (Second ed.). Cambridge, UK: Cambridge University Press.

Bougault, V., Loubaki, L., Joubert, P., Turmel, J., Couture, C., Laviolette, M., Chakir, J.Boulet, L. P. (2012). Airway remodeling and inflammation in competitive

swimmers training in indoor chlorinated swimming pools. *J Allergy Clin Immunol, 129*(2), 351-358, 358 e351.

Brunelli, D. T., Rodrigues, A., Lopes, W. A., Gaspari, A. F., Bonganha, V., Montagner, P. C., Borin, J. P.Cavaglieri, C. R. (2014). Monitoring of immunological parameters in adolescent basketball athletes during and after a sports season. *J Sports Sci, 32*(11), 1050-1059.

Buchheit, M.Laursen, P. B. (2013). High-intensity interval training, solutions to the programming puzzle. Part II: anaerobic energy, neuromuscular load and practical applications. *Sports Med, 43*(10), 927-954.

Burtis, C.Ashwood, E. (1999). *Tietz TextBook of Clinical Chemistry* (3th ed.): W.B.Saunders Company.

Bury, T., Marechal, R., Mahieu, P.Pirnay, F. (1998). Immunological status of competitive football players during the training season. *Int J Sports Med, 19*(5), 364-368.

Busso, T. (2003). Variable dose-response relationship between exercise training and performance. *Med Sci Sports Exerc, 35*(7), 1188-1195.

Campbell, J. P., Riddell, N. E., Burns, V. E., Turner, M., van Zanten, J. J., Drayson, M. T.Bosch, J. A. (2009). Acute exercise mobilises CD8+ T lymphocytes exhibiting an effector-memory phenotype. *Brain Behav Immun, 23*(6), 767-775.

Conover, W. J. (1999). *Practical Nonparametric Statistics* (Third Edition ed.). New York: John Wiley & Sons.

Cordova, A., Sureda, A., Tur, J. A.Pons, A. (2010). Immune response to exercise in elite sportsmen during the competitive season. *J Physiol Biochem, 66*(1), 1-6.

Costill, D. L., Kovaleski, J., Porter, D., Kirwan, J., Fielding, R.King, D. (1985). Energy expenditure during front crawl swimming: predicting success in middle-distance events. *Int J Sports Med, 6*(5), 266-270.

Costill, D. L., Thomas, R., Robergs, R. A., Pascoe, D., Lambert, C., Barr, S.Fink, W. J. (1991). Adaptations to swimming training: influence of training volume. *Med Sci Sports Exerc, 23*(3), 371-377.

Cox, A. J., Gleeson, M., Pyne, D. B., Callister, R., Hopkins, W. G.Fricker, P. A. (2008). Clinical and laboratory evaluation of upper respiratory symptoms in elite athletes. *Clin J Sport Med, 18*(5), 438-445.

Craig, N. P., Norton, K. I., Conyers, R. A., Woolford, S. M., Bourdon, P. C., Stanef, T.Walsh, C. B. (1995). Influence of test duration and event specificity on maximal accumulated oxygen deficit of high performance track cyclists. *Int J Sports Med, 16*(8), 534-540.

Del Giacco, S. R., Scorcu, M., Argiolas, F., Firinu, D.Del Giacco, G. S. (2014). Exercise training, lymphocyte subsets and their cytokines production: experience of an Italian professional football team and their impact on allergy. *Biomed Res Int, 2014*, 429248.

Denguezli, M., Ben Chiekh, I., Ben Saad, H., Zaouali-Ajina, M., Tabka, Z.Abdelkrim, Z. (2008). One-year endurance training: effects on lung function and airway inflammation. *J Sports Sci, 26*(12), 1351-1359.

Dias, R., Frollini, A. B., Brunelli, D. T., Yamada, A. K., Leite, R. D., Simoes, R. A., Salles, G. S., Trevisan, D., Pellegrinotti, I. L., de Castro Cesar, M., Alves, S. C., Verlengia, R., Borin, J. P., Prestes, J.Cavaglieri, C. R. (2011). Immune parameters, symptoms of upper respiratory tract infections, and training-load indicators in volleyball athletes. *Int J Gen Med, 4*, 837-844.

Dill, D. B.Costill, D. L. (1974). Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. *J Appl Physiol, 37*(2), 247-248.

Dimitrov, S., Lange, T.Born, J. (2010). Selective mobilization of cytotoxic leukocytes by epinephrine. *J Immunol, 184*(1), 503-511.

Dinarello, C. A. (1997). Role of pro- and anti-inflammatory cytokines during inflammation: experimental and clinical findings. *J Biol Regul Homeost Agents, 11*(3), 91-103.

Dunn, O. J. (1964). Multiple comparisons using rank sums. *Technometrics, 6*(3), 241-252.

Fabbri, M., Smart, C.Pardi, R. (2003). T lymphocytes. *Int J Biochem Cell Biol, 35*(7), 1004-1008.

Ferrer, M. D., Tauler, P., Sureda, A., Tur, J. A.Pons, A. (2009). Antioxidant regulatory mechanisms in neutrophils and lymphocytes after intense exercise. *J Sports Sci, 27*(1), 49-58.

Foss, M. L.Keteyian, S. J. (1998). *Fox's Physiological Basis for Exercise and Sport* (6th ed.): McGraw-Hill.

Fragala, M. S., Kraemer, W. J., Denegar, C. R., Maresh, C. M., Mastro, A. M.Volek, J. S. (2011). Neuroendocrine-immune interactions and responses to exercise. *Sports Med, 41*(8), 621-639.

