Evidence of immunometabolic dysregulation and airway dysbiosis in athletes susceptible to respiratory illness

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Summary

Background Respiratory tract infection (RTI) is a leading cause of training and in-competition time-loss in athlete health. The immune factors associated with RTI susceptibility remain unclear. In this study, we prospectively characterise host immune factors in elite athletes exhibiting RTI susceptibility.

Methods Peripheral blood lymphocyte flow cytometry phenotyping and 16S rRNA microbial sequencing of oropharyngeal swabs was performed in a prospective elite athlete cohort study (n = 121). Mass cytometry, peripheral blood mononuclear cell (PBMC) stimulation and plasma metabolic profiling was performed in age-matched highly-susceptible (HS) athletes (≥4 RTI in last 18 months) (n = 22) compared to non-susceptible (NS) (<1 RTI in last 18 months) (n = 23) athletes. Findings were compared to non-athletic healthy controls (HC) (n = 19).

Findings Athletes (n = 121) had a reduced peripheral blood memory T regulatory cell compartment compared to HC (p = 0.02 (95%CI:0.1,1.0)) and reduced upper airway bacterial biomass compared to HC (p = 0.032, effect size r = 0.19). HS athletes (n = 22) had lower circulating memory T regulatory cells compared to NS (n = 23) athletes (p = 0.005 (95%CI:1.5-0.15)) and HC (p = 0.002 (95%CI:1.9-0.3)) with PBMC microbial stimulation assays revealing a T-helper 2 skewed immune response compared to HC. Plasma metabolomic profiling showed differences in sphingolipid pathway metabolites (a class of lipids important in infection and inflammation regulation) in HS compared to NS athletes and HC, with sphingomyelin predictive of RTI infection susceptibility (p = 0.005).

Interpretation Athletes susceptible to RTI have reduced circulating memory T regulatory cells, metabolic dysregulation of the sphingolipid pathway and evidence of upper airway bacterial dysbiosis.

Funding This study was funded by the English Institute of Sport (UK).

Keywords: Exercise; Respiratory tract infection; T regulatory cell; Sphingolipid; Microbiome

Introduction Respiratory tract illness (RTI) is a key issue in elite athlete health and a leading cause of training and in-competition time loss. Some elite athletes can be classified as having increased susceptibility to RTI compared to the general population, suffering recurrent infections throughout the year, with considerable implications for their health, athletic availability.
Perturbations in host-specific defence mechanisms are likely to be relevant; preparation for elite level sport necessitates high intensity, frequent and prolonged exercise, often interspersed with periods of altered recovery and nutritional intake and other challenges including international travel and psychological stress. The impact of vigorous daily physical activity on immune function remains to be fully determined. Although, engagement in regular exercise has been shown to result in beneficial augmentation of T regulatory cell responses and a reduction in immune senescence, strenuous exercise may reduce peripheral circulating lymphocytes and lead to impaired mucosal immunity, potentially leading to infection susceptibility. There is, an increasing appreciation of the immuno-metabolic impact of exercise on the T cell response, with studies implicating strenuous exercise in the development of an immunosuppressive state.

A further potentially relevant consideration in athletes who partake in endurance sport is the influence of prolonged periods of heightened ventilatory stress on the upper airway and potentially microbial carriage. A high prevalence of airways disease and laryngeal-related issues is evident in elite endurance athletes. In the general population, airways disease has been shown to be associated with microbial dysbiosis, reduced airway microbial diversity and dysregulation of microbial-related immunological processes. In addition, it is additionally increasingly recognised that through their metabolites, mucosal-associated bacteria exert profound effects on host immune responses.

Despite several studies detailing component aspects of immune dysregulation in athletes, there is still controversy over the role of host immune factors underpinning RTI susceptibility in elite athletes. Specifically, there has been a lack of prospective studies with commensurate measurement of multiple physiological, microbial and immune parameters in this context.

In this study, we prospectively analysed host immune factors in a large cohort (n = 121) of elite Olympic athletes comprehensively clinically phenotyped through a systematic respiratory health assessment including susceptibility to RTI. Peripheral blood immune compartments and oropharyngeal microbiome were analysed with further mass cytometry, peripheral blood mononuclear cell immune response and plasma metabolomic analysis performed to compare RTI-susceptible athletes to non-susceptible athletes and healthy controls. Our study reveals that RTI-susceptible athletes are characterised by a reduction in circulating memory T regulatory cells compared to non-susceptible athletes and healthy controls, alongside a propensity to Th2 inflammation. Elite athletes are additionally shown to have evidence of upper airway microbial dysbiosis compared to healthy controls, with RTI-susceptible athletes characterised by a dysregulated plasma sphingolipid metabolism compared to non-susceptible athletes, with sphingomyelin predictive of infection susceptibility.

Our study reinforces the presence of an RTI-susceptible athlete endotype characterised by a persistent reduction in memory T regulatory cells and evidence of microbial dysbiosis and immune metabolic dysregulation in particular within the sphingolipid pathway. These findings present plausible future therapeutic targets in this cohort and extend current understanding and insight into the implication of elite training and exercise on host immune function.

