Open Access
eISSN: 2508-9056

Exercise Medicine

Exercise Medicine 2021; 5:1 | https://doi.org/10.26644/em.2021.001

Brief Report

Provisional Designation of the Interleukin-6 Receptor (IL-6R) as a Novel Marker Gene for Exercise Tolerance

Richard Webb[1*], Alison Early[1], Bethan Scarlett[1], Jack Andrew Clark[1], Jumo Doran[1], Daniel Nash[1], Michael G Hughes[1], Lee R Butcher[1]

[1] *School of Sport & Health Sciences, Cardiff Metropolitan University, Cardiff CF5 2YB, United Kingdom*

Article Information

History:

Received: May 26, 2021
Accepted: September 13, 2021
Published: September 14, 2021

Keywords:

Interleukin-6 receptor (IL-6R);
Physical activity;
Single nucleotide polymorphism;

ABSTRACT

Objectives: Genomic markers linked to exercise-associated health benefits and/or sporting performance are increasingly used to guide decision-making in healthcare and sport/exercise science. This project investigated whether the IL-6R SNP "rs2228145" might be provisionally designated a novel physical activity/exercise marker. rs2228145 results in an Aspartate[358]/Alanine[358] change adjacent to the site where the IL-6R protein is cleaved into two fragments, resulting in ~two-fold increases in blood-borne levels of soluble IL-6R ['sIL-6R'].

Methods: Cohorts of staff/students at Cardiff Metropolitan University donated/completed: [i] finger-prick capillary blood samples (subjected to ELISAs for sIL-6R, the associated signalling protein sgp130, and the IL-6/sIL-6R complex); [ii] cheek-swab samples containing buccal epithelial cell DNA (subjected to PCR-based IL-6R/rs2228145 genotyping assays); [iii] International Physical Activity Questionnaires (to estimate physical activity levels in the week preceding sample donation).

Results: As expected, we observed significant genotype-dependent differences in blood-borne sIL-6R levels (CC (44.1±21.7ng/mL) vs. AC (28.6±7.3ng/mL) vs. AA (19.9±6.5ng/mL; $P<0.05$)); Importantly, AA homozygotes undertook significantly more physical activity than AC heterozygotes (6318±899 v. 3904±2280 MET-mins/week; $P<0.01$). Genotype was significantly associated with physical activity levels ($P<0.05$), and sIL-6R ($P=0.197$) and sgp130 ($P=0.160$) showed non-significant correlations with physical activity levels.

Conclusions: These data suggest that IL-6R/rs2228145 genotype may influence participation in physical activity/exercise, perhaps by impacting on abilities to tolerate activity without experiencing adverse-effects. Although more research is required to confirm these preliminary findings, designation of IL-6R/rs2228145 as a novel marker, and determination of participants' IL-6R/rs2228145 genotypes, may in future be useful tools to aid exercise-providers in designing personalised exercise programmes matched to clients/patients.

INTRODUCTION

Interleukin-6 (IL-6), a pleiotropic cytokine that mediates many physiological and pathophysiological processes, is often regarded as a pro-inflammatory cytokine secreted by inflammatory cells, with elevated plasma concentrations observed in conditions where inflammation is present [1]. Conversely, although exercise-associated IL-6 secretion from leukocytes is negligible [2], IL-6 is released as a "myokine" from contracting muscles to enable muscle-organ cross-talk, which is important in the body's systemic anti-inflammatory response to exercise [1,2].

