

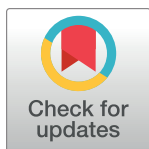
RESEARCH ARTICLE

What is the effect of a Mediterranean compared with a Fast Food meal on the exercise induced adipokine changes? A randomized cross-over clinical trial

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Abstract

Background

Adipose tissue-derived adipokines are pro-inflammatory cytokines involved in metabolic-related diseases and can be influenced by diet and exercise. We aimed to compare the effect of a Mediterranean (MdM) compared with Fast Food (FFM) meal on the exercise induced adipokines changes.

Methods

In a double blinded cross over trial, 46 participants were randomly assigned to one of two standardized iso-energy pre-exercise meals: FFM or MdM-type. Three hours after each meal, participants completed a treadmill exercise test (EC). Serum adiponectin, resistin, PAI-1, lipocalin-2/NGAL and adipsin were determined by Luminex magnetic bead immunoassay. Wilcoxon signed rank test compared changes before/after meal and before/after EC and a linear mixed model evaluated the effect of meals on the adipokine response to exercise, adjusted for confounders.

Results

Thirty-nine participants (mean age of 25, with a standard deviation of 5 years) completed the trial (56% females). For both interventions, a significant reduction of adipsin after each meal and a significant increase of lipocalin, PAI-1, adipsin and resistin, after exercise was observed. When exercise was preceded by a MdM meal a higher increase in adipsin levels was seen.

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Conclusion

Acute exercise induced an increase of circulatory levels of adipisin, resistin, lipocalin and PAI-1, but not adiponectin. A pre-exercise Mediterranean meal potentiated the increase of adipisin after the exercise test, which possibly relates to the immune regulatory role of adipisin. These changes suggest a cross-talk between the immune and metabolic immediate response to exercise and its modulation by the pre-exercise diet composition.

Introduction

Unhealthy dietary intake and sedentary behavior in a genetically susceptible individual have been associated with adipokine dysregulation resulting both in adverse metabolic and immune responses [1]. Adiposity modulates inflammation and metabolic balance, both through adipokines, like adiponectin, as well as cytokines produced within the non-adipocyte tissue fraction, namely resistin [2]. These biomarkers might mediate the effect of obesity on other inflammatory diseases, namely in asthma [3]. This pro-inflammatory profile may be counteracted by exercise-induced fatness decrease [4]. Changes in dietary content, towards an increase of omega-3-fatty acid intake, induced an increase in adiponectin levels [5]. Taken together, evidence suggests that dietary habits and physical activity, in a long-term perspective, may modulate these adipokine influences on the metabolic and immune profile. Nevertheless, the effects of short-term interventions such as a single meal and an exercise bout remain poorly recognized.

Traditional Mediterranean diet is the dietary pattern that has been linked to a number of metabolic and health benefits, including reduced mortality risk and lower incidence of cardiovascular disease [6, 7] and reducing airway inflammation in asthma [8]. The benefits of even short periods of adherence to Mediterranean diet on inflammatory markers have been shown [9] and the adipose tissue inflammatory response, measured by IL-6, MCP-1, leptin and adiponectin gene expression [10], can be modulated by a single meal. A study comparing fast food with organic beef or turkey meal showed that the lesser the quantity of saturated and trans fatty acids, the higher the decrease in LDL-cholesterol [11].

Long-term aerobic and resistance training have also known metabolic and inflammatory positive effects [12]. Recently, it has been shown that higher intensity interval training seems to be more beneficial in improving cardiometabolic outcomes than moderate long-term exercise [13]. Adiponectin increased with exercise training both in adults [14] and children [15]. However, acute exercise did not show consistent results and only few studies have studied other adipose tissue-derived factors in the acute response to exercise [12]. A single exercise bout induces consistent immune changes [2] and increases serum inflammatory markers, particularly in the obese individuals [16]. Immune response to acute exercise may reflect a transient and time-dependent redistribution of immune cells to peripheral tissues, resulting in a heightened state of immune surveillance and regulation [17]. These beneficial effects might correlate with changes in the adipokine profile. Feeding status before moderate-intensity exercise influenced the adipose tissue adipokine gene expression, but did not affected circulating levels of adiponectin or leptin [18]. The effect of different meals on the adipokine response to acute high-intensity exercise has not been exhaustively studied. Therefore, we aimed to evaluate the effect of a Mediterranean (MdM) compared with a Fast Food (FFM) iso-energy meal on the acute exercise-induced adipokines changes.

Methods

Study design

This is a randomized crossover clinical trial, comparing the effect a high fat micronutrient poor meal, fast-food like meal (FFM) versus an iso-energy similar, micronutrient rich, Mediterranean like meal (MdM) in the immediate adipokine response to an exercise challenge (EC). A wash-out period of 7 days was performed after which participants crossed over to the alternate sequence (Fig 1). Study length was selected to reach complete normalization of post prandial inflammation and plasma lipid levels and avoid carry-over of the exercise response [19, 20].

Participants were evaluated at baseline for sociodemographic, lifestyle habits, as well as previous medical history. Physical activity and diet were assessed during the 7 days washing-out period. On the days of interventions participants were requested to have the same breakfast, avoiding caffeine and abstain smoking 12h before the meal. Main outcomes were evaluated immediately before, three hours after each meal and immediately after EC. This trial was previously registered in clinicaltrials.gov NCT02027675 [21]. All procedures were approved by the ethics committee of Centro Hospitalar de São João, E.P.E. (CES 318–12) and written informed consent was obtained from the participants.

Participants

Subjects aged between 18 and 35 years old were eligible and invited to participate through public advertising, accordingly to the previously described recruitment strategy [21]. Exclusion criteria were previously detailed and, briefly were: suffer from any major systemic disease [21]; pregnant or breastfeeding; under dietary restrictions or weight losing diet the last 3 months.

Randomization, allocation and sample size

Participants were randomly assigned to the intervention order in a double-blinded fashion [21], stratified by asthma diagnosis. Outcomes were measured blinded to the participant's allocation order.

Sample size was not specifically calculated to assess adipokine effect of meal-exercise challenge, as this was a secondary outcome of the study protocol. The power to evaluate a difference was calculated using liquid meal challenge differences for resistin in comparison with fasting [22], as no other previous studies have addressed the adipokine response to EC, assuming a minimal detectable difference in means of -0.6, a within-patient standard deviation of 1 with a sample of 40 participants the probability to detect treatment difference, using a paired sample t-test, at a two-sided 0.05 significance level will reach 74% power.

Meal and exercise challenge

Meals were built to be energetically similar, respecting the Dietary Reference Intakes for macronutrient distribution. The FFM, consisted on a burger, BigMac, with medium size portion of French fries and a cola-drink and the MdM, consisted of vegetable soup, pasta, tomato, olive oil, herbs, garlic, bread, sardines, fruit and water. Design, preparation and detailed composition of the meals were previously detailed [21]. Meals nutritional composition are summarized in S1 Table. After each meal, participants were not allowed to eat or drink.

Exercise challenge was performed with a progressive multistage exercise testing, accordingly to Bruce Protocol, in a treadmill, until exhaustion [23]. Ventilation and respiratory gas analysis were measured via dynamic breath-by-breath measurement using the Oxycon Pro Metabolic Cart, Jaeger, Hochberg, Germany. There were no differences after FFM or MdM in

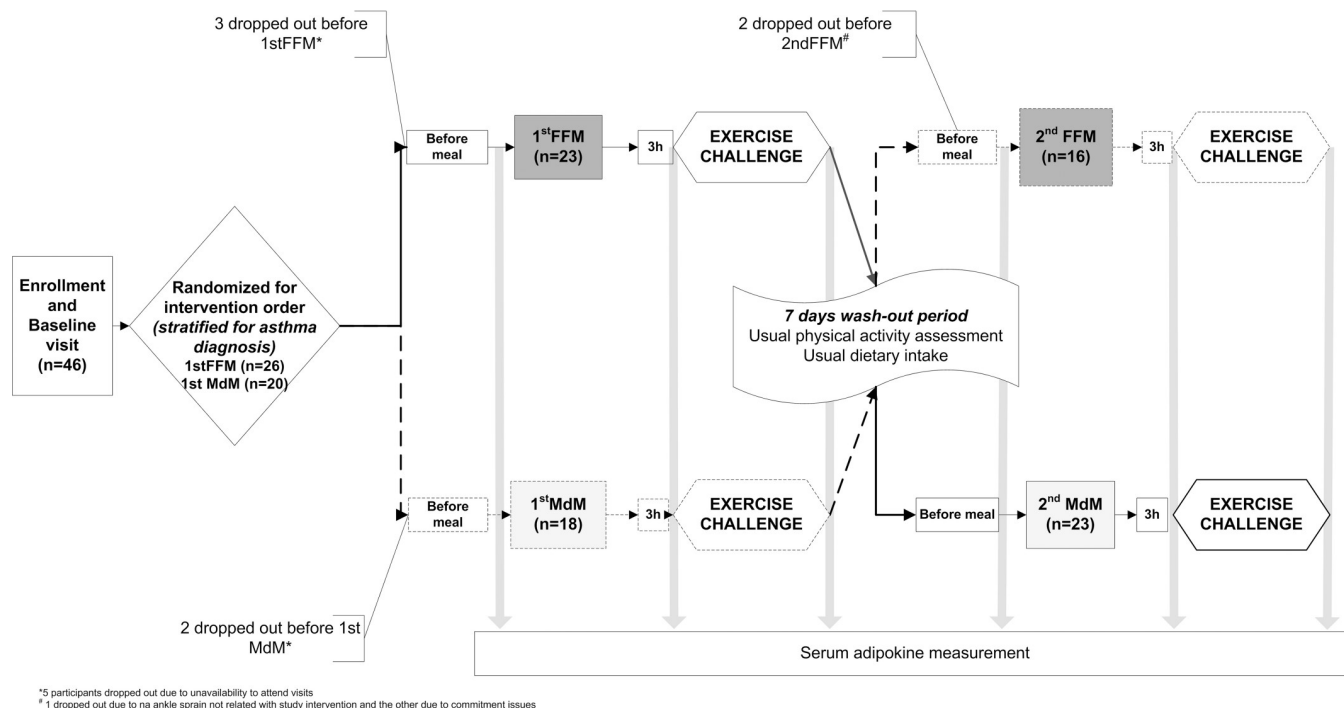


Fig 1. Flowchart of study participants.

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each group for all the ventilatory, respiratory parameters and in median duration of the EC, as previously published [21].

Adipokine and cortisol measurement

Blood was obtained by venipuncture from an antecubital vein and collected into two Terumo Venosafe serum-gel tubes 10 ml. Samples were centrifuged for 10 min at 400xg and the serum stored at -80°C. Adipokines were measured in serum, analyzed in duplicates using a bioassay multiplex technology, based on Luminex xMAP with a human adipokine panel (HADK1-MAG-61K, EMD Millipore) that included adiponectin, resistin, PAI-1 (total), lipocalin-2/NGAL and adipsin, as previously described [24]. The intra-assay coefficients of variation (CV) ranged from 6% for adiponectin, adipsin and lipocalin to 10% for resistin and PAI-I. The inter-assay CV, evaluated in the repeated samples, ranged from 16% for adipsin, 26% for lipocalin, 27% for resistin and adiponectin and 29% for PAI-1.

Serum cortisol was measured in duplicate using a solid phase enzyme-linked immunosorbent assay (DRG Cortisol ELISA Kit) in the automatic ELISA Triturus analyser.

Anthropometric, diet and physical activity evaluations

Height was measured using a portable stadiometer (SECA model 214). Weight and body composition using a digital scale Tanita BC-418. Usual dietary intake was assessed during wash-out period using a three-consecutive day food diary, two weekdays and one day during the weekend [25]. Nutrient analysis was performed using Food Processor, complemented with Portuguese Food Composition Table [26].

Physical activity was evaluated during the wash out-period in seven consecutive days by accelerometry (ActigraphWGT3Xmonitor) [21]. Troiano 2008[27] cut points were used. Data was presented as average time spent in moderate and in vigorous physical activity per day.

Lung function, asthma and rhinitis diagnosis

Clinical asthma and rhinitis diagnosis were determined at baseline, as well as asthma pattern, accordingly to Global Initiative for Asthma recommendations [28] and diagnosis was established by a physician. Those individuals under regular asthma treatment were requested to maintain treatment. Lung function was evaluated by spirometry (Spirobank, Winspiro pro software, MIR) [29]. Asthma and rhinitis control assessed using Control of Allergic Rhinitis and Asthma Test presented a good overall control of the diseases.

Skin prick tests with common aeroallergens (*Dermatophagoides pteronyssinus*, grass pollen and weed pollen mixture, cat and dog epithelium, Alternaria) (Hal-allergy) were performed to define atopy if any positive test was found [30].

Table 1. Baseline characteristics of the study participants.

	Total (n = 46)
Age years, mean(SD)	25 (5)
Female, n(%)	26(57)
Caucasian, n(%)	43(94)
Body mass index (BMI)	23.6[21.2;27.8]
Free fat mass (kg)	49.2[44.3;63.9]
Body fat (%), mean(SD)	22.3(7.2)
Smoking habits, active smokers, n(%)	7(15)
Atopy, n(%)	32(70)
Rhinitis diagnosis, n(%)	19 (41)
Asthma diagnosis, n(%)	13 (28)
CARAT score	24.5[23.0;29.0]
Lung function	
FVC %prev, mean(SD)	105(14)
FEV1%prev, mean(SD)	101(10)
FEV1/FVC %	104 [99;108]
FeNO,ppb	24 [15;47]
Cortisol(ng/ml)	144.0[100.1;193.0]
Dietary Intake	
Energy kcal	2392(586)
Carbohydrates, %TEV	51.2(6.6)
Protein, % TEV	16.7(3.4)
Lipids, % TEV	30.1(5.4)
Fiber, g/day	17.0[14.0;24.1]
Physical activity	
Average MVPA(min/day) mean(SD)	46.7(20.2)
Total time in sedentary activity (min) mean(SD)	2997(525)
Total time in moderate activity(min)] mean(SD)	241(110)
Total time in vigorous activity(min)	7 [0;37]

Data is presented as median and interquartile range [IQR], except if otherwise specified. MVPA- moderate vigorous physical activity; SD- standard deviation; TEV-total energy value

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Statistics

All statistical analyses were performed using the SPSS 24.0[31] or STATA 15[32] software. All graphics were made with GraphPad Prism 7. Normality of data distribution was assessed by the Kolmogorov-Smirnov test and QQ plot distribution. Results were presented as mean and standard deviation or as median and inter-quartile range, if data was not normally distributed, and in discrete variables as count and percentage. Count and percentage were analyzed with Pearson chi-square or Fisher exact test. For comparison between groups, non-parametric tests were used for paired samples Wilcoxon signed rank test was applied. For mixed model analysis, in data with skewed distributions (adiponectin, resistin and lipocalin) logarithmic transformation was done, as normal distribution was achieved analysis were made with this change. An analysis by intention to treat was planned. For missing data, Little's MCAR test was performed, as missing completely at random was verified, a complete case analysis was done. Analysis for the carryover effect was performed using a two-way mixed ANOVA. A linear mixed model was performed with a random intercept for intervention order, and an autoregressive variance-covariance structure of order 1 used. The model was adjusted for the following confounders: age, gender, race, asthma diagnosis, BMI, FFM, smoking habits, moderate and vigorous physical activity, cortisol and diet. Data from the model was presented as marginal mean estimate with 95% confidence interval. Spearman correlations were performed to evaluate the relation between the several adipokine responses.

Results

After recruitment strategy, from participants that met the eligibility criteria, 61 attended the screening visit. A total of 46 participants were included, of those 5 withdraw before any of the interventions and 2 before the second meal (Fig 1). Baseline demographic and clinical characteristics of the participants are presented in Table 1.

Cortisol

No differences in serum cortisol changes existed between meals (MdM median -39.10 ng/ml 95% CI [-88.35;7.60] and FFM -31.75 [-81.13; -0.50]). An increase after exercise bout was seen, though being more pronounced after the FFM no difference between meals were observed (MdM 13.30 CI 95% [-17.05;65.40], $p = 0.03$ and FFM 32.90 CI 95% [-12.25;86.13], $p = 0.02$).

Adipokines

No differences on the exercise induced adipokine changes existed between MdM and FFM meals using non-adjusted data (Table 2). When adjusted for confounders, MdM caused a significantly exercise induced increase of adiponectin response (Fig 2).

Meals did not significantly affect the levels of adiponectin, resistin, lipocalin or PAI-1 (Table 2). After exercise, levels of resistin, lipocalin or PAI-1 increased (Table 2). When assessed with the linear mixed model, after adjustment for confounders, the effect of exercise remained significant, independently of the pre-exercise meal (Fig 2). No carry-over effects were seen, and no interaction occurred between intervention order and time for all adipokine measured (S2 Table).

Adipokines moderately correlated with each other in their response to the MdM (Table 3). When FFM was the intervention, adiponectin only correlated to adiponectin and adiponectin lost its correlation with lipocalin and resistin. Lipocalin and resistin responses were strongly and significantly correlated both in their response to each meal as well as to the exercise challenge (Table 3).