Fry, R. W., Morton, A. R., Crawford, G. P.Keast, D. (1992a). Cell numbers and in vitro responses of leucocytes and lymphocyte subpopulations following maximal exercise and interval training sessions of different intensities. *Eur J Appl Physiol Occup Physiol, 64*(3), 218-227.

Fry, R. W., Morton, A. R.Keast, D. (1992b). Acute intensive interval training and T-lymphocyte function. *Med Sci Sports Exerc, 24*(3), 339-345.

Gabriel, H., Kullmer, T., Schwarz, L., Urhausen, A., Weiler, B., Born, P.Kindermann, W. (1993). Circulating leucocyte subpopulations in sedentary subjects following graded maximal exercise with hypoxia. *Eur J Appl Physiol Occup Physiol, 67*(4), 348-353.

Gabriel, H., Schwarz, L., Steffens, G.Kindermann, W. (1992a). Immunoregulatory hormones, circulating leucocyte and lymphocyte subpopulations before and after endurance exercise of different intensities. *Int J Sports Med, 13*(5), 359-366.

Gabriel, H., Urhausen, A.Kindermann, W. (1992b). Mobilization of circulating leucocyte and lymphocyte subpopulations during and after short, anaerobic exercise. *Eur J Appl Physiol Occup Physiol, 65*(2), 164-170.

Giraldo, E., Garcia, J. J., Hinchado, M. D.Ortega, E. (2009). Exercise intensity-dependent changes in the inflammatory response in sedentary women: role of neuroendocrine parameters in the neutrophil phagocytic process and the pro-/anti-inflammatory cytokine balance. *Neuroimmunomodulation, 16*(4), 237-244.

Gleeson, M. (2000). Mucosal immunity and respiratory illness in elite athletes. *Int J Sports Med, 21 Suppl 1*, S33-43.

Gleeson, M. (2006). *Immune function in sports and exercise*. Edinburgh: Churchill Livingstone Elsevier.

Gleeson, M. (2007). Immune function in sport and exercise. *J Appl Physiol, 103*(2), 693-699.

Gleeson, M., Bishop, N., Oliveira, M., McCauley, T.Tauler, P. (2011). Sex differences in immune variables and respiratory infection incidence in an athletic population. *Exerc Immunol Rev, 17*, 122-135.

Gleeson, M.Bishop, N. C. (2005). The T cell and NK cell immune response to exercise. *Ann Transplant, 10*(4), 43-48.

Gleeson, M., McDonald, W. A., Cripps, A. W., Pyne, D. B., Clancy, R. L.Fricker, P. A. (1995). The effect on immunity of long-term intensive training in elite swimmers. *Clin Exp Immunol, 102*(1), 210-216.

Gleeson, M., McDonald, W. A., Pyne, D. B., Clancy, R. L., Cripps, A. W., Francis, J. L.Fricker, P. A. (2000). Immune status and respiratory illness for elite swimmers during a 12-week training cycle. *Int J Sports Med, 21*(4), 302-307.

Gleeson, M., Pyne, D. B.Callister, R. (2004). The missing links in exercise effects on mucosal immunity. *Exerc Immunol Rev, 10*, 107-128.

Gleeson, M.Williams, C. (2013). Intense exercise training and immune function. *Nestle Nutr Inst Workshop Ser, 76*, 39-50.

Goldsby, R. A., Kindt, T. J., Osborne, B. A.Kuby, J. (2003). The inflammatory process *Immunology* (5th ed.). New York: W.H. Freeman and Company.

Green, K. J., Croaker, S. J.Rowbottom, D. G. (2003). Carbohydrate supplementation and exercise-induced changes in T-lymphocyte function. *J Appl Physiol, 95*(3), 1216-1223.

Green, K. J., Rowbottom, D. G.Mackinnon, L. T. (2003). Acute exercise and T-lymphocyte expression of the early activation marker CD69. *Med Sci Sports Exerc, 35*(4), 582-588.

Hackney, A. C. (2013). Clinical management of immuno-suppression in athletes associated with exercise training: sports medicine considerations. *Acta Med Iran, 51*(11), 751-756.

Halson, S. L.Jeukendrup, A. E. (2004). Does overtraining exist? An analysis of overreaching and overtraining research. *Sports Med, 34*(14), 967-981.

Harris, M. D. (2011). Infectious disease in athletes. *Curr Sports Med Rep, 10*(2), 84-89.

Hellard, P., Avalos, M., Guimaraes, F., Toussaint, J. F.Pyne, D. B. (2015). Training-related risk of common illnesses in elite swimmers over a 4-yr period. *Med Sci Sports Exerc, 47*(4), 698-707.

Hellard, P., Avalos, M., Hausswirth, C., Pyne, D., Toussaint, J. F.Mujika, I. (2013). Identifying Optimal Overload and Taper in Elite Swimmers over Time. *J Sports Sci Med, 12*(4), 668-678.

Hellard, P., Avalos, M., Millet, G., Lacoste, L., Barale, F.Chatard, J. C. (2005). Modeling the residual effects and threshold saturation of training: a case study of Olympic swimmers. *J Strength Cond Res, 19*(1), 67-75.

Henson, D. A., Nieman, D. C., Blodgett, A. D., Butterworth, D. E., Utter, A., Davis, J. M., Sonnenfeld, G., Morton, D. S., Fagoaga, O. R.Nehlsen-Cannarella, S. L. (1999). Influence of exercise mode and carbohydrate on the immune response to prolonged exercise. *Int J Sport Nutr, 9*(2), 213-228.

Holmer, I., Stein, E. M., Saltin, B., Ekblom, B.Astrand, P. O. (1974). Hemodynamic and respiratory responses compared in swimming and running. *J Appl Physiol, 37*(1), 49-54.