IL-6's apparently opposing actions may be attributed to its divergent signalling pathways. IL-6 directly signals through its cell-surface receptor (membrane-bound IL-6R ['mbIL-6R']), which initiates downstream intracellular responses via glycoprotein-130 ('gp130') [3,4]. This pathway, labelled "classical signalling", only occurs in mbIL-6R-expressing cell-types (eg. myocytes, neutrophils, monocyte-macrophages) [3,4]. However, mbIL-6R can be cleaved into two fragments, one of which is lost from cells as soluble IL-6R

***Correspondence:** Richard Webb, Principal Lecturer, School of Sport & Health Sciences, Cardiff Metropolitan University, Cardiff CF5 2YB, United Kingdom
Tel: +44-29-2020-5559
E-mail address: rwebb@cardiffmet.ac.uk

['sIL-6R']) [3,4]. Circulating IL-6 binds to sIL-6R; the resulting IL-6/sIL-6R "active complex" can bind to gp130 (which is ubiquitously expressed, but which does not bind to IL-6 alone, or sIL-6R alone) [3], and trigger "trans-signalling" responses independent of mbIL-6R [4]. Finally, a circulating soluble fragment of gp130 ('sgp130') binds to IL-6/sIL-6R, forming an "inactive (or buffer) complex" and impairing trans-signalling [3]. Thus, whether IL-6 signals via classical or trans-signalling is determined by the respective levels of IL-6, sIL-6R, and sgp130.

The IL-6R gene contains a single clinically-relevant single-nucleotide-polymorphism ('SNP'): A→C at nucleotide position-1073 (NCBI Accession Code rs2228145) [5]. This results in an Aspartate→Alanine change at position-358 in the IL-6R protein (adjacent to the IL-6R cleavage site); approximately two-fold increases in cleavage result, with rs2228145 being responsible for ~50% of the variability in blood-borne sIL-6R levels [5].

Neutralisation of IL-6 trans-signalling using anti-sIL-6R antibody therapy has been approved as an effective treatment of rheumatoid arthritis [6] (and is currently undergoing clinical trials regarding the treatment of asthma [7] and COVID-19 [8]). rs2228145 is linked with several inflammatory conditions [9,10], and it seems plausible that rs2228145-inflammatory disease associations may be linked to excessive pro-inflammatory trans-signalling due to elevated sIL-6R levels, particularly in individuals possessing the CC genotype. We have previously reported that blood-borne sIL-6R levels are positively associated with adverse reactions (upper respiratory symptoms, perceived stress, worse mood, fatigue, poor sleep-quality – collectively referred to as 'over-reaching') in athletes undertaking prolonged periods of intense/high-volume exercise training [11]. Therefore, we hypothesised that rs2228145 may, via its impact on blood-borne sIL-6R levels, influence one's ability to tolerate physical activity (PA) without experiencing adverse effects. Accordingly, we aimed to undertake preliminary studies exploring the relationships between rs2228145 genotype; blood-borne sIL-6R, IL-6/sIL-6R and sgp130; and self-selected PA levels in healthy adults.

METHODS

Ethics/Governance/Participant Recruitment

In this exploratory pilot study, two small participant cohorts were recruited from staff/students at Cardiff Metropolitan University. Exclusion criteria were underlying inflammatory disease, or circumstances preventing normal habitual PA levels.

Group-1: 12 participants (8 male; 4 female; age=26±9yrs) donated one set of samples each.

Group-2: 6 participants (4 male; 2 female; age=39±14yrs) underwent a longitudinal study, donating samples via five sampling points over a period of several months.

Ethical approval was obtained from Cardiff Metropolitan University School of Sport and Health Sciences ethics committee; all procedures conformed to the Declaration of Helsinki.

Sample Collection/Processing.

All participants refrained from undertaking exercise for >12h before sample donation. Capillary blood was collected from the fingertip using heparinized microvette capillary blood collection-tubes (Sarstedt, Numbrecht, Germany). The resulting samples were fractionated using a double-centrifugation method (2x1min; 6000rpm) to obtain acellular plasma samples, which were stored at -80°C for subsequent batched analysis. To collect DNA samples, each participant swilled 10ml ddH2O around his/her mouth for 1min, before spitting the contents into a tube. After centrifugation (3000rpm; 3min), pellets (containing buccal epithelial cells) were resuspended in 350µl Chelex/4µl Proteinase K (10ng/µl; both from Sigma, Poole, UK). Samples were incubated (56°C; 30min), heated (98°C; 15min), and centrifuged (13000rpm; 3min). The buccal epithelial cell DNA content in the resulting supernatant samples was determined using Nanodrop spectrophotometry (Fisher-Scientific, Loughborough, UK); samples were stored at -80°C for subsequent batched analysis.