Table 2. Comparison between Mediterranean Meal(MdM) and Fast Food Meal (FFM) effects on serum adipokine response before and after each meal and after exercise challenge (EC) (n = 39).

								p Δ meals	p EC
	Before	After Meal	p	After exercise	p*	Δ Exercise-baseline	p#		
Adiponectin (μg/ml)								0.971	0.429
MdM	19.86 [14.83;47.40]	18.57 [15.45;35.85]	0.390	20.75 [15.10;39.31]	0.536	0.89 [-2.50;2.42]	0.840		
FFM	20.98 [16.25;35.49]	18.96 [15.51;35.51]	0.350	20.94 [15.73;33.01]	0.180	0.88 [-3.46;4.78]	0.301		
Adipsin (ng/ml), mean (SD)								0.814	0.168
MdM	3721.08(1342.67)	3353.26(1320.62)	0.049	4214.80(1849.98)	<0.001	493.72(1266.97)	0.017		
FFM	3600.09(1325.07)	3191.47(1123.51)	<0.001	3727.13(1256.79)	<0.001	127.04(905.11)	0.386		
Lipocalin (ng/ml)								0.587	0.833
MdM	541.90 [421.90;1015.86]	545.39 [401.10;1016.64]	0.953	887.04 [558.50;1149.74]	0.007	186.81 [-13.17;457.17]	0.002		
FFM	494.40 [364.61;858.3]	612.87 [358.67;957.89]	0.199	890.26 [523.93;1035.00]	0.009	184.67 [-37.10;509.96]	0.001		
PAI-1 (ng/ml), mean (SD)								0.618	0.573
MdM	82.78(40.77)	80.56(42.31)	0.622	95.36(49.40)	0.005	12.58(28.61)	<0.001		
FFM	78.22(41.27)	78.28(44.61)	0.930	100.66(44.75)	<0.001	22.44(34.46)	<0.001		
Resistin (ng/ml)								0.811	0.777
MdM	35.59 [27.34;43.88]	36.23 [23.83;44.53]	0.683	38.14 [28.67;55.23]	0.010	7.51 [0.38;15.10]	0.007		
FFM	31.87 [24.19;41.86]	32.7 [25.70;39.94]	0.766	41.79 [3097;54.57]	<0.001	9.28 [0.53;20.33]	<0.001		

Data is expressed in median and interquartile range, except if otherwise specified. p values were performed using Wilcoxon signed rank test, except for adipsin and PAI-1 where paired sample t-test were used; SD-standard deviation

* between after and before exercise challenge(EC)

between EC and baseline

<https://doi.org/10.1371/journal.pone.0215475.t002>

When considering the response to an exercise bout, correlations were weaker and lost statistical significance after MdM-exercise challenge between adiponectin and resistin or lipocalin and between the associations of PAI-1 and Lipocalin or adipsin (**Table 3**). After FFM-exercise challenge adiponectin showed a mild correlation with resistin and lipocalin. No strong correlations were seen between adipokine and cortisol changes with exercise stimulus (**S3 Table**).

Discussion

Our study shows that a Mediterranean meal (MdM) may blunt the adipsin immediate response and potentiate its exercise induced increase in comparison with a fast food meal (FFM). MdM attenuated, non-significantly, the exercise induced cortisol increase. Exercise challenge(EC) induced an acute inflammatory response represented by an increase of lipocalin, PAI-1 and resistin independent of the previous type of meal. These findings highlight the importance of the pre-exercise dietary intake on both the immune and metabolic response to acute exercise.

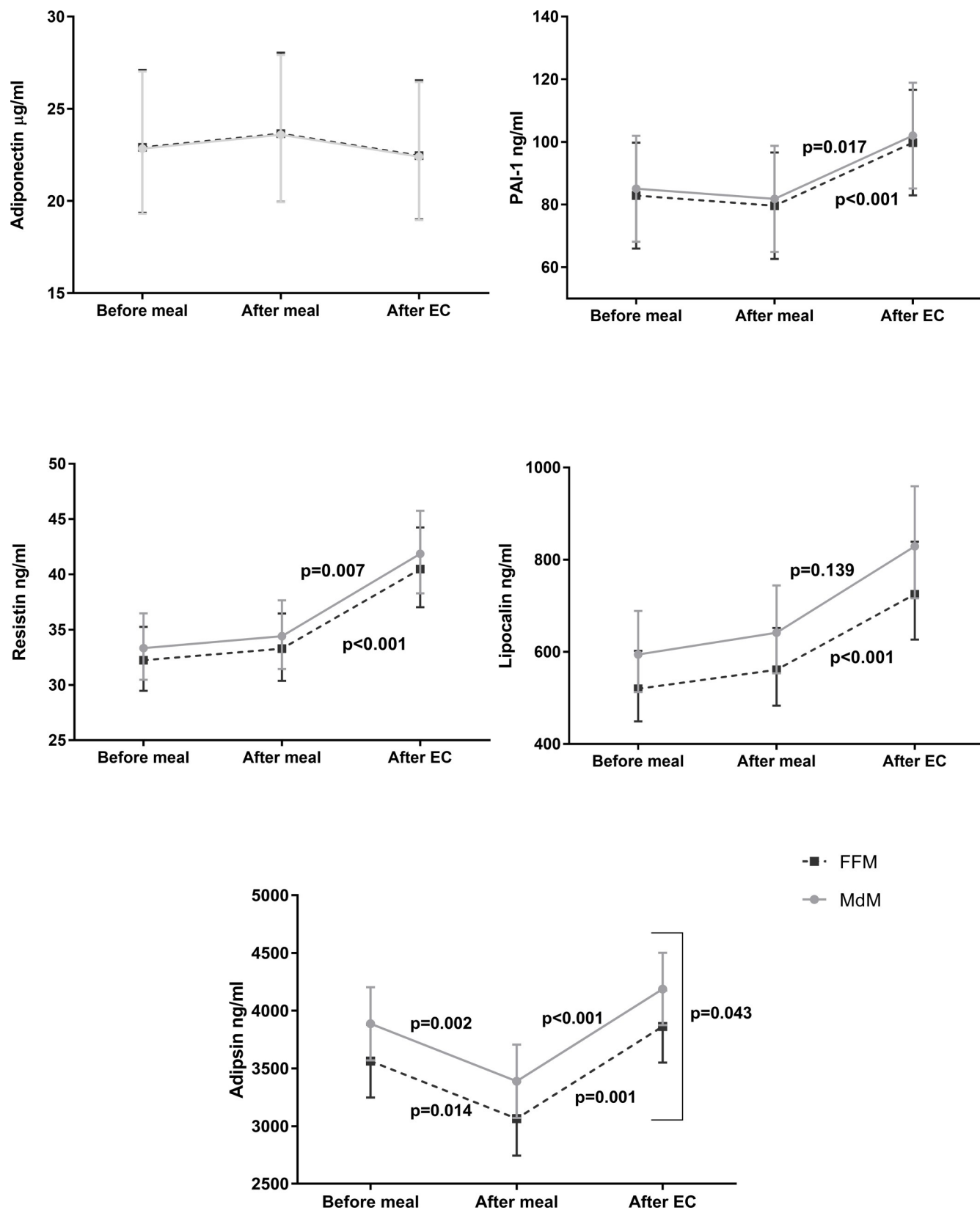


Fig 2. Points and connecting dots with error bar comparing Mediterranean and Fast Food meals effects on adipokine response to meal and exercise challenge using linear mixed model. Data is expressed in marginal mean estimate and 95% CI. EC- Exercise challenge.

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Table 3. Correlation coefficients matrix between adipokines responses to Mediterranean Meal(MdM) and Fast Food Meal(FFM) and exercise challenge.

	MdM					FFM				
	Adiponectin	PAI-1	Resistin	Lipocalin	Adipsin	Adiponectin	PAI-1	Resistin	Lipocalin	Adipsin
Δ Meal										
Adiponectin	1	0.496*	0.515*	0.438*	0.564*	1	0.286	0.197	0.075	0.530*
PAI-1	-	1	0.617*	0.477*	0.609*	-	1	0.411#	0.443*	0.458*
Resistin	-	-	1	0.796*	0.408*	-	-	1	0.789*	0.029
Lipocalin	-	-	-	1	0.437*	-	-	-	1	0.056
Adipsin	-	-	-	-	1	-	-	-	-	1
Δ Meal-Exercise										
Adiponectin	1	0.359#	0.259	-0.041	0.459*	1	0.25	0.481*	0.342#	0.584*
PAI-1	-	1	0.336#	0.004	0.29	-	1	0.371#	0.433*	0.134
Resistin	-	-	1	0.719*	0.422*	-	-	1	0.866*	0.589*
Lipocalin	-	-	-	1	0.330#	-	-	-	1	0.492*
Adipsin	-	-	-	-	1	-	-	-	-	1

*p<0.01

#p<0.05

<https://doi.org/10.1371/journal.pone.0215475.t003>

Our findings are limited by a few reasons. Participants were normal weight, though obese or overweight were not excluded. Still, BMI and free fat mass were considered in the model. The number of individuals was insufficient to compare participants with or without asthma, although targeted by recruitment strategy, to minimize this effect, lung function was controlled. Furthermore, asthma control was assessed and the applied exercise stress test was not designed to induce exercise bronchoconstriction. A population with interest in achieving a healthier lifestyle could be more prone to engage in our trial and might have specific physical activity and dietary behaviors. Therefore, both characteristics were monitored and controlled. Despite the demanding protocol, attrition rate was below 20%. Previous studies were scarce to perform a sample size calculation accordingly to the adipokines assessed and it is possible that with a larger number of participants differences between meals would be reached, namely for lipocalin. It is not possible to exclude that a larger effect could be found in an obese population. The immediate response to exercise might differ in recreational versus elite athletes [33]. Therefore, it is not possible to generalize between these populations. A graded exercise test was used instead of an exercise session, this choice allowed a better control of the challenge, although this type of test would be dependent on the cardiorespiratory fitness, this was controlled and pairwise comparison were performed. Adipokine changes might last until 4 hours after a meal [34], which might interfere with the EC. However, as we aimed to address the effect of a pre-exercise meal, a longer period would not allow to assess the targeted intervention and a shorter one would not induce a response [34]. Repeated evaluations could overcome these limitations and provide more information regarding the response through time, but this would escalate the complexity of the protocol and increase the drop-outs.

The evaluation of a meal-exercise intervention is one of the study strengths as it elucidates the response to a meal, but also to a stressor. The assessment of the effect of a MdM has rarely been addressed and this challenge provides a more real-life intervention, assessing the interactions between nutriment and not of a specific supplement. The study was controlled for main confounders, namely adiposity, physical activity, diet and cortisol. Another strength is the evaluation of adipokines with close relation with immune response, namely adipsin, but also PAI-

1 and lipocalin which allows to monitor the cross-talk between immune and adipokine responses with exercise that probably exceeds the obesity-related inflammation.

Physical activity modulates the inflammatory cytokine response from the adipose tissue. Adipsin links the immune and adipose tissue, mediated by its activity as Factor D, a catalyzer of the alternative pathway of complement activation, being one of the major proteins expressed in the adipose tissue [35]. Recently, it has been shown to link fat cells and obesity to β -cell function, as adipsin increases insulin secretion mediated by the production of peptide complement 3a [36]. In our trial, a higher quantity of saturated fat was present in FFM, which is compatible with previous results that have shown a decrease in adipsin after a high fat meal [37]. In contrast to other studies, where a resistance exercise induced adipsin decrease [38], an increase after EC was observed. This may be explained by the use of an aerobic exercise bout. Although quantity of fat was similar between meals, type of fat, micronutrients, fibers and salt differed, it is possible that type of exercise and meal components modulated adipsin behavior.

A beneficial effect of high-intensity aerobic exercise training in adipokine profiles, has been suggested for overweight/obese women [39]. We failed to observe any significant adiponectin changes. However, our study differs in type of participants, exercise spectrum and on the pre-exercise meal. Nevertheless, our results were consistent with other findings where no changes after acute exercise were found [40]. Meals did not interfere with adiponectin levels, as occurred in a previous study comparing high versus low fat meals [41]. Therefore, although the quantity of a meal fat does not seem to have an impact, the type of fat might have a role, as in a three iso-energy meals challenge study, the one which was walnut-enriched lead to a higher postprandial adiponectin response [42]. A 4-week Mediterranean Diet reduced adiponectin levels [9]. A long term, one-year, randomized controlled trial lead to different results, as newly diagnosed type 2 participants that where adherent to the Mediterranean diet had lower C-reactive protein and higher circulating adiponectin levels [43]. Still, moderate correlations between adiponectin and other tested adipokines after MdM were seen.

Exercise challenge induced immediate changes in PAI-1, resistin and lipocalin. Though a MdM versus FFM did not modulated this response, lipocalin tended to have a different response after MdM. Lipocalin and resistin correlated strongly, which could be explained by their pro-inflammatory profile [44]. Resistin has potent proinflammatory properties [44] and has a role in glucose homeostasis, lipid metabolism and insulin action [15], it has showed to have an inverse association with levels of physical activity. In a recent meta-analysis, regular exercise induced a decrease in resistin levels [15]. Our study differed, showing an increase, consistent with studies evaluating acute high-intensity exercise [45]. It is not possible to exclude the effect of the meal on resistin increase, although contradictory results occurred in the literature with similar meal tests, as outcomes were assessed at different time-points [22, 34]. Therefore, an inflammatory response to a strenuous stimulus is more likely.

Lipocalin-2 is a glycoprotein that has a role in the modulation of inflammation [46], acts in metabolic homeostasis and, as an acute phase protein, it has been use as potential biomarker in inflammatory diseases [46]. This adipokine has a role in the metabolic homeostasis being evaluated also in obesity-related metabolic diseases [46]. High-fat overfeeding did not induce any change in lipocalin-2 levels [47], similarly to our study. Consistent with previous reports [48], EC increased lipocalin levels. However, when adjusted for confounders after the MdM this difference lost significance. Despite no conclusions could be drawn for the effect of MdM, controlling both for age, gender, asthma diagnosis, physical activity and free-fat mass changed this relationship. As adipose tissue, namely fat mass quantity, might modulate the response to the different meals, studies with larger samples and in obese individuals might elicit different responses. Adipokines dysregulation appears to be associated with an increase of sympathetic nervous system activation [49]. Adiponectin altered gastric vagal afferent satiety signals in

animal models[50]. Another axis is the potential modulation by exercise of the autonomic nervous system.

Acute stress, mediates sympathetic nervous system response and promotes increase of circulating PAI-1 [51]. PAI-1 inhibits fibrinolysis, is an acute-phase reactant and acts as an adipokine, as it is expressed in the adipose tissue. Two iso-energy meals that differed only on type of proteins showed a decrease on PAI-1 levels through time [52], a tendency also seen in our data. Another study, measuring the effect of an oral fat-challenge, did not found differences in PAI-1 [53]. EC promoted a significant response in PAI-1, independently of type of meal. Increased PAI-1 immediately after high-intensity EC have been seen in participants with untreated hypertension [54], although a decrease on PAI-1 activity was also reported[55]. This response, independent of fitness, in hypertensive [56], might be explained by the increased shear stress and blood pressure during exercise that correlates with the blood lymphocyte increase following acute exercise[17].

The mechanism of shear stress induced by acute exercise could explain the increase of other adipokines, namely lipocalin, which was already seen in combination of heat and exercise [57]. Furthermore, resistin release from adipose tissue is stimulated by steroid hormones [58], although no correlation between cortisol and resistin was seen.

In conclusion, a pre-exercise MdM potentiated the increase of adipisin after EC which might be related to the immune-regulatory role of adipisin. Acute exercise induced pro-inflammatory response to resistin, lipocalin and PAI-1, but not to adiponectin. This might represent a cross-talk between the immediate inflammatory and metabolic response to exercise, which could be addressed in other studies including the autonomic response.

Supporting information

S1 CONSORT Checklist.

(DOC)

S1 Table. Characterization of meals nutritional composition for Mediterranean Meal (MdM) and Fast Food Meal (FFM).

(DOCX)

S2 Table. Adipokine response accordingly to treatment sequence and carry-over effect analysis.

(DOCX)

S3 Table. Correlation coefficients matrix between cortisol and adipokines responses to Mediterranean Meal(MdM) and Fast Food Meal(FFM) and exercise challenge.

(DOCX)

S1 Protocol. Study protocol.

(PDF)

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CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	3
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	4
	2b	Specific objectives or hypotheses	5
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	6,8
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	
Participants	4a	Eligibility criteria for participants	6
	4b	Settings and locations where the data were collected	6
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	8,9
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	9,10 [1]
	6b	Any changes to trial outcomes after the trial commenced, with reasons	
Sample size	7a	How sample size was determined	8
	7b	When applicable, explanation of any interim analyses and stopping guidelines	8
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	8
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	8[1]
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	8 [1]
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	10
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	10
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	Figure 1
	13b	For each group, losses and exclusions after randomisation, together with reasons	Figure 1
Recruitment	14a	Dates defining the periods of recruitment and follow-up	6,7
	14b	Why the trial ended or was stopped	
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	7; Table 1
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	10
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	11,12 table 2
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	12,13 table 3, figure 2
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	Figure 1
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	15
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	15
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	16,17
Other information			
Registration	23	Registration number and name of trial registry	6
Protocol	24	Where the full trial protocol can be accessed, if available	6
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	Submission process

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming; for those and for up to date references relevant to this checklist, see www.consort-statement.org.

1. Silva D, Moreira R, Sokhatska O, Beltrão M, Montanha T, Garcia-Larsen V et al. Meal-exercise challenge and physical activity reduction impact on immunity and inflammation (MERIIT trial). Contemporary Clinical Trials Communications. 2018;10:177-89. doi:<https://doi.org/10.1016/j.conctc.2018.05.010>.

Supplementary table 1. Characterization of meals nutritional composition for Mediterranean Meal(MdM) and Fast Food Meal(FFM).