Horn, P. L., Pyne, D. B., Hopkins, W. G.Barnes, C. J. (2010). Lower white blood cell counts in elite athletes training for highly aerobic sports. *Eur J Appl Physiol, 110*(5), 925-932.

Ibfelt, T., Petersen, E. W., Bruunsgaard, H., Sandmand, M.Pedersen, B. K. (2002). Exercise-induced change in type 1 cytokine-producing CD8+ T cells is related to a decrease in memory T cells. *J Appl Physiol, 93*(2), 645-648.

Ihalainen, J., Walker, S., Paulsen, G., Hakkinen, K., Kraemer, W. J., Hamalainen, M., Vuolteenaho, K., Moilanen, E.Mero, A. A. (2014). Acute leukocyte, cytokine and adipocytokine responses to maximal and hypertrophic resistance exercise bouts. *Eur J Appl Physiol, 114*(12), 2607-2616.

Issurin, V. B. (2010). New horizons for the methodology and physiology of training periodization. *Sports Med, 40*(3), 189-206.

Jones, A. M.Carter, H. (2000). The effect of endurance training on parameters of aerobic fitness. *Sports Med, 29*(6), 373-386.

Kakanis, M. W., Peake, J., Brenu, E. W., Simmonds, M., Gray, B., Hooper, S. L.Marshall-Gradisnik, S. M. (2010). The open window of susceptibility to infection after acute exercise in healthy young male elite athletes. *Exerc Immunol Rev, 16*, 119-137.

Kargotich, S., Keast, D., Goodman, C., Crawford, G. P.Morton, A. R. (1997). The influence of blood volume changes on leucocyte and lymphocyte subpopulations in elite swimmers following interval training of varying intensities. *Int J Sports Med, 18*(5), 373-380.

Kindt, T., Goldsby, R.Osborne, B. (2007). *Kuby Immunology* (6 ed.). New York: W. H. Freeman and Company.

Kruger, K., Lechtermann, A., Fobker, M., Volker, K.Mooren, F. C. (2008). Exercise-induced redistribution of T lymphocytes is regulated by adrenergic mechanisms. *Brain Behav Immun, 22*(3), 324-338.

Kruger, K.Mooren, F. C. (2014). Exercise-induced leukocyte apoptosis. *Exerc Immunol Rev, 20*, 117-134.

Kruijsen-Jaarsma, M., Revesz, D., Bierings, M. B., Buffart, L. M.Takken, T. (2013). Effects of exercise on immune function in patients with cancer: a systematic review. *Exerc Immunol Rev, 19*, 120-143.

Laires, M. J.Monteiro, C. (2008). Exercise, magnesium and immune function. *Magnes Res, 21*(2), 92-96.

Lancaster, G. I. (2006). Methods of assessing immune function. In M. Gleeson (Ed.), *Immune function in sports and exercise* (pp. 91-113). Edinburgh: Churchill Livingstone Elsevier.

Lancaster, G. I., Halson, S. L., Khan, Q., Drysdale, P., Wallace, F., Jeukendrup, A. E., Drayson, M. T.Gleeson, M. (2004). Effects of acute exhaustive exercise and chronic exercise training on type 1 and type 2 T lymphocytes. *Exerc Immunol Rev, 10*, 91-106.

Lancaster, G. I., Khan, Q., Drysdale, P., Wallace, F., Jeukendrup, A. E., Drayson, M. T.Gleeson, M. (2005). The physiological regulation of toll-like receptor expression and function in humans. *J Physiol, 563*(Pt 3), 945-955.

Lewicki, R., Tchorzewski, H., Denys, A., Kowalska, M.Golinska, A. (1987). Effect of physical exercise on some parameters of immunity in conditioned sportsmen. *Int J Sports Med, 8*(5), 309-314.

Lewis, S. M., Bain, B. J.Bates, I. (2006). *Dacie and Lewis Practical Haematology* (10 ed.): Churchill Livingstone Elsevier.

Lopes, R. F., Osiecki, R.Rama, L. M. P. L. (2011). Biochemical markers during and after an olympic triathlon race. *JEPonline, 14*(4), 87-96.

Mackinnon, L. T. (1997). Immunity in athletes. *Int J Sports Med, 18 Suppl 1*, S62-68.

Mackinnon, L. T. (2000). Chronic exercise training effects on immune function. *Med Sci Sports Exerc, 32*(7 Suppl), S369-376.

Maglischo, E. W. (2003). *Swimming Fastest – The essential reference on technique, training and program designing*: Human Kinetics.

Malina, R. M., Bouchard, C.Bar-Or, O. (2004). *Growth, Maturation and Physical Activity* (Second ed.). Champaign, Illinois: Human Kinetics.

Mars, M., Govender, S., Weston, A., Naicker, V.Chuturgoon, A. (1998). High intensity exercise: a cause of lymphocyte apoptosis? *Biochem Biophys Res Commun, 249*(2), 366-370.

Martin, R.Saller, K. (1957). *Lehrbuch der anthropologie*. Stuttgart: Fischer.

McCarthy, D. A., Grant, M., Marbut, M., Watling, M., Wade, A. J., Macdonald, I., Nicholson, S., Melsom, R. D.Perry, J. D. (1991). Brief exercise induces an immediate and a delayed leucocytosis. *Br J Sports Med, 25*(4), 191-195.

McCarthy, D. A., Macdonald, I., Grant, M., Marbut, M., Watling, M., Nicholson, S., Deeks, J. J., Wade, A. J.Perry, J. D. (1992). Studies on the immediate and delayed leucocytosis elicited by brief (30-min) strenuous exercise. *Eur J Appl Physiol Occup Physiol, 64*(6), 513-517.

