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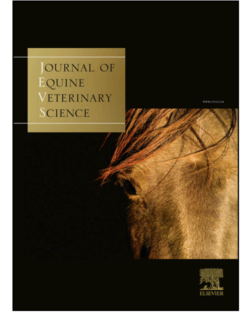

The effect of race training on the basal gene expression of alveolar macrophages derived from Standardbred racehorses

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1 **The effect of race training on the basal gene expression of alveolar macrophages derived**
2 **from Standardbred racehorses**

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22 **ABSTRACT**

23 Mild to moderate equine asthma is prevalent in young racehorses, particularly early in their
24 training period. Although the precise aetiopathogenesis remains undetermined, it is possible
25 that the susceptibility of this population might partly reflect an exercise-associated immune
26 derangement at the level of the airway. We performed a genome-wide basal gene expression
27 scan on alveolar macrophages (AMs) isolated from Standardbred racehorses prior to and after
28 commencement of competition race training with a view to identifying any exercise-
29 associated gene expression modulation consistent with functional alterations which might
30 reflect training-associated immunological derangement. Microarray technology was used to
31 analyse the basal gene expression profiles of bronchoalveolar fluid-derived AMs, harvested
32 from six systemically healthy Standardbred racehorses prior to (T0) and following (T1) entry
33 into training. Additionally, AM LPS-induced TNF-α and IL-10 release at T0 and T1 was
34 assessed. Although the data revealed significant inter-horse heterogeneity in relation to the
35 magnitude of individual gene expression at each time-point, within each horse, several
36 inflammatory related genes (e.g. chemokine ligands, interferons and NFKB) declined in
37 expression from T0 to T1. Entry into training did not significantly alter AM LPS-induced
38 TNF-α or IL-10 release. The data support a direct effect of training on AM basal gene
39 expression, particularly with respect to immune-related genes. The pattern of training-
40 associated differential gene expression may indicate relative downregulation of
41 inflammatory-related genes, consistent with an immunosuppressive effect of training and an
42 increased susceptibility to opportunistic pathogens.

43

44 **Keywords**: immunity; racehorse; lungs; transcriptomics; microarray; alveolar macrophage

45 **1. Introduction**

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46 Mild to moderate equine asthma (previously termed inflammatory airway disease) is a
47 chronic airway inflammatory disease, characterised by chronic coughing, increased mucus
48 production, inflammatory cell infiltration of the airways and poor performance [1, 2]. Based
49 upon the diversity of inflammatory cell populations and the disparity in both the direction and
50 degree of airway T-helper cell polarisation associated with the syndrome [1, 3-6], it is likely
51 that different causative and predisposing factors contribute to varying degrees in different
52 horse populations. Evidence exists of an association between bacterial isolation from the
53 trachea and the presence of mucus, an important criterion in disease definition [7-12]. This
54 association, when considered along with the apparent reduction in disease risk with
55 increasing time in training may support an infectious aetiology with the subsequent
56 development of immunity [7, 13]. However, the inverse association with time in training
57 remains even after adjustment for bacterial detection, potentially supporting alternative
58 aetiopathogenic mechanisms [14]. Adaptation of the innate immune response to repetitive
59 airborne challenge with non-infectious agents, such as that recognised in organic dust
60 induced respiratory disease in man [15, 16], could also potentially result in a similar inverse
61 association with time in training. Despite such hypotheses, the influence of training on
62 immune function in horses has received comparatively little attention [17, 18].
63
64 Despite a clear association between high intensity exercise in humans and symptoms of
65 respiratory infection, infectious causes are generally identified in only 35-45% of cases
66 exhibiting symptoms [19-21]. Several immunological derangements are associated with
67 intense exercise, including effects on cell migration and margination, mucosal IgA secretion,
68 cellular function and cell surface receptor expression [22-25]. Although most studies on
69 innate immune cells in humans and animals have focused on circulating monocytes, [19, 22,
70 26-28], exercise also elicits alterations in alveolar macrophages (AM). In humans [29], mice

71 [30-32] and horses [33], intense exercise reduced AM MHC-II expression [31], antigen
72 presentation capacity [30], phagocytic properties [33] and impaired responses to various
73 stimuli [23, 32].

74 Previous studies have used microarray technology to investigate the effect of intense exercise
75 on global gene expression of peripheral blood monocytes [34, 35]. In addition, recent RNA-
76 sequencing has also identified a number of novel candidate regulators [36]. Although such
77 work has provided valuable information on exercise-induced alterations in immunity, it offers
78 limited insight into exercise- and/or training-associated changes in resident macrophage
79 function and related tissue level immune responses [25]. Previous equine studies have
80 investigated the effect of intense exercise on the functional properties of harvested AMs [33,
81 37, 38], revealing an impairment of phagocytic capacity and an increase in oxidative burst
82 activity [33, 38]. More recently, using qPCR, Frellstedt *et al* (2014) reported the
83 downregulation of *TLR3* mRNA expression in both AMs and monocytes in response to
84 training, without normalisation during the recovery period, as well as an exercise-associated
85 reduction in the level of viral-induced *TNF* and *IFNβ* expression in AMs [39]. In the present
86 study, we aimed to extend these observations by examining the gene expression profiles of
87 AMs in Standardbred horses before and after entry into training.

