Impaired Cell Cycle Regulation in a Natural Equine Model of Asthma

Alicja Pacholewska1,2*, Vidhya Jagannathan2, Michaela Drögemüller2, Jolanta Klukowska-Rötzler1,2,3, Simone Lanz1, Eman Hamza4,5, Emmanouil T. Dermitzakis6,7, Eliane Marti4, Tosso Leeb2☯, Vincent Gerber1☯

1 Swiss Institute of Equine Medicine, Vetsuisse Faculty, University of Bern and Agroscope, Bern, Switzerland, 2 Institute of Genetics, Vetsuisse Faculty, University of Bern, Bern, Switzerland, 3 Division of Pediatric Hematology/Oncology, Department of Pediatrics, Bern University Hospital, Bern, Switzerland, 4 Clinical Immunology Group, Department of Clinical Research and Veterinary Public Health, University of Bern, Bern, Switzerland, 5 Department of Zoonoses, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt, 6 Department of Genetic Medicine and Development, University of Geneva Medical School, Geneva, Switzerland, 7 Institute of Genetics and Genomics in Geneva, Swiss Institute of Bioinformatics, Geneva, Switzerland

☯ These authors contributed equally to this work.
* alicja.pacholewska@vetsuisse.unibe.ch

Abstract

Recurrent airway obstruction (RAO) is a common and potentially debilitating lower airway disease in horses, which shares many similarities with human asthma. In susceptible horses RAO exacerbation is caused by environmental allergens and irritants present in hay dust. The objective of this study was the identification of genes and pathways involved in the pathology of RAO by global transcriptome analyses in stimulated peripheral blood mononuclear cells (PBMCs). We performed RNA-seq on PBMCs derived from 40 RAO affected and 45 control horses belonging to three cohorts of Warmblood horses: two half-sib families and one group of unrelated horses. PBMCs were stimulated with hay dust extract, lipopolysaccharides, a recombinant parasite antigen, or left unstimulated. The total dataset consisted of 561 individual samples. We detected significant differences in the expression profiles between RAO and control horses. Differential expression (DE) was most marked upon stimulation with hay dust extract. An important novel finding was a strong upregulation of CXCL13 together with many genes involved in cell cycle regulation in stimulated samples from RAO affected horses, in addition to changes in the expression of several HIF-1 transcription factor target genes. The RAO condition alters systemic changes observed as differential expression profiles of PBMCs. Those changes also depended on the cohort and stimulation of the samples and were dominated by genes involved in immune cell trafficking, development, and cell cycle regulation. Our findings indicate an important role of CXCL13, likely macrophage or Th17 derived, and the cell cycle regulator CDC20 in the immune response in RAO.
Introduction

According to the World Allergy Organization 300 million people suffer from asthma. Asthma is a public health problem worldwide and its prevalence is still increasing [1]. There are several types of asthma described, which vary regarding their pathogenesis, molecular mechanisms and clinical phenotype. Specifically, cytokine expression profiles and the types of T helper cells implicated in the immune response after allergenic stimulation show considerable variation, as reviewed in [2].

Asthma has an estimated heritability of more than 60% [3,4], but in accordance with the considerable immunological and phenotypical variation, it is genetically heterogenous [5] and more than 200 genes have been shown to be related to asthma [6]. Furthermore, there are also important environmental risk factors, including indoor and outdoor allergens, such as mites or pollens, and irritants like lipopolysaccharides (LPS).

Animal, predominantly murine, models are extensively studied to improve our understanding of the complex immunogenetic background of asthma [6]. However, murine models have important limitations, since mice have a short life span and do not spontaneously develop asthma, but must undergo sensitization protocols with allergen challenges [7]. Apart from humans, allergic asthmatic conditions occur naturally only in cats and horses [8]. The asthma-like disease in horses is called recurrent airway obstruction (RAO), which is a debilitating and incurable respiratory disease affecting stabled mature horses worldwide [9–11]. RAO-affected horses develop marked airway obstruction due to bronchospasm, inflammation, mucus accumulation and remodelling resulting in severe clinical signs such as coughing, respiratory distress and increased breathing effort during periods of exacerbation, which are triggered by hypersensitivity reactions to allergens and irritants mainly from hay dust [11–14]. Affected horses may be treated medically or by allergen avoidance and can live longer than 25 years. These characteristics make RAO a unique animal model for human asthma [11,15].