McFarlin, B. K., Flynn, M. G., Stewart, L. K.Timmerman, K. L. (2004). Carbohydrate intake during endurance exercise increases natural killer cell responsiveness to IL-2. *J Appl Physiol, 96*(1), 271-275.

Meeusen, R., Duclos, M., Foster, C., Fry, A., Gleeson, M., Nieman, D., Raglin, J., Rietjens, G., Steinacker, J., Urhausen, A., European College of Sport, S.American College of Sports, M. (2013). Prevention, diagnosis, and treatment of the overtraining syndrome: joint consensus statement of the European College of Sport Science and the American College of Sports Medicine. *Med Sci Sports Exerc, 45*(1), 186-205.

Menicucci, D., Piarulli, A., Mastorci, F., Sebastiani, L., Laurino, M., Garbella, E., Castagnini, C., Pellegrini, S., Lubrano, V., Bernardi, G., Metelli, M., Bedini, R., L'Abbate, A., Pingitore, A.Gemignani, A. (2013). Interactions between immune, stress-related hormonal and cardiovascular systems following strenuous physical exercise. *Arch Ital Biol, 151*(3), 126-136.

Mignini, F., Traini, E., Tomassoni, D., Vitali, M.Streccioni, V. (2008). Leucocyte subset redistribution in a human model of physical stress. *Clin Exp Hypertens, 30*(8), 720-731.

Miles, M. P., Kraemer, W. J., Nindl, B. C., Grove, D. S., Leach, S. K., Dohi, K., Marx, J. O., Volek, J. S.Mastro, A. M. (2003). Strength, workload, anaerobic intensity and the immune response to resistance exercise in women. *Acta Physiol Scand, 178*(2), 155-163.

Millard, A. L., Valli, P. V., Stussi, G., Mueller, N. J., Yung, G. P.Seebach, J. D. (2013). Brief Exercise Increases Peripheral Blood NK Cell Counts without Immediate Functional Changes, but Impairs their Responses to ex vivo Stimulation. *Front Immunol, 4*, 125.

Molinari, L.Gasser, T. (2004). The human growth curve: distance, velocity and acceleration. In R. C. Hauspie, N. Cameron, & L. Molinari (Eds.), *Methods in Human Growth Research* (pp. 27-54). Cambridge, UK: Cambridge University Press.

Mooren, F. C., Bloming, D., Lechtermann, A., Lerch, M. M.Volker, K. (2002). Lymphocyte apoptosis after exhaustive and moderate exercise. *J Appl Physiol, 93*(1), 147-153.

Morgado, J. M., Rama, L., Silva, I., de Jesus Inacio, M., Henriques, A., Laranjeira, P., Pedreiro, S., Rosado, F., Alves, F., Gleeson, M., Pais, M. L., Paiva, A.Teixeira, A. M. (2012). Cytokine production by monocytes, neutrophils, and dendritic cells is hampered by long-term intensive training in elite swimmers. *Eur J Appl Physiol, 112*(2), 471-482.

Morgado, J. P., Monteiro, C. P., Matias, C. N., Alves, F., Pessoa, P., Reis, J., Martins, F., Seixas, T.Laires, M. J. (2014). Sex-based effects on immune changes induced by a maximal incremental exercise test in well-trained swimmers. *J Sports Sci Med, 13*(3), 708-714.

Mujika, I., Busso, T., Lacoste, L., Barale, F., Geyssant, A.Chatard, J. C. (1996a). Modeled responses to training and taper in competitive swimmers. *Med Sci Sports Exerc, 28*(2), 251-258.

Mujika, I., Chatard, J. C., Busso, T., Geyssant, A., Barale, F.Lacoste, L. (1995). Effects of training on performance in competitive swimming. *Can J Appl Physiol, 20*(4), 395-406.

Mujika, I., Chatard, J. C.Geyssant, A. (1996b). Effects of training and taper on blood leucocyte populations in competitive swimmers: relationships with cortisol and performance. *Int J Sports Med, 17*(3), 213-217.

Natale, V. M., Brenner, I. K., Moldoveanu, A. I., Vasiliou, P., Shek, P.Shephard, R. J. (2003). Effects of three different types of exercise on blood leukocyte count during and following exercise. *Sao Paulo Med J, 121*(1), 9-14.

Nehlsen-Cannarella, S. L., Nieman, D. C., Balk-Lamberton, A. J., Markoff, P. A., Chritton, D. B., Gusewitch, G.Lee, J. W. (1991a). The effects of moderate exercise training on immune response. *Med Sci Sports Exerc, 23*(1), 64-70.

Nehlsen-Cannarella, S. L., Nieman, D. C., Jessen, J., Chang, L., Gusewitch, G., Blix, G. G.Ashley, E. (1991b). The effects of acute moderate exercise on lymphocyte function and serum immunoglobulin levels. *Int J Sports Med, 12*(4), 391-398.

Nemet, D.Eliakim, A. (2010). Growth hormone-insulin-like growth factor-1 and inflammatory response to a single exercise bout in children and adolescents. *Med Sport Sci, 55*, 141-155.

Nemet, D., Mills, P. J.Cooper, D. M. (2004). Effect of intense wrestling exercise on leucocytes and adhesion molecules in adolescent boys. *Br J Sports Med, 38*(2), 154-158.

Nielsen, H. B., Secher, N. H., Christensen, N. J.Pedersen, B. K. (1996). Lymphocytes and NK cell activity during repeated bouts of maximal exercise. *Am J Physiol, 271*(1 Pt 2), R222-227.

Nieman, D. C. (1994). Exercise, upper respiratory tract infection, and the immune system. *Med Sci Sports Exerc, 26*(2), 128-139.