**Methods**

**Study participants and design**

Elite athletes, from all 24 UK Sport-funded Olympic World Class Programmes, were invited to participate as part of a prospective ‘Systematic approach to the

**Evidence before this study**

Respiratory tract illness (RTI) is a key issue in elite athlete health and a leading cause of training and in-competitive time loss. A proportion of elite athletes have increased susceptibility to RTI compared to the general population, suffering recurrent infections throughout the year, with considerable implications for their health, athletic availability, career progression and level of competitive achievement. Despite several studies detailing component aspects of immune dysregulation in athletes, there is still controversy over the role of host immune factors underpinning RTI susceptibility in elite athletes. Specifically, there has been a lack of prospective studies with commensurate measurement of multiple physiological, microbial and immune parameters in this context.

**Added value of this study**

In this study, we prospectively analysed host immune factors in a large cohort (n = 121) of elite Olympic athletes comprehensively clinically phenotyped through a systematic respiratory health assessment including susceptibility to RTI. Peripheral blood immune compartments and oropharyngeal microbiome were analysed with further mass cytometry, peripheral blood mononuclear cell immune response and plasma metabolomic analysis performed to compare RTI-susceptible athletes to non-susceptible athletes and healthy controls. Our study reveals that RTI-susceptible athletes are characterised by a reduction in circulating memory T regulatory cells compared to non-susceptible athletes and healthy controls, alongside a propensity to Th2 inflammation. Elite athletes are additionally shown to have evidence of upper airway microbial dysbiosis compared to healthy controls, with RTI-susceptible athletes characterised by a dysregulated plasma sphingolipid metabolism compared to non-susceptible athletes, with sphingomyelin predictive of infection susceptibility.

Implications of all evidence available

Our study reinforces the presence of an RTI-susceptible athlete endotype characterised by a persistent reduction in memory T regulatory cells and evidence of microbial dysbiosis and immune metabolic dysregulation in particular within the sphingolipid pathway. These findings present plausible future therapeutic targets in this cohort and extend current understanding and insight into the implication of elite training and exercise on host immune function.
assessment of respiratory athlete health (SARAH) study as previously published.7 Athletes aged <18 years, current smokers and those with a history of cardiovascular and/or metabolic disease were excluded. 19 age- and sex-matched, non-athletic (exercise <4 h/week) healthy controls (HC) with normal range body mass index (BMI) were additionally recruited. Healthy control participants had ≤2 RTI in the last 12 months and had not had a RTI in the last 4 weeks at time of sampling.

After participants were consented for the study, and prior to sampling, all athletes completed an electronic Respiratory health questionnaire. All sampling was carried out in the morning (between 7.30 and 11 am) of sampling to allow for appropriate storage and processing of samples. Prior to sampling athletes were requested to partake in no high intensity exercise, including weight training, within 4 h of testing. Athletes were required to abstain from caffeine for 4 h prior to sampling and consume no food for 60 min prior to sampling. Athletes were required to be illness free for two weeks, and free form chest infections within 4 weeks.

To analyse athlete RTI susceptibility, athletes self-reported infection frequency with cross-check against an athlete health electronic medical record. An RTI was defined as a period of respiratory illness that resulted in at least 2 days of restricted training. Athletes were stratified by RTI frequency in the last 18 months with athletes in the upper quartile (≥4 RTI in last 18 months) termed highly-susceptible (HS) and athletes from the lower quartile (≤1 RTI in last 18 months) termed non-susceptible (NS). Athletes were additionally classified by training status at time of sampling, with high intensity (HI) defined if the athlete was undertaking a full program of training aimed at producing positive adaptations in their physical capacity during the 3–4 weeks prior to sampling. Asthma was defined through fraction of exhaled nitric oxide (FeNO), spirometry, indirect bronchoprovocation testing (eucapnic voluntary hyperpnoea [EVH] test) and validated questionnaire for atopy (AQUA-allergy questionnaire for athletes).

**Physiological measurements.** As completed a systematic assessment of their respiratory health, including evaluation of atopy (with questionnaires) and airways disease (with measurement of exhaled nitric oxide (FeNO) and spirometry (FEV1 and FVC) with indirect bronchoprovocation testing).

Asthma was defined through fraction of exhaled nitric oxide (FeNO), spirometry, indirect bronchoprovocation testing (eucapnic voluntary hyperpnoea [EVH] test) and validated questionnaire (MiniAHLQ).

**Sample collection and PBMC isolation.** Venous blood was collected in lithium heparin blood tubes and transported at room temperature whilst EDTA tubes were transported at 4 °C. Plasma was isolated from EDTA tubes following centrifugation at 1200g for 10 min, with plasma aspirated and stored at -80 °C. Venous blood from lithium heparin tubes was diluted 1:1 with PBS (Sigma Aldrich, 866352), layered over histopaque (Sigma Aldrich, 10771) and PBMCs isolated following density gradient centrifugation.