While the immunogenetic background of RAO is still not completely understood, several studies have shown that interactions of innate and adaptive immune responses play an important role [11]. Importantly, we have previously shown strong genetic effects. The prevalence of RAO is 3-5-fold increased among offspring from affected stallions compared to controls, and the heritability is high with a complex mode of inheritance involving several major genes suggesting genetic heterogeneity of RAO [14,16,17]. Moreover, we have demonstrated an association of RAO in horses with an augmented resistance against intestinal strongylids manifested by decreased shedding of parasite eggs [10,13,18]. These studies showed that RAO-affected horses had a significantly lower likelihood of egg shedding compared to unaffected horses. However, this association appears to depend on the genetic background with a marked relationship between RAO and strongylid egg shedding in one, but not another high-prevalence family.

Gene expression studies may potentially highlight genes that play a key role in the response to RAO-related antigens. Some studies showed contradictory results regarding the involvement of cytokines characteristic for Th1 or Th2 type immune response and it has been suggested that cytokine profiles reflecting both types of Th responses are observed at different time-points after antigen challenge [9]. In addition, an increased expression level of IL17, which is characteristic for Th17 cells, was shown to be associated with RAO [19]. However, only limited sets of specific candidate genes have been investigated in RAO so far [15,18,20–24].

In this study, we therefore chose a more comprehensive approach and compared global gene expression levels between RAO-affected and control horses. We hypothesized that antigen challenge in RAO-affected horses has not only local effects in the lungs but can also elicit systemic responses that would be reflected in the gene expression profiles of peripheral blood.
mononuclear cells (PBMCs). We were particularly interested in the differences in gene expression upon stimulation with hay dust extract (HDE) as the main causative environmental factor in RAO. HDE is a mixture of molecules (e.g. mold spores, inorganic dust, plant fragments) [25] and it contains LPS [26,27]. We also performed cell stimulations with LPS alone to be able to distinguish between LPS-mediated and allergen-specific effects of HDE. In addition, we analysed differences in the immune response with respect to strongylid parasite antigen (recombinant cyathostomin antigen, RCA) in RAO compared to non-affected horses. Finally, we investigated the influence of the host genetics on RAO by studying the expression profiles of three cohorts of horses with different genetic backgrounds.

Material and Methods

Ethics Statement

All animal experiments were performed according to the local regulations and with the consent of the horse owners. This study was approved by the Animal Experimentation Committee of the Canton of Bern, Switzerland (BE33/07, BE58/10 and BE10/13). The sample collection was previously described in detail in an earlier publication [18].