ELISA

Duoset ELISA kits (dy227, dy8139, dy228; R&D Systems, Abingdon, UK) were used according to manufacturers' instructions to determine sIL-6R, IL-6/sIL-6R complex, and sgp130 levels respectively, in plasma samples (diluted 1:100 v/v, to bring readings within the range of the standard curve provided). Inter-assay coefficients of variability were calculated from readings obtained from different ELISA plates for each experiment, and were found to be <15%. Post-hoc analyses determined ratios of [IL-6/sIL-6R]:[total sIL-6R] and [total sIL-6R]:[sgp130] in each case, to estimate proportions of sIL-6R in 'active complexes' or 'inactive complexes', respectively.

Genotyping Assay

Primers flanking the rs2228145 site were designed using Primer-BLAST software (https://www.ncbi.nlm.nih.gov/primer-blast): Forward: 5'-TGACAGCACCAGCTAAGT-3'; Reverse: 5'-ACAATGGCAATGCAGAGGAG-3'. Primers plus 1.78µg DNA template were included within 25µl PCR reactions (95°C/3min; 35 cycles of 95°C/30s, 61°C/30s, 72°C/60s; 72°C/5min). PCR products were subjected to HinfI restriction digests (5µl CutSmart buffer (10x); 1µl HinfI; 1µg DNA, within 50µl reaction volumes; 37°C; 60min). Given the presence of the HinfI consensus sequence (GATTC) in PCR product from AA homozygoytes, but its absence in CC homozygotes (GCTTC), samples' banding patterns in agarose-gels (Fig1A) reflected the genotype of the sample donor: either an intact PCR product (183bp [CC homozygotes]), a pair of fragments (101bp and 82bp [AA homozy-

gotes]), or a triplet of bands (183bp, 101bp, and 82bp [AC heterozygotes]).

International Physical Activity Questionnaires (IPAQ)

Prior to sample donation, all participants completed IPAQs to estimate self-selected PA levels in the week preceding sample donation. In each case, IPAQ results were processed to produce a total PA score (MET-mins/week).

Statistical Analysis

All statistical analyses were performed using MiniTab19 software (MiniTab Ltd, Coventry, UK). Shapiro-Wilks tests were carried out to determine whether parameters were normally-distributed. 2-sample t-tests or 1-way ANOVA (plus Dunnett's post-hoc comparisons) were used for analysis of normally-distributed parameters within two-sample or multiple-sample datasets, respectively. Because IPAQ was not normally distributed, non-parametric analyses (Mann-Whitney or Mood's median test) were carried out for two-sample or multiple-sample datasets, respectively. Regression analysis was used to evaluate potential associations between two numerical parameters within the same dataset. Data were expressed as mean±standard deviation, with significance set at $P<0.05$.

RESULTS

Group-1: Agarose-gel banding patterns revealed that the cohort comprised 6 AA homozygotes (50.0%), 4 AC heterozygotes (33.3%), and 2 CC homozygotes (16.7%), which showed significant similarity with previously-reported rs2228145 genotype frequencies (eg. International HapMap Project [2007; https://www.genome.gov/10001688/international-hapmap-project]). Also in agreement with previous reports [3-6], sIL-6R levels were in the 20-80ng/ml range, with significant genotype-dependent differences in blood-borne sIL-6R levels (CC (44.1±21.7ng/mL) *vs.* AC (28.6±7.3ng/mL) *vs.* AA (19.9±6.5ng/mL); Fig 1B).