88

89 **2. Materials and Methods**

90 *2.1 Horses, sample collection and sample time-points*

91 Six Standardbred racehorses (2 males and 4 females; age range 3-6 years), from a racing yard
92 under the care of the Nantes Veterinary School Equine Clinic (Oniris), were included in this
93 study. The Veterinary Ethical Review Committee of the College of Veterinary Medicine at
94 Nantes approved all protocols involving animal use. Standard welfare procedures were

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95 followed and informed owner consent was obtained for inclusion on the study. Prior to
96 sample collection, the absence of clinical abnormalities was confirmed by two of the authors
97 (AC, MD), both equine veterinary medicine specialists.

98 **Bronchoalveolar lavage fluid (BALF) samples were collected at two different time-**
99 **points: prior to (T0) and following (T1) entry into the training program. All samples**
100 **were collected between 0600h and 0700h, prior to feeding and $\geq$ 24h since the previous**
101 **training session. The standard training programme consisted of an initial three week**
102 **period of light work (jogging), followed by an increase in work intensity which included**
103 **two days per week of continuous aerobic exercise to a point whereby the blood lactate**
104 **concentration reached 3-4mmol/L. Details of the sampling time points for each animal**
105 **used in the study are included in Supplementary data 1. Briefly, in 3 horses, T1 samples**
106 **were obtained following a 1.5 week period of jogging from the commencement of entry**
107 **into the training programme; namely, mid-way through the initial period of light work.**
108 **In the remaining 3 horses, T1 samples were obtained following a 7 week period within a**
109 **standardised full training program; namely, 4 weeks following completion of the initial**
110 **3 weeks light work and commencement of the intense aerobic exercise (see above).**
111 BALF was collected, a differential cell count performed and BALF-derived cells isolated and
112 cryopreserved as previously described [40, 41]. Horses were considered free from mild to
113 moderate equine asthma based on the differential cell ratios not exceeding the following cut
114 off values: neutrophils - 10%; mast cells - 5%; eosinophils - 2% [42, 43].
115
116 *2.2 Cell culture*

117 Cells were seeded in duplicate ($1x10^6$ cells/mL) in petri dishes in complete medium: (RPMI-
118 1640 medium supplemented with GlutaMAX™-I (Invitrogen Ltd, Paisley, UK) supplement,

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119 penicillin/streptomycin (Invitrogen Ltd, Paisley, UK) and 10% heat-inactivated Horse Serum
120 (HS, cat no: H1138' Sigma-Aldrich, Dorset, UK) and incubated at 37°C and 5% CO_2
121 overnight. The following day, non-adherent cells were removed and fresh complete medium
122 was added before adherent cells were stimulated with LPS (100ng/mL) from *Salmonella*
123 *enterica* serotype Minnesota Re 595 (L9764, Sigma-Aldrich, Dorset, UK). Supernatant from
124 the plates was collected prior to and 6 h following LPS stimulation. Following overnight
125 culture and removal of non-adherent cells, more than 90% of adherent cells were identified
126 morphologically as macrophages.

127 *2.3 TNFα and IL10 assay*

128 To assess AM LPS responsiveness, culture supernatant TNFα and IL10 concentration was
129 measured by ELISA before and 6h following LPS stimulation at both time-points (T0, T1),
130 using the DuoSet ELISA kit (R&D systems, Minneapolis) according to manufacturer's
131 instructions. Optical density was read (Multiskan v2.6' Thermo Scientific, Wilmington, NC,
132 USA) at 540 nm and data analysed using Ascent software.

133 *2.4 RNA extraction*

134 RNA was extracted using 1mL RNA-Bee according to manufacturer's instructions and RNA
135 concentration and purity measured using ND-1000 Nanodrop (Thermo Scientific,
136 Wilmington, NC, USA) spectrophotometer. RNA integrity was confirmed with RNA 6000
137 Nano Assay (Agilent Technologies, Waldbronn, Germany); an RNA integrity number (RIN)
138 >7 was considered appropriate for microarray analysis.

139 *2.5 Microarray analysis*

140 Microarrays (Affymetrix, Santa Clara, CA) were processed by Edinburgh Genomics as
141 previously described [44]. Briefly, total RNA (50ng) was amplified by the Nugen Pico SL kit

142 (Agilent, the Netherlands). Two and a half micrograms of the cDNA produced were biotin
143 labelled using the Nugen Encore labelling kit. Biotin labelled transcripts were prepared for
144 hybridisation following the Nugen protocol for Gene Titan hybridisation (Affymetrix, Santa
145 Clara, CA), using the Affymetrix Gene Titan Hybridization Wash and Stain Kit for WT
146 Array Plates (PN 901622). The samples were hybridised to Equine Gene 1.0 ST Array Strips
147 from Affymetrix, including 30,559 probe sets, each interrogating a specific transcript of a
148 gene, using the appropriate Hyb-Wash Scan protocol and the Gene Titan Hyb-Wash Stain Kit
149 for the reagents (Affymetrix, Santa Clara, CA). The arrays used were oligonucleotide
150 microarrays that consisted of probes corresponding to exons along the whole length of each
151 transcript (25 probes per probe set representing a transcript).