Horses and Phenotyping

A total of 85 animals were selected from three cohorts of Warmblood horses: two half-sibling families, consisting of the descendants of two RAO-affected sires (Family 1, Family 2) and one group of unrelated horses (Unrelated). These animals were already used in earlier studies [18,28]. Family 1 consisted of 9 RAO-affected (RAO) and 8 control (CTL) horses. Family 2 consisted of 8 RAO and 9 CTL, and Unrelated of 23 RAO and 28 CTL horses. Horses in Family 1 and Family 2 were paternal half-siblings with different mothers. Based on pedigree records comprising 7 ancestral generations, the horses in the families had inbreeding levels of less than 1%, calculated with MTDFREML [29]. The group of unrelated horses consisted of a subset of horses described in Kehrli et al [30]. The horses did not share parents and, as far as feasible based on the available pedigree information, were also unrelated at the grandparent level. The RAO phenotype (RAO/CTL) was assigned according to the Horse Owner Assessed Respiratory Signs Index (HOARSI), as described previously by Ramseyer et al. [31] and validated by Laumen et al. [32]: Control horses had HOARSI 1, defined by the absence of coughing or nasal discharge episodes; all RAO-affected horses had HOARSI 3 or 4, showing moderate to severe clinical signs of respiratory disease. Case definition for HOARSI 3 is abnormal breathing, regular or frequent coughing, or both. HOARSI 4 is defined by poor performance in addition to the same clinical signs as HOARSI 3. The intermediate milder phenotype of HOARSI 2 was not included in this study. The classification refers to the period when the horses were exhibiting their most severe clinical signs. At the time of the study, the RAO-affected horses were kept in a “low dust” environment and were in complete or partial remission showing either no or only mild clinical signs (mainly mildly increased breathing effort). All horses underwent a thorough clinical examination to exclude any systemic or localized infectious diseases. None of the horses had received any medication for treatment of RAO or any anthelmintic treatment within at least three months preceding the examination and blood collection to avoid any effects on the responses to stimulations.

Samples, Sequencing, and Data Preparation

The blood sample collection, isolation of peripheral blood mononuclear cells, and RNA purification were performed as described in Lanz et al. [18]. We used the RNA TruSeq Sample Preparation Kit v2 for library preparation (guide Part #15026495 Rev.D, Illumina) and sequenced the
libraries on the Illumina HiSeq2000 platform using 2 x 50 bp paired-end sequencing cycles. For this study we used RNA-seq data derived from PBMCs stimulated for 24 h with LPS (250 ng/ml), HDE (12, 9, or 6 μg/ml), RCA (4 or 1 μg/ml), or unstimulated (mock) as described in Pacholewska et al. [28]. Some of the cell stimulations or RNA extractions failed. Therefore, the RNA-seq data were generated from the 561 available RNA libraries out of the full set of 595 samples (94%). The exact number of biological replicates per group is shown in Table 1.

We prepared non-stranded libraries and collected 17 million read pairs per library on average. The reads mapped to the horse genome reference (EquCab2, [33]) with high efficiency (more than 90% mapped reads). All 561 binary alignment files (BAMs) were used previously for an assembly of the equine PBMC transcriptome [28] and are available from the European Nucleotide Archive database (http://www.ebi.ac.uk/ena/data/view/PRJEB7497). Reads uniquely mapped to annotated transcripts (Ensembl, release 72) were counted gene-wise with the HTSeq software [34]. More details on sequence quality control and mapping can be found in Pacholewska et al. [28].

### Data Quality Control

In order to look for an overall experiment effect or any batch effect on the clustering of the samples, we performed a principal component analysis. The raw counts were normalized using variance-stabilizing transformation included in the DESeq2 R package [35]. The 500 most variable genes were used for this analysis. The samples were then plotted across the first three principal components.

Based on multidimensional scaling (MDS) plots the decision of merging different concentrations of the same agent was made, as explained in the Results section (S1 Fig).

### DE Analysis

We used the R package edgeR for the analyses of DE, since this package offered the most comprehensive generalized linear model, which was essential for the analysis of our multifactorial experiment [36]. The R source codes are available in S1 File.

Genes with an expression level less than 1 cpm at least in the mean number of samples per group (8 for Family 1 and Family 2; 25 for Unrelated) were excluded from further analysis. Raw counts were then normalized with trimmed means of M values included in the edgeR package. The data were fitted using the generalized linear model (GLM), which allows for multiple comparisons using only one design model, and tested for DE.
Based on Fisher’s exact test we observed significant differences in the number of differentially expressed (DE) genes (false discovery rate threshold: FDR < 0.05) among the families and therefore cohort was taken as factor influencing the gene expression level in the experimental design (S2 Fig).