**B and T lymphocyte staining for flow cytometry analysis.** Peripheral blood mononuclear cells (PBMC) at baseline were stained with fluorescently conjugated antibodies for B and T cell analysis using flow cytometry as previously described. For B cell analysis, 1 × 10^6 cells were stained with, α-CD38 APC-H7 (65314), α-CD27 BV421 (742731), α-IgG FITC (554200), α-IgM PerCP-Cy5.5 (561285), α-CD3 BV510 (740202) (BD Biosciences), α-CD19 BV711 (AB_2562062), α-CD24 BV605 (AB_2563464), α-IgD PE/Dazzle 594 (AB_256989), α-CD21 PE (AB_2561406), α-CD14 BV510 (AB_2716228) (Biolegend), α-IgA APC (150-116-997) (Miltenyi Biotec) and Live/Dead stain (L34963) (ThermoFisher). For T cell analysis, 1 × 10^6 cells were stained with α-HLA-DR BV711 (563696), α-CD27 BV605 (753676), α-CD3 APC-H7 (562275), α-CD25 BV421 (562442), α-CD4 FITC (435768), α-CD8 PerCP-Cy5.5 (81049), α-CD127 PE CF594 (563297), α-CCR7 PE (552176), α-CD19 BV510 (562947) (BD Bioscience), α-CD45RA APC (550855), α-CD14 BV510 (AB_2716228) (Biolegend) and Live/Dead stain (L34963) (ThermoFisher). After staining for 30 min at room temperature, cells were washed with PBS and fixed with BD cytofix (AB_2869010) (BD Biosciences), before storing in PBS at 4 °C until acquisition on the LSRII flow cytometer (BD Bioscience) using FACS Diva software (BD Biosciences). FCS files were analysed using FlowJo v10.2 (FlowJo). Compensation matrices were calculated for each experiment, using the single stained compensation controls, and applied to samples. T and B cell populations were gated using surface markers and fluorescence minus one and unstained controls where necessary.

**Mass cytometry (CyTOF).** Three x 10^6 freshly isolated PBMCs re stained using the Fluidigm MaxPar Direct Immune Profiling (DIP) Kit (201325) (Fluidigm). CD39 165Ho, CD95 169Tm and CD122 175Lu antibodies (201319) (conjugated by the BRC Flow Cytometry Platform, NIHR Guy’s and St Thomas’ Biomedical Research Centre), as well as FoxP3 159Tb (3159309A) (Fluidigm). Cells were Fc blocked using TruStain FcX (422301) (Biolegend) and transferred to a labelled DIP assay tube with CD39 and CD95 also added. These were subsequently incubated at room temperature for 30 min followed by washing in Maxpar Cell Staining Buffer (201068), with fixation and permeabilization.
using FoxP3 Fix/Perm (00-5523-00) (eBioscience) following the manufacturer’s protocol. Cells were intracellularly stained with CD152 75Lu and FoxP3 159Tb for 30 min at room temperature. Following washing, cells were incubated in 1.6% formaldehyde for 10 mins on a rocker, washed and incubated overnight at 4 °C in MaxPar Fix and Perm buffer with Intercalator-Ir. The following morning cells were washed and frozen in 150µl FBS (10% DMSO) using Mr Frosty freezing containers (Thermofisher Scientific) and stored at -80 °C. Stained cells were shipped on dry ice to the Botnar Research Institute, University of Oxford for acquisition on the Helios 2 (Fluidigm). FSC files were imported into and analysed using Cytobank (Beckmann Coulter). Following initial gating on CD45+CD3+CD4+ cells (Fig. S3), viSNE analysis was performed. The viSNE seed was random, and the analysis was set with 1000 iterations and a perplexity of 30.

Peripheral blood mononuclear cell immune response to microbial stimuli. PBMCs were stimulated with lipopolysaccharide (LPS) (10 ng/ml) (L293) (Sigma-Aldrich), R848 (144875-48-9) (250 ng/ml), polyinosinic:polycytidylic acid (Poly I:C)) (1 ug/mL) (42424-50-0) (Sigma-Aldrich) and phorbol 12-myristate 13-acetate/ Ionomycin (50ng/mL and 1ug/mL, respectively) (P1585, I3909) (Sigma-Aldrich), and an unstimulated control (Thermofisher Scientific) and stored at -80 °C. Stained samples were incubated in 1.6% formaldehyde for 10 mins on a rocker, washed and incubated overnight at 4 °C until processing. DNA extraction was carried out using a modified CTAB, phenol chloroform extraction as previously outlined. Bacterial qualification was carried out in triplicate using SYBR green qPCR assay. The most abundant sample, from qPCR results, from each athlete (n = 119) were taken forward for bacterial sequencing on the Illumina MiSeq using the V4 region of the 16S rRNA gene as previously outlined. Mock communities, extraction controls and PCR negative controls were included on each sequencing run for control and decontamination purposes. All sequences were submitted to the European nucleotide database under project number PRJEB45648.

Sequence processing of 16S rRNA gene sequencing data was carried out using QIIME1.9 as described previously. After initial sequencing processing through QIIME2 data was transferred to R version 4.0.3 for all further analysis. Unassign OTUs, those assigned to Chloroplast or Mitochondria, and OTUs with less than 10 reads were removed before further decontamination. Potential contaminants were identified by first applying a spearman correlations between OTUs and bacterial biomass. Decontam version 1.4.0 was then applied using both the “either” method to identify most likely contaminants. OTUs identified were removed from further analysis.