Nieman, D. C. (2000a). Exercise immunology: future directions for research related to athletes, nutrition, and the elderly. *Int J Sports Med, 21 Suppl 1*, S61-68.

Nieman, D. C. (2000b). Special feature for the Olympics: effects of exercise on the immune system: exercise effects on systemic immunity. *Immunol Cell Biol, 78*(5), 496-501.

Nieman, D. C. (2001). *Does Exercise Alter Immune Function and Respiratory Infections?* Washington, D.C.: President's Council on Physical Fitness and Sports.

Nieman, D. C., Berk, L. S., Simpson-Westerberg, M., Arabatzis, K., Youngberg, S., Tan, S. A., Lee, J. W.Eby, W. C. (1989). Effects of long-endurance running on immune system parameters and lymphocyte function in experienced marathoners. *Int J Sports Med, 10*(5), 317-323.

Nieman, D. C., Brendle, D., Henson, D. A., Suttles, J., Cook, V. D., Warren, B. J., Butterworth, D. E., Fagoaga, O. R.Nehlsen-Cannarella, S. L. (1995a). Immune function in athletes versus nonathletes. *Int J Sports Med, 16*(5), 329-333.

Nieman, D. C., Buckley, K. S., Henson, D. A., Warren, B. J., Suttles, J., Ahle, J. C., Simandle, S., Fagoaga, O. R.Nehlsen-Cannarella, S. L. (1995b). Immune function in marathon runners versus sedentary controls. *Med Sci Sports Exerc, 27*(7), 986-992.

Nieman, D. C., Kernodle, M. W., Henson, D. A., Sonnenfeld, G.Morton, D. S. (2000). The acute response of the immune system to tennis drills in adolescent athletes. *Res Q Exerc Sport, 71*(4), 403-408.

Nieman, D. C., Miller, A. R., Henson, D. A., Warren, B. J., Gusewitch, G., Johnson, R. L., Davis, J. M., Butterworth, D. E.Nehlsen-Cannarella, S. L. (1993). Effects of high- vs moderate-intensity exercise on natural killer cell activity. *Med Sci Sports Exerc, 25*(10), 1126-1134.

Nieman, D. C., Nehlsen-Cannarella, S. L., Donohue, K. M., Chritton, D. B., Haddock, B. L., Stout, R. W.Lee, J. W. (1991). The effects of acute moderate exercise on leukocyte and lymphocyte subpopulations. *Med Sci Sports Exerc, 23*(5), 578-585.

Nieman, D. C., Nehlsen-Cannarella, S. L., Fagoaga, O. R., Henson, D. A., Utter, A., Davis, J. M., Williams, F.Butterworth, D. E. (1998a). Effects of mode and carbohydrate on the granulocyte and monocyte response to intensive, prolonged exercise. *J Appl Physiol, 84*(4), 1252-1259.

Nieman, D. C., Nehlsen-Cannarella, S. L., Fagoaga, O. R., Henson, D. A., Utter, A., Davis, J. M., Williams, F.Butterworth, D. E. (1998b). Effects of mode and carbohydrate on the granulocyte and monocyte response to intensive, prolonged exercise. *J Appl Physiol (1985), 84*(4), 1252-1259.

Noguchi, K., Gel, Y. R., Brunner, E.Konietschke, F. (2012). nparLD: An R package for nonparametric analysis of longitudinal data for factorial designs. *Journal of Statistical Software, 50*(12), 1-23.

Ortega, E., Collazos, M. E., Maynar, M., Barriga, C.De la Fuente, M. (1993). Stimulation of the phagocytic function of neutrophils in sedentary men after acute moderate exercise. *Eur J Appl Physiol Occup Physiol, 66*(1), 60-64.

Ottaviani, E.Franceschi, C. (1996). The neuroimmunology of stress from invertebrates to man. *Prog Neurobiol, 48*(4-5), 421-440.

Parslow, T., Stites, D., Terr, A.Imboden, J. (1997). *Medical Immunology* (10 ed.): McGrawHill.

Peake, J. M., Markworth, J. F., Nosaka, K., Raastad, T., Wadley, G. D.Coffey, V. G. (2015). Modulating exercise-induced hormesis: does less equal more? *J Appl Physiol (1985)*, jap 01055 02014.

Pedersen, B. K., Steensberg, A.Schjerling, P. (2001). Exercise and interleukin-6. *Curr Opin Hematol, 8*(3), 137-141.

Pyne, D. B. (1994). Regulation of neutrophil function during exercise. *Sports Med, 17*(4), 245-258.

Pyne, D. B.Gleeson, M. (1998). Effects of intensive exercise training on immunity in athletes. *Int J Sports Med, 19 Suppl 3*, S183-191; discussion S191-184.

Radbruch, A. (2000). *Flow Cytometry and Cell Sorting* (2nd ed.). New York: Springer.

Rama, L., Teixeira, A. M., Matos, A., Borges, G., Henriques, A., Gleeson, M., Pedreiro, S., Filaire, E., Alves, F.Paiva, A. (2013). Changes in natural killer cell subpopulations over a winter training season in elite swimmers. *Eur J Appl Physiol, 113*(4), 859-868.

Rebelo, A. N., Candeias, J. R., Fraga, M. M., Duarte, J. A., Soares, J. M., Magalhaes, C.Torrinha, J. A. (1998). The impact of soccer training on the immune system. *J Sports Med Phys Fitness, 38*(3), 258-261.

Reid, V. L., Gleeson, M., Williams, N.Clancy, R. L. (2004). Clinical investigation of athletes with persistent fatigue and/or recurrent infections. *Br J Sports Med, 38*(1), 42-45.