For the comparison of the RAO effect in the different horse cohorts upon each of the stimulations, we used a model with coefficients for every group studied. In total, we had 24 groups: 2 conditions (RAO, control) * 4 stimuli (mock, LPS, HDE, RCA) * 3 cohorts (Family 1, Family 2, Unrelated). The gene-wise negative binomial generalized linear model was used for fitting the data. The fitted data were then tested for differential expression between RAO and control horses using likelihood ratio tests and appropriate contrasts (S1 File). To determine genes differently regulated in RAO samples, relative to control samples, upon at least one of the stimulations in at least one of the cohorts we applied a statistical model with coefficients for RAO status, stimulation, and cohort with interactions (S1 File).

DE genes were then used for pathway analysis with the GeneGo software (MetaCore by Thomson Reuters) and GeneCodis (Gene Ontology (GO) Annotations Human (EBI), version 106) [37–40] using the orthologous human genes annotations. Human gene symbols were assigned to the Ensembl equine gene IDs using Ensembl Biomart (version 72) [41]. In GeneGo the probability for a random intersection of an input list with a set IDs in ontology groups is calculated as using the hypergeometric distribution p-value.

Results

Experimental Design & Data Collection

For a global transcriptome analysis we isolated PBMCs from RAO-affected (RAO) and control (CTL) horses (2 conditions) belonging to two different half-sib families or a cohort of unrelated horses (3 cohorts). PBMCs were stimulated with HDE, LPS, RCA or left unstimulated (4 treatments). In total, the material consisted of 561 RNA samples derived from 85 horses. More details on the sample numbers and design of the analysis are given in Table 1.

Data Quality Assessment

The MDS plot showed clear separation between the stimulation groups and concordance among group replicates. The analysis showed that RCA-stimulated samples were the least distant and the HDE-stimulated samples were the most distant samples from the unstimulated samples (Fig 1). Although the HDE-stimulated samples were less distant from the LPS-stimulated samples, they created a separate cluster, indicating that HDE-specific factors affect the gene expression profile of PBMCs.

The MDS analysis revealed that samples derived from the same horse, but stimulated with different concentrations of the same agent, tightly clustered together (S1 Fig). Therefore, different concentrations of the same agent were merged for the subsequent analysis and treated as a single stimulation. This resulted in more robust DE analysis with more samples used.

Of the 26,991 equine genes annotated in Ensembl, 13,265 had more than 1 cpm (read count per million reads) at least in the mean number of samples per group to be included in the DE analysis (S1 File).

Evaluation of Stimulation with LPS

LPS is a potent stimulator of innate immunity and has been studied in detail in several cells and immune related tissues [42–44]. As an additional quality control experiment we compared the expression profiles of LPS-stimulated and unstimulated PBMCs from control horses. The
identified 3,787 statistically significantly DE genes (FDR < 0.05) consisted of several cytokines in addition to previously identified LPS response genes like IL1B, IL6, IL8, IL15, IFNG and chemokines like CCL22, CXCL2, CXCL6 [42]. Also the DE genes consisted of LPS upregulated protein kinases like STAT3 and cell division proteins like CD80, CD86 as shown in Deifl et al. [45].

In addition, we subjected the DE genes with HGNC symbols to enrichment analysis using GenoGo Metacore. The most significantly enriched GeneGo pathway was the naive CD4+ T cell differentiation (FDR = 6.92e-6), which includes the highly upregulated IL6 (log 2 fold change = 2.90). The expression of IL6 is known to be induced by LPS through the nuclear factor κB (NF-κB) transcription factor [46]. GenoGo provides a list of GO processes significant for a given pathway map. Nine out of the ten most significant pathways enriched for DE genes showed significance for the GO process cellular response to LPS (GO:0071222). These results were in agreement with our expectations and confirmed successful stimulation of cells with LPS.