Metabolomics
1ml plasma aliquots were used for global metabolomics analysis by Metabolon Inc. Samples were prepared using the automated MicroLab STAR® system from Hamilton Company and analysed as previously described. After quality control, a total of 990 metabolites were included for further analysis. Raw data and scaled imputed values were provided by Metabolon. Metabolites with zero variance were removed from further study.

Statistical analysis
Analysis was performed using Prism 8 (GraphPad) (PBMC analysis), R version 4.0.3 or SPSS (microbiome and metabolomic analysis). Microbiome analysis was carried out using Phyloseq version 1.34 and vegan version 2.5. For parametric comparisons Brown-Forsythe and Welch ANOVA were used, with Games-Howell’s or Dunnetts T3 multiple comparison test. For non-parametric analysis Kruskall-Wallis test with Dunn’s multiple comparison test was applied. Spearman correlations were used to explore associations between all metabolites, OTUs and PBMCs. All values were corrected using Benjamini-Hochberg procedure for multiple comparisons. Automatic Linear Modelling (ALM) was used to explore RTI frequency in SPSS, PBMC and the top 50 OTUs were included as covariates. Partial Least Squares (PLS) regression models were used to explore changes metabolites between healthy controls (HC), highly susceptible (HS) and non-susceptible (NS) athletes using rplvs version 1.22 in R. Principal components analysis (PCA) was performed in r using the log transformed metabolite data to explore differences between groups.

Ethics
Ethical approval for the study was obtained from University College London Research Ethics Committee (12513/001) with all study participants providing written consent.

Role of funders
This study was funded by the English Institute of Sport (EIS). The study was designed in collaboration with the EIS who assisted with sample collection from athletes. EIS did not have a direct role in study analysis, interpretation or writing of report.
Results

B and T cell lymphocyte composition of athletes susceptible to RTI

121 athletes were included in the initial immune phenotyping analysis with a mean age of 24.4 (±3.89) and a prevalence of asthma diagnosis ≤30%. Initial immune phenotyping analysing T cell populations revealed differences in the memory Treg compartment (Figures 1 and S1) (defined as CD4+CD25hiCD127dimCD45RAdimHLADRhi; see Figs. S2, S3 for gating strategy) between athletes and health control groups. Athletes had lower circulating CD4+ memory T regulatory cell compartment (p = 0.02 (95%CI 0.1,1.0), Brown-Forsythe test) compared to healthy controls (Figure 1a), with sensitivity analysis excluding athletes with asthma showing persistent differences (p = 0.03 (95%CI -1.0,-0.06)) (See Fig. S1). There was additionally increased circulating CD8+ central memory (CM) (p = 0.009 (95%CI -3.5,-0.5)) cell populations in athletes compared to healthy controls (HC) (Figure 1b). Further differences were noted when analysing the CD8 CM to CD4+ memory Treg ratio between athletes to healthy controls (p = 0.008 (95%CI -0.8,-0.12) (Figure 1f). No differences were noted in the B cell population frequencies between the athlete and HC group (see Fig. S4).

Given the wide variation in infection frequency in the athlete cohort (median RTI frequency in 18 months - 2 (IQR-1,4)), analysis was stratified by age-matched HS (upper quartile; ≥4 RTI in last 18 months) and NS (lower quartile; ≤1 RTI in last 18 months) athletes (Figure 1c and Table 1). A female preponderance was evident in HS athletes however no significant differences were seen with regards to age, training intensity, presence of atopy or asthma. When exploring athlete susceptibility in relation to sporting discipline (Table S1), no significant difference in number of infections was observed between sports however athletes considered to take part in an aquatic based sports had significantly more RTIs than non-aquatic sports (W=1155.5, p = 0.04, Wilcoxon signed ranked test).

HS athletes had lower circulating CD4+ memory T regulatory (Treg) cell populations compared to NS athletes (p = 0.005 (95%CI -1.5,-0.15), Brown-Forsythe test) and HC (p = 0.002 (95%CI -1.5,-0.3)). Although HS athletes had increased circulating CD8+ central memory (CM) (p = 0.004 (95%CI 1.00,5.8)) and CD8 CM to CD4+ memory Treg ratio (p = 0.001 (95%CI -1.3,-0.3)) compared to HC, no difference was noted in CD8+ CM T cell populations between HS and NS athlete groups (p = 0.41 (95%CI -3.4,1.4)) (Figure 1).

Mass cytometry (Cytof)

To validate cross-sectional immune phenotype findings, mass cytometry (CyTOF) profiling of PBMCs and computational unsupervised clustering focussed on CD4+ T cells were performed on a subset of HS (n = 7), NS (n = 12) athletes and HC (n = 10) at a further single repeat testing (see Fig. S5 for gating strategy). Automated subset identification of the CD4+ T cell population using viSNE stratified by infection susceptibility revealed density differences between HS athletes and HC (Fig. S6). Heat maps were used to identify 2 FoxP3+, CD4+ T cell populations (Figure 2a), with differential expression patterns. FoxP3+ population 2 was noted to be CD45ROhi CD45RAdim CD95hi identifying it as a memory Treg subset (Figure 2b). On further analysis of FoxP3+ population frequency as a percentage of the total CD4+ T cell population, HS athletes were shown again to have a reduced FoxP3+ population 2 compared to NS athletes (p < 0.001 (95%CI -2.4,-0.63)) and HC (p = 0.002 (95%CI -2.46,-0.42), Brown-Forsythe test) (Figure 2f). Further expression marker analysis of FoxP3+ population 2 between susceptibility groups (e.g. CD25, CD39, CD95 and CD152) showed no difference.