152 Analysis performed by Partek Genomic Suite 6.6 software. A two way ANOVA (with time-
153 point: fixed effect; horse ID: random effect) was performed to identify differentially
154 expressed genes within the group of horses between the sampling time-points. Consistent
155 with cut-off values widely used in microarray data analysis, fold changes of ≥ 2 and a p value
156 of 0.05 were applied.

157 A network analysis of expression data was performed in BioLayout *Express*^{3D} [45], whereby
158 pairwise Pearson correlation coefficients were calculated and a threshold of $r \geq 0.9$ chosen for
159 transcript to transcript comparisons across the array samples. The resulting network graph
160 consists of nodes, representing transcripts, and edges, representing correlations between the
161 expression patterns of the transcripts above the threshold. The Markov cluster algorithm
162 (MCL) was used with an inflation value of 2.2 [45] to identify groups of tightly co-expressed
163 genes. Clusters are numbered according to the number of transcripts they contain (i.e. Cluster
164 1 has the greatest number of transcripts). Transcripts with dynamic range less than 1.5 were
165 removed from analysis. Only clusters showing consistent up or downregulation of genes

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166 across all samples in the group were analysed. Any clusters resulting from aberrant
167 expression in a single horse were excluded.

168 *2.6 Functional annotation*

169 DAVID (Database for Annotation, Visualisation, and Integrated Discovery) 6.7 software, was
170 used to determine the biological processes of the genes included in the gene lists. The
171 enrichment score of the DAVID package is a modified Fisher exact p value calculated by the
172 software. The higher the enrichment score, the more enriched the cluster. An enrichment
173 score of >1 reflects over-expression of the functional category.

174

175 **3. Results**

176 *3.1 Cell recovery and populations*

177 Harvested cell viability exceeded 80%. Total cell counts [200($^{\pm}$23)/µl for T0 and 255($^{\pm}$43)/µl
178 for T1] did not significantly differ between time-points. There was no statistical difference in
179 BALF differential cell ratios (**Fig. 1**) between time-points and a degree of inter-horse
180 heterogeneity was evident regarding training associated alterations in BALF neutrophil,
181 eosinophil and mast cell ratios. With the exception of horse 5, neutrophil ratios remained
182 relatively unaltered. With the exception of horses 2 and 5, the eosinophil ratios remained low.
183 With respect to BALF mast cell ratios, horses 2 and 5 showed a mild training associated
184 increase and Horse 1 showed a marked training associated decrease.

185

186 *3.2 The effect of race training on AM basal and LPS-induced TNFα and IL10 production*

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187 AM basal TNFα production remained low at both T0 and T1. LPS stimulation resulted in a
188 significant increase in 6h AM TNFα production at both T0 and T1 **(Fig. 2)**. LPS stimulation
189 failed to induce detectable AM IL10 production at either T0 or T1 (*data not shown*).

190 *3.3 Transcriptomic analysis of equine AMs before and during the training period*

191 RNA extraction of adhered cells prior to and during the training period yielded an average of
192 1.7µg of RNA /10^6 cells (± 0.5). Selection of appropriate samples for subsequent microarray
193 analysis was based on the RNA derived from pre-LPS treated samples having a RIN > 7. Full
194 data are available in the Gene Expression Omnibus [GEO] database;
195 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE107494). Exploration of gene
196 expression by PCA revealed sample separation into two groups corresponding to T0 and T1
197 **(Fig. 3)**. However, no statistically significant difference was observed in gene expression
198 between T0 and T1 ($p < 0.05$, Fold change ≥ 2).

199 Network analysis of normalised expression data created a graph of 12,860 nodes connected
200 by 104,012 edges. The clustering algorithm MCL with an inflation value of 2.2 was used to
201 identify groups of tightly co-expressed genes and create 4,117 clusters containing a minimum
202 of three nodes [46]. **Fig. 4** shows the network graph of all the genes based on co-expression,
203 in which clusters of genes with greater expression at either T0 or T1 were observed. Despite
204 significant inter-horse heterogeneity in the magnitude of individual gene expression at each
205 time-point, within each horse, several inflammatory related genes (e.g. chemokine ligands,
206 interferons and *NFKB1*) were expressed to a lesser degree at T1, compared to T0 **(Fig. 5)**.

207 Using DAVID software, GO annotation analysis (Biological Processes) of genes associated
208 with the different clusters yielded a gene list of 76 annotated genes with lower expression at
209 T1 (Cluster 2, 5, 16, 21, 39, **Supplementary data 2**). This list included genes involved in cell

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210 activation and immune system processes, implying that there was less immune activity at T1
211 compared to T0 **(Table 1)**.

Annotation cluster	Biological process	Enrichment score	Gene count
1	Response to stimulus	7.92	50-58
2	Immune system process	2.7	11-17
3	Immune system development	2.68	3-17
4	Immune response activating cell surface receptor signalling pathway	1.89	3-27
5	Cell development-differentiation	1.26	8-46
6	Response to virus/biotic stimulus	1.04	4-8

212

213 **Table 1:** The top 6 out of 25 annotation clusters created by DAVID software that were
214 related with the list of 117 genes found higher at T0. The enrichment score is a modified
215 Fisher exact p value calculated by the software.