	MdM [*]	FFM [#]
Energy (kcal)	1070	1015
Protein (g)	45.0	31.0
Protein (% TEV)	16.8	12.2
Carbohydrates (g)	137.1	126.0
Carbohydrates (%)	51.3	49.7
Sugars (g)	31.3	52.0
Fiber (g)	23.0	7.0
Fiber (% TEV)	4.3	1.4
Fat (g)	37.9	43.0
Fat (% TEV)	33.2	37.7
saturated (g)	7.4	12.0
saturated (% TEV)	6.2	9.3
MUFA (g)	12.2	NA
MUFA (%TEV)	0.13	NA
PUFA (g)	8.4	NA
PUFA (%TEV)	0.09	NA
n-3 (g)	4.0	NA
n-3 (%TEV)	0.04	NA
n-6 (g)	2.3	NA
n-6 (%TEV)	0.02	NA
Trans (g)	0.2	NA
Trans (%TEV)	0.17	NA
Salt (g)	3.0	2.9

TEV- total energy value for the meal; NA- not available

Supplementary table 2. Adipokine response accordingly to treatment sequence and carry-over effect analysis.

Treatment period						
	MdM			FFM		
Treatment sequence	BM	AF	AE	BM	AF	AE
Log Adiponectin						
MdM then FFM						
Mean (SD)	1.51(0.30)	1.50(0.31)	1.51 (0.37)	1.50(0.30)	1.43 (0.26)	1.46(0.32)
Sample size	15	15	15	15	15	15
FFM then MdM						
Mean (SD)	1.27(0.33)	1.27 (0.37)	1.27(0.30)	1.25 (0.29)	1.25(0.29)	1.30(0.33)
Sample size	22	22	22	22	22	22
Adipsin						
MdM then FFM						
Mean (SD)	3533.85(1549.12)	2906.12(1005.87)	3948.84(1950.14)	3590.45(1488.69)	3034.34(1200.58)	3500.85(1567.10)
Sample size	15	15	15	15	15	15
FFM then MdM						
Mean (SD)	3881.03(1268.28)	3707.67(1486.76)	4534.81(1851.42)	3599.60(1273.30)	3293.9(1085.29)	3293.94(1085.297)
Sample size	23	23	23	23	23	23
Log Lipocalin						
MdM then FFM						
Mean (SD)	2.78(0.26)	2.76(0.31)	2.88(0.27)	2.66(0.31)	2.74(0.37)	2.83(0.30)
Sample size	15	15	15	15	15	15
FFM then MdM						
Mean (SD)	2.82(0.27)	2.81(0.32)	2.94(0.22)	2.74(0.30)	2.74(0.28)	2.90(0.26)
Sample size	23	23	23	23	23	23
PAI-I						
MdM then FFM						

Mean (SD)	79.94(42.99)	76.98(40.90)	94.02(46.73)	75.53(42.80)	64.81(41.18)	92.40(36.69)
Sample size	15	15	15	15	15	15
FFM then MdM						
Mean (SD)	86.19(41.34)	83.42(45.00)	97.88(53.60)	79.34(41.95)	87.06(45.42)	101.22(44.67)
Sample size	23	23	23	23	23	23
Log Resistin						
MdM then FFM						
Mean (SD)	1.52(0.12)	1.51(0.16)	1.60(0.15849)	1.4974(0.11255)	1.5103(0.14524)	1.5745(0.16921)
Sample size	15	15	15	15	15	15
FFM then MdM						
Mean (SD)	1.55 (0.15)	1.54 (0.19)	1.61(0.22)	1.50 (0.15)	1.50 (0.16)	1.65(0.18)
Sample size	23	23	23	23	23	23

Adiponectin analysis of carry-over effect: There was homogeneity of variances, as assessed by Levene's test of homogeneity of variance ($p > 0.05$).

The Box test was significant. Mauchly's test of sphericity indicated that the assumption of sphericity was violated for the two-way interaction. $\chi^2(2) = 3.343$, $p = 0.008$. There was no statistically significant interaction between intervention order and treatment time periods $F(2.725270; 95.384440) = 0.934003$, $p = 0.420406$, partial $\eta^2 = 0.2472$ using Greenhouse-Geisser.

Adipsin carry-over effect: There was homogeneity of variances, as assessed by Levene's test of homogeneity of variance ($p > 0.05$). There was not homogeneity of covariances. as assessed by Box's test of equality of covariance matrices ($p = 0.042$), nevertheless analysis was continued. Mauchly's test of sphericity indicated that the assumption of sphericity was violated for the two-way interaction, $\chi^2(2) = 41.979$, $p < 0.001$. Therefore, it was used estimate this adjustment and Greenhouse-Geisser. There was no statistical significant interaction between time and intervention order $F(3.362, 12.033) = 1.205$, $p = 0.312$ partial $\eta^2 = 0.032$, using Greenhouse-Geisser.

Lipocalin carry-over effect: There was homogeneity of variances, as assessed by Levene's test of homogeneity of variance ($p > 0.05$). There was homogeneity of covariances. as assessed by Box's test of equality of covariance matrices ($p = 0.104$).

Mauchly's test of sphericity indicated that the assumption of sphericity was violated for the two-way interaction, $\chi^2(2) = 30.221$, $p = 0.007$. Therefore, it was used estimate this adjustment and Greenhouse-Geisser. There was no statistical significant interaction between time and intervention order $F(3.919, 141.077) = 0.242$, $p = 0.911$ partial $\eta^2 = 0.007$.

PAI-I carry over effect: There was homogeneity of variances, as assessed by Levene's test of homogeneity of variance ($p > 0.05$). There was homogeneity of covariances, as assessed by Box's test of equality of covariance matrices ($p = 0.168$). Mauchly's test of sphericity indicated that the assumption of sphericity was met for the two-way interaction. $\chi^2(2) = 15.083$, $p = 0.374$. There was no statistical significant interaction between time and intervention order $F(5.180) = 0.833$, $p = 0.258$ partial $\eta^2 = 0.023$.

Resistin carry-over effect: There was homogeneity of variances, as assessed by Levene's test of homogeneity of variance ($p > 0.05$). There was homogeneity of covariances, as assessed by Box's test of equality of covariance matrices ($p = 0.919$). Mauchly's test of sphericity indicated that the assumption of sphericity was violated for the two-way interaction. $\chi^2(2) = 27.764$, $p = 0.016$. Therefore, it was used the adjustment by Greenhouse-Geisser. There was no statistical significant interaction between time and intervention order $F(3.713.133.681) = 0.833$, $p = 0.500$ partial $\eta^2 = 0.037$.

Supplementary table 3. Correlation coefficients matrix between cortisol and adipokines responses to Mediterranean Meal(MdM) and Fast Food Meal(FFM) and exercise challenge.

	MdM					FFM				
	Adiponectin	PAI-1	Resistin	Lipocalin	Adipsin	Adiponectin	PAI-1	Resistin	Lipocalin	Adipsin
Δ Meal										
Cortisol	-0.104	-0.087	-0.002	-0.146	-0.046	0.353*	-0.169	-0.129	-0.356*	0.212
Δ Meal-Exercise										
Cortisol	0.197	0.081	-0.195	-0.327*	-0.155	-0.097	-0.064	-0.130	-0.139	-0.294

*p<0.01; #p<0.05

**Department of Allergology and Clinical Immunology
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**PHYSICAL ACTIVITY AND DIET AS
DETERMINANTS OF IMMUNE FUNCTION**

Study protocol

**Diana Silva
Doctoral Program in Clinical Investigation and Health Services
Research**

10 December 2012

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PROJECT IDENTIFICATION

Title

Physical activity and diet as determinants of immune function
A dieta e atividade física e o seu impacto no sistema imunológico

Authors

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Main scientific domain

Exercise Immunology
Secondary domain: Diet and neuro-immuno-endocrine modulation

Key-words

Exercise, immune function, immune-suppression, diet, obesity, asthma

Project start and duration

From 1st September 2010 to 1st January 2015

LIST OF PUBLICATIONS

I Cross-cultural translation and validation of a respiratory infection symptoms questionnaire

II Upper respiratory tract infection susceptibility in athletes – a 3 months follow-up study

III Effect of physical training on respiratory infections in the elderly

IV Effect of an high fat versus healthy diet meal on neuro-immune-endocrine response to exercise

V Effect of physical activity on immunity

SUMMARY

Background: Exercise is related to several acute and long-term changes in the immune system[1]. Respiratory infections have also been studied in relation to physical activity, being those with more sedentary behavior and the population that performs high intensity exercise more susceptible[2]. Moreover different characteristics interfere with this relationship, namely physical fitness, age and also diet. Indeed diet modulates immune functions and inflammatory processes [2, 3]. Specific populations namely obese and asthmatic have an intrinsic relation with this changes, but the exercise effect after a meal have never been studied in these particularly susceptible population[4, 5].

Main Problem: In a society where exercise is prescribed by physicians to increase physical condition, improve outcomes and reduce cardiovascular risk, the impact of exercise, acute or chronic, in the immune system is unknown. Specific populations can have different responses towards acute and chronic exercise and specific conditions like the type of ingredients ingested, namely fat meals, can be correlated with this responses. The true effect of exercise in the immune system is unknown.

Research question: Understand the effect of physical activity and diet as determinants of immune function.

Aims: Obtain a valid instrument to evaluate respiratory infections in Portuguese population; to find characteristics related to a illness-prone profile in high competition athletes during a bout of exercise; evaluate the impact of a moderate exercise program in a susceptible population (elderly); Correlate the effect of diet (high fat meal) in acute exercise response in a specific obese and asthma population; ascertain the physical inactivity impact on the immune system.

Methods:

Study design: An observational study with validation methodology will be applied to evaluate respiratory infections; in prospective cohort study of 12 weeks a group of athletes under high competition levels, respiratory infections and potential risk factors accordingly to literature will be evaluated; an prospective case-control with a moderate- exercise program intervention will compare the impact in respiratory infections in elderly under exercise training or not; a randomized, placebo controlled cross-over trial will evaluate the impact of a high fat/high energy meal challenge in an acute exercise challenge; randomized clinical trial will be used to ascertain the impact of physical activity reduction in the immune function

Selection of participants: For questionnaire validation will be selected participants with respiratory infections; athletes will be selected from a high elite pre-competition level team; elderly will be selected from general population, namely from senior universities and nursing homes; asthma and obese participants will be selected from an university population sample

Sample Size: 281 patients will be chosen as target for evaluation in respiratory tract infections validation; 40 patients will be evaluated in total in swimmers illness-prone study trial; 40 participants will be selected to evaluate exercise program in the elderly; 30 participants, with a

equal number of obese, asthma, obese and asthma and a control will be allocated to the meal interventions (60 participants in total) and a target of 60 participants will be randomized to the physical inactivity intervention.

Measurements: Upper respiratory tract infections number, severity and quality of life impact, immune system markers, complete blood cell count, pro-inflammatory and anti-inflammatory mediators, namely cytokines, and metabolomic.

Main Outcomes: Respiratory Infection Risk in Susceptible Population, Immune markers parameter related with acute exercise and physical inactivity interventions (balance between pro-inflammatory and anti-inflammatory cytokines).

Expected results: Obtaining a valid method for upper respiratory infections assessment, evaluate the risk factors related with respiratory infections in a athletes population, namely physical fitness, nutrition and stress showing the multifactorial side of immunosuppression in athletes; risk of respiratory infections in a physical activity and inactivity intervention as well as immune mediators impact related with an acute exercise challenge and the interference with diet previously to the exercise. Demonstrate the effect of exercise in immune system in particularly susceptible population.

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RESUMO

Introdução: O exercício está relacionado com alterações agudas e a longo prazo no sistema imune. Estudos anteriores relacionaram infeções respiratórias com a prática de exercício, estando as populações com comportamento sedentário e que realizam atividade física intensa mais susceptíveis. Além disso, diferentes características interferem com esta relação, nomeadamente aptidão física, idade e dieta. De facto, a dieta tem uma função moduladora dos processos imunes e inflamatórios. Populações específicas como obesos e asmáticos têm uma relação intrínseca com estas alterações, mas o efeito do exercício após uma refeição nunca foi estudado nestas populações mais susceptíveis.

Principal problema: na sociedade moderna, onde o exercício é prescrito para aumentar a condição física e diminuir o risco cardiovascular, o impacto do exercício no sistema imunitário é desconhecido. Populações específicas podem ter diferentes respostas em relação ao exercício agudo e crónico e condições específicas como tipo de ingredientes ingeridos, como refeições hipercalóricas, podem estar relacionadas com estas respostas. O verdadeiro efeito do exercício no sistema imune é desconhecido.

Pergunta de investigação: perceber o efeito da actividade física e dieta como determinantes da função imune.

Objectivos: Obter um instrumento válido para avaliar infeções respiratórias na população Portuguesa; encontrar características de um perfil predisponente à doença em atletas de alta competição durante o exercício; avaliar o impacto de um programa de exercício moderado numa população susceptível; correlacionar o efeito da dieta (hipercalórica) na resposta aguda ao exercício numa população específica de obesos e asmáticos; avaliar o impacto da inactividade física no sistema imune.

Métodos:

Desenho do estudo: um estudo observacional com metodologia de validação vai ser aplicado para avaliar infeções respiratórias; num estudo de coorte prospectivo de 12 semanas de um grupo de atletas de alta competição irão ser avaliadas infeções respiratórias e potenciais factores de risco de acordo com literatura; um estudo caso controlo prospectivo com intervenção de exercício moderado irá comparar o impacto das infeções respiratórias num grupo de idosos ; um ensaio randomizado, controlado com placebo estilo cross-over irá avaliar o impacto de uma refeição hipercalórica na resposta aguda ao exercício; um ensaio randomizado irá avaliar o impacto da redução da actividade física na função imune.

Participantes: para validação do questionário foram selecionados participantes com infeções respiratórias; atletas foram selecionados a partir de uma equipa de natação de alta-competição; idosos foram selecionados da população geral, nomeadamente universidades seniores e lares; participantes com asma e obesidade foram selecionados a partir de uma população universitária.

Tamanho amostra: 281 doentes foram escolhidos para avaliação da validação do questionário de infeções respiratórias; 40 doentes foram avaliados no estudo de nadadores de alta-competição para avaliar a propensão à doença; 40 participantes foram selecionados para avaliar o programa de exercício em idosos; 30 participantes, em grupos iguais de obesos, asmáticos, obesos e asmáticos e controlo serão alocados para a intervenção com refeição (60 participantes no total) e um alvo de 60 participantes serão randomizados para a intervenção da inatividade física.

Medidas: número de infeções respiratórias superiores, impacto na qualidade de vida, marcadores sistema imune, hemograma, mediadores pró-inflamatórios e anti-inflamatórios (citocinas, metaboloma).

Principais resultados: Risco de infeção respiratória em populações susceptíveis, parâmetros de marcadores imunes relacionados com exercício agudo e inatividade física (balanço entre citocinas pro-inflamatórias e anti-inflamatórias)

Resultados esperados: Obter um método válido para avaliar infeções respiratórias, avaliar os fatores de risco relacionados com infeções respiratórias em atletas (aptidão física, nutrição e stress) de forma a evidenciar o aspeto multifatorial da imunossupressão em atletas; risco de infeções respiratórias na atividade e inatividade física bem como o impacto nos mediadores imunes relacionado com uma prova de exercício agudo e interferência com dieta prévia ao mesmo. Demonstrar o efeito do exercício no sistema imune particularmente em populações susceptíveis.

1. RATIONALE

Physical activity is a generic term to any body movement, mediated by skeletal muscles activity and is performed by anyone in order to sustain life [6]. Exercise is frequently used interchangeably with physical activity, but implies an activity performed for a specific purpose such as improvement of physical condition or competition [6]. Acute and chronic exercise induces changes in both number and function of the immune system [1]. Also mucosa immunity, namely that mediated by specific IgA in saliva is depressed in periods of long and intensified training, which could be related to an increase susceptibility to infections[1]. The main focus of exercise and immune impact has been studied in the susceptibility to upper respiratory tract infections (URTI)[2].

Respiratory tract infections are very common and interfere with daily life. Diagnosis is clinical and, until recently, physician confirmation of an infection cause of the symptoms was considered the gold standard. Nowadays this paradigm has been questioned at light of the laboratory identification of pathogens [7]. The use of these techniques are sometimes expensive and time-consuming [1]. Surveys to access respiratory infections have been created, the first of all was the Jackson scale, and more recently the Wisconsin Upper Respiratory Symptoms Survey, which also has an evaluative illness-specific quality of life outcome instrument [8, 9]. These questionnaires have been proven useful to monitor respiratory infections in research conditions[10]. Still, no Portuguese version exists. This type of tool would enable physicians to monitor disease with patients at home and provide a long-term and distant monitoring of this condition and relate it with individual characteristics, physical activity and impact on their lives. Furthermore it would be useful to ascertain the relationship between specific risk factors and immune system susceptibility, namely exercise interventions.

A relationship has been proposed between respiratory tract infections and exercise proposed by a J-shaped model, still not supported by high quality of evidence [1, 2]. This model suggests that moderate exercise may lower the risk for URTI compared to sedentary individuals, but those under high intensity exercise and strenuous exercise periods may raise risk for URTI. It has been proposed a hypotheses that further factors should be considered in this model, namely physical fitness of the individual, concomitant allergy, inflammation, concomitant disease or infection, environmental factors and nutritional status [1, 2, 11]. The sum of these characteristics can define an illness prone profile, specific for each population.