Peripheral blood mononuclear cell immune response to microbial stimuli

To understand the functional impact of the differences identified in the peripheral T regulatory cell compartment, further immune response analysis of HS (n = 7), NS (n = 12) athletes and HC (n = 10) was performed at repeat testing using in vitro PBMC stimulation with microbial stimuli. This revealed HS athletes have a comparative pro-inflammatory cytokine response with increased IL-6 (H(2) = 8.16, p = 0.016) and TNF-α (H(2) = 9.29, p = 0.007, Kruskall-Wallis test) production (Figure 3) when stimulated with PMA/Ionomycin with similar non-significant trends noted for microbial stimuli. HS athletes additionally had increased production of IL-4 (H(2)=6.02, p = 0.049) and IL-13 (H(2)=13.5, p = 0.004) compared to HC when stimulated with Poly(I:C) (H(2)=6.60, p = 0.04) and R848 (H(2)= 7.15, p = 0.028) and increased IL-13 production with lipopolysaccharide (LPS) (H(2)= 10.2, p = 0.006) and Poly(I:C) (H(2)=6.60, p = 0.035) stimulation. IL-10 and IFN-γ production were not different between groups upon stimulation in any condition.

16S rRNA microbiome analysis of RTI susceptible athletes

16S rRNA microbiome analysis of the oropharynx was performed to understand the impact of the resident mucosal microbiome on athlete RTI susceptibility and circulating immune compartments. Analysis of the total athlete cohort (n = 117) revealed a reduction in bacterial biomass in athletes (mean±SE = 5.4 × 106 (+/-1.3 × 106)) compared to HC (n= 10, mean±SE = 7.7 × 106 (+/-3.0 × 106)).
Figure 1. T cell population frequencies in an elite athletic cohort. Memory T regulatory cell percentage of CD4+ T cells (a) and Central Memory (CM) CD8+ T cell percentage populations (b) using flow cytometry are shown in an elite athlete cohort (n = 121) compared to a healthy control cohort (n = 19). RTI infection susceptibility frequency over the last 18 months is shown in the total elite athlete cohort with red lines indicating the upper quartile (RTI frequency of ≥4) and lower quartile (RTI frequency of ≤1) (c). Memory T reg percentage of CD4+ T cells (d), CM CD8+ T cell percentage (e) and CM CD8+ T cell to memory T reg ratio (f) is shown in athletes phenotyped by infection susceptibility: Highly susceptible (HS) (n = 22); non-susceptible (NS) (n = 23) and healthy controls (HC) (n = 19). Welch's ANOVA with multiple comparisons was used to test for statistical differences between groups. * indicate statistical significance (* = <0.05; ** = <0.01).
Bacterial biomass was found to have a weak positive Spearman’s correlation with Simpson’s diversity ($r = 0.23$, $p = 0.008$). Pielou evenness ($r = 0.23$, $p = 0.016$) and Berger-parker dominance ($r = 0.29$, $p < 0.001$). Athletes were found to have lower bacterial diversity than HC when considering Simpson’s diversity ($p = 0.04$, effect size $r = 0.201$) (Figure 4), alongside a significant increase in bacterial dominance observed in the athlete population ($p = 0.01$, effect size $r = 0.236$) (Figure 4). Bacterial community composition was found to be different between athletes and controls however, the explanation was found to be lower ($R^2 = 0.02$, $p = 0.005$). A positive correlation of memory Treg percentage of CD4+ T cells with bacterial diversity was observed (Shannon’s; $r = 0.18$, $p = 0.050$, Simpsons; $r = 0.18$, $p = 0.049$). No significant correlation was found between bacterial biomass and CD4+ or CD8+ T cell populations ($p > 0.05$).

An Automatic Linear Modelling (ALM) using SPSS was further used to explore the effects of the peripheral immune cell compartments and the top 50 OTUs in the total athlete cohort ($n = 117$) on RTI frequency. No significant associations were observed with any OTUs and number of RTIs, however, memory Treg % of CD4+ T cells were found to be associated with increased RTI susceptibility. The association was confirmed in a linear regression ($R^2 = 0.041$, $p = 0.019$). Age and sex were included as covariates, and had no effects.

When stratifying athlete susceptibility by RTI infection frequency, as detailed above in Table 1, no difference in bacterial biomass was observed between HS ($n = 22$) and NS ($n = 23$) athletes. However, HS athletes ($\text{mean(+/SE)} = 5.5 \times 10^6 \ (+/ -3.6 \times 10^6)$, $p = 0.012$) and NS athletes ($\text{mean(+/SE)} = 9.0 \times 10^5 \ (+/ -3.4 \times 10^5)$, $p = 0.006$) remained significantly different from healthy controls ($n = 10$) (Figure 4b). Additionally, no difference was found in community composition between HS and NS athletes ($R^2 = 0.015$, $p = 0.796$, Figure 4).