![Figure 1. Quality control of the RNA-seq data. The figure shows a principal component analysis of the individual samples plotted across the three most variable components. The circles on the MDS plot represent the individual samples and are colored according to the 4 different stimulations: no stimulating factor (mock), lipopolysaccharides (LPS), recombinant cyathostomin antigen (RCA, both concentrations), and hay dust extract (HDE, all three concentrations).](image-url)
RAO-status Effect

After all quality control experiments had yielded satisfactory results, we started to address the differences between RAO-affected and control horses. Pairwise comparisons revealed the greatest difference in the gene expression in RAO samples upon stimulation with HDE. Stimulation with LPS revealed a similar number of RAO-regulated DE genes as in the unstimulated cells (Fig 2A). In Family 2 the number of DE genes was smaller than in the other cohorts (Fig 2B–2E). The whole list of DE genes and the detailed results of the gene enrichment analysis are given in S1, S2 and S3 Tables.

The GeneGo tool “compare experiments workflow” intersects DE gene lists in terms of their mapping to gene ontologies and pathway maps. Hence this tool can be used to study differential pathway enrichment in different DE gene lists. This tool subclassifies the overlapping gene lists as “common” set for all genes found in each of the lists, “similar” set comprising all partial intersections and a “unique” set for every list.

Ontology enrichment and canonical pathway analysis for common genes suggested several functions and pathways involved mainly in cell cycle regulation and immune response (S3 Table). Interesting to note in pathway analysis for LPS/HDE/RCA stimulation was “role of anaphase-promoting complex (APC) in cell cycle regulation” (Fig 3).

While the pairwise comparisons revealed almost 3,900 DE genes between RAO and control samples upon at least one of the stimulations and cohorts (Fig 2, S1 Table), the overall RAO effect regulated 3,508 genes (S2 Table). This overall RAO-effect represents differences in gene expression in RAO compared to control samples, regardless of stimulation or cohort. The GeneGo map folder consists of pathways grouped into regulatory, metabolic, disease, toxicity and drug action sections. Enrichment analysis with map folders with differentially expressed genes regulated by overall RAO effect showed the ‘asthma’ folder as the top statistically significant folder with the regulation of epithelial-to-mesenchymal transition and extracellular matrix remodelling as top enriched pathways.

Additional factors altering RAO-regulated genes

The gene regulation by RAO depended on the stimulation agent and/or cohort that further modified the gene ‘response’ with respect to the RAO baseline effect, i.e. in unstimulated cells in the Unrelated group of horses (Table 2).

The RAO regulated genes comprised of genes involved in e.g. immune response, cell differentiation, and response to hypoxia as shown in Fig 4.

Discussion

In this study, we investigated the influence of different antigenic and irritant stimuli on the gene expression profiles of PBMCs from genetically distinct groups of horses affected by RAO, an asthma-like condition. In contrast to the rodent models typically used in asthma research, equine RAO is a wide-spread naturally occurring disorder. The results of our study show that stimulation with HDE strongly affects the gene expression profile of equine PBMCs. The observed RAO-dependent effects of allergenic and irritant stimuli on PBMCs support the hypothesis that this asthma-like disease causes a systemic immune response. HDE, which is believed to be the major trigger of RAO, induced differential gene expression in a much broader set of genes than stimulation with LPS, which is one of the components of hay dust and is also known to provoke a strong immune response. Genes that are affected by RAO status (regardless of the specific stimulation) may be of importance for our understanding of the pathomechanisms involved in RAO.
Fig 2. Venn diagrams illustrating the number of DE genes between RAO and control horses. Panel A illustrates the number of DE genes upon each stimulation across all horses. The panels B-E further differentiate this dependent on the three different cohorts. (B) Unstimulated cells (mock), followed by cells stimulated with (C) recombinant cyathostomin antigen (RCA), (D) lipopolysaccharides (LPS), and (E) hay dust extract (HDE). The numbers represent the number of significantly up (\(^{\uparrow}\)) and down (\(^{\downarrow}\)) regulated genes in RAO samples compared to control samples.

doi:10.1371/journal.pone.0136103.g002
We also observed the impact of the RAO condition in response to parasite antigen with almost 300 DE genes altered by both HDE and RCA, providing further evidence that RAO and other equine hypersensitivity disorders like urticarial reactions and multiple hypersensitivities [10,13,30] are associated with altered defence against parasites.