Athletes are an especially susceptible population to respiratory infections, which have an important impact in their performance in high competition challenge. An “open window theory” of altered immunity has been used to explain this susceptibility[12]. After each bout of prolonged exercise it was demonstrated a period of 3 to 72 hours of altered immunity[13]. The acute exercise induces immune system responses, similar to those that occur in the infection period, increased neutrophils, monocytes, natural killer, and a higher concentration of stress hormones, namely catecholamines, growth hormones and cortisol [4, 14]. Furthermore it enhances release of anti-inflammatory and pro-inflammatory mediators [15]. These changes

support the theory of increased susceptibility[4]. Nonetheless the true measure of immune function and its association with immune-suppression is still an unmet need, as cited in the Walsh et al position paper [1]. The main issue is to understand if an athlete is an immune-compromised host and what is the true role of intense and prolonged exercise training in their susceptibility as well as other risk factors that might be involved, namely stress and nutrition[2, 3, 16, 17]. Athletes have reported less episodes of respiratory infections when under normal training comparing with their sedentary peers[3]. In a recent large study, in physically fit and active adults not in high intense training programs showed a reduction of the number and severity of respiratory infections[18].

Exercise and immune system changes are probably in relation with the subject susceptibility. Elderly are frequently recommended to perform physical activity and exercise due to its multiple positive effects on health-related problems[19] and it has been seen as a countermeasure to immune-senescence[20, 21]. It has been shown that the less physically active elderly have an increase on respiratory tract infections[22]. Most of the previous literature focuses on immune mediators response to exercise intervention in elderly, contradictory effects have been seen between cross-sectional and longitudinal studies as it was reported by a recent thorough literature review [21, 23]. This suggests that perhaps other markers should be evaluated, namely specifically patient related outcomes. Only one study performed in postmenopausal women evaluated disease as a main outcome after a 12 months moderate exercise intervention and reported a reduced incidence of common colds[24]. Further investigation is needed to evaluate the true impact of chronic regular exercise in restoring immune function and which factors can be effectively associated to potentiate it. Also, acute exercise might also have a deleterious effect and could limit the beginning of a regular physical activity in illness-prone subjects. This potential effect can be supported by the previously open window theory and immune changes related to them [13]. Acute exercise effect has seldom been studied in populations other than athletes or healthy physical active populations [12, 25, 26]. In Mills et al[27] study proposed that being physically fit is protective of inflammatory responses so the previously seen results might be more significant in sedentary and in persons with chronic inflammatory systemic diseases. Possibly, reducing the physical activity will be associated also with a decreased immune function response. This was previously demonstrated by recent studies of Pedersen group, where there was seen after a short reduction of physical activity a decrease in insulin sensitivity and cardiovascular fitness. Effect of this intervention on immune function has never been studied[28-30].

Populations like asthma patients, obese and asthma-obese also benefit from physical training and it is nowadays widely recommended [5]. Both populations are associated with a chronic inflammatory profile and an increase susceptibility to infections, namely respiratory tract infections[31, 32]. Especially in asthma it is questioned that this seen susceptibility is only an epiphenomenon, infections appear more severe in patients with underlying respiratory disease, or instead a reflection of altered host susceptibility[32]. No previous study has been performed in evaluating acute exercise challenge in this specific population, as epidemiologically the

patient related outcomes are more difficult to relate, there is a need to understand how inflammatory, endocrine and immune function markers behave with acute exercise. Nowadays new forms of inflammation and metabolic assessment appeared which are more informative, namely metabolomic and microbiome, these new mediators can open the knowledge of the exercise impact in this populations[33-35]. This project aims to further study the impact of acute exercise, using immune and new inflammatory biomarkers in these susceptible populations. As previously stated other aspects can be involved in the respiratory infection susceptibility, namely diet.

Dietary interventions have been mainly studied in athletes, namely to be used as an intervention to decrease exercise-induced immune-depression and also to improve performance[4]. High fat meal challenge has been recently studied in a obese asthmatic population and it showed an increase of airway inflammation, mediated by neutrophils and even a decrease in bronchodilator response[36]. In these susceptible populations it will be interesting to evaluate the effects of a high fat diet on post-exercise immunity. Through these results a specific recommendation can be posed to susceptible populations to start exercise activity and do exercise more safely and decrease respiratory infection susceptibility if a healthier diet is chosen. This project aims widen exercise immunology understanding skipping to an individual context susceptibility in order to understand expected results and make the future recommendations on how to prescribe exercise more safely.

2.BACKGROUND

Research has been intensified in athlete population to understand their respiratory infection susceptibility. In a systematic review by Moreira et al[2], it is agreed that moderate activity may enhance immune function, but high-intensity exercise temporarily impairs the immune competence. Still areas of controversy are suggested by this systematic review; relationship between exercise and upper respiratory infections is affected by poorly known individual determinants such as genetic, fitness, nutritional status and atopy. Mills et al compared physical fit and less fit individuals and showed that in acute response to exercise fit individuals had an attenuation of leukocyte-endothelial adhesion in response to exercise, suggesting a protected inflammatory response to exercise. Reduced physical activity intervention, has recently been used by Pedersen team to evaluate metabolic responses, and showed that this intervention reduced peripheral insulin sensitivity and body composition, furthermore it also showed that this short intervention reduced cardiovascular fitness evaluated by VO₂ max [13, 28-30].

Relationship between physical fitness, diet, exercise and immune suppression has been mainly studied in athlete or highly physically active population as reported in the recent published systematic review of Gunzer et al[4]. No conclusion has been withdrawn due to limited research evidence on how to reduce post-exercise upper respiratory symptoms with single macronutrients, namely in high fat nutrition meals [4]. In asthma patients a recent randomizes study by Wood et al, non-obese subjects with asthma were compared with obese subjects who were submitted to a high fat meal challenge versus a low fat meal. It was shown that the participants allocated to the high fat meal showed 4h post-meal an increase in sputum neutrophil percentage, toll-like receptor 4 and a lower relationship of FEV₁/FVC, thereby relating the fat consumed to an increase inflammation status. In another study by Meksawan, sedentary men (n=5) and women(n=6) had dietary fat intake during 3 weeks (19% calories and 50% calories) followed by an acute maximal exercise effort test. Evaluated inflammatory mediators of the immune system associated the bout of exercise to an increase of pro-inflammatory mediators which was aggravated by the fat meal for the previous 3 weeks in comparison with normal fat or high fat diet in this study the increase in pro-inflammatory mediators were not seen with a 50% caloric balance.

3. RESEARCH QUESTION AND AIMS

The research question of this project is: how physical activity and diet interfere with the immune system functions, namely respiratory infection susceptibility and illness prone profile.

The main outcomes are:

- Evaluate the use of a respiratory infections questionnaire to diagnose and characterize respiratory infections and their impact on lives in Portuguese patients with respiratory infections over 16 years old. Perform a cross-cultural translation and validation of WURSS-21;
- Evaluate the upper respiratory tract infections risk factors (demographics, fitness profile, inflammatory, metabolomic, neuro-endocrine and environment) in a population with a high level of exercise intensity;
- Test the effect of a controlled, moderate intensity long-term training program in an elderly population;
- Evaluate the effect of a high fat meal compared to a healthy diet meal on the neuro-immune-endocrine response to exercise;
- Study the effect of physical activity reduction on neuro-immune-endocrine mediators and respiratory infections.

4.METHODS

4.1. Participants and Study Design

4.1.1. Cross-cultural translation and validation of a respiratory infection symptom questionnaire (I)

This is a trans-sectional case control validation research project of a respiratory symptoms questionnaire to be applied in a three months follow-up period during the seasonal peak of upper respiratory infection from January to March 2012 in patients over 16 years old with and without respiratory infections.

Participants

All patients that require an unscheduled appointment due to acute illness in a primary health care center (USF Progresso, Perafita, Unidade Local de Saúde de Matosinhos) and from the Department of Allergy and Clinical Immunology from Centro Hospitalar de São João will be recruited. The inclusion criteria for respiratory infections cases are: over or equal 16 years of age, physician diagnosis of respiratory tract infections, with onset of symptoms within 48h and with Jackson score for respiratory infections over 3 or higher. In Jackson score eight symptoms (sneezing, nasal discharge, nasal obstruction, sore throat, cough, headache, malaise and chilliness) are classified as 0=absent, 1=mild, 2=moderate or 3=severe and presence of at least one of the first four cold specific symptoms is required for diagnosis. All those patients that attend to the primary care center or allergy department for other reason except respiratory symptoms/infections will be proposed to enroll the study project as controls. Exclusion criteria are: under 16 years of age, all patients with basal or throat symptoms that started more than 48h before they searched medical care. Results provided from patients with allergy and those without allergy complains will be compared for changes in respiratory symptoms score and in quality of life changes.

Procedures

The Wisconsin Upper Respiratory Symptom Survey (**Attachment 1**) is an empirically derived patient-oriented illness specific quality-of-life evaluative outcomes instrument. It is a validated questionnaire in English and is now translated into Spanish, Japanese, French, German, Korean, Russian, and Ukrainian, with high correlation of previous questionnaires aimed to evaluate the presence of upper respiratory symptoms. It is also designed to reflect the impact of colds in quality of life and evaluate the severity through time [9, 37, 38]. This questionnaires correlate with laboratory assessed measures in a induced respiratory infections clinical trial[39]. This is a 21 items questionnaire, based on a 7 point Likert-type severity scale. To those will also be associated the items for headache, body-ache and fever [10, 40]. Questionnaire, WURSS-21 will be given to the included cases and controls, to be completed during 6 days and at the third

day it will be also filled in a validated questionnaire in Portuguese, MOS SF-36 (**Attachment 2**) [41, 42] of quality of life for convergent validity evaluation.

Demographic characteristics (age, gender, education, smoking habits, allergic diseases) will be collected when the questionnaire is delivered, written informed consent will be obtained if the patient decides to fill the questionnaire.

Cross-cultural translation

Cross-cultural translation will be provided by investigators that contact daily with patients with respiratory infections and follow their course. This procedure will be based in the previous published guidelines by Baiardini et al.[43] and all items will be included in the cross cultural translation process (namely questionnaire instructions, items and response choices). The linguistic validation will consist on three steps:

1. Forward translation (translate from source language, English, to target language, Portuguese) by two translators, native speakers in the target language and bilingual in the source language, supported by an experienced specialist in respiratory infection treatment and diagnosis; translators will independently produce a forward translation of the questionnaire, then a combined version, obtained by the agreement on a single reconciled version with all elements (translators and physician)
2. Backward translation (translation of the first reconciled forward version of the questionnaire back into the source language): requires a translator with no previous access to the questionnaire, native speaker of the source language and bilingual in the target language, this version will be sent to the authors for review and comment; then a comparison will be made with the original source version to detect any misunderstandings, mis-translations or inaccuracies in the forward version of the questionnaire
3. Patient testing (comprehension test): the final consensus version will be administered to a sample of 10 respondents, native speakers of the target language, over 16 years old, in a face to face interview, during which participants will be inquired if any difficulties were found in understanding the questionnaire and check the interpretation of all items; if any problem is encountered the interviewer should ask the person to propose alternatives and test alternative translations. This is aimed to determine whether the translation is acceptable, understood and if the language is simple/ appropriate. During interview, data will be collected: number of interviewed, age, difficulties and solutions.

From this report a third and final version will be produced

Validation

Content validity of the Wisconsin questionnaire survey was previously studied in the validated versions of WURSS-44 and the short form WURSS-21[9, 38]. In the short form, will be assessed 7 different domains: global day severity, global change since yesterday, cough, throat, nasal, tiredness, functional/activity and other (head congestion). It will be also included

headache, body ache and fever to these domains, which has been used for WURSS-24 [10, 40].

Convergent validity will be assessed by comparison with a health related quality of life questionnaire, SF-36, already validated, which will be filled in at the third day of disease.

For criteria validity, the questionnaire will be compared between patients with respiratory infections and without respiratory infections diagnosed by physicians (which is the gold standard for respiratory infections diagnosis). Furthermore, inclusion criteria use the previously known Jackson criteria widely applied for respiratory infections diagnosis[8]. Both patients will fill WURSS during 6 days and SF-36 on the third day.

Reliability will be evaluated by the application of the same questionnaire through a 6 day period, first three days will be compared using internal consistency measures. As a respiratory infection symptom will change through time (patients will be asked if comparing to the previous day they feel that my cold is very much better, somewhat better, a little better, the same, a little worse, somewhat worse and very much worse) responsiveness to the questionnaire will be evaluated through already published methods[9, 44], namely Guyatt's responsiveness index. This index demands the calculation of Minimal important difference, the mean value of day-to-day changes. Also the mean squared error of the scores will also be needed, which is based on those subjects reporting the same state.

4.1.2. Respiratory Infection susceptibility in athletes-illness prone profile (II)

This is a prospective, three month follow-up, cohort study to evaluate the risk factors for respiratory infection. A group of swimmers from a Porto sports team (Futebol Clube do Porto), from a high competition level and active during the follow-up period, which is coincident with a pre-competition season and higher incidence of respiratory infections (from January to March). During this time participants will be followed for respiratory infections episodes through a daily respiratory infections questionnaire, which is filled in when they began respiratory infections symptoms and then everyday during the scheduled-time illness. Acute respiratory infection number, duration severity of each episode will be characterized. All potential risk factors for respiratory infections will be evaluated at baseline and at the end of the study, except for urine analysis which will be collected every week and during the respiratory infection episode to establish an eventual association between changes of urinary metabolome in and outside the infection period.

Participants

All elite swimmers of Porto Sports Club from the main swimming team who are annually screened for asthma and atopy at Allergy Asthma and Sports Unit will be invited to participate in the project. The inclusion criteria will be: competitive level swimmer; aged above 16 year-old; either sex and of any race. A potential subject who meets any of the following criteria will be excluded from participation in this study: neurological or psychiatric illness which enable them to fill in the formulary/ and questionnaires and anything that, in the opinion of the investigator, would place the subject at increased risk or preclude the subject's full compliance with or completion of the study. All these participants were previously included in the study, "*Mechanisms of airway damage in elite swimmers*" that was previously submitted and accepted in the Ethical Commission for Health of Centro Hospitalar de São João. For being included in this project written information about this follow-up will be provided and informed consent will be signed.

Procedures

Participants recruited in the previous project "*Mechanisms of airway damage in elite swimmers*" and all those that fill in the inclusion criteria, accept to participate and sign the informed consent will be evaluated at baseline.

In baseline the following characteristics and potential risk factors for respiratory infections will be screened:

- demographic data; full clinical history (namely asthma, respiratory or cardiovascular disease); chronic medication (any medication taken oral or inhaled); time of exercise practice and intensity of the trainings; immunization status and influenza vaccine; use of pro-biotics or vitamin supplements

- anthropometric and bio-impedance measures;
- nutritional state assessment and nutritional questionnaires (see description in measurements)
- sleep quality assessment questionnaires (**Attachment 3**) and anxiety symptoms questionnaire (Zung Self Rate Anxiety Scale- **Attachment 4**)
- lung function (espirometry), airway inflammation evaluated by induced sputum evaluation, airway hyper-responsiveness (methacholine provocation testing), bronchial challenge with inhaled capsaicin, exhaled nitric oxide measurement (*performed in the “Mechanisms of airway damage in elite swimmers” project-accepted by the ethical commission Centro Hospitalar de São João Attachment 5*)
- cholinergic-parasympathetic system measure by pupillometry response (*performed in the “Mechanisms of airway damage in elite swimmers” project-accepted by the ethical commission of Centro Hospitalar de São João Attachment 5*)
- blood withdraw for inflammatory cytokines measurement
- exhaled breath condensate, exhaled breath
- urine collection for metabolome measurements

During the follow-up it will be monitored through a respiratory infections questionnaire the episodes of respiratory infections, duration, severity and quality of life impact. A diary of all missed trainings will be kept during the follow-up period. Urine samples will be obtained every week and during the infections period.

Participants will also be submitted to a nutritional 24h dietary recall interview twice a month. The first one will occur at baseline and will be performed face-to-face with the investigator, so that the participant understand the purpose of the interview and also could be trained in the procedure. The following recalls will be assessed by telephone.

At the end of the follow-up period participants will perform:

- anthropometric and bio-impedance measures;
- nutritional state assessment and nutritional questionnaires;
- sleep quality assessment questionnaires and anxiety symptoms questionnaire (Zung Self Rate Anxiety Scale-- **Attachment 4**);

All variables will be assembled at the end of the study and individuals with respiratory infections during this period will be compared with those without the outcome. Associations will be performed *a posteriori* between urine metabolome analysis and the days with respiratory infections and before/after an infection episode.

4.1.3. Effect of Physical training on respiratory tract infection in the elderly (III)

This study was previously presented and accepted by the ethical commission of Centro Hospitalar de São João (Attachment 6)

The study was a non-randomized, un-blinded, controlled trial targeting patients over 60 years recruited by the Exercise Health for the Elderly program, coordinated in Sports Faculty of Porto University, from senior universities and nursing homes from Northern Portugal area. The selected participants performed a 36 week, 3 times per week, exercise-training program in the Sports University of Porto, with aerobic and anaerobic components using an individualized protocol by trained sports personal. During this period of time, respiratory tract infections were monitored through a symptoms diary and respiratory infections were defined as severe, moderate or mild accordingly to the symptoms assembly. Previously to the intervention a global physical condition was assessed (weight, height, heart rate, functional fitness and blood pressure were measured), co-morbidities were registered (hypertension, dyslipidemia, diabetes, ischemic heart disease, previous stroke, previous cancer diseases, osteo-articular and osteoporosis), as well as smoking habits, influenza immunization status and if they previously practiced exercise.