Metabolomic profiling of RTI susceptible athletes

We subsequently performed global untargeted metabolomic analysis of plasma to identify metabolomic signatures in athletes with increased infection susceptibility (HS athletes ($n = 22$), NS ($n = 23$) athletes and HC ($n = 10$)). Principal components analysis (PCA) of the log10 transformed data revealed no clear separation between the groups (Fig. S6). As the variance explained by each principal component was low, further exploration was carried out at metabolite level between the individual groups. 12 metabolites were found to be different between groups with a false discovery rate (FDR) of less than 10% (Figure 5A). Nine of the 12 metabolites identified were members of the lipid super pathway. Comparing HS and NS athletes, 10 metabolites were found to be significantly different, of which the predominant metabolite pathways were the sphingomyelin and ceramide sub pathways (Figure 5B). PLS models were applied to explore differences in metabolites between HS athletes, NS athletes and HC (Fig. S7). When exploring all metabolites in the data set the model showed a $R^2$ value of 0.679, however the $Q^2$ was low ($Q^2 = 0.147$). To further explore this, a total of 159 metabolites indicated as different between athlete susceptibility groups.

### Table 1: Study cohort demographics. Key demographics of the elite athlete and healthy control study cohort for baseline and repeat testing.

| Description | Complete SARAH Cohort | Highly Susceptible athletes (HS) | Non-Susceptible athletes (NS) | p (HS vs NS) |
|-------------|-----------------------|---------------------------------|-----------------------------|-------------|
| n           | 121                   | 22                              | 23                          |             |
| Age (mean (SD)) | 24.40 (3.89)          | 23.95 (4.21)                    | 24.04 (4.04)                | 0.943       |
| BMI (mean (SD))  | 22.89 (2.18)          | 22.59 (1.80)                    | 23.08 (1.92)                | 0.378       |
| Sex = Male (%) | 67 (55.4)             | 7 (31.8)                        | 15 (65.2)                   | 0.052       |
| AQUA (mean (SD)) | 2.25 (1.99)           | 2.86 (2.27)                     | 1.74 (1.51)                 | 0.062       |
| FEV1 % predicted (mean (SD)) | 103                 | 105                          | 105                          | 0.999       |
| Biggest %fall in FEV1 (mean (SD)) | 7 (0.12)            | 6.7                         | 6.7                         | 0.972       |
| Prop. of FeNO $>$ 25ppb (mean (SD)) | 43                | 9                        | 7                          | 0.94        |
| Asthma (%) | 38 (31.4)             | 7 (35.0)                        | 4 (17.4)                    | 0.104       |
| Training intensity (%)** | 75 (64.1)        | 12 (57.1)                      | 14 (63.6)                   | 0.118       |

* - For athletes completing eucapnic voluntary hyperpnoea.
** - Training intensity: High training intensity defined as athlete undertaking a full program of training aimed at producing positive adaptations in their physical capacity during the 3–4 weeks prior to sampling. Modified training including; predominantly technical training blocks with low volume and intensity of physical conditioning elements, post competition or post-season recovery block or early phase injury rehabilitation was defined as low intensity training.
(p ≤ 0.1), without correction for multiple testing, were taken forward into the PLS model. Despite improvements in the model, the predictive power of metabolites to distinguish between groups was low (r = 0.608, Q2 = 0.253).

The effect of all metabolites, PBMCs and top 20 OTUs on athlete RTI susceptibility was further explored using SPSS ALM (22) with the top 10 variables based on model importance shown in Table 2. These were taken into a forward selected stepwise binomial regression model. Results again identified sphingomyelin (d18:1/17:0, d17:1/18:0, d19:1/16:0), (importance=0.052) as a significant predictor of athlete RTI susceptibility (p = 0.005). Other metabolites identified as significant predictors of RTI infection susceptibility are shown in Table 2 and Figure 5.

Further associations were explored using Spearman’s correlations between metabolites and the top 20 OTUs, bacterial biomass and the T-cell populations (Figure 6). Sixty metabolites were found to be correlated with one or more of the top 20 OTUs, bacterial biomass or peripheral T cell compartments with less than 10% FDR (Figure 6). A positive correlation of members of the sphingolipid pathways to central memory CD8+ to memory T reg ratio was noted, however, this did not reach significance after multiple correction.

Discussion

In this study, commensurate immune phenotyping delivered through a multi-omic approach, with multiparameter flow cytometry of clinically phenotyped athletes revealed significant perturbations in peripheral blood lymphocyte compartments and immuno-metabolic dysregulation in athletes with heightened susceptibility to RTI. RTI susceptible athletes are shown to have a reduction in circulating memory T regulatory cells alongside dysbiosis of airway microbiome and metabolic dysregulation in particular of their sphingolipid metabolism.