In addition, we also observed differences in the number of DE genes between RAO and CTL samples between the cohorts, which support our hypothesis that differences in the genetic role of anaphase-promoting complex (APC) in cell cycle regulation. The thermometers indicate the object regulating genes were up- (red) or downregulated (blue) by the RAO condition upon stimulation with 1) lipopolysaccharides (LPS), 2) recombinant cyathostomin antigen (RCA), and 3) hay dust extract (HDE). Lines indicate activation (green), inhibition (red) or unspecified (grey) interactions between the molecules. The object shapes correspond to molecule type and are described in S3 Fig and at https://portal.genego.com/legends/MetaCoreQuickReferenceGuide.pdf.

doi:10.1371/journal.pone.0136103.g003

We also observed the impact of the RAO condition in response to parasite antigen with almost 300 DE genes altered by both HDE and RCA, providing further evidence that RAO and other equine hypersensitivity disorders like urticarial reactions and multiple hypersensitivities [10,13,30] are associated with altered defence against parasites.

In addition, we also observed differences in the number of DE genes between RAO and CTL samples between the cohorts, which support our hypothesis that differences in the genetic role of anaphase-promoting complex (APC) in cell cycle regulation. The thermometers indicate the object regulating genes were up- (red) or downregulated (blue) by the RAO condition upon stimulation with 1) lipopolysaccharides (LPS), 2) recombinant cyathostomin antigen (RCA), and 3) hay dust extract (HDE). Lines indicate activation (green), inhibition (red) or unspecified (grey) interactions between the molecules. The object shapes correspond to molecule type and are described in S3 Fig and at https://portal.genego.com/legends/MetaCoreQuickReferenceGuide.pdf.

doi:10.1371/journal.pone.0136103.g003
Table 2. Genes regulated by RAO-status with further dependencies on stimulating agent and/or cohort. For every effect the number of DE genes and the top ten DE genes are listed. The overall “conditionRAO” effect includes significant genes due to the RAO effect in at least one cohort and stimulation. Mock-stimulated samples from control horses of the Unrelated unstimulated group were used as a reference group. Baseline “conditionRAO” effect represents differences between RAO and control samples from the Unrelated unstimulated group. Colons “:” indicate an interaction effect, e.g. “conditionRAO:Family1” effect represents differential changes in the RAO effect in Family 1 in mock compared to RAO effect in Unrelated group in mock.

| Effect | # DE genes | Top ten genes |
|--------|------------|---------------|
| conditionRAO—overall | 3,508 | GNPNMB, MMP3, ENSECAG00000003774, SERPINH1, CXCL13, CD163L1, IFNG, APOBEC3Z1B, NDUF4A4L2, MMP-1 |
| conditionRAO—baseline (Unrelated, mock) | 382 | VNN1, CCL24, GPT2, MMP1, FER1L6, SLIT3, APOBEC3Z1B, CCL26, COL4A1, SLC22A3, UHRF1 |
| conditionRAO:cohortFamily1 | 357 | MMP3, CXCL17, ENSECAG00000001949, ENSECAG00000003099, GPT2, MARCO, PPP1R36, TG, CCL24, APOBEC3Z1B |
| conditionRAO:cohortFamily2 | 175 | MMP3, SELP, TGFB3, FBPI, VNN1, CCL24, APOBEC3Z1B, CCL26, ENSECAG00000023871, ENSECAG00000024588 |
| conditionRAO:stimulusHDE | 428 | MMP3, EREG, CXCL13, MARCO, CXCL6, CD163L1, IFNG, NDUFA4L2, MMP-1, CCL13 |
| conditionRAO:stimulusLPS: | 140 | GPR1, EREG, STAB1, MRC1, MMP19, CXCL13, MARCO, TFPI2, CCL24, CCL13 |
| conditionRAO:stimulusRCA: | 97 | MMP3, ENSECAG00000001910, EREG, NTRK1, IFNG, ENTPD3, FBPI, CCL26, ENSECAG00000023871, CCL13 |
| conditionRAO:stimulusRCA: | 68 | MMP3, PTX3, SERPINH1, CXCL6, ALDH1A2, FBPI, CCL24, MMP-1, RNase_MRP, 5_8S_rRNA |
| conditionRAO:stimulusRCA: | 20 | G-CSF, MMP19, MARCO, TREM2, IL8, ENSECAG00000015885, FBPI, CCL24, F3, CLEC4F |
| conditionRAO:stimulusRCA: | 21 | MMP3, PTX3, SERPINH1, NTRK1, ALDH1A2, TGFB3, ENTPD3, FBPI, CCL13, ENSECAG00000024588 |
| conditionRAO:stimulusRCA: | 15 | MMP3, MARCO, SLC47A1, CXCL6, ENSECAG0000013872, ENSECAG0000015885, SPP1, MMP12, FABP4, ENSECAG00000023871 |
| conditionRAO:stimulusRCA: | 14 | MMP3, ENSECAG00000003801, SERPINH1, SLC27A6, CD5L, AMPP, SEPP1, RN18S, RN18S, ENSECAG00000027634 |