Participants

Recruitment occurred from September until November 2010 through Exercise Health for the Elderly program, organized by The Research Centre in Physical Activity, Health and Leisure, at Faculty of Sport Sciences and Physical Education at the University of Porto, which receives patients from the Porto metropolitan area, Senior Universities from Porto and also nursing homes and daily care centers for the elderly in Northern region of Portugal. Participants were contacted by phone or personal contact at the universities or nursing homes. Intervention group participants belonged mainly from Porto metropolitan area, as they had the ability to travel to the training sessions that occurred at Faculty of Sport Sciences and Physical Education at the University of Porto. Control group were recruited from nursing rooms and daily care centers from Paredes de Coura, and all of those from Porto metropolitan area which were not able to participate in the intervention group by schedule incompatibilities. Participants were eligible if they had: over sixty years old, a medical report describing that they were physically able to practice aerobic and anaerobic exercise of moderate intensity and did not participated in previous physical exercise programs. Exclusion criteria included all patients with uncontrolled disease, namely hypertension, patients with cardiac or orthopedic prosthesis, patients with a psychological, familial or sociological problem that might compromise their compliance to the exercise program. Participants were provided with written information about the exercise training program and symptoms diary data collection. A total of 80 patients were recruited for

the study, 12 did not meet the eligibility criteria and 30 refused to participate, so 38 patients were selected. Participants were then assigned to the training group and control group accordingly to the availability and proximity to the exercise training group.

Procedures

Exercise program

Exercise program was performed during 36 weeks and consisted in 3 times per week in non-consecutive days (Mondays, Wednesdays and Fridays). Each session was performed in group with all elements and included a 30 minutes aerobic training and 45 min of anaerobic training. Aerobic training consisted in 30 min walking at moderate intensity reaching 60-80% of the maximum heart rate (*monitored by polar team system, Finland*). Resistance or anaerobic training was performed during 45 min aiming 9 different muscular groups (leg press, chest press, leg extension, seated row, seated leg curl, abdominal flexion, low-back extension, biceps curl and triceps extension), each exercise was performed twice with 10 to 12 repetition at 60-80 % of repetition maximum (repetition maximum for each patient was previously calculated on base of *Epley formula*). Training was standardized, but adapted to each participant difficulties or handicaps, namely accordingly to previous osteoarticular conditions.

Intervention and control group were submitted first to a demographic, previous history of cardiovascular, osteoarticular, cancer or respiratory diseases, smoking habits, influenza immunization and history of previously practiced exercise. Baseline weight and height was also collected.

Respiratory Infections Symptoms Diary

For respiratory infections measure it was used a symptom diary, based in previously published symptoms diaries and common cold assessment questionnaires based in the Jackson index, Common Cold Questionnaire and Wisconsin diary symptoms short form-survey[45, 46]. The diary was built to answer to the specific demands of the population, namely age, real life conditions of the study as no patients with chronic previously reported respiratory diseases were excluded. Through this symptom diary it would be possible to quantify respiratory infections, their severity and duration. The questionnaire was subdivided in five main symptoms categories, similarly as occurred in the common cold questionnaire, but also including ear and eye symptoms. Furthermore allergy symptoms were included, namely nose and eye itching to exclude allergic symptoms in the evaluation of the symptoms diary. Patients were also requested to fill in if they had any symptoms and those symptoms reported in every and most of the days during the intervention study were excluded, as they were probably reported by patients with chronic respiratory diseases. The five domains were: general symptoms (shivering, muscle pain, tiredness, chest pain, dispnea), nasal symptoms (runny nose, obstructed nose, sneezing, nose itch), eye symptoms (itching eyes), throat symptoms (sore throat, itching throat, cough and hoarseness) and ear symptoms (ear pain) and body temperature. Temperature was

registered if the absolute value met above 37.5°C. Each symptom would be classified accordingly to the severity in a likert scale from 0 to 7.

A pilot study was performed with an elderly population already in an exercise program and due to difficulties in filling the Likert scale it was adapted to classification of the symptoms when present in mild, moderate or severe.

All participants was given a symptom diary, and explained individually how it should be filled, also a written explanation was provided in the questionnaire itself. Symptoms diary should be filled daily if any of the symptoms was experienced. A contact to the investigator was provided if any questions or doubts were present. All participants were contacted telephonically each month to maintain compliance and questioned if they were still in the trial, if they had been sick or had any problem filling in the questionnaire. The questionnaire could also be filled by a third person if the participant was not able to read. In the nursing home the responsible for the elderly was also trained to know how the questionnaire should be filled.

4.1.4. Effect of an high fat meal on neuro-immune-endocrine response to an acute exercise challenge (IV)

This is a randomized cross-over trial, with a wash out period of 7 days, single-blinded, placebo controlled. Study design is specified in figure 1. Participants will be recruited from advertisement and four different groups will be compared: obese-asthmatic; asthmatic non-obese; obese without asthma and non-asthmatic and non-obese. Stratification will be done accordingly to having asthma and $BMI \geq 30 \text{ kg/m}^2$. All patients will be allocated to a meal challenge in which a high fat meal will be followed by a low fat meal or vice-versa, meal challenge order will be randomized and participants blinded to the intervention. Three hours after it will be followed by an exercise challenge, which is aimed to induce acute immune changes. The main outcome will be exercise-immune function (immune function markers) changes after a diet challenge. As secondary outcomes stress/inflammatory markers, endocrine mediators, as well as blood fatty acids will be measured. Lung function, VO_2 and airway inflammation will be assessed by exhaled breath condensate inflammatory markers and eNO. Any adverse events of the challenge will be registered, namely respiratory infections, gastro-intestinal symptoms and muscular ache.

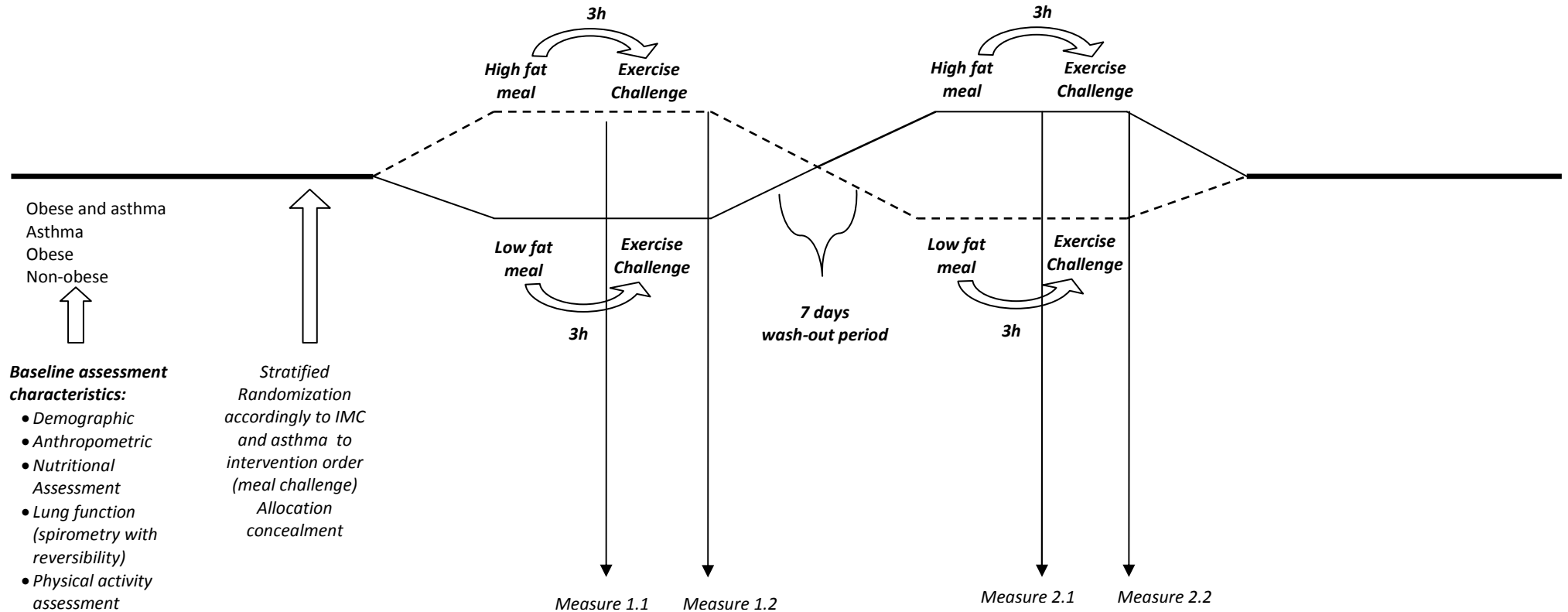


Figure1. Randomized cross-over study trial, single-blinded to meal intervention. Measures (1.1; 1.2;2.1;2.2) immediately before and after exercise challenge include: Immune-endocrine-metabolomic response (blood, urine and saliva samples); Pupilometry; Lung-function and VO₂ consumption; skin prick histamine reactivity

Participants

Participants will be recruited from March to September 2013 by public advertisement in the Porto metropolitan Area from be from Porto University Faculties, by journal, media and through the academic organization information. In the advertisement a simple explanation will be provided about the study. Eligibility criteria will be: target age from 18 to 35 years old, have controlled asthma (which answered positively to the question have you ever been diagnosed with asthma by a medical physician? and controlled assessed by Asthma Control Questionnaire e o CARAT- **Attachment 7**), obese and non-obese and healthy participants for control group.

Groups will be stratified by having asthma and by their IMC in 4 groups: Asthmatic and obese; asthmatic non-obese; obese and non-asthmatic and non-obese. Patients will be considered as obese if they have $BMI \geq 30 \text{ kg/m}^2$

Exclusion criteria will be: smoking in the past 6 months, abnormal electrocardiogram screening, pregnancy, diabetes mellitus, cardiac arrhythmia, angina, congestive heart failure, renal or hepatic failure and systemic disease, mal-absorption disease history, intestinal inflammatory disease, unable to adhere to full intervention or complete follow-up, unable to perform lung function testing and impaired mental status that makes difficult to follow or understand the instructions. Non-obese healthy controls need to have: no respiratory symptoms, had never a doctor's diagnosis asthma, normal lung function and have to be steroid-naïve.

Procedures

After recruitment an interview will be performed to verify if patient verifies the inclusion criteria. This interview will assess weight and height, previous history of asthma and its control, presence of co-morbidities and availability to the study intervention and follow-up. If included a baseline assessment will be performed in the same day.

Baseline assessment will consist in obtaining information about: demographic characteristics, assessment of co-morbidities, anthropometric measure, blood pressure, rest heart rate, pupilometry, nutritional assessment, lung function measured by spirometry and bronchodilation, atopy assessed by skin prick test, basal physical activity assessment through a questionnaire, patients will also use an accelerometer for 7 days to evaluate their daily physical activity.

Randomization and blinding

Patients will be informed of the procedure but they will be blinded to which is the intervention (high fat or low fat meal). Stratified blocked randomization will be performed accordingly to being asthmatic or obese. This methodology was chosen despite acute changes are the aim, to exclude and evaluate the intervention order in the studied outcomes. Equal number of participants will be allocated to each group.

Allocation concealment will be prevailed as investigator will only know the intervention order for that participant at the moment the meal is provided. Exercise challenge and outcome assessment, namely urine, blood sample collection, exhaled breath condensate and lung function will only be performed by another investigator blinded to the meal order.

Intervention:

Meal challenge will be of high fat and low fat meal. Both meals composition will be controlled and built by a nutritionist team. All food left in the plate will be evaluated and registered to understand the caloric ingestion of each participant. No restrictions will be imposed previously neither diet control, as acute changes are the main outcome of this study and a daily diet diary will be provided. Only patients with controlled asthma can start the intervention, in the day of the first challenge they will return the accelerometer.

After the meal challenge, participants will not be allowed to eat or drink, in exception to water, and cannot perform any physical exercise. Then they will be taken to an exercise challenge laboratory, at Sports Faculty of Porto University, there 3 hours after the meal challenge blood, urine sample and exhaled breath condensate will be collected, heart rate at rest registered, skin test reactivity with histamine and spirometry performed. Exercise challenge, after the measurements 1 or 2 (figure 1) will be done. During the exercise heart rate response, exhaled VO₂, CO₂ and symptoms will be systematically registered. A physician and a cardiopulmonary technician will follow the challenge. Immediately after, a spirometry will be done and then at 5, 10 and 15 min after stopping the run, induced bronchoconstriction, pupillometry will be also measured and skin test with histamine and new blood and urine samples will be taken. If a fall in FEV₁ over 10% occurs, bronchodilation will be done and patient treated. The exercise challenge will take at minimum 8 min, and patient will be asked to perform maximum effort, as the aim is to maintain for 4 min the heart rate at 95% of the target heart rate (220-rest heart rate).

Blood, exhaled air condensate, urine and saliva obtained before and after the exercise challenge will be used to analyze: metabolomic markers, immune function and inflammatory measures (in ex. leukocytes, lymphocytes, neutrophils, lymphocytes subpopulations and cytokine reduction, like IL-1, IL-6 and IgA in the saliva), stress mediators/hormones (ex. blood cortisol, growth hormone and epinephrine), endocrinological markers (ex. insulin, resistin, adiponectin, PAI-1, leptin) and fatty acids level (ex. monounsaturated, polyunsaturated and saturated fatty acids). Skin test sensitivity will be evaluate by histamine reactivity.

Patient will then go through a wash-out period of a week to avoid carry-over effect, as previously published intense acute exercise inducts changes in immune parameter that are maintained elevated for hours. It was also suggested an association between creatine kinase and IL-6 levels and the first can stay increased after exercise for almost 4 days after acute exercise challenge and by that the IL-6 level could be increase if shorter period of wash-out would be selected [14, 25].

4.1.5. Physical activity reduction effect on immune response (V)

This is a randomized controlled trial where participants, after a run-in period and eligibility evaluation, will be randomized to an intervention consisting in a reduction of physical activity by 50% or maintaining habitual physical activity for two weeks. Participants will be recruited from the same population as the meal-exercise challenge study and stratification will be done accordingly to having asthma and $BMI \geq 30 \text{ kg/m}^2$ in order for both characteristics could be balanced between groups.

Participants will be at baseline characterized for their nutritional status, dietary habits and physical activity through questionnaire and basal assessment of all sports activity practiced during a week. Also generic quality of life questionnaire will be applied. A pedometer and accelerometer will be used to monitor physical activity. After this run-in period, if they have an adequate physical activity they will be allocated randomly to one of the interventions. Calculation of physical activity reduction will be performed individually, and a daily steps target will be demanded for each group. Both groups will use a pedometer during 3 weeks, except during the night or at circumstances that the device might be damaged (bath). They will be able to monitor if they achieved or exceeded the target number of steps for that day. Keeping the same regular diet without any new changes will be requested and new recall dietary assessment will be also performed in the end of the study. Immune function mediators will be the primary outcome to be assessed before and after the intervention period in both groups. As secondary outcomes endocrine and metabolomic evaluation will be performed.

Participants

Participants will be recruited from March to September 2013 by public advertisement in the Porto metropolitan Area from Porto University Faculties, by journal, media and through the academic organization information. Same participants recruited for the meal-exercise challenge trial will be invited to participate in the physical activity reduction. Eligibility criteria will be 18 to 35 years old, able to perform exercise. In this study a run-in period will be demanded to evaluate inclusion criteria. In all participants daily-life physical activity will be evaluated and use a pedometer for a 7 day period to evaluate the mean number of steps/day. Only those with more than 3500 steps per day will be included for randomization. Inclusion criteria will be: target age 18 to 35 years, healthy, nonsmokers, revealed no physical abnormalities and were physically active (performed >3500 steps/day). Groups will be balanced in the characteristics: having asthma and if they are obese ($BMI \geq 30 \text{ kg/m}^2$) or non-obese.

Exclusion criteria will be: smoking in the past 6 months, pregnancy, diabetes mellitus, cardiac arrhythmia, angina, congestive heart failure, renal or hepatic failure and systemic disease, unable to adhere to full intervention or complete follow-up, impaired mental status that makes difficult to follow or understand the instructions.

Procedures

After recruitment an interview will be performed to verify if patient meets the inclusion criteria for study inclusion. This interview will assess weight and height, previous history of asthma and its control, presence of co-morbidities and availability to the study intervention and follow-up. If eligible, a run-in period will be performed that include: baseline assessment and a 7 week daily assessment of physical activity by a pedometer and accelerometer.

Baseline assessment will consist in: nutritional assessment, quality of life questionnaire (**Attachment 2**), asthma control questionnaire in asthmatic patients (**Attachment 7**), blood pressure, rest heart rate measurements, pupillometry, lung function measured by spirometry and bronchodilation, atopy assessed by skin prick tests, basal physical activity evaluated by Baecke (**Attachment 8**) and International Physical Activity questionnaire(**Attachment 9**). Physical activity will be also accessed by accelerometer and pedometer; only subjects that perform more than 3500 steps/day will be included. All other participants that are not eligible will receive dietary and physical activity written recommendations.

Randomization

Stratified blocked randomization will be performed accordingly to being asthmatic or obese. This methodology was chosen in order for both groups to be balanced for intervention comparison. Neither the participants or investigator team will be blind to the intervention procedure as there is a need to monitor number of steps/day to be compliant with the prescribed physical activity diary (keeping the same mean steps or reducing by 50% the number of steps/day). Equal number of participants will be allocated to each group.

Allocation concealment will be prevailed as investigator and participant will only know the intervention that they will be submitted at the second visit.

Intervention:

At baseline the included subjects will all collect blood samples, urine and exhaled air condensate. Then they will receive a list of indications adapted to their usual physical activity previously assessed in order for them to reduce by 50% their mean number of steps per day. As an example, they will be advised to: take the elevators instead of the stairs and riding car instead of walking or bicycling, reduce daily exercise training. The other participants will keep the same activity, also a number of target steps will be performed. Participants will not have knowledge of the mean number of steps that they usually performed, so both groups will receive specific written indications, but they will not know which will represent the intervention. No other contact will be done, but contact availability will be possible at anytime with investigators for any doubt. During the intervention the respiratory symptoms questionnaire will be given for them to fill in if they had an respiratory infection (**Attachment-1**)

After intervention all participants will deliver the pedometer. Blood, exhaled air condensate and urine will be collected. A questionnaire to evaluate nutritional assessment, quality of life (SF-36) and asthma control in patients with asthma will be performed.