216

217 **4. Discussion**

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218 To our knowledge, this study is the first to report on the findings of microarray analysis of
219 equine AMs harvested before and during a period of training, thus permitting an assessment
220 of the effect of training on the basal gene expression of the principal innate immune cell of
221 the equine airway. We considered this a justifiable approach due to (a) the high prevalence of
222 airway inflammation in racehorses in training, (b) the undetermined role of opportunistic
223 bacterial and/or viral infection in this population and (c) the apparent parallels with the well-
224 recognized phenomenon of exercise-associated respiratory symptoms in humans. Despite the
225 limited sample size, our analysis supports a direct effect of training on racehorse AM gene
226 expression, particularly of immune-related genes, that may play a role in increasing
227 susceptibility to opportunistic infection and suggest that larger population studies would be
228 more informative.

229

230 Principal component analysis revealed a distinct separation between the two time-points,
231 supporting a universal effect of training on AM gene expression. However, there was marked
232 horse-to-horse variation that may have had an effect in the magnitude of altered gene
233 expression. Although this may have had a genetic basis, the data set is clearly not large
234 enough to permit an expression quantitative trait locus analysis. In humans, the large majority
235 of LPS-inducible genes show evidence of heritable variation in their expression [47].
236 Accordingly, a much larger dataset would be required to demonstrate the significance of any
237 impact of training against a substantial background of inter-individual variation.

238 Despite that, a subset of genes was apparently down-regulated during the training period.
239 These included genes encoding several chemokines and interferons and the transcription
240 factors *STAT4* and *NFKB1*, thus complementing the findings of Frellstedt *et al* (2014), who
241 reported a significant training-associated decrease in equine AM basal *IFNB* expression, also

242 reported in the current study. Similar results have also been reported in human PBMCs,
243 where prolonged intense training suppressed the NFKB signaling pathway, indicating an
244 immunosuppressive effect of training on blood cell compartments [48]. This decreased AM
245 IFN expression could predispose to lower airway viral infection. Interestingly, orally
246 administered IFN has previously shown clinical efficacy in Standardbred racehorses with
247 mild to moderate asthma [49]. Similarly, as *STAT4* is integral to the induction of IL12
248 signaling and the Th1 response [50], its downregulation could increase susceptibility to
249 opportunistic bacterial infection. Many of the other downregulated genes (*CXCL9*, *CXCL10*,
250 *STAT4*, *GBP5*) are known IFN target genes, and likely to be down-regulated consequent to
251 reduced IFN signalling [51]. Downregulation of these genes is therefore suggestive of a
252 phenotypic shift in AMs representing an overall immunosuppressive effect of exercise, as
253 previously suggested [28, 33, 38].

254 In agreement with previous reports, training was not associated with altered AM morphology
255 [52, 53] or LPS responsiveness, as assessed by TNFα release [39]. Exercise-induced
256 abrogation of LPS-induced TNFα release is recognised in humans and partly attributed to
257 muscle-derived IL-6 release [54]. Others have shown a post-race reduction in LPS-induced
258 inflammatory gene (e.g. *IFNB* and *CXCL10*) expression in whole blood cells of marathon
259 runners [55]. In light of the proposed contributory role of housing-associated increases in
260 airborne endotoxin exposure in mild to moderate asthma [2], "entry into training" could
261 potentially induce a degree of acquired tolerance to endotoxin exposure, as recognized in
262 human organic dust-induced disease. Our study provided no evidence for this proposal. With
263 one exception, all horses fulfilled BALF cytological, although not clinical, criteria consistent
264 with mild to moderate equine asthma, at T0 and/or T1. As previously reported, there was no
265 consistent relationship between training and the temporal change in total or differential BALF
266 cell counts [24, 33].

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267 **5. Conclusion**

268 In keeping with the “one health” initiative, there is increasing awareness of the potential
269 benefits of the bi-directional cross-species translational application of biological data between
270 horse and human; indeed, we have recently published work supporting this concept [44]. It is
271 particularly feasible that data derived from a natural athlete such as the horse could make
272 relevant contributions to the increasing body of evidence relating to human exercise
273 immunology. Furthermore, compared with humans, the relative ease by which AMs can be
274 harvested from equine athletes, within the context of standard clinical investigation, offers a
275 potentially valuable resource for such comparative studies. Together, our study, which
276 exploited the infrequent availability of lower airway derived immune cells from a small
277 cohort of racehorses, revealed unique, albeit limited, data on compartmentalised training-
278 associated immune effects. However, future longitudinal studies involving larger groups of
279 animals, including those with evidence of pathology, are required to expand on our results
280 and produce additional insights into disease susceptibility.