doi:10.1371/journal.pone.0136103.t002

background of RAO affect the immunological response to specific stimuli (Fig 2B–2F). Family 1 and the Unrelated horses showed more genes affected by RCA compared to Family 2. Interestingly, this is in accordance with earlier results demonstrating an association of RAO with
increased parasite resistance in Family 1 and unrelated RAO-affected horses, but not in Family 2 based on strongylid egg counts in faecal samples [10,13].

One of the top genes altered by the condition RAO:cohortFamily1 effect was CCL24 that is located in a genomic region previously identified to be associated with RAO in this family [14]. This gene was also differentially expressed in Family 2, however, the effect was smaller than in Family 1. We did not observe significant changes in the expression of the SOCS5 gene that has been associated with RAO condition in Family 2.

These results further support the hypothesis of genetic heterogeneity in RAO, which was previously postulated based on genetic and gene expression data [14,18,20]. The effect of the genetic background on RAO needs further investigation, preferably, including genomic data.

A previous expression study showed an increased expression of IL4, IL4R, and IL10 upon stimulation with HDE in RAO compared to non-affected horses [18]. We could confirm the upregulation of IL10 in our data. IL4R was also upregulated in RAO samples, with stronger upregulation in Family 1 compared to Family 2, albeit below the significance threshold (Fig 5).

In contrast, the RAO-regulation of IL4 in contrast to the previous study showed a differential expression in the opposite direction. The IL4 gene had overall low expression levels across the samples and DE analysis of RNA-seq data has been shown to present problems with lowly expressed genes [47–49]. Moreover, the previously published qRT-PCR expression study measured IL4 gene expression in comparison to 18S rRNA as a standard. In the present data rRNA was removed during poly(A)-selection in our library preparation and hence we could not investigate this aspect in detail.

The overall RAO effect showed 3,508 genes to be up- or downregulated regardless of stimulation or cohort. These genes were mostly enriched for pathways related to human asthma supporting the idea of RAO in horses being a good natural model for studies on asthma.

Interestingly, the pathway “role of APC in cell cycle regulation” has not been listed as significant asthma-related pathway in the GeneGo collection yet, as only one object of this pathway (RASSF1) has been described to be associated with asthma [50]. Our data, however, showed that this pathway is significantly related to RAO-response with many objects upregulated,
especially upon stimulation with the RAO-related antigen HDE (Fig 3). APC can be activated by one of two activators: cell division cycle 20 (CDC20) or its homolog, cadherin 1 type 1 (CDH1). These two factors act at different stages of the cell cycle to regulate distinct functions. CDC20 is active from metaphase through anaphase and promotes separation of sister chromatids, whereas CDH1 acts from the end of mitosis and prevents premature entry into S phase, as reviewed in [51–53]. The upregulated CDC20 in RAO samples may thus suggest increased cell proliferation in RAO-samples (Fig 6).