The mediators that will be evaluated are: metabolomic markers, immune function and inflammatory measures (leukocytes, lymphocytes, neutrophils, lymphocytes subpopulations and cytokine reduction, IL-1, IL-6), endocrinological markers (insulin, resistin, adiponectin, PAI-1, leptin).

At the end of the study each patient will receive an individualized nutritional plan and physical activity indications performed by a nutritionist and a physician.

4.2. Measurements

4.2.1. Respiratory infection symptoms (I, II,III)

Data collection

Data will be collected throughout the study. In the validation study physicians will follow the inclusion criteria and each patient that meets possible inclusion for case or control, after accepting study inclusion demographic and clinical characteristics (age, gender, education, smoking habits, allergic diseases) will be collected before delivering the questionnaires. This information will be kept. The 6 days questionnaire of respiratory infections (**Attachment1**), and the SF-36(**Attachment 2**) and an pre-paid envelope with the Hospital/health center address will be given to the participant, explaining to him how it should be filled and that they could send it to the investigators by mail or left in the institution.

In the respiratory infection in athletes(I) a daily questionnaire will be provided and participants will fill in the questionnaire if they fill sick and daily until the symptoms have ceased. At the end of the 3 months follow-up questionnaires will be retrieved.

In the elderly population a simplified symptoms diary was provided, where they everyday coated which of the respiratory symptoms they had.

Respiratory infections symptoms questionnaire (I, II)

Respiratory infections symptoms questionnaire has 21 items, organizes in a 7 point Likert-type severity scale (from 1, very mild to 7, severe and one 0, without symptoms). Two items are used to classify the global-severity-today (how sick do you feel today and compared to yesterday I fell my cold is...). The severity score includes 10 items classifying in the last 24h respiratory symptoms (runny nose, plugged nose, sneezing, sore throat, scratchy throat, cough, hoarseness, head congestion, chest congestion, feeling tired) and 9 items of quality of life impact (think clearly, sleep well, breath easily, walk, climb stairs, accomplish daily activities, work outside home, work inside home, interact with other, live your personal life) [10, 40].

An acute respiratory infection episode is defined by having all these items for at least 2 days:

- answering "Yes" to either: "Do you think you have a cold?" or "Do you think you are coming down with a cold?"
- reporting at least 1 of 4 cold symptoms (or synonyms) (nasal discharge (runny nose); nasal obstruction (plugged or congested); sneezing; or sore (scratchy) throat)
- scoring at least 2 points on the Jackson scale (Jackson score is calculated by summing 8 symptom scores (sneezing, headache, malaise, chilliness, nasal discharge, nasal obstruction, sore throat and cough), rated as 0=absent, 1=mild, 2=moderate, and 3=severe.

From the first day of acute respiratory infection day and forward each participant fills out a daily WURSS until they answer "No" to the question "Do you think that you are still sick with this respiratory infection?" for 2 days in a row. The last day the participant answers "Yes" and fills out a WURSS instrument will be the last day classified as acute respiratory infection.

Daily WURSS summary scores are calculated by summing scores of individual item scores, excluding the first and last items, which have categorically different reference domains, and are analyzed separately. Summary scores of ARI illness episodes are calculated by summing daily scores.

Respiratory infections symptoms diary (III)

Symptom diary was built accordingly to the previously published respiratory infection assessment and after a pilot study to search for comprehensibility and adaptability to the population age [45, 46]. It was subdivided in five main symptoms categories: general symptoms (shivering, muscle pain, tiredness, chest pain, dyspnea), nasal symptoms (runny nose, obstructed nose, sneezing, nose itch), eye symptoms (itching eyes), throat symptoms (sore throat, itching throat, cough and hoarseness) and ear symptoms (ear pain) and body temperature. Temperature was registered, if the absolute value was above 37.5°C. Each symptom would be classified accordingly to the severity in mild, moderate or severe by the participant.

Respiratory infections were defined accordingly to symptoms assembly and period of time. Duration of respiratory infection was calculated accordingly to the symptoms that were correspondent to infection episode duration. Severity of the respiratory infections was classified as mild, moderate or severe, based in the type of symptoms and if they affected general health state of the individual.

Probable respiratory infection was defined by obtaining in at least three consecutive days, one of the four different settings:

- moderate or severe symptoms in two of five symptom categories (excluding the allergic symptoms namely nasal or ocular pruritus);
- mild/moderate symptoms in more than 3 symptom categories
- cough associated to at least 3 symptoms of 2 different symptom categories
- fever with other respiratory symptom.

Severity of respiratory infection was classified in: severe (participant had a systemic infection and/or severe local symptoms); moderate (mild systemic symptoms with moderate local symptoms) and mild (mild to moderate local symptoms).

4.2.2. Health state and quality of life measurements (I,II)

Health state and quality of life questionnaire (MOS SF-36v2)

At the third day of illness in the respiratory infection questionnaire validation it will be also filled in a validated questionnaire in Portuguese, MOS SF-36 (**Attachment2**), of health state. Quality of life was assessed using the SF-36, a self administered, widely used questionnaire, and validated to quantify subjective reports of health in terms of functional status, emotional status, and general well being[41, 42]. This generic instrument is composed of 36 questions, distributed

into eight different dimensions of health: physical function, role limitations related to role physical, bodily pain, general health perception, vitality, social functioning, role limitations due to emotional problems, and mental health. Each dimension is reported on a scale from 0 to 100 with a higher score reflecting better quality of life[41, 42].

Anxiety questionnaire

The Zung self-rating anxiety scale is a questionnaire aimed to evaluate a state of anxiety more than a anxious personality[47]. It has been validated in portuguese and is composed by 20 items that explore affective (5 items) and somatic (15 items) aspects of anxiety, on a 4-level scale[47, 48]. The total point score can range from 20 to 80. A high score indicate a high degree of anxiety. (**Attachment 4**).

Sleep and sleep quality questionnaire

To understand and evaluate sleep quality a questionnaire will be applied, asking sleep habits and sleep quality, both addressed by the Pittsburgh Sleep Quality Index. This is a self-rated validated questionnaire that assesses sleep quality and sleep disturbances over a 1- month time interval. A score >5 is used to define a 'poor sleeper'. (**Attachment 3**)

4.2.3. Nutritional Assessment (II, VI)

Anthropometric measures (II,IV)

Anthropometric measures will include weight, height and specific measures to determine body composition, namely a triceps skinfold thickness and medial calf skinfold thickness.

Weight will be measured to the nearest 0,1 kilogram and height will be measured to the nearest 0,5 centimeters. Subjects will be lightly clothed and bare-footed.

After height and weight, it will be calculated body mass index (BMI) - weight (kilograms) divided by square of height (meters).

Individuals will be classified according to World Health Organization (WHO) classifications: normal weight, 18.5 to 24.9 kg/m²; overweight, 25.0 to 29.9 kg/m²; obesity, 30.0 kg/m² or plus.

Skinfold thickness measurement will be taken using a slim guide skinfold calipers and according to procedures described by International Society for the Advancement of Kinanthropometry (ISAK)[49]. Three measurements will be taken, at each site (+/- 0,1 mm). Percentage body fat will be calculated using prediction equations (Males: % body fat = 0,735 * (triceps + calf) + 1.0 / Females: % body fat = 0,610 * (t+c) + 5.1), as previously published [50].

Waist circumference will be measured according to procedures described on ISAK[49]. Individuals will be classified in abdominal overweight or obese, according to the following sex-specific cut-offs: females - abdominal overweight 80 - 88 cm, abdominal obesity 88 cm or plus; males - abdominal overweight 94 - 102 cm, abdominal obesity 102 cm or plus[51].

Dietary Reports (II, IV)

Dietary intake will be assessed by a repeated 24-hour recall. Participants will be asked to provide detailed information about their dietary intake, through a 24-hour dietary recall method. This method will be conducted in four steps: first, individuals will be asked to inform the interviewer about all foods and beverages they've consumed in the previous 24 hours; second it will be questioned for foods that are documented as being frequently forgotten; third, individuals will be asked about the time and occasion at which foods were consumed; finally, the interviewer will ask questions about food description and amounts.

Participants of study IV, will provide their reports on the first and second meetings.

4.2.4. Airway Inflammation (III)

Sputum collection and analysis will be performed as described previously in literature [52]. Patients will perform previously bronchodilation with salbutamol followed by a protocol of inhalation of hypertonic saline (5%NaCl) generated by an ultrasonic nebulizer during 5 min periods for 20 min. Sputum will then be obtained. After the sample is collected it will be selected in a Petri dish, and the selected sample treated with dithiothreitol (Sputolysin; Calbiochem Corporation, San Diego, CA, USA). The suspension will be centrifuged and the cell pellet re-suspended. Cytospins will be prepared and stained using May-Grünwald/Giemsa. Differential cell counts will be made by counting a minimum of 500 nonsquamous cells. Sputum eosinophilia will be defined as a sputum differential of eosinophil count over 4%.

Metabolomics is the study of small molecules (0,1 kDa) generated from cellular metabolic activity and it seems to be a potential biomarker for airway inflammation[33]. Exhaled breath has potential to be a matrix with relevant metabolomic information to characterize asthma[34, 53]. Comprehensive two-dimensional gas chromatography–time of flight mass spectrometry (GC ×GC–ToFMS) has been used to on exhaled breath in patients with asthma and a pattern of six compounds belonging to the alkanes were found that characterized the asthmatic population: nonane, 2,2,4,6,6-pentamethylheptane, decane, 3,6-dimethyldecane, dodecane, and tetradecane. This profile can be useful for distinguishing patients with asthma from other different inflammatory airway disease and possibly new profiles can be withdrawn from this technique, namely characterizing an illness prone profile [34, 53]. The breath sampling will be obtained as previously published [34, 53]. Briefly, in a previously clean 1 L Tedlar® bags and after washing the mouth with water will exhale deeply into the Tedlar® bag previously holding their breath for 5 seconds. All bags will be analyzed for a maximum of 6 h after the collection [34, 53]. This analysis can also be performed in exhaled air condensate, this is obtained accordingly to the previous published guidelines. Extraction/concentration will be done with HS-SPME methodology to coating fiber, this will then be introduced into the GCx GC–ToFMS, which consists of an Agilent GC 7890A gas chromatograph, with a dual stage jet cryogenic modulator. Detection will then be performed by a high-speed ToF mass spectrometer. In order

to identify the different compounds, the mass spectrum of each compound detected will be compared to those in mass spectral libraries databases[34, 53].

4.2.5. Lung function and airway responsiveness (III, IV,V)

Spirometry will be carried out according to the American Thoracic Society criteria[54]. Lung function measurements will be repeated 15 minutes after 400 µg of salbutamol in aerochamber to assess reversibility.

Non-specific bronchial hyper-responsiveness will be measured by methacholine challenge, according to the previous published guidelines. Methacholine will be delivered by inspiration triggered by a dosimeter, five-breath will be needed and PD20 (provocative dose causing a 22% fall in forced expiratory volume in one second (FEV1)) determined.

The capsaicin challenge will be performed accordingly to the previous guidelines [55]. The procedure will be performed in a ventilated area. Fresh dilutions of capsaicin solution (capsaicin in powder (30.54 mg) dissolved in 1 ml of Tween 80, 1 ml of ethanol and 8 ml of normal saline) are performed with physiological saline in the laboratory, previous to the challenge and stored at 4°C. Dilutions of 0.49, 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250 and 500 µM are prepared. Three ml of each solution are provided by nebulizer through a dosimeter. Subjects perform a baseline spirometry and then start the protocol: inhaling a control solution of normal saline followed by progressively increasing concentrations of capsaicin solutions at 2 minutes intervals. A breath-activated nebulizer controlled by a dosimeter (Optineb®, Air Liquide), set to nebulize for 0.9s will be used. Subjects are asked to cough freely and the concentrations inducing 2 and 5 coughs during the 30 seconds period after capsaicin administration will be registered. The test is finished when 5 coughs are elicited or when the maximal dose (500 µM) is achieved. At the end of the challenge, a new spirometry is performed.

4.2.6. Allergic and inflammatory systemic markers (III, IV, V)

Skin prick tests will be carried out in accordance international guidelines with a standard battery of commercial extracts for common aeroallergens[56, 57]. Histamine dihydrochloride (10mg/ml) and diluent will be as used as positive and negative controls, respectively. The tested extracts will be stored at +2 to +8°C when not in use. A small drop of each testing solution will be placed on the volar forearm surface of the patient arm and then pressed against the skin in the centre of the allergen drop for at least 1 s without causing bleeding. Results will be recorded after 15 min. The largest and perpendicular diameter of the wheal for each of the allergens is measured and the following value calculated: largest + perpendicular diameter/2. A subject is defined as atopic in the presence of at least one positive result (regarded if the value calculated was ≥3 mm and controls showed adequate reactions) [56, 57].

Inflammatory systemic markers, namely urine metabolome (study II), will be performed by the same methodology already described in the metabolomic analysis of the exhaled breath condensate on point 4.2.4.

4.2.7. Parasympathetic activity measure (III,IV)

Pupillary measurements will be taken with a portable infrared PLR-200™ Pupillometer (NeuroOptics Inc, CA, USA). This comprises in two major parts, a hand-held optical unit and an electronic board. The subjects will be asked to spend at least 15 min in a room to allow their eyes to adjust to the low lighting levels before any measurements are undertaken on their pupils. Each subject will sit upright on a chair and the measurements are performed by the operator by resting the pupillometer optical unit against the subject's face. Two pairs of infrared-emitting diodes of an 880-nm wavelength (programmed to adjust intensity automatically for papillary pigmentation and ambient light) will illuminate the eye. The measurement takes about 3 seconds. At the end of the measurement cycle, a graph of the pupil diameters as a function of time will appear on the host screen. Five pupil light response curves will be recorded. The following parameters will be calculated: initial diameter (Init Dia), minimum diameter (Min Dia), final diameter (Final Dia), time to minimum diameter (TTM), reflex amplitude (RA= Init Dia_Min Dia), constriction velocity (CV), reflex amplitude as percentage of initial diameter (%RRA), maximum constriction velocity (Max CV), time to maximum constriction velocity (TM MCV), maximum redilation velocity (Max RV), time to maximum redilation velocity (TM MRV), relative redilation residue as percentage (%RRR = Init Dia_Final Dia/Init Dia_Min Dia), and time at which pupil has re-dilated 75% of the reflex amplitude (TM 75%).

Heart rate after 15 min rest will be measured , during the procedure verbal instructions and a practice period of paced breathing will be provided.

4.2.8. Meal challenge (IV)

All individuals will be asked to consume two different meals, an high fat/high energy meal and another low fat/low energy. The high-fat/high energy meal will by characterize by: total energy (TE) approximately of 1600kcal, consisted of 40g protein (9,6% TE), 218g carbohydrate (55% TE), and 60g total fat (34% TE) and 18g saturated fat (10% TE). The other meal will be according to the Portuguese guide - Roda dos Alimentos[58], characterized with low-fat/low energy meal: TE approximately with 660kcal (1/3 of 2200kcal), consisted of 50 to 70% TE of carbohydrate, 30% TE of total fat and less than 10% of saturated fat.

4.2.9. Exercise challenge (IV)

Protocol will be performed accordingly as previously published and aimed to access physical fitness, exercise induced bronchoconstriction [59, 60].

Contra-indications to perform exercise testing will be: moderate to severe airflow limitation (predicted FEV1<60% or <1,5L), heart attack or stroke in the last 3 months, uncontrolled hypertension (systolic BP>200 or diastolic BP>100), malignant arrhythmias, know aortic aneurysm, orthopedic limitations to exercise, inability to perform acceptable-quality spirometry, pregnancy, nursing mothers, current use of cholinesterase inhibitor medication (myasthenia gravis).

The challenge will be performed in controlled conditions, ambient temperature between 20-25°C, with low relative humidity (less or equal to 50%). Heart rate will be monitored during all test by a reliable measurement (three lead electrocardiographic configuration or pulse oximeter), nasal clips will be used during the test and blood pressure will be taken before the challenge.

Exercise challenge will be done in a treadmill with starting with a slope of 5.3% and consists: in a 2 min warm up until the heart rate achieved 70-80% of the estimated HR peak, calculated as $(220 - \text{age [years]})$, (ventilation can also be used to monitor and should reach 40-60% of the predicted maximum voluntary ventilation, estimated by $\text{FEV}_1 \times 35$). The speed will then be adjusted during the first 4 minutes to achieve a workload corresponding to the maximum speed subjects will be able to sustain for the last 4 min, about 95% of the estimated HR. This test usually requires 6 to 8 min, but longer period of time will be needed and will be adjusted to an individual basis and the patient physical condition. At the end of the test VO_2 , VE, RER, and HR will be measured as well as lung function, namely maximal forced expiratory flow volume loops at 1, 3, 5, 10, 15 and 20 minutes after the challenge. In all patients bronchodilation with salbutamol will be performed after 15 min.

4.2.10. Immune-Endocrine and metabolomic mediators assessment (II, IV, V)

Urine, blood and saliva samples will be collected to assess immune, endocrine and metabolomic assessment markers.

In urine samples metabolomic analysis will be performed with the same protocol described for exhaled breath condensate. But, samples will be collected and immediately frozen at -80°C, then only those samples that have been related to the outcome, respiratory infections in the swimmers cohort or those that completed all protocol of the clinical trials will be used.