281

282 **Conflict of Interest Disclosure**

283 The authors declare no conflict of interest.

284

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296

297 **References**

298 [1] Couetil LL, Hoffman AM, Hodgson J, Buechner-Maxwell V, Viel L, Wood JLN, et al.
299 Inflammatory airway disease of horses. Journal of Veterinary Internal Medicine.
300 2007;21:356-61.
301 [2] Couetil LL, Cardwell JM, Gerber V, Lavoie JP, Leguillette R, Richard EA. Inflammatory
302 Airway Disease of Horses--Revised Consensus Statement. 2016;30:503-15.
303 [3] Lavoie JP, Maghni K, Desnoyers M, Taha R, Martin JG, Hamid QA. Neutrophilic airway
304 inflammation in horses with heaves is characterized by a Th2-type cytokine profile.
305 American Journal of Respiratory and Critical Care Medicine. 2001;164:1410-3.
306 [4] Hughes KJ, Nicolson L, Da Costa N, Franklin SH, Allen KJ, Dunham SP. Evaluation of
307 cytokine mRNA expression in bronchoalveolar lavage cells from horses with inflammatory
308 airway disease. Veterinary Immunology and Immunopathology. 2011;140:82-9.
309 [5] Beekman L, Tohver T, Léguillette R. Comparison of cytokine mRNA expression in the
310 bronchoalveolar lavage fluid of horses with inflammatory airway disease and
311 bronchoalveolar lavage mastocytosis or neutrophilia using REST software analysis. Journal
312 Of Veterinary Internal Medicine / American College Of Veterinary Internal Medicine.
313 2012;26:153-61.
314 [6] Richard EA, Depecker M, Defontis M, Leleu C, Fortier G, Pitel PH, et al. Cytokine
315 Concentrations in Bronchoalveolar Lavage Fluid from Horses with Neutrophilic
316 Inflammatory Airway Disease. Journal of Veterinary Internal Medicine. 2014;28:1838-44.
317 [7] Cardwell JM, Smith KC, Wood JL, Newton JR. Infectious risk factors and clinical
318 indicators for tracheal mucus in British National Hunt racehorses. Equine Vet J. 2014;46:150-
319 5.
320 [8] Burrell MH, Wood JLN, Whitwell KE, Chanter N, Mackintosh ME, Mumford JA.
321 Respiratory disease in thoroughbred horses in training: The relationships between disease and
322 viruses, bacteria and environment. Veterinary Record. 1996;139:308-13.
323 [9] Wood JL, Burrell MH, Roberts CA, Chanter N, Shaw Y. Streptococci and Pasteurella spp.
324 associated with disease of the equine lower respiratory tract. Equine Vet J. 1993;25:314-8.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

325 [10] Wood JL, Newton JR, Chanter N, Mumford JA. Association between respiratory disease
326 and bacterial and viral infections in British racehorses. Journal of clinical microbiology.
327 2005;43:120-6.
328 [11] Chapman PS, Green C, Main JPM, Taylor PM, Cunningham FM, Cook AJC, et al.
329 Retrospective study of the relationships between age, inflammation and the isolation of
330 bacteria from the lower respiratory tract of thoroughbred horses. Veterinary Record.
331 2000;146:91-5.
332 [12] Christley RM, Hodgson DR, Rose RJ, Wood JL, Reids SW, Whitear KG, et al. A case-
333 control study of respiratory disease in Thoroughbred racehorses in Sydney, Australia. Equine
334 Vet J. 2001;33:256-64.
335 [13] Newton JR, Wood JLN. Evidence of an association between inflammatory airway
336 disease and EIPH in young Thoroughbreds during training. In: Hinchcliff KW, Geor RJ,
337 Pagan JD, editors.: Equine Veterinary Journal; 2002. p. 417-24.
338 [14] Cardwell JM, Smith KC, Wood JL, Newton JR. A longitudinal study of respiratory
339 infections in British National Hunt racehorses. The Veterinary record. 2013;172:637.
340 [15] Kuchuk OO, Rosyns'ka LM, Basanets AV. [The early clinical signs of
341 bronchopulmonary pathology in workers exposed to organic dust]. Likars'ka sprava. 2005:75-
342 9.
343 [16] Seifert SA, Von Essen S, Jacobitz K, Crouch R, Lintner CP. Organic dust toxic
344 syndrome: a review. Journal of toxicology Clinical toxicology. 2003;41:185-93.
345 [17] Wilsher S, Allen WR, Wood JLN. Factors associated with failure of Thoroughbred
346 horses to train and race. Pferdeheilkunde. 2006;22:503-4.
347 [18] Cardwell JM. Risk factors for inflammatory airway disease in UK National Hunt
348 racehorses. Proceedings of the 4th World Equine airways Symposium,wwwivisorg. 2009.
349 [19] Kakanis MW, Peake J, Brenu EW, Simmonds M, Gray B, Hooper SL, et al. The open
350 window of susceptibility to infection after acute exercise in healthy young male elite athletes.
351 Exercise Immunology Review. 2010;16:119-37.
352 [20] Cox AJ, Gleeson M, Pyne DB, Callister R, Hopkins WG, Fricker PA. Clinical and
353 laboratory evaluation of upper respiratory symptoms in elite athletes. Clinical Journal of
354 Sport Medicine. 2008;18:438-45.
355 [21] Gleeson M. Mucosal immune responses and risk of respiratory illness in elite athletes.
356 Exercise Immunology Review. 2000;6:5-42.
357 [22] Buschmann H, Baumann M. Alterations of cellular immune response during intensive
358 training of event horses. Zentralblatt fur Veterinarmedizin Reihe B Journal of veterinary
359 medicine Series B. 1991;38:90-4.
360 [23] Mignot CC, Pirottin D, Farnir F, de Maffarts B, Molitor C, Lekeux P, et al. Effect of
361 strenuous exercise and ex vivo TLR3 and TLR4 stimulation on inflammatory gene expression
362 in equine pulmonary leukocytes. Veterinary Immunology and Immunopathology.
363 2012;147:127-35.
364 [24] Ainsworth DM, Appleton JA, Eicker SW, Luce R, Flaminio MJ, Antczak DF. The effect
365 of strenuous exercise on mRNA concentrations of interleukin-12, interferon-gamma and
366 interleukin-4 in equine pulmonary and peripheral blood mononuclear cells. Veterinary
367 Immunology and Immunopathology. 2003;91:61-71.
368 [25] Walsh NP, Gleeson M, Shephard RJ, Gleeson M, Woods JA, Bishop NC, et al. Position
369 Statement Part one: Immune function and exercise. Exercise Immunology Review.
370 2011;17:6-63.
371 [26] Rossdale PD, Burguez PN, Cash RS. Changes in blood neutrophil/lymphocyte ratio
372 related to adrenocortical function in the horse. Equine Veterinary Journal. 1982;14:293-8.
373 [27] Nesse LL, Johansen GI, Blom AK. Effects of racing on lymphocyte proliferation in
374 horses. American Journal of Veterinary Research. 2002;63:528-30.