Group-2: PA levels of the participants (2 AA homozygotes; 4 AC heterozygotes) did not significantly vary during the 6-month sampling period, although non-significant trends towards lower PA in January compared to August were apparent (Fig2A). Conversely, sIL-6R levels were significantly higher in the November-January period than in August (Fig2B). As for Group-1, AA homozygotes had lower plasma sIL-6R levels than AC heterozygotes across the entire 6-month period, albeit without this difference achieving statistical significance [data not shown]. No monthly differences in sgp130 or IL-6/sIL-6R were observed (Figs2C-D), but the proportion of sIL-6R complexed with IL-6 (ie. in 'active complexes') underwent a significant decrease in January (Fig2E). The proportion of sIL-6R complexed with sgp130 (ie. in 'inactive complexes') underwent a non-significant increase ($P=0.096$) in January (Fig2F). Importantly, when average PA readings from across the 6-month time-window were calculated, AA homozygotes undertook significantly more PA than AC heterozygotes (6318±899 v. 3904±2280 MET-mins/week; Fig3A).

Overall: Data from the two cohorts were pooled into a composite dataset (age=30±12yrs; 12 AA homozygote

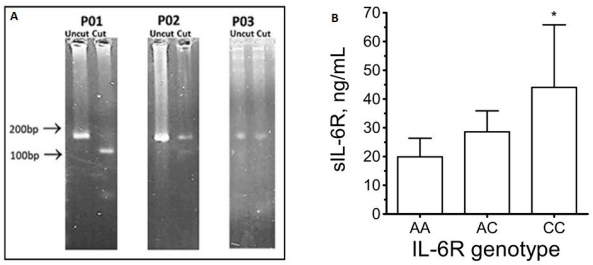

Figure 1. Determination of IL-6R rs2228145 genotype and plasma levels of sIL-6R in individuals from Cohort 1
[A] Representative agarose gel images from DNA samples subjected to PCR-based genotyping assay. Primers plus 1.78µg DNA template were included within 25µl PCR reactions. PCR products were subjected to HinfI restriction digests (5µl CutSmart buffer (10x); 1µl HinfI; 1µg DNA, within 50µl reaction volumes; 37°C; 60min). P01 [AA homozygote], with PCR product cleaved into 2 fragments; P02 [AC heterozygote], with PCR product partially cleaved, yielding 3-band pattern; P03 [CC homozygote], with PCR product remaining intact]. [B] Capillary blood samples (diluted 1:100 v/v) were subjected to ELISA in order to determine blood-borne sIL-6R levels. Data are displayed depending on IL-6R rs2228145 genotyping results for the participants in question (n=12; 6xAA; 4xAC; 2xCC. * denotes $P<0.05$ by 1-way ANOVA with AA IL-6R genotype being used as 'control' in the accompanying Dunnett's post-hoc comparison analysis.

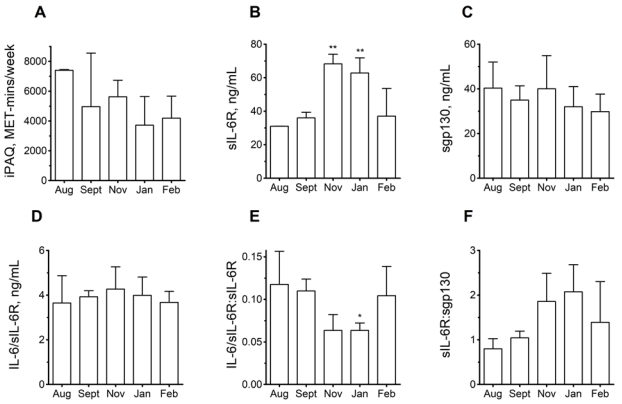

Figure 2. Variation in IPAQ, sIL-6R, sgp130, IL-6/sIL-6R complex in individuals from Cohort 2, over a 6-month sampling period
[A] Physical activity levels (MET-mins/week) in the week preceding each sampling point were estimated using IPAQ questionnaires. [B]-[F] Capillary blood samples (diluted 1:100 v/v) were subjected to ELISA to determine blood-borne levels of sIL-6R [B], sgp130 [C], and IL-6/sIL-6R [D]; ratios of IL-6R/sIL-6R-to-sIL-6R [E], and sIL-6R-to-sgp130 [F] were also calculated. In all cases, data are displayed according to the month on which they were collected (** denotes $P<0.01$ and * denotes $P<0.05$; 1-way ANOVA, with August being used as 'control' in the accompanying Dunnett's post-hoc comparison analysis).