T regulatory cells expressing the transcription factor forkhead box P3 (FoxP3), which form 5–10% of the peripheral CD4+ T cell population, are critical to maintain immune homeostasis and are able to modulate the immune response to infection alongside polarisation between T helper 1 (Th1) and T helper 2 (Th2) responses.23 Recently, there has been an increased appreciation of the presence and role of the memory T regulatory cell compartment, postulated to regulate potent memory effector responses.24 The effects of exercise on the circulating T regulatory cell population are thought to be biphasic, with moderate exercise leading to a beneficial increase with suppression of Th2 responses, but maximal exercise resulting in a reduced circulating Treg population.25,26 Whether the effects of repeated strenuous exercise on circulating lymphocyte populations is associated with basal immune
Figure 3. Cytokine production by stimulated PBMCs. Supernatants from PBMCs stimulated using LPS (10 ng/ml), R848 (250 ng/ml), Poly IC (1 ug/ml) and PMA/ionomycin (50 ng/ml and 1 ug/ml) were tested for IL-4, IL-10 and IL-13, IFNg, IL-6 and TNF-a cytokines using MSD proinflammatory panel 1 kit (MSD). Kruskal-wallis ANOVA and Dunns multiple comparison test were used to identify significant differences between susceptibility groups; highly susceptible (HS, n = 7), non-susceptible (NS, n = 11) and healthy controls (HC, n = 10).
Figure 4. Athlete microbiome. (a) Stacked bar chart indicating the top 20 most abundant OTUs in each sample obtained from athletes and healthy controls. Bars are coloured by bacterial genus. Samples are grouped into healthy controls (n = 10) and athletes (n = 111, 6 samples were removed due to lack of validated data on number of RTIs in these individuals) and ordered by number of RTIs in the past year indicated by bar shown below the stacked bar plot. Blue bars above indicate individuals with asthma diagnosis. (b) Comparison of bacterial biomass between athletes (n = 117) and healthy controls (n = 10) (p = 0.032) and between susceptibility groups and healthy controls (highly susceptible; p = 0.012, non-susceptible; p = 0.005). (c) Differences in Simpsons diversity between athletes (n = 117) and healthy controls (n = 10) (p = 0.023) and between susceptibility groups and healthy controls (highly susceptible; p = 0.07, non-susceptible; p = 0.1). (d) Differences in Berger Parker dominance between athletes (n = 117) and healthy controls (n = 10) (p = 0.008) and between susceptibility groups and healthy controls (highly susceptible; p = 0.07, non-susceptible; p = 0.08).
compromise and increased susceptibility to infection has however been debated.27

In our study, we show that elite athletes have a reduction in their circulating memory T regulatory cell compartment compared to HC. Further, HS athletes stratified by infection frequency have a lower circulating memory T regulatory cell compartment compared to NS athletes suggesting an infection susceptible immune endotype. This finding was validated at a repeat time point using an unbiased computational gating strategy and was not associated with training intensity or presence of asthma. Although a female preponderance in HS athletes was seen (with female sex known to associate with a reduced circulating T regulatory cell compartment), automatic linear modelling adjusted for age and sex again highlighted an association of memory T regulatory cell percentages to infection susceptibility in athletes.28 Further in vitro PBMC immune response assays in athletes showed a pro-inflammatory, skewed Th2 response in HS athletes, suggesting a dysregulation of immune homeostasis. This is similar to previous research using whole blood challenge where significant increased IL-4 and IL-10 were noted in endurance athletes with recurrent RTI.29 These findings support an RTI-susceptible athlete endotype associated with aberrant immune homeostasis and a reduced circulating memory T regulatory cell compartment with ineffective suppression of excessive Th2 responses.10

Given the importance of commensal microbial metabolites in T regulatory cell generation and function, alongside the known impact of strenuous exercise on the microbiome, we analysed the role of bacterial dysbiosis in athlete RTI-susceptibility.31,32 We reveal evidence of upper respiratory tract bacterial dysbiosis in elite athletes compared to healthy controls with a reduction in bacterial biomass and reduced Simpsons’ diversity, but no significant difference in community composition between HS and NS athletes. The aetiology of the reduced bacterial biomass in athletes requires further investigation, but dietary recommendations to elite athletes with a low consumption of plant-based polysaccharides have previously been shown to result in reduced gut and oral microbiota diversity and functionality.33,34 Given the non-significant difference in bacterial community composition between RTI-susceptible athletes compared to non-susceptible athletes, how this impacts T regulatory cell function in athletes and subsequent infection susceptibility is unclear. Bacterial biodiversity however is critical in the development and maintenance of T regulatory cell populations, with antibiotic treated mice with depleted microbiota leading to drastic reductions in T regulatory cell frequency, and rural environments in early life with high bacterial microbial diversity resulting in asthma-protective effects.35-37 Our findings highlight the need for further research to understand the implication of reduced bacterial biomass in elite athletes on mucosal T regulatory cell populations and effects on RTI-susceptibility.