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375 [28] Robson PJ, Alston TD, Myburgh KH. Prolonged suppression of the innate immune
376 system in the horse following an 80 km endurance race. Equine Veterinary Journal.
377 2003;35:133-7.
378 [29] Fehr HG, Lötzerich H, Michna H. Human macrophage function and physical exercise:
379 phagocytic and histochemical studies. European Journal of Applied Physiology and
380 Occupational Physiology. 1989;58:613-7.
381 [30] Ceddia MA, Voss EW, Woods JA. Intracellular mechanisms responsible for exercise-
382 induced suppression of macrophage antigen presentation. Journal of Applied Physiology.
383 2000;88:804-10.
384 [31] Woods JA, Ceddia MA, Kozak C, Wolters BW. Effects of exercise on the macrophage
385 MHC II response to inflammation. International Journal of Sports Medicine. 1997;18:483-8.
386 [32] Kohut ML, Davis JM, Jackson DA, Colbert LH, Strasner A, Essig DA, et al. The role of
387 stress hormones in exercise-induced suppression of alveolar macrophage antiviral function.
388 Journal of Neuroimmunology. 1998;81:193-200.
389 [33] Raidal SL, Love DN, Bailey GD, Rose RJ. The effect of high intensity exercise on the
390 functional capacity of equine pulmonary alveolar macrophages and BAL-derived
391 lymphocytes. Research in Veterinary Science. 2000;68:249-53.
392 [34] Barrey E, Mucher E, Robert C, Amiot F, Gidrol X. Gene expression profiling in blood
393 cells of endurance horses completing competition or disqualified due to metabolic disorder.
394 Equine veterinary journal Supplement. 2006;38:43-9.
395 [35] Capomaccio S, Cappelli K, Barrey E, Felicetti M, Silvestrelli M, Verini-Supplizi A.
396 Microarray analysis after strenuous exercise in peripheral blood mononuclear cells of
397 endurance horses. Animal Genetics. 2010;41:166-75.
398 [36] Capomaccio S, Vitulo N, Verini-Supplizi A, Barcaccia G, Albiero A, D'Angelo M, et al.
399 RNA Sequencing of the Exercise Transcriptome in Equine Athletes. PLoS ONE.
400 2013;8:e83504.
401 [37] Wong CW, Thompson HL, Thong YH, Thornton JR. Effect of strenuous exercise stress
402 on chemi-luminescence response of equine alveolar macrophages. Equine Veterinary Journal.
403 1990;22:33-5.
404 [38] Raidal SL, Rose RJ, Love DN. Effects of training on resting peripheral blood and BAL-
405 derived leucocyte function in horses. Equine Veterinary Journal. 2001;33:238-43.
406 [39] Frellstedt L, Waldschmidt I, Gosset P, Desmet C, Pirottin D, Bureau F, et al. Training
407 Modifies Innate Immune Responses in Blood Monocytes and in Pulmonary Alveolar
408 Macrophages. American Journal of Respiratory Cell and Molecular Biology. 2014;51:135-42.
409 [40] Depecker M, Richard EA, Pitel P-H, Fortier G, Leleu C, Couroucé-Malblanc A.
410 Bronchoalveolar lavage fluid in Standardbred racehorses: Influence of unilateral/bilateral
411 profiles and cut-off values on lower airway disease diagnosis. The Veterinary Journal.
412 2014;199:150-6.
413 [41] Karagianni AE, Kapetanovic R, McGorum BC, Hume DA, Pirie SR. The equine alveolar
414 macrophage: Functional and phenotypic comparisons with peritoneal macrophages.
415 Veterinary Immunology and Immunopathology. 2013;155:219-28.
416 [42] Richard EA, Fortier GD, Lekeux PM, Van Erck E. Laboratory findings in respiratory
417 fluids of the poorly-performing horse. Veterinary Journal. 2010;185:115-22.
418 [43] Koblinger K, Nicol J, McDonald K, Wasko A, Logie N, Weiss M, et al. Endoscopic
419 Assessment of Airway Inflammation in Horses. Journal of Veterinary Internal Medicine.
420 2011;25:1118-26.
421 [44] Karagianni AE, Kapetanovic R, Summers KM, McGorum BC, Hume DA, Pirie RS.
422 Comparative transcriptome analysis of equine alveolar macrophages. Equine Vet J. 2016.