Reis, J. F., Alves, F. B., Bruno, P. M., Vleck, V.Millet, G. P. (2012). Oxygen uptake kinetics and middle distance swimming performance. *J Sci Med Sport, 15*(1), 58-63.

Robson, P. J., Blannin, A. K., Walsh, N. P., Castell, L. M.Gleeson, M. (1999). Effects of exercise intensity, duration and recovery on in vitro neutrophil function in male athletes. *Int J Sports Med, 20*(2), 128-135.

Rose, P. (2002). *Forensic Speaker Identification.* London & New York: Taylor and Francis.

Rowbottom, D. G.Green, K. J. (2000). Acute exercise effects on the immune system. *Med Sci Sports Exerc, 32*(7 Suppl), S396-405.

Sa, C. S., Boaventura, R. A.Pereira, I. B. (2011). Analysis of trihalomethanes in water and air from indoor swimming pools using HS-SPME/GC/ECD. *J Environ Sci Health A Tox Hazard Subst Environ Eng, 46*(4), 355-363.

Sargent, C., Halson, S.Roach, G. D. (2014). Sleep or swim? Early-morning training severely restricts the amount of sleep obtained by elite swimmers. *Eur J Sport Sci, 14 Suppl 1*, S310-315.

Sharp, R. L. (1993). Prescribing and Evaluating Interval Training Sets in Swimming: A Proposed Model. *Journal of Swimming Research, 9*, 36-40.

Shephard, R. J. (2003). Adhesion molecules, catecholamines and leucocyte redistribution during and following exercise. *Sports Med, 33*(4), 261-284.

Shephard, R. J. (2010). Development of the discipline of exercise immunology. *Exerc Immunol Rev, 16*, 194-222.

Shephard, R. J.Shek, P. N. (1999). Effects of exercise and training on natural killer cell counts and cytolytic activity: a meta-analysis. *Sports Med, 28*(3), 177-195.

Simpson, R. J., Cosgrove, C., Chee, M. M., McFarlin, B. K., Bartlett, D. B., Spielmann, G., O'Connor, D. P., Pircher, H.Shiels, P. G. (2010). Senescent phenotypes and telomere lengths of peripheral blood T-cells mobilized by acute exercise in humans. *Exerc Immunol Rev, 16*, 40-55.

Simpson, R. J., Florida-James, G. D., Whyte, G. P., Black, J. R., Ross, J. A.Guy, K. (2007). Apoptosis does not contribute to the blood lymphocytopenia observed after intensive and downhill treadmill running in humans. *Res Sports Med, 15*(3), 157-174.

Simpson, R. J., McFarlin, B. K., McSporran, C., Spielmann, G., o Hartaigh, B.Guy, K. (2009). Toll-like receptor expression on classic and pro-inflammatory blood monocytes after acute exercise in humans. *Brain Behav Immun, 23*(2), 232-239.

Smith, L. L. (2004). Tissue trauma: the underlying cause of overtraining syndrome? *J Strength Cond Res, 18*(1), 185-193.

Sothern, M. S., Loftin, M., Suskind, R. M., Udall, J. N.Blecker, U. (1999). The health benefits of physical activity in children and adolescents: implications for chronic disease prevention. *Eur J Pediatr, 158*(4), 271-274.

Spence, L., Brown, W. J., Pyne, D. B., Nissen, M. D., Sloots, T. P., McCormack, J. G., Locke, A. S.Fricker, P. A. (2007). Incidence, etiology, and symptomatology of upper respiratory illness in elite athletes. *Med Sci Sports Exerc, 39*(4), 577-586.

Spencer, M. R.Gastin, P. B. (2001). Energy system contribution during 200- to 1500-m running in highly trained athletes. *Med Sci Sports Exerc, 33*(1), 157-162.

Starkie, R. L., Rolland, J., Angus, D. J., Anderson, M. J.Febbraio, M. A. (2001). Circulating monocytes are not the source of elevations in plasma IL-6 and TNF-alpha levels after prolonged running. *Am J Physiol Cell Physiol, 280*(4), C769-774.

Steensberg, A., Toft, A. D., Bruunsgaard, H., Sandmand, M., Halkjaer-Kristensen, J.Pedersen, B. K. (2001a). Strenuous exercise decreases the percentage of type 1 T cells in the circulation. *J Appl Physiol, 91*(4), 1708-1712.

Steensberg, A., Toft, A. D., Schjerling, P., Halkjaer-Kristensen, J.Pedersen, B. K. (2001b). Plasma interleukin-6 during strenuous exercise: role of epinephrine. *Am J Physiol Cell Physiol, 281*(3), C1001-1004.

Suda, Y., Umeda, T., Watanebe, K., Kuroiwa, J., Sasaki, E., Tsukamoto, T., Takahashi, I., Matsuzaka, M., Iwane, K.Nakaji, S. (2013). Changes in neutrophil functions during a 10-month soccer season and their effects on the physical condition of professional Japanese soccer players. *Luminescence, 28*(2), 121-128.

Suwa, T., Hogg, J. C., English, D.Van Eeden, S. F. (2000). Interleukin-6 induces demargination of intravascular neutrophils and shortens their transit in marrow. *Am J Physiol Heart Circ Physiol, 279*(6), H2954-2960.

Suzui, M., Kawai, T., Kimura, H., Takeda, K., Yagita, H., Okumura, K., Shek, P. N.Shephard, R. J. (2004). Natural killer cell lytic activity and CD56(dim) and CD56(bright) cell distributions during and after intensive training. *J Appl Physiol (1985), 96*(6), 2167-2173.