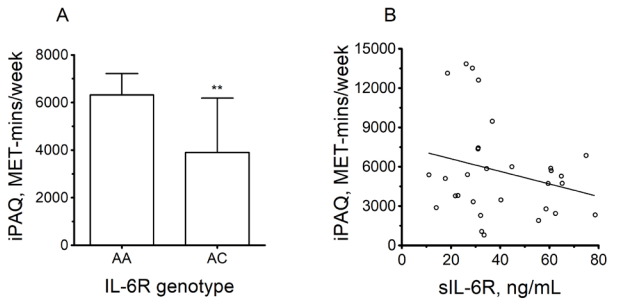

Figure 3. Physical activity levels of sIL-6R in individuals from Cohorts 2 and 3

Physical activity levels (MET-mins/week) of Cohort 2 participants (n=6; 2xAA; 4xAC) in the week preceding each sampling point were estimated using IPAQ questionnaires. Data are displayed stratified by IL-6R rs2228145 genotype. ** denotes P<0.01 by Mann-Whitney test. [B] Scatter graph illustrating (for each genotype within Cohort 3 [n=30; 12xAA; 16xAC; 2xCC]): [i] physical activity levels (MET-mins/week) in the week before each sampling point, as estimated using IPAQ questionnaires; [ii] blood-borne sIL-6R levels, as determined by subjecting capillary blood samples (diluted 1:100 v/v) to ELISA.

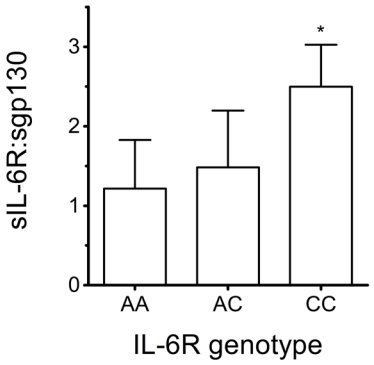

Figure 4. The ratio of sIL-6R to sgp130 in the plasma of individuals from Cohort 3, stratified by rs2228145 genotype

Capillary blood samples (diluted 1:100 v/v) were subjected to ELISA in order to determine blood-borne levels of sIL-6R and sgp130, and the ratio of these 2 analytes calculated in each case. Data are displayed stratified by IL-6R rs2228145 genotype (n=30; 12xAA; 16xAC; 2xCC). * denotes *P*<0.05 by 1-way ANOVA with AA IL-6R genotype being used as 'control' in the accompanying Dunnett's post-hoc comparison analysis.

samples [40.0%]; 16 AC heterozygote samples [53.3%]; 2 CC homozygote samples [6.7%]). Again, these observed frequencies showed significant similarity to previously-reported rs2228145 frequencies. Ratios of IL-6/sIL-6R complex-to-total sIL-6R were low across all three genotypes (4.1±1.1ng/ml [complex] *vs.* 39.4±18.9ng/ml [sIL-6R]), indicating that only ~10% of sIL-6R molecules were in 'active complexes'. This was expected, as IL-6 release as a myokine should be minimal given that participants had refrained from exercising for >12hrs before sampling points [2]. In contrast, sIL-6R:sgp130 ratios were significantly higher for CC than for AA homozygotes (Fig4).

Weekly PA levels showed similar genotype-dependent variation as those seen for Group-2 (*P*<0.05; data not shown). Non-significant inverse correlations were observed between plasma sIL-6R and PA levels (*P*=0.197, r -0.25; Fig3B), and between plasma sgp130 and PA levels (P=0.160, r=-0.27; data not shown). We and others [4,11] have previously observed similar inverse correlations, which have been tentatively attributed to exercise-associated regulatory mechanisms controlling either expression of proteases responsible for sIL-6R 'shedding' [12], or influx of leukocyte subpopulations (as sources of sIL-6R 'shedding') into the vicinity of damaged muscle tissue post-exercise [4].