Saliva will be collected as previously published[61], subjects will set quietly and with an initial swallow to empty the mouth, un-stimulated whole saliva will be collected by expectoration into a pre-weighted vial for 2 min with eyes open, head tilted slightly forward and making minimal oro-facial movement and saliva flow rate (ml/min) will be determined by weighing saliva density. Duplicate saliva samples can be analyzed for secretory IgA (S-IgA) using ELISA kit. The S-IgA secretion rate will be calculated by multiplying the S-IgA concentration by saliva flow rate

Blood will be obtained by venepuncture from an antecubital vein and will be collected into two Vacutainer tubes containing EDTA, 7ml and two serum-standard tubes 10 ml. Two of the samples with serum and plasma will be centrifuged and then stored -20°C. Hematological parameter, total and differential leukocyte counts will be performed in an automated cell-counter. Lymphocyte subpopulations (CD3, CD4, CD8, CD19, CD56) can be determined by flow cytometry with a three-color flow, in order to enumerate total T cell, T helper, T cytotoxic, B

and NK cells. Side-scatter and forward scatter will be use to gate lymphocyte population by morphology and at least 10 000 lymphocyte events will be acquired per analysis.

Serum Immunoglobulins IgA, IgG, IgM can be measured by nephelometry. Cytokines, IL-1, IL4, IL8 IL-10 also could be measured using commercial ELISA. Insulin, adiponectin, leptin, resistin, tumor necrosis factor- α , serpin/type-1 plasminogen activator inhibitor and interleukin-6 measured by multiplexed bead immunoassay, as previously described[62]. Plasma fatty acids concentration will possibly be analyzed using gas chromatography as previously described[63].

Plasma epinephrine and norepinephrine concentrations might be measured by high-performance liquid chromatography. Plasma adrenocorticotrophic hormone concentrations could be measured by an electrochemiluminescence immunoassay while plasma cortisol concentrations radioimmunoassay will be used.

4.2.11. Physical activity measurement and intervention (IV,V)

Physical activity will be estimated by: Baecke questionnaire (**Attachment 8**), and International physical activity questionnaire (**Attachment 9**) and by the mean number of steps/day performed during one week assessed by a pedometer.

Baecke questionnaire(**Attachment 8**) is a reliable and valid instrument and has been previously used in the Portuguese population[64, 65]. It is made up of 16 items that call for a Likert-type response (from 1 to 5), designed to assess different categories of the broad concept of physical activity (work/school, sport and leisure). The work/school index was calculate by eight questions (sat,stood, walke, Lifted heavy loads, sweat, work level. Sport index were reported in 4 questions, as well as leisure time index. Total physical activity score could range from 3 to 15. The International Physical Activity questionnaire (IPAQ) (**Attachment 9**) is a general measure of assessment of physical activity and it complements the Baecke questionnaire in physical activity assessment. It is also a valid and reliable measure is several countries including Portugal and has a comparable validity and reliability to the accelerometer and other questionnaires[66].

IPAQ reports separately vigorous-intensity, moderate-intensity and walking in terms of frequency and duration of each specific type of activity, in the past 7 days. This instrument also reports time spent sitting in an ordinary week day. IPAQ will be an expressed according to the guidelines for data processing and analysis in as MET-min/week (metabolic equivalent). After which they will be classified in three categories, low PA level, moderate PA level and high PA [67] .

Physical activity will be evaluated at baseline by two parameter, a pedometer and accelerometer. The accelerometer (Computer Science Application Actigraph), one of the most widely used accelerometers in physical activity research, uses a uni-axial accelerometer that

measures vertical acceleration and deceleration in 1-min epochs. Participants will put it on the right mid-axillary line of the hip on an elastic belt or clip and wear during one week period and then remove it for sleep or any activity that could cause harm to either the monitor or another person. This can be used to monitor light moderate, hard and very hard activity [68]: light intensity will be considered as <3 metabolic equivalents (METs), moderate 3–5.99 METs, hard 6–8.99 METs, and very hard >8.99 METs. Activity counts cut-off, will correspond to MET levels by the equation $(\text{cnts}/\text{min}) = (5.99 - 1.439008) / 0.000795$ [68]. Pedometer is also a validated method to evaluate physical activity it will be used in association with an accelerometer for baseline physical activity assessment. To register the number of daily steps the subjects will be instructed to wear the pedometer placed in the waistline above the knee, as previously published [29] and it will be worn for 1 week, being removed for sleep or other activity that could damage the device, namely bath.

Pedometer will be further used for intervention of reduced physical activity (V). In this study, accordingly to the previous baseline number of steps per day were instructed to reduce daily steps in more than 50%. For that accordingly to the assessed physical activity in baseline evaluation in those that performed >3500 steps/day, will be recommended to use elevators instead of stairs, riding in cars instead of walking or bicycling during 2 weeks period. A limit of steps/day will be calculated for each participant and they will be able to see if the limit was achieved. All steps will be recorded in the display and stored in the device for posterior computer download.

4.3. Statistical analysis (I-V)

All statistical analyses will be performed using the SPSS 20.0 software (Chicago, Ill) and Excel, Microsoft Office, for data assembly and graphical analysis. Normality of data distribution will be assessed by the Kolmogorov-Smirnov test, histogram and QQ plot distribution. Results will be expressed as median and inter-quartile range or mean and standard deviation and in discrete variables as count and percentage. If a normal distribution is described t-test-student would then be used in continuous variables. For non-normal data, non-parametric tests, namely Mann-Whitney will be used, to compare groups. Measures with discrete distributions are expressed as counts (%) and analyzed with Pearson-Chi Square or Fisher exact test. The minimum threshold for statistical significance will be $p \leq 0.05$. In data with skewed distributions, logarithmic transformation will be done and if appropriate (normal distribution is achieved) comparisons will be made with this change.

4.3.1. Cross-cultural translation and Validation of WURSS (I)

For statistical analysis of questionnaire validation factorial analysis will be used for content validity evaluation using the 7 identified domains of WURSS-21 plus headache, body-ache and fever, which are included in WURSS-24. This approach combines exploratory and confirmatory procedures, using weighted least square estimates employing diagonal weight matrix techniques to seek common factors between the domains; all questioned items will be included in the analysis. Convergent validity will be provided by Pearson correlations between WURSS-21 scores in the first day and SF-36 scores in the third, as this measures health state in the last 4 weeks. For criterion validity sensitivity, specificity and predictive positive values will be calculated, as well as area under the curve. For internal consistency Cronbach's alpha coefficient in each domain based on the average of scores from the first three days will be calculated and also comparison between the first and last day. To evaluate each day, responsiveness index will be calculated which is equal to minimal important differences (MID)/ $\sqrt{(2 \text{ mean squared error})}$.

Sample size calculation

Sample size was calculated assuming the worst proportion for an item, based in the previous published literature, which was chest congestion, that occurred in a prevalence of 75,7%, then assuming a confidence interval of 0,95% and a $\alpha=0,05$ (with a standard normal deviate for a two-sided α), the required number would be 280 participants. This sample is adequate for the target factorial analysis in which 24 items will be used (WURSS-21 + headache, body ache and fever). For controls comparison and predictive validity evaluation, assuming that WURSS has a Sensitivity of 0,85 and Specificity of 0,44, and the fact that 70% of the population experiences a cold in a given year[9, 39], for a desired precision of 0,05 for the sensitivity and for a specificity of 0,1 the sample size population needed will be 281 participants. The largest population of 281 patients will be chosen as target for evaluation.

4.3.2. Respiratory Infection susceptibility in athletes and elderly (II,III)

All potential risk factors for respiratory infections will be analyzed for two different in risk populations, elderly and athletes (II,III). Elderly clinical trial will be analyzed as a completed-protocol (III).

Descriptive values for each risk factor will be calculated and Relative Risk to Respiratory Infection with the respective confidence intervals at 95% assessed.

Multiple linear regression and logistic regression models will be used as appropriate to modulate for possible confounders, namely respiratory infections and their severity will be given by questionnaire scores. Univariate and multiple logistic regression models will be developed using independent variables as risk factors for having respiratory tract infections. Results will be presented as odds ratio (OR) with [95% confidence interval (CI)].

Variables potentially included in the model for swimmers illness profile (II) will be: gender, age, BMI (variable categorized), anthropometric changes, history of respiratory tract disease, smoking habits, previous influenza immunization, probiotics consumption, vitamins consumption, sleep quality, anxiety, nutritional status, urinary metabolomic profile, asthma, bronchial hyper-responsiveness, airway inflammation presence and immunological mediators.

Variables used in the elderly intervention model will included: gender, age, BMI, history of respiratory tract disease, diabetes, smoking habits, previous influenza immunization and previous practiced exercise training.

Models will be performed by data driven and progressively adjusted considering its goodness-of-fit (assessed by Hosmer-Lemeshow test) and predictive power (evaluated by ROC curve analysis).

For the moderate exercise intervention trial in elderly (II) sample power was calculated as infrastructure conditions only allowed a maximum sample of 40 persons in each group to be included. Relative risk of having an infection in the training group and in control group will be used. In the previous published literature assessing postmenopausal women [24], having an infection during an moderate-exercise intervention, 48% had at least one episode of respiratory infections compared to control group, which had at 30% in the second. Assuming an alpha of 0.05 on exercise group had 48% change of having infection and 30% of having infections in the non-exercising group the power would be 30%.

Sample size calculation

In the athletes population, all swimmers under competition in the Porto's team Swimmers will be eligible to the study, a population of 40 participants in total can be evaluated for this study purposes. Using previous studies comparing the rate number of respiratory infections for this specific population, elite swimmers under 12 weeks high intensity period before competitions 40% have a respiratory infection, with an alpha of 0,05 the power will be 35%.

4.3.3. Acute exercise and meal challenge (IV)

In the randomized cross-over study, includes all randomized subjects which took at least one meal challenge and exercise intervention and who had at least one post-baseline immune marker variable measurement. Primary and secondary endpoints will be analyzed for the presence of a carry-over effect in the meal challenge study [69].

Changes in the immune biomarkers and endocrine will be presented as mean $\pm$ standard deviation. Data obtained from high fat and low fat meal challenge will be compared using paired t-tests. Repeated-measures analysis of variance (ANOVA) were used to analyze the effects of the challenges.

Sample size calculation

This study is a 2-intervention, 2-period cross over design. Each subject received each meal once in one of the two periods in an order dependent on the randomization, and consequently comparisons were made within-subject. Estimates of between-subject variability have been derived for from previous studies evaluating meal high carbohydrate ingestion in physically active subjects, before an exercise challenge[70]. Standard-deviation was estimated for interleukin-6, more specifically to be 1.3 of standard deviation. This cannot be generalized to this population as they are not physically activ, but no study was previously performed in asthma patients or obese. Only in one previous study performed an high fat challenge in asthma and obese patients and showed a standard deviation in IL-6 of 0.5[63]. Assuming a range difference of 1, it was calculated a value over 80% power to demonstrate (at the 5% level of significance) a difference between interventions of 1 pg/ml in the IL-6, in a sample size of 25 patients in each sequence. To allow for dropouts the study was designed to recruit 30 patients in total, 15 in each group asthma and obese.

4.3.4. Reducing physical activity (V)

The analysis was conducted using the “intention-to-treat” (ITT) approach, including all randomized subjects which took at least one dose of the study preparation/or intervention and who had at least one post-baseline efficacy variable measurement. Changes between baseline and after intervention period was analyzed with paired t-tests. The absence of studies evaluating the immunological outcomes precluded the power analysis.

5. Safety

An Adverse Event (AE) is any untoward medical occurrence in a patient regardless of its causal relationship to the study treatment. An AE can therefore be any unfavorable and unintended sign, symptom or disease, temporally associated with the trial. The occurrence of an AE may come to the attention of study personnel during the study interviews or procedures or upon review by a study monitor. All AEs including local and systemic reactions not meeting the criteria for “serious adverse events” will be registered (event description, time of onset, clinician’s assessment of severity), relationship to study procedures will be searched. All AEs will be followed to adequate resolution. Any medical condition that is present at the time that the subject is screened will be considered as baseline and not reported as an AE. However, if it deteriorates at any time during the study, it will be recorded as an AE. All AEs will be assessed by the clinician using a protocol defined grading system: mild, events require minimal or no treatment and do not interfere with the subject’s daily activities; moderate, events result in a low level of inconvenience or concern with the therapeutic measures and severe, events interrupt a subject’s usual daily activity and may require systemic drug therapy or other treatment. As relationship to the study is concerned an AE will be classified in: associated, event is temporally related to the administration of the study product and no other etiology explains the event and not associated, event is temporally independent of study product and/or the event appears to be explained by another etiology.

A serious adverse event is defined as an AE that meets one of the following conditions: death during the period of protocol-defined surveillance; life-threatening event (defined as a subject at immediate risk of death at the time of the event); inpatient hospitalization or prolongation of existing hospitalization during the period of protocol defined surveillance and any other important medical event that may not result in death, be life threatening, or require hospitalization, but based upon appropriate medical judgment, the event may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed above.

Safety

No safety issues will come from the participation in the respiratory symptoms survey study (I), as only observational data will be retrieved. Data confidentiality will be preserved and the questionnaires will be identified by a number.

For the illness-prone evaluation profile (II), baseline assessment procedure as well as measures were previously submitted and accepted by the ethical commission (**Attachment-5**).

For lung function evaluation and testing procedures the following risk and safety measures will be considered:

- Lung function will be assessed by spirometry and bronchodilation response will be evaluated by salbutamol, all good practice guidelines will be followed[54]. These are

safe procedures only a possible discomfort related with forced expiratory maneuvers can be felt, but no adverse reactions are expected.

- In capsaicin challenge in a recent review adverse events were investigated and only minor side effects like throat irritation, self-solved feeling was reported during the procedure [71]. Published contra-indications will be respected[55].
- Methacoline challenge is a standardized procedure widely use for bronchial hyper-responsiveness assessment[60], accordingly to guidelines, a medical trained doctor trained in evaluating and treating bronchospasm will be present at all times and appropriate resuscitation equipment available. Contra-indications published in the guidelines will be respected[60].The procedure will be performed in total safety conditions starting from very small doses and in stepwise, as soon as positive criteria is achieve or any symptom occurs procedure is stopped and will be given inhaled salbutamol to reverse bronchial constriction. Transient symptoms can occur namely dyspnea, cough and chest tightness but will be immediately treated. Participant will be discharged when he returns to their baseline spirometry values and his symptomless.
- Exhaled nitric oxide, exhaled breath condensate measurements were not associated in the literature to safety problems, risks or complications[72].
- Induced sputum is a safe procedure, pre-treatment with salbutamol is used to avoid potential side effects and lung function will be monitored during the procedure with peak expiratory flow, when symptoms occur or a drop occurs in lung function the participant is immediately treated and broncho-constriction reversed. There are no reports of death or need for hospital admission in patients undergoing sputum induction[73].

Pupillometry (II, IV) is a non-invasive method, through an extensive search through databases for “safety”, “risks”, “complications”, and “safety” associated with “pupillometry” retrieved no results concerning potentials risks for this procedure were found. Contraindications, which will be strictly followed, include avoidance when the orbit structure is damaged or surrounding soft tissue has an open lesion or edema.

Blood sampling (II, III, IV and V) will be performed accordingly to the published recommendations and hygiene measures will be followed[74]. Risks related to phlebotomy have been previously described namely infection risk, light-headiness and local hematoma. All potential adverse effects will be registered and oriented by the researcher team. No adverse effects, except the inconvenience will be related with urine and saliva samples collection. The prick-puncture test is safe[57]. Systemic reactions with skin prick tests for inhalant extracts are rare and have decreased dramatically to an overall risk below 0.02% for anaphylactic reactions and no fatalities with aeroallergen commercial extracts have been reported[57]. Mild reactions are expected to occur in 15 minutes (defining a positive test) in a skin prick test local wheal, itching, and erythema. Delayed or systemic reactions are unexpected.

Exercise testing (IV), namely maximal symptom-limited exercise testing is a relatively safe procedure, especially in otherwise healthy individuals. Fatality risk is related to medical complications to the underlying disease and the rate of death per patients during exercise testing is 2 to 5 per 100.000[75]. Strict contra-indications will be followed. A licensed physician and an cardio-respiratory technician will observe the participant during exercise and the recovery period and watch for undue stress (e.g., severe wheezing, chest pain, lack of coordination) or adverse signs (e.g., ECG abnormalities, falling blood pressure, decrease in O₂ saturation), if it occurs the test will be immediately stopped and any adverse event treated. A resuscitation cart will be immediately available. The population tested in this study will be young and otherwise healthy without know disease, only chronic co-morbidity will be asthma and obesity. Exercise induced bronchoconstriction is assessed by exercise challenge and it is a recommended and widely used method to evaluate asthma patients. Furthermore these population will be under controlled asthma. If any adverse event occurs, namely bronchoconstriction it will be immediately reversed with bronchodilation.

Exercise intervention in the elderly population (III), was already submitted and accepted by the ethical commission (**Attachment 6**). Moderate exercise program will be performed and adapted to each elderly participant, co-morbidities and difficulties and physical condition and anaerobic resistance for exercise strength will be evaluated previously to the training by resistance maximum. Exercise will then be adapted to each individual condition and progressively increased at each participant rhythm. The exercise will be guided by sports professors trained in exercise teaching and monitoring. Potential adverse conditions can occur, namely muscle and body ache which will be self-limited and prevented by this progressive adaptation to exercise. All elderly will have an individual insurance if any adverse event occurs, namely falls during training or any unavoidable accident.