Suzuki, K., Nakaji, S., Yamada, M., Liu, Q., Kurakake, S., Okamura, N., Kumae, T., Umeda, T.Sugawara, K. (2003). Impact of a competitive marathon race on systemic cytokine and neutrophil responses. *Med Sci Sports Exerc, 35*(2), 348-355.

Sweetenham, B.Atkinson, J. (2003). *Championship Swim Training*: Human Kinetics.

Tanner, J. M. (1962). *Growth at Adolescence*. Oxford: Blackwell Scientific Publications.

Tanner, J. M. (1978). Human growth standard: construction and use. In L. Gedda & P. Parisi (Eds.), *Auxology: human growth in health disorders* (2nd ed., pp. 109-121). London: Academic Press.

Tauler, P., Ferrer, M. D., Romaguera, D., Sureda, A., Aguilo, A., Tur, J.Pons, A. (2008). Antioxidant response and oxidative damage induced by a swimming session: influence of gender. *J Sports Sci, 26*(12), 1303-1311.

Teixeira, A. M., Rama, L., Carvalho, H. M., Borges, G., Carvalheiro, T., Gleeson, M., Alves, F., Trindade, H.Paiva, A. (2014). Changes in naive and memory T-cells in elite swimmers during a winter training season. *Brain Behav Immun, 39*, 186-193.

Timmons, B. W.Bar-Or, O. (2007). Evidence of sex-based differences in natural killer cell responses to exercise and carbohydrate intake in children. *Eur J Appl Physiol, 101*(2), 233-240.

Timmons, B. W., Hamadeh, M. J., Devries, M. C.Tarnopolsky, M. A. (2005). Influence of gender, menstrual phase, and oral contraceptive use on immunological changes in response to prolonged cycling. *J Appl Physiol, 99*(3), 979-985.

Timmons, B. W., Hamadeh, M. J.Tarnopolsky, M. A. (2006a). No effect of short-term 17beta-estradiol supplementation in healthy men on systemic inflammatory responses to exercise. *Am J Physiol Regul Integr Comp Physiol, 291*(2), R285-290.

Timmons, B. W., Tarnopolsky, M. A.Bar-Or, O. (2004). Immune responses to strenuous exercise and carbohydrate intake in boys and men. *Pediatr Res, 56*(2), 227-234.

Timmons, B. W., Tarnopolsky, M. A.Bar-Or, O. (2006b). Sex-based effects on the distribution of NK cell subsets in response to exercise and carbohydrate intake in adolescents. *J Appl Physiol, 100*(5), 1513-1519.

Timmons, B. W., Tarnopolsky, M. A., Snider, D. P.Bar-Or, O. (2006c). Immunological changes in response to exercise: influence of age, puberty, and gender. *Med Sci Sports Exerc, 38*(2), 293-304.

Toussaint, H. M.Beek, P. J. (1992). Biomechanics of competitive front crawl swimming. *Sports Med, 13*(1), 8-24.

Verde, T., Thomas, S.Shephard, R. J. (1992). Potential markers of heavy training in highly trained distance runners. *Br J Sports Med, 26*(3), 167-175.

Vleck, V., Millet, G. P.Alves, F. B. (2014). The impact of triathlon training and racing on athletes' general health. *Sports Med, 44*(12), 1659-1692.

Walsh, N. P., Gleeson, M., Shephard, R. J., Woods, J. A., Bishop, N. C., Fleshner, M., Green, C., Pedersen, B. K., Hoffman-Goetz, L., Rogers, C. J., Northoff, H., Abbasi, A.Simon, P. (2011). Position statement. Part one: Immune function and exercise. *Exerc Immunol Rev, 17*, 6-63.

World Medical Association. (2008). Declaration of Helsinki - Ethical Principles for Medical Research involving Human Subjects. *WMJ, 54*(4), 122-125.

Yamada, M., Suzuki, K., Kudo, S., Totsuka, M., Simoyama, T., Nakaji, S.Sugawara, K. (2000). Effect of exhaustive exercise on human neutrophils in athletes. *Luminescence, 15*(1), 15-20.

Zamparo, P., Capelli, C.Pendergast, D. (2011). Energetics of swimming: a historical perspective. *Eur J Appl Physiol, 111*(3), 367-378.

Zhang, X., Matsuo, K., Farmawati, A., Higashi, Y., Ogawa, K., Nagata, K.Nagatomi, R. (2006). Exhaustive exercise induces differential changes in serum granulysin and circulating number of natural killer cells. *Tohoku J Exp Med, 210*(2), 117-124.

APPENDICES

Appendix A: Informed consent

Universidade de Lisboa
FACULDADE DE MOTRICIDADE HUMANA
Laboratório de Fisiologia e Bioquímica do Exercício

CONSENTIMENTO INFORMADO

O estudo intitulado *Variações Imunitárias em Nadadores de Competição: Resposta ao Exercício Agudo e ao Treino*, pretende analisar as alterações induzidas pelo exercício agudo e crónico em indicadores associados ao sistema imunitário e endócrino. O estudo decorrerá durante a época competitiva de Natação Pura Desportiva 2011/2012 sendo a participação voluntária.

Aos participantes serão aplicados questionários maturacionais e de controlo da percepção subjectiva de esforço e de recuperação. Efectuar-se-á o registo de sintomas associados a episódios de Infecções do Tracto Respiratório Superior e também do consumo energético e nutricional. Solicitar-se-á a recolha de amostras de saliva e amostras sanguíneas, através de venopunção, ao acordar e após a realização de sessões de treino enquadradas em diferentes momentos de preparação da época desportiva. Nestas alturas serão também avaliadas as características físicas e antropométricas e a Variabilidade da Frequência Cardíaca.