DISCUSSION

Genomic markers linked to biological processes relevant to exercise-associated health benefits and/or sporting performance are used to guide decision-making in healthcare and sport/exercise science [13]. This project aimed to investigate whether the IL-6R gene (and specifically one's rs2228145 genotype) can be provisionally designated a novel marker for PA/exercise tolerance.

Longitudinal data showed low PA levels coinciding with high sIL-6R levels in November and January, while the converse was the case during periods when PA was higher. This appears to support our previous proposal that sIL-6R could represent a marker of athletes' exercise/training levels that is sensitive to changes on weekly/monthly bases [11], and to extend this principle by applying it to cohorts of non-elite athletes.

In contrast to previous studies (which reported that sgp130 levels exceed those of sIL-6R [3]), our data indicates that sIL-6R and sgp130 are present in capillary blood at approximately equivalent levels (see Fig2). Importantly, the genotype-dependent pattern seen in Fig4 suggests sIL-6R:sgp130 ratios of ~1:1 for AA, but ~2:1 for CC. Given the hexameric 2:2:2 IL-6:sIL-6R:sgp130 stoichiometry of the 'inactive complex' [3], our data suggest that lower proportions of sIL-6R molecules may be bound within 'inactive complexes' in CC individuals (thus implying greater potential for trans-signalling). Additionally, decreased mbIL-6R cell-surface expression, and hence decreased responsiveness to classical signalling, have been associated with the CC genotype [14]. This suggests that the balance of trans- versus classical-signalling may differ in a genotype-dependent manner (ie. favouring trans-signalling in C-allele bearers). Importantly, this may provide a mechanism underpinning the genotype-dependent variation in PA levels illustrated in Fig3A.

As stated earlier, associations between blood-borne sIL-6R levels and 'over-reaching' [11] suggest that rs2228145 genotype may influence participation in exercise, perhaps by impacting on one's ability to tolerate PA without

experiencing adverse effects. As an exploratory pilot-study, the current study has numerous limitations (eg. small cohort sizes; reliance on questionnaire responses concerning self-reported PA levels; no recording of participants' perceptions regarding undertaking of exercise). Nevertheless, we hope that the current study provides a starting-point from which future investigations may confirm our initial findings; determine whether rs2228145 genotype is associated with severity of over-reaching symptoms; and investigate which symptoms are most affected, and why?

Future studies could also investigate specific physiological contexts in which rs2228145 genotype may impact on exercising participants' wellbeing. For example, sIL-6R's ability to cross the blood-brain barrier and elicit neuroinflammatory trans-signalling responses in CNS cells has been linked to cognitive responses that present as fatigue, reduced power output, and termination of exercise [15]. Interestingly, we have previously reported that exercising participants' self-reported sensations of fatigue have been reported to correlate with their plasma sIL-6R levels [11]. Thus, it may be that triggering of such neuroinflammatory signalling, and possibly perceptions of fatigue, are more marked in individuals such as CC homozygotes with high blood-borne sIL-6R levels, and this may influence their choices regarding incorporation of exercise into their lifestyles. Similarly, rs2228145 significantly impairs lung function (specifically FVC and FEV) in CC homozygotes with asthma [10]. While these effects failed to reach statistical significance in non-asthma sufferers [10], one may speculate that weaker sub-clinical effects at rest may pre-dispose non-asthmatic CC homozygotes to episodic upper respiratory symptoms when exercising, and so discourage participation in exercise.

CONCLUSIONS

In conclusion, this preliminary study's findings suggest associations between rs2228145 genotype, sIL-6R, and self-selected PA levels in healthy adults. Although more research is required to confirm and extend the above findings, designation of IL-6R as a novel marker gene, and determination of clients' rs2228145 genotypes, may in future aid design of personalised exercise programmes matched to exercise-providers' clients, in both elite sport and exercise-referral settings [13].