ACCEPTED MANUSCRIPT

423 [45] Freeman TC, Goldovsky L, Brosch M, Van Dongen S, Maziere P, Grocock RJ, et al.
424 Construction, visualisation, and clustering of transcription networks from Microarray
425 expression data. Plos Computational Biology. 2007;3:2032-42.
426 [46] van Dongen S, Abreu-Goodger C. Using MCL to extract clusters from networks.
427 Methods in molecular biology (Clifton, NJ). 2012;804:281-95.
428 [47] Fairfax BP, Humburg P, Makino S, Naranbhai V, Wong D, Lau E, et al. Innate Immune
429 Activity Conditions the Effect of Regulatory Variants upon Monocyte Gene Expression.
430 Science. 2014;343.
431 [48] Sousa e Silva T, Longui CA, Rocha MN, Faria CDC, Melo MR, Faria TG, et al.
432 Prolonged Physical Training Decreases mRNA Levels of Glucocorticoid Receptor and
433 Inflammatory Genes. Hormone Research in Paediatrics. 2010;74:6-14.
434 [49] Moore BR, Krakowka S, McVey DS, Cummins JM, Robertson JT. Inflammatory
435 markers in bronchoalveolar lavage fluid of Standardbred racehorses with inflammatory
436 airway disease: Response to interferon-alpha. Equine Veterinary Journal. 1997;29:142-7.
437 [50] Deng JC, Zeng X, Newstead M, Moore TA, Tsai WC, Thannickal VJ, et al. STAT4 Is a
438 Critical Mediator of Early Innate Immune Responses against Pulmonary Klebsiella Infection.
439 The Journal of Immunology. 2004;173:4075-83.
440 [51] Hume DA, Freeman TC. Transcriptomic analysis of mononuclear phagocyte
441 differentiation and activation. Immunol Rev. 2014;262:74-84.
442 [52] Huston LJ, Bayly WM, D. LH, S. MN. Alveolar macrophage function in Thoroughbreds
443 after strenuous exercise. Equine Exercise Physiology 2. 1986;Eds: J.R. Gillepsie and N.E.
444 Robinson, ICEEP Publications, Davis, California:234-52.
445 [53] Adamson EJ, Slocombe RF. Flow cytometric studies of equine phagocytes following
446 strenuous exercise. Equine Veterinary Journal. 1995;27:37-42.
447 [54] Starkie R, Ostrowski SR, Jauffred S, Febbraio M, Pedersen BK. Exercise and IL-6
448 infusion inhibit endotoxin-induced TNF-alpha production in humans. Faseb Journal.
449 2003;17:884-6.
450 [55] Abbasi A, Hauth M, Walter M, Hudemann J, Wank V, Niess AM, et al. Exhaustive
451 exercise modifies different gene expression profiles and pathways in LPS-stimulated and un-
452 stimulated whole blood cultures. Brain Behav Immun. 2014;39:130-41.

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Figure Legends

Fig. 1: BALF differential cell count (%; mean +/- SEM) derived from six horses before (T0) and after (T1) entry into training.

Fig. 2: Supernatant TNFα concentration (pg/ml; mean +/- SEM) in LPS treated AMs harvested from six horses before (T0) and after (T1) entry into training.

Fig. 3: Principle component analysis. Principle component analysis of AMs (6 racehorses at T0 and T1). Yellow and pink ellipsoid-webs represent T0 and T1 AMs, respectively. Each individual represented by a different coloured sphere. Analysis performed by Partek Genomic Suite 6.6 software.

Fig. 4: Network graph analysis of response of equine AMs to training using Biolayout *Express*^{3D}. Graph (a): gene expression of AMs (6 racehorses at T0 and T1. Image (b): nodes involved in the clusters with an expression profile shown in (c) and (d). Graphs (c) and (d): pattern of gene clusters detected higher at T0 and T1, respectively (x-axis = horse ID at T0 and T1; y-axis = normalised expression level based on microarray intensity).

Fig. 5: Expression profiles of immune related genes downregulated at T0 and considered to have potential biological significance. X-axis shows each horse ID at T0 (blue) and T1 (red); y-axis shows the normalised relative intensity of gene expression. Dark and light green columns represent the mean (+SEM) normalised relative intensity of expression (n=6) at T0 and T1, respectively. P values provided where statistical differences (paired t-test) were detected between T0 and T1 (Mini Tab 16, Minitab UK). Statistical significance assumed at $p<0.05$.