Physical inactivity intervention (V) was previously studied to evaluate potential endocrinological impact [28-30]. This intervention does not aim to promote inactivity but an reduction of the ambulatory usual activity. During these trials the population was physically active and healthy, as it occurs in the studied population, and no long term adverse events were described. As for immediate side effects, only was seen a reduction of insulin sensitivity, cardiovascular fitness evaluated by VO₂ and lean leg mass[28]. No adverse events are expected during the duration of the intervention. At the end of the study a series of recommendations and educational advice will be provided also to the participant which will counteract with any potential adverse events of the well known sedentary behavior. Furthermore, a dietary counseling will be provided by a nutritionist which will also be beneficial for the participant.

High fat meal challenge followed by exercise has not previously been studied in this population. An isolated meal is not associated neither with immediate nor long term adverse events. Meal will be made accordingly to the appropriate hygiene rules and after confection will immediately

be provided to the participant. Only in a previous study high fat challenge increase airway inflammation and reduced bronchodilator recovery, but both of the effects were transitory and no side-effects were reported in the tested asthma and obese population[36].

Unanticipated Problems

Unanticipated problem is any incident, experience, or outcome that meets all of the following criteria: unexpected (in terms of nature, severity, or frequency) given the research procedures that are described in the protocol-related documents; related or possibly related to participation in the research and related with the research places, subjects or others that puts a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized. Any incident, experience, or outcome that meets the three criteria above, generally will warrant consideration of substantive changes in order to protect the safety, welfare, or rights of subjects or others. Corrective actions or substantive changes that might need to be considered in response to an unanticipated problem include: changes to the research protocol initiated by the investigator prior to obtaining Ethical Commission approval to eliminate apparent immediate hazards to subjects; modification of inclusion or exclusion criteria to mitigate the newly identified risks; implementation of additional procedures for monitoring subjects; suspension of enrollment of new subjects; suspension of research procedures in currently enrolled subjects; modification of informed consent documents to include a description of newly recognized risks; provision of additional information about newly recognized risks to previously enrolled subjects.

All unanticipated problems will be recorded and reported throughout the study.

6. Ethical Considerations

The investigator will ensure that this study is conducted in full conformity with the principles set in the Declaration of Helsinki. The protocol will be submitted for Ethical Commission approval.

Exercise intervention in the elderly population (III), was already submitted and accepted by the ethical commission (**Attachment 6**).

For the illness-prone evaluation profile (II), baseline assessment procedure as well as measurements were previously submitted and accepted by the ethical commission in association with the project - Mechanisms of airway damage in elite swimmers (**Attachment 5**).

The investigator will invite the participant to enroll the study participation after explaining the aims and goals of the study. A written information about each study will be provided to the participant (**Attachments V and VI** - presented in the ethical submission dossier). Consent forms and patient information will be given and the subject will be asked to read and review the documents carefully, the participant don't need to decide at that moment and time will be provide for reflecting about the decision to enroll the study. Upon reviewing the consent form and information, the investigator will explain the research study to the subject and answer any questions that may arise. The subject will then sign the informed consent document prior to any procedures being done specifically for the study. The subjects may withdraw consent at any time throughout the course of the trial. A copy of the informed consent document will be given to the subjects for their records. The rights and welfare of the subjects will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study. No minors will participate in these studies.

Subject confidentiality is strictly held in trust by the participating investigators. This confidentiality is extended to the clinical information relating to participating subjects.

The study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party. In the event of studies' discontinuation, patients will not be affected.

Confidentiality of the data will be preserved by the investigation groups and the acquired information will only be used for the purposes of this project. Study will end when the time limit for sending the questionnaires is achieved. The final aim of this study will be scientific publication.

Data Handling and Record Keeping

The investigator is responsible to ensure the accuracy, completeness, legibility, and timeliness of the data reported. All source documents should be completed in a neat, legible manner to ensure accurate interpretation of data. When making changes or corrections, cross out the original entry with a single line, and initial and date the change.

Copies of the electronic clinical form will be provided for use as source documents and maintained for recording data for each subject enrolled. Data reported in the electronic clinical form derived from source documents should be consistent with the source documents or the discrepancies should be explained. All source documents and laboratory reports must be reviewed by the clinical team and data entry staff, who will ensure that they are accurate and complete.

Data collection is the responsibility of the clinical trial staff. During the study, the investigator must maintain complete and accurate documentation for the study.

Clinical data (including demographic data, questionnaires, outcome measures, adverse events, concomitant medications, and expected adverse reactions data) as well as laboratory data will be entered into a database in a data system. This data system includes password protection and internal quality checks, such as automatic range checks, to identify data that appear inconsistent, incomplete, or inaccurate.

7. MILESTONES

I	Cross-cultural translation and validation of Wisconsin Upper Respiratory Symptom Survey (WURSS-21) for upper respiratory tract infections monitoring	Cross-cultural translation and validation of a measuring tool to be applied in upper respiratory tract infection assessment. Validation of a measure that can be further used in respiratory tract infection assessment.
Month 28	Cross-cultural translation concluded	
Month 34	Questionnaire validation	
II	Upper respiratory tract infection susceptibility in athletes – a 3 months follow-up study	Total number of participants assembled for follow-up procedure. Laboratory analysis will be performed in association with statistic analysis as only the urine that was obtained during an infection period will be analyzed
Month 28	Recruitment and baseline evaluation concluded	
Month 31	Follow-up termination and urine collection	
Month 37	Laboratory analysis and statistical analysis concluded	
III	Effect of physical training on respiratory tract infections in the elderly	Total number of participants assembled for intervention procedure Laboratory analysis will be done a posteriori and statistical analysis will occur at the same time in order to evaluate which patients had respiratory infections
Month 4	Recruitment and allocation concluded	
Month 10	Exercise program intervention and respiratory symptoms questionnaire assembly	
Month 18	Laboratory and statistical analysis concluded	
Month 30	Study I, II, III publication	
IV	Effect of an high fat versus healthy diet meal on neuroimmunendocrine response to exercise	Randomization procedures will be performed alongside the recruitment and baseline achievement. After recruitment intervention can be performed. Both interventions will be done in parallel but independent from one another despite coming from same recruited population
V	Effect of physical activity on immunity	
Month 39	Recruitment concluded	
Month 39	Randomization to meal and physical activity	
Month 42	Meal challenge followed by exercise intervention concluded	
Month 44	Reducing physical activity intervention concluded	
Month 45	Assessment of safety and adverse events concluded, in order to evaluate long term changes	
Month 50	Laboratory analysis concluded Statistical analysis concluded	
Month 52	Theses presentation, Study IV and V publication	

8. TIMELINE

Project title: PHYSICAL ACTIVITY AND DIET AS DETERMINANTS OF IMMUNE FUNCTION
 Time line starting in September 2010

Time line starting in September 2010		2010				2011				2012				2013				2014											
Study	Task Denomination	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D
I	1 Study enrollment																												
	2 Cross Cultural Translation																												
	3 Questionnaire application																												
	4 Statistical Analysis																												
	5 Data handling and record keeping																												
II	6 Recruitment																												
	7 Baseline Evaluation																												
	8 Questionnaires application and urine samples																												
	9 Laboratorial analysis																												
	10 Statistical Analysis																												
	11 Data handling and record keeping																												
III	12 Recruitment and Allocation to intervention/control groups																												
	13 Exercise Program Intervention																												
	14 Respiratory symptoms questionnaire assembly																												
	15 Laboratorial analysis																												
	15 Statistical analysis																												
	16 Data handling and record keeping																												
	IV,V	18 Recruitment																											
19 Randomization to meal intervention																													
20 Meal challenge followed by exercise challenge																													
21 Reducing physical activity by 30%																													
22 Assessment of safety and adverse events																													
23 Laboratorial analysis																													
24 Statistical Analysis																													
8 Data handling and record keeping																													

Em anexo ficheiro em excel.

9. HUMAN AND MATERIAL RESOURCES

The research team will be constituted by four physicians, one of those is the main investigator, two nutritionist, who will take part in the nutritional evaluation and food challenge composition, one professor in sports science and one investigator in sports science and physical activity, which will be supporting the physical activity interventions. None of those elements will receive any income from the project participation, but will share publications that come from this project results.

All materials used will be acquired by the study group, namely personal computers and software, letters, stamps and costs of sending the questionnaires to the patients.

For further testing and laboratorial analysis the investigators will apply for funding, until then samples will be stored in proper conditions.

10. EXPECTED RESULTS AND OUTCOMES

Expected results

To obtain a valid method for upper respiratory infections assessment that will permit its monitoring in the clinical and experimental setting.

To evaluate the risk factors related with respiratory infections in a athletes population, namely physical fitness, nutrition and stress showing the multi-factorial side of immunosuppression in high intensity exercise.

To assess risk of respiratory infections in a physical activity and inactivity intervention as well as immune mediators impact. Evaluate the interference of high fat diet previously to acute exercise challenge and demonstrate the effect of this association in immune system in a particularly susceptible population (asthma-obese patients). This will permit to understand the effects of exercise in the immune system allowing physicians and trainers to adapt its intensity to specific populations.

Outcomes

Publication of the validation study of a respiratory infections questionnaires that will allow its use in the clinical and experimental setting.

To establish the risk factors for respiratory infection in susceptible populations (elderly, athletes, asthma-obese patients) and to study the immune markers parameter related with acute exercise and physical inactivity interventions (balance between pro-inflammatory and anti-inflammatory cytokines). These will permit the design of a illness-prone profile in the populations studied and a first understanding on the effect of exercise in the immune function.

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ATTACHMENTS

Attachment 1

Wisconsin Upper Respiratory Symptom Survey-21

Attachment 2

SF-36 v2

Attachment 3

ÍNDICE DE QUALIDADE DE SONO DE PITTSBURGH

Attachment 4

ESCALA DE ANSIEDADE DE AUTO-AVALIAÇÃO DE ZUNG

Attachment 5

Parecer da Comissão de Ética da avaliação basal efetuada para o projeto II (Upper respiratory tract infection susceptibility in athletes – a 3 months follow-up study)

Attachment 6

Parecer da Comissão de Ética da avaliação basal efetuada para o projeto- Effect of physical training on respiratory infections in the elderly/ Infecção Respiratória & Exercício Físico em Idosos)

Attachment 7

Questionários de Controlo da Asma e da Rinite (Asthma Control Questionnaire e CARAT)

P1 – Durante as últimas 4 semanas , quanto tempo é que a asma o/a impediu de fazer as suas tarefas habituais no trabalho, na escola/universidade ou em casa?				
1 – Sempre	2 – A maior parte do tempo	3 – Algum tempo	4 – Pouco tempo	5 – Nunca
P2 – Durante as últimas 4 semanas , quantas vezes teve falta de ar?				
1 – Mais de uma vez por dia	2 – Uma vez por dia	3 – 3 a 6 vezes por semana	4 – Uma a duas vezes por semana	5 – Nunca
P3 – Durante as últimas 4 semanas , quantas vezes os sintomas de asma (pieira, tosse, falta de ar, aperto ou dor no peito) o/a fizeram acordar de noite ou mais cedo do que é costume de manhã?				
1 – 4 ou mais noites por semana	2 – 2 a 3 noites por semana	3 – Uma vez por semana	4 – Uma ou duas vezes	5 – Nunca
P4 – Durante as últimas 4 semanas , quantas vezes usou os seus medicamentos para alívio rápido, em inalador ou nebulizador, como por exemplo salbutamol?				
1 – 3 ou mais vezes por dia	2 – 1 ou 2 vezes por dia	3 – 2 ou 3 vezes por semana	4 – Uma vez por semana ou menos	5 – Nunca
P5 – Como avaliaria o seu controlo da asma nas últimas 4 semanas ?				
1 – Não controlada	2 – Mal controlada	3 – Mais ou menos controlada	4 – Bem controlada	5 – Completamente controlada

Attachment 8

Questionário Baecke para avaliação da atividade física habitual

Attachment 9

Questionário internacional de Atividade Física

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 25.3.2013

Exmo. Senhor

Presidente do Conselho de Administração do

Centro Hospitalar S. João – EPE

AUTORIZADO

CONSELHO DE ADMINISTRAÇÃO @ REUNIÃO DE 25 MAR 2013

Presidente do Conselho de Administração

(Prof. Doutor António Ferreira)

Directora Clínica (Dra. Margarida Tavares)

Enfermeira Directora (Enfermeira Eulália Portela)

Vogal Executiva (Dra. Mariana Pereira)

Vogal Executivo (Dr. João Oliveira)

Assunto: Pedido de autorização para realização de estudo/projecto de investigação

Nome do Investigador Principal: Diana Pereira da Silva

Título do projeto de investigação: Programa Doutoral em Investigação Clínica e em Serviços de Saúde - "PHYSICAL ACTIVITY AND DIET AS DETERMINANTS OF IMMUNE FUNCTION"

Pretendendo realizar no(s) Serviço(s) de Imunoalergologia do Centro Hospitalar S. João – EPE em colaboração com a Faculdade de Medicina, Nutrição e de Desporto da Universidade do Porto e Unidade Local de Saúde de Matosinhos o projeto de investigação em epígrafe, solicito a V. Exa., na qualidade de Investigador, autorização para a sua efetivação.

Para o efeito, anexa toda a documentação referida no dossier da Comissão de Ética do Centro Hospitalar S. João respeitante a estudos/projectos de investigação, à qual endereçou pedido de apreciação e parecer.

Com os melhores cumprimentos.

Porto, 10 / Dezembro / 2012

O INVESTIGADOR/PROMOTOR

Diana Pereira da Silva

Comissão de Ética para a Saúde do HSJ

Parecer

Projecto de investigação intitulado “Physical activity and diet as determinants of immune function”

Projeto de investigação multicêntrico que se propõe vir a ser desenvolvido no Serviço de Imunoalergologia e Serviço de Imunologia do Centro Hospitalar São João EPE, pela Dr^a Diana Pereira da Silva, no âmbito do desenvolvimento do programa doutoral do PDICS sob a orientação do Prof. André Moreira.

Do ponto de vista científico, o projeto visa avaliar o impacto da prática de exercício físico e/ou da atividade física e fatores associados como a dieta, na função imune e na susceptibilidade às infeções respiratórias. O estudo prevê ainda: a) validar um questionário de infeções respiratórias para aplicar na observação dos fatores de risco de infeções em populações susceptíveis; b) estudar o efeito de uma refeição calórica vs. normal seguida de prova de exercício no sistema imune, sob a forma de resposta aguda e c) avaliar o efeito da redução da atividade física em marcadores imunológicos, na qualidade de vida e em infeções respiratórias.

São referidos uma série de benefícios do estudo para os participantes, designadamente relacionados com o melhor acompanhamento e a avaliação otimizada de que serão alvo. Como riscos e incómodos invocam-se os decorrentes das deslocações dos participantes e os associados à realização de exames complementares, relativamente aos quais estão clarificadas as condições de segurança em que serão efectuados, designadamente no que respeita à assistência médica.

Serão realizados questionários, dos quais se anexam as respetivas cópias que *não estão anonimizadas em todos os casos*.

A investigadora dispõe da competência científica para a realização do estudo, que está autorizado pelo diretor do Serviço de Imunoalergologia, Dr. José Luis Plácido.

Os dados clínicos dos doentes serão colhidos no respeito pela confidencialidade e anonimato, sem necessidade de FCA.

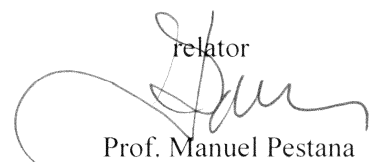
Está prevista a obtenção de consentimento informado e de uma *informação escrita para o participante, que não foi disponibilizada*.

Prevê-se que os exames complementares a realizar no âmbito do estudo, venham a ser suportados por *financiamento ainda não disponível*. O estudo só deverá ser iniciado quando o *financiamento for disponível e do mesmo deve ser dado conhecimento à CES*.

Em face da análise do projeto proponho que a sua aprovação pela CES do CHSJ fique dependente da resposta da resposta da investigadora às questões em itálico.

Porto, 20 de Dezembro de 2012

*face à resposta da investigadora
proponho a aprovação do projeto
pela CES do CHSJ 24.01.2013*

relator

Prof. Manuel Pestana

8. TERMO DE RESPONSABILIDADE

Eu, Diana Pereira da Silva, abaixo-assinado, na qualidade de Investigador Principal, declaro por minha honra que as informações prestadas neste questionário são verdadeiras. Mais declaro que, durante o estudo, serão respeitadas as recomendações constantes da Declaração de Helsínquia (com as emendas de Tóquio 1975, Veneza 1983, Hong-Kong 1989, Somerset West 1996 e Edimburgo 2000) e da Organização Mundial da Saúde, no que se refere à experimentação que envolve seres humanos. Aceito, também, a recomendação da CES de que o recrutamento para este estudo se fará junto de doentes que não tenham participado em outro estudo no decurso do actual internamento ou da mesma consulta.

Porto, 10 / Dezembro / 2012

A Comissão de Ética para a Saúde tendo aprovado o parecer do Relator, aguarda que o Investigador/Promotor esclareça as questões nele enunciadas para que possa emitir parecer definitivo.

Diana Pereira da Silva

O Investigador Principal

Prof. Doutor Filipe Almeida
Presidente da Comissão de Ética

2012.12.21
Prof. Doutor Filipe Almeida

PARECER DA COMISSÃO DE ÉTICA PARA A SAÚDE DO CENTRO HOSPITALAR DE S. JOÃO

Considerando que foram com estes os esclarecimentos prestados pelo investigador

A Comissão de Ética para a Saúde APROVA por unanimidade o parecer do Relator, pelo que nada tem a opor à realização deste projecto de investigação.

2013.01.28

Prof. Doutor Filipe Almeida
Presidente da Comissão de Ética

emitido na reunião plenária da CES

de