ACKNOWLEDGEMENTS

We would like to thank all participants for their engagement in this study, and also acknowledge the excellent technical support of Gareth Walters, Leighton Jenkins, Paul Jones, Laura Watkeys, Sam Hooper, and Steve Potter. This project was supported by a Cardiff Metropolitan University 'Global Academies' grant. Also, DN is the recipient of an EU Social Fund KESS2 PhD studentship (home institution: Cardiff Metropolitan University; external partner: Sport Wales).

Conflicts of Interest

The authors declare no conflict of interest.

REFERENCES

1. Pedersen BK, Febbraio MA. Muscle as an endocrine organ: focus on muscle-derived interleukin-6. Physiol Rev. 2008; 88(4):1379-406.
2. Abbasi A, Fehrenbach E, Hauth M, et al. Changes in spontaneous and LPS-induced ex vivo cytokine production and mRNA expression in male and female athletes following prolonged exhaustive exercise. Exerc Immunol Rev 2013; 19:8-28.
3. Lokau J, Agthe M, Garbers C. Generation of Soluble Interleukin-11 and Interleukin-6 Receptors: A Crucial Function for Proteases during Inflammation. Mediators Inflamm. 2016:1785021.
4. Robson-Ansley P, Cockburn E, Walshe I, Stevenson E, Nimmo M. The effect of exercise on plasma soluble IL-6 receptor concentration: a dichotomous response. Exerc Immunol Rev. 2010; 16:56-76.
5. Galicia JC, Tai H, Komatsu Y, Shimada Y, Akazawa K, Yoshie H. Polymorphisms in the IL-6 receptor (IL-6R) gene: strong evidence that serum levels of soluble IL-6R are genetically influenced. Genes Immun 2004; 5(6):513-6.
6. Tanaka T, Kishimoto T. Targeting interleukin-6: all the way to treat autoimmune and inflammatory diseases. Int J Biol Sci. 2012; 8(9):1227-36. doi: 10.7150/ijbs.4666.
7. Esty B, Harb H, Bartnikas LM, et al. Treatment of severe persistent asthma with IL-6 receptor blockade. J Allergy Clin Immunol Prac 2019; 7(5):1639-1642.e4.
8. Guaraldi G, Meschiari M, Cozzi-Lepri A, et al. Tocilizumab in patients with severe COVID-19: a retrospective cohort study. Lancet Rheumatol. 2020; 2(8):e474-e484. Ahmed S, Hussain S, Ammar A, Jahan S, Khaliq S, Kaul H. Interleukin 6 Receptor (IL6-R) Gene Polymorphisms Underlie Susceptibility to Rheumatoid Arthritis. Clin Lab. 2017; 63(9):1365-1369.
9. Hawkins GA, Robinson MB, Hastie AT, et al. The IL6R variation Asp(358)Ala is a potential modifier of lung function in subjects with asthma. J Allergy Clin Immunol 2012; 130(2):510-5.e1.
10. Cullen T, Thomas AW, Webb R, Phillips T, Hughes MG. sIL-6R is related to weekly training mileage and psychological wellbeing in athletes. Med Sci Sport Exercise, 2017; 49(6):1176-1183.
11. Somineni HK, Boivin GP, Elased KM. Daily exercise training protects against albuminuria and angiotensin converting enzyme 2 shedding in db/db diabetic mice. J Endocrinol. 2014; 221(2):235-51.
12. Jayal A, McRobert A, Oatley G, O'Donoghue P. Sports Analytics. Routledge Press, London, UK. 2018. ISBN 9780415789431.
13. Ferreira RC, Freitag DF, Cutler AJ, et al. Functional

IL6R 358Ala allele impairs classical IL-6 receptor signaling and influences risk of diverse inflammatory diseases. PLoS Genet. 2013; 9(4):e1003444.

14. Vargas N, Marino F. Neuroinflammation, cortical activity, and fatiguing behaviour during self-paced exercise. Pflugers Arch. 2018; 470(2):413-426.