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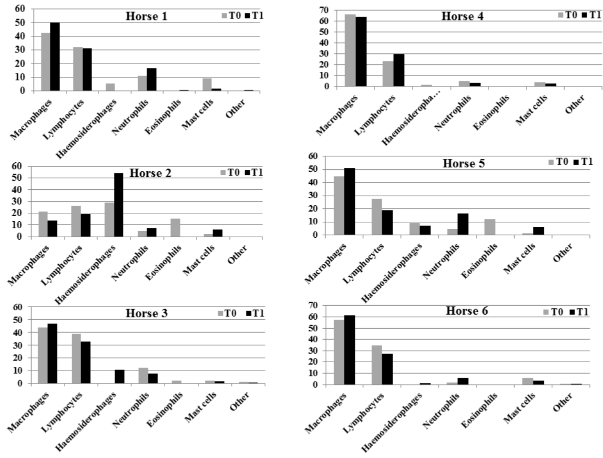

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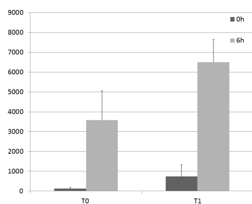

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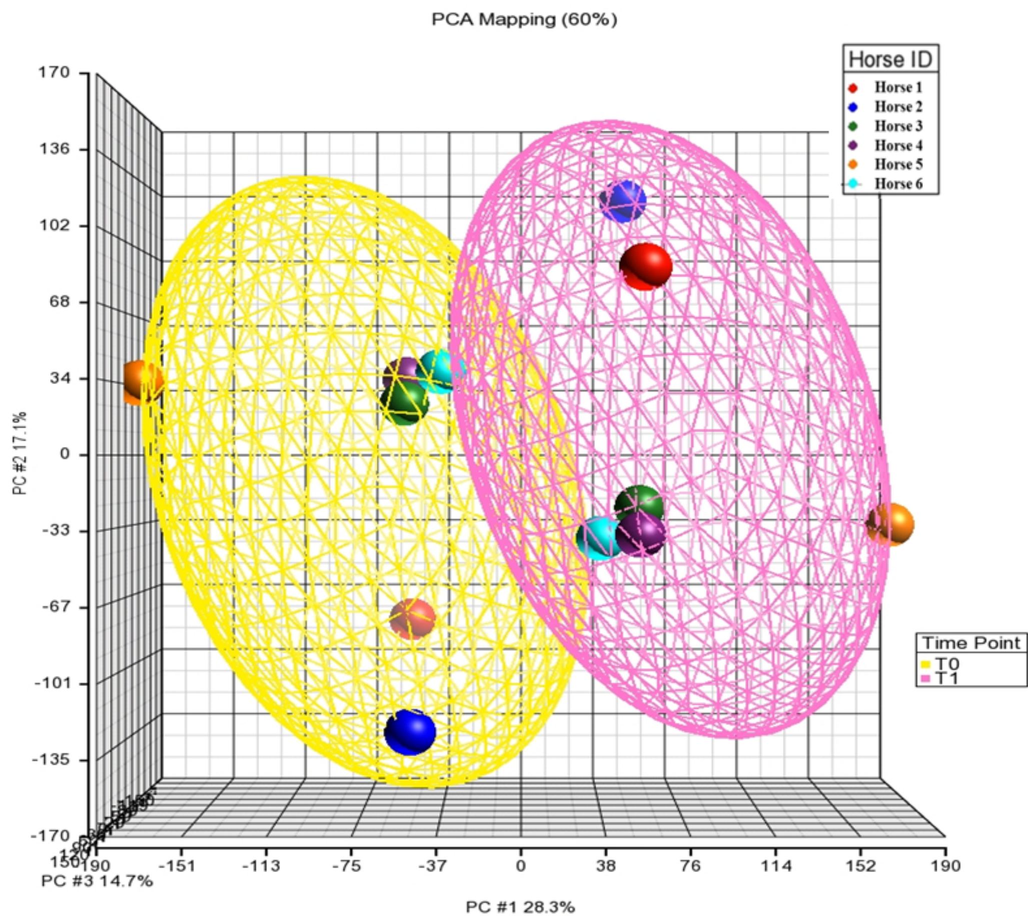

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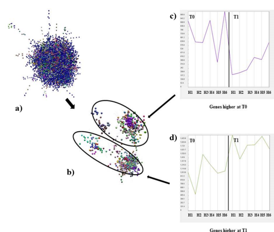

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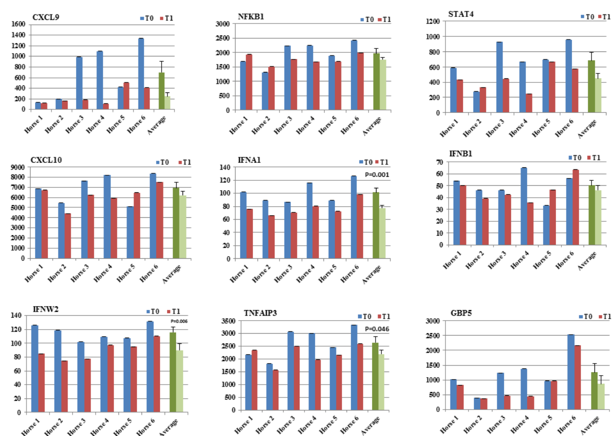

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Highlights

- Microarray analysis of alveolar macrophage gene expression of racehorses.
- Training resulted in a change in alveolar macrophage basal gene expression.
- This was potentially reflective of a degree of immunosuppression.
- Results complement previous findings derived from human and equine-based studies.

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