Asseguramos que a totalidade dos procedimentos utilizados na recolha dos dados são perfeitamente inofensivos do ponto de vista clínico e que serão realizados por profissionais habilitados para o efeito. Os resultados serão apenas utilizados para investigação não sendo divulgados a não ser ao próprio. Este estudo enquadra-se no âmbito do projecto de doutoramento com o mesmo nome, aprovado pela Comissão de Ética da Faculdade de Motricidade Humana – FMH. Todas as dúvidas serão esclarecidas com os investigadores participantes ou com o coordenador do projecto.

O grupo de investigação agradece a sua participação neste estudo.

DECLARAÇÃO

Declaro que, voluntariamente, aceito participar neste estudo e que estou esclarecido quanto aos objectivos e procedimentos do mesmo.

_____   _____
O participante   O Encarregado de Educação

Appendix B: Tanner questionnaires

CARACTERÍSTICAS SEXUAIS FEMININAS
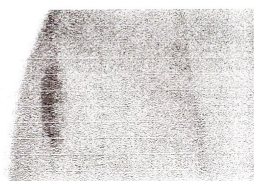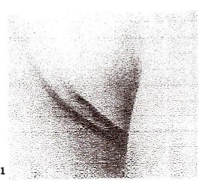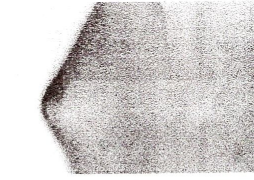
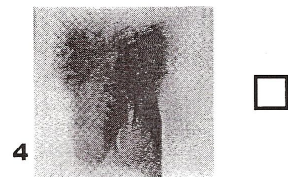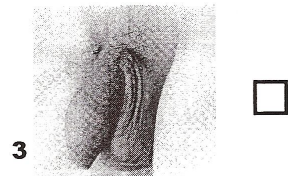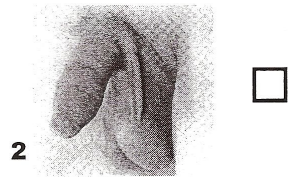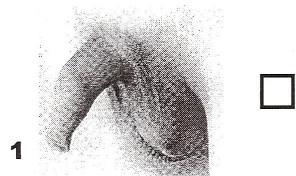ASSINALA COM ☒ O ESTÁDIO DE MATURAÇÃO EM QUE TE ENCONTRAS

Nº DE IDENTIFICAÇÃO: _____   DATA DA MENARCA: _____ / _____
                                                        (mês)   (ano)

(1)	Ausência de pêlos púbicos e de desenvolvimento da mama	☐
(2)	Ausência de pêlos e existência de desenvolvimento mamário sem menarca	☐
(2)	Existência de pêlos e ausência de desenvolvimento mamário sem menarca	☐
(3)	Existência de pêlos púbicos e desenvolvimento da mama mas sem menarca	☐
(4)	Existência de pêlos púbicos e desenvolvimento da mama, com menarca entre 0 - 3 anos	☐
(5)	Mais de 3 anos após a ocorrência da menarca	☐

DESENVOLVIMENTO MAMÁRIO   **QUANTIDADE DE PÊLOS PÚBICOS**

CARACTERÍSTICAS SEXUAIS MASCULINAS

ASSINALA COM ⊠ O ESTÁDIO DE MATURAÇÃO EM QUE TE ENCONTRAS

Nº DE IDENTIFICAÇÃO: _____

(1)   Ausência de pêlos púbicos e de alteração de voz   ☐
(2)   Existência de pêlos púbicos sem alteração de voz   ☐
(3)   Existência de pêlos púbicos e de alteração de voz há menos de 2 anos   ☐
(4)   Existência de pêlos púbicos e de alteração de voz entre 2-3 anos   ☐
(5)   Existência de pêlos púbicos e de alteração de voz há mais de 3 anos   ☐

DESENVOLVIMENTO GENITAL   **QUANTIDADE DE PÊLOS PÚBICOS**
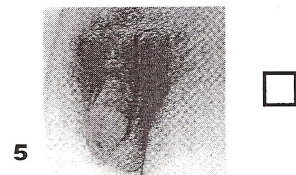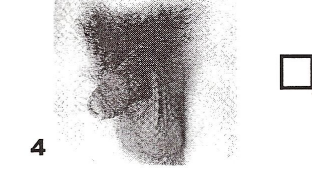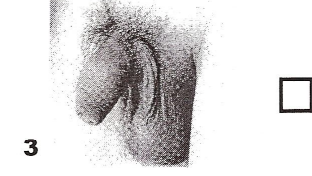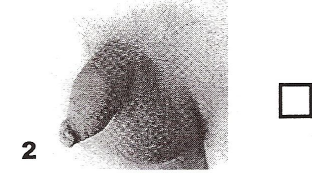
Appendix C: Upper Respiratory Symptoms daily logbook

Natação –   Juvenis, Juniores e Seniores

Nome: **Código:**

Semana de _ _ _ _ a _ _ _ _ de _ _ _ _ _ _ _ _ _

Diário de Registo de Ocorrências (S- sim; N-não)

Sintomas de ITRS / Dias da semana	Segunda	Terça	Quarta	Quinta	Sexta	Sábado	Domingo
Dores de cabeça							
Febre							
Tosse							
Náuseas/Vómito							
Otite/Dôr de ouvidos							
Faringite/Amigdalite							
Bronquite							
Asma							
Expectoração							
Comichão nos olhos							
Diarreia							
Corrimento nasal/ nariz entupido							
Prescrições médicas/Medicação/Suplementos (marca e quantidade)							
Data da última *Menstruação*							

Faculdade de Motricidade Humana - FMH   Laboratório de Fisiologia e Bioquímica do Exercício