Given the additional significant effects of strenuous exercise on host metabolism, and the importance of T cell metabolic reprogramming to determine functionality, to analyse the immuno-metabolic phenotype of HS athletes, we performed global untargeted metabolomic analysis of plasma and compared to NS athletes and HC. Although the predictive power of regression modeling was limited due to sample size, analysis of variance revealed significant metabolite differences between HS athletes, NS athletes and HC. Further analysis of variance revealed significant metabolite differences between HS athletes, NS athletes and HC. In particular, sphingolipid pathway metabolites were strongly represented in differences between HS and NS athletes. There has been a significant increase in our understanding of sphingolipid metabolism, with sphingolipid metabolites such as ceramide and sphingosine-1-phosphate known to be signalling molecules that regulate a diverse range of cellular processes important within host immune response and inflammation.38 T cells are activated through cell-surface receptors such as the T-cell receptor and the co-stimulatory molecule CD28 which are in close contact to surrounding phospholipids of which ~30% are sphingolipids. Sphingolipids are not chemically inert but are further metabolized by acid sphingomyelinase (ASM), with the most prominent and complex sphingolipid, sphingomyelin, reversibly cleaved into ceramide and phosphocholine by sphingomyelinas. Ceramide molecules can self-aggregate forming ceramide-rich platforms and be further metabolized into sphingosine (which can be phosphorylated to sphingosine-1-phosphate) and fatty acids with wide-ranging biological activity.38 Previous studies have shown the importance of ASM in regulating T regulatory cell numbers in humans alongside the development of allergic asthma.39,40 Sphingosine-1-phosphate (SIP) has further been shown to reciprocally regulate pro-inflammatory Th1 and anti-inflammatory T regulatory cell differentiation in addition to the development of central memory through effects on mitochondrial respiration.41,42 Therefore, our finding of significant sphingolipid pathway differences in elite athletes compared to healthy individuals, and in particular within HS athletes presents a plausible hypothesis to understand the mechanism of increased susceptibility to infection in elite athletes. This is supported by a positive correlation of a number of sphingolipid pathway metabolites to central memory CD8 to memory T reg ratio in athletes suggesting a role in immune homeostasis. SIP has previously been shown to be modulated by exercise, but further analysis is required to understand and validate the longitudinal effects of repeated strenuous exercise in athletes on the sphingolipid pathway and T regulatory cell function.43 Previous studies highlighting the beneficial effect of fatty acid supplementation in elite athlete airway inflammation additionally reinforces the potential importance of metabolic reprogramming on
Figure 5. Metabolites with significantly associated with susceptibility group. (a) Plot of metabolite effect size of at 10% false discovery rate (FDR) calculated using ANOVA (left), heat map indicating the mean log normalized imputed value for each metabolite between the three study groups (right). (b) Box plots of the top 10 metabolites significantly different between non-susceptible and highly-susceptible athlete groups using Wilcoxon rank sum test. P values presented are prior to correction for FDR.
Our results also note a number of other metabolites associated with infection susceptibility including tryptophan, glycerophospholipid and androstenediol, all of which have been shown to potentially affect T cell function and require further study. Steroid metabolism has been shown to be dysregulated in elite athletes with our significant female preponderance in HS athletes additionally likely having a significant confounding effect.

Our study has a number of limitations. Although stringently clinically phenotyped, our measure of RTI-susceptibility in elite athletes is not based on microbiological confirmation and hence may be inaccurate. Nevertheless, this study represents an in-depth phenotypic respiratory characterisation of elite athletes with results strongly suggestive of a RTI-susceptible elite athlete endotype. Although our healthy control group was age and gender matched, further prospective studies with more stringent matching and phenotyping is required to fully analyse RTI-susceptibility in athletes compared to non-athletes. Further work is additionally required to understand whether specific sport disciplines have increased RTI susceptibility and the underlying mechanism given the increased frequency seen in aquatic sports. Prospective definitive testing to confirm viral infection and analyse the virome in athletes will be additionally important in longitudinal studies to objectively understand RTI-susceptibility and endotype accurately. This, however, is often practically challenging due to the demanding competition and training schedule of elite athletes with novel remote point-of-care testing platforms perhaps providing a solution. In addition, in our study we have focussed on peripheral immune subsets which may not accurately represent the mucosal immune response, with previous studies although showing correlation of peripheral central memory T regulatory cells to lung resident cells, additionally highlighting some differences in function.

Although immunological findings were validated at a separate single time point, our study highlights the need for further longitudinal analysis with greater power to delineate mechanistic causality of infection susceptibility. Lastly, given the immune metabolic in particular lipid pathway differences seen in highly susceptible athletes and the higher prevalence of female gender, further research is also required to explore the role of athlete nutrition alongside sex hormones (e.g. androgens and oestrogen) which are known to regulate immune function in athlete infection susceptibility.

In summary, in this study we identify a RTI-susceptible athlete endotype characterised by a persistent reduction in circulating memory T regulatory cells, propensity to Th2 inflammation, metabolic dysregulation of the sphingolipid pathway and evidence of upper airway bacterial dysbiosis indicating a potential mechanism for aberrant T regulatory cell function and susceptibility to respiratory tract infection.

**Contributors**

JH, CR, ML, AS, MM and WC conceived the study design. AJ performed sample acquisition. LC and ST performed experiments. ST, LC, AS, PK, MM and WC performed data interpretation, verification and analysis. All authors contributed to manuscript drafting and revision and have read and approved the final version of the manuscript.

**Data sharing**

De-identified participant data collected as part of the study including immune parameter, microbiome and metabolomic analysis will be made available on request. Microbiome sequences have already been submitted to the European nucleotide database under project number PRJEB45648.
Declaration of Competing Interest
LC, ST, AJ, CR, ML, PK, MM, WC, JH and AS have no conflict of interest to disclose.

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Supplementary materials
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