The most RAO-downregulated gene upon stimulation with HDE was the interferon gamma gene (IFNG). Interferon gamma is crucial for the function of both innate and adaptive immune responses e.g. due to its stimulatory function on macrophages [54–56]. Indeed, we observed

---

**Fig 6. Expression of RAO-relevant genes.** Expression levels of IFNG, CXCL13, CDC20, and NDUFA4L2 are shown. The x-axes represent the stimulations: no stimulating factor (mock), lipopolysaccharides (LPS), recombinant cyathostomin antigen (RCA), and hay dust extract (HDE). On the logarithmic scaled (log 2) y-axes mean expression values are given in normalized counts per million. Error bars represent 95% confidence intervals. The stars (*) represent significant changes between expression levels of RAO (red dots and lines) and control (green dots and lines) samples at false discovery rate < 0.05.

doi:10.1371/journal.pone.0136103.g006
both types of immune response to be affected by the RAO condition, and therefore the decrease in IFNG in RAO horses could be involved in the RAO pathomechanism (Fig 6).

Innate Response

The HDE stimulation resulted in a significant upregulation of genes involved in the innate immune response, e.g. complement system proteins (C1S, C1R, C1QC, C2) and complement component receptors (C3AR1, C5AR2) in the samples of RAO-affected compared to control horses. The role of the complement system in asthma has previously been described with the main focus on C3 and C5 complexes [57–61].

We also observed increased levels of IL34 mRNA in RAO samples treated with LPS or HDE. This cytokine supports the viability of monocytes and promotes the formation of macrophages [62]. It is crucial for the differentiation and maintenance of Langerhans cells and tissue resident macrophages and with its receptor, colony-stimulating factor-1 receptor, represents a novel target for the treatment of several chronic inflammatory conditions [63,64].

In addition, the upregulated chemokines (CXCL12, CXCL14, CCL14) also attract and/or activate monocytes [65–68]. CXCL13 is known as a B-cell attractant in germinal centres [69,70], but monocytes and macrophages are the major source of CXCL13 [70]. This chemokine was the most significantly upregulated DE gene when RAO samples stimulated with HDE were compared to controls stimulated with HDE (Fig 6, S1 Table). Moreover, it was upregulated following stimulation with LPS, downregulated upon RCA stimulation, and no significant changes were observed in unstimulated cells (Fig 6, S1 Table). Secretion of CXCL13 protein seems to occur only after cell activation, and circulating monocytes have been shown to express CXCL13 when stimulated by LPS [70].

Th1/Th2 Types of Adaptive Response

Our data did not indicate any bias towards either a Th1 or a Th2 type response. Previous studies investigating the type of response in RAO have found conflicting results. The time point of sampling and the duration of stimulation, respectively, may influence results [9]. In the present study, we observed downregulation of both Th1 type (IFNG) and Th2-type cytokines (IL4, IL13) in RAO cells stimulated with HDE. This may be explained by increased levels of the interleukin IL10 in the HDE-stimulated RAO samples, which is known to inhibit the expression of Th1- and Th2-type cytokines [71] and has previously been reported to be upregulated in RAO [18,72]. Interestingly, increased IL10 is associated with decreased IL4 expression in parasitized horses [73].

Although it was shown that CXCL13 is not produced by either Th1 nor Th2 cells, it is produced by human Th17 cells [74]. The Th17 cell subset is known for its role in autoimmune and inflammatory diseases [58,75]. In asthmatic patients the number of Th17 cells in blood and BALF is increased, positively correlating with asthma severity and airway remodelling [76,77]. Since our study was performed on cell mixtures, we cannot rule out the possibility that the increase in CXCL13 might be due to an expansion of Th17 cells within the PBMCs rather than the above mentioned monocyte activation.

Response to Hypoxia

The overall RAO effect revealed a significant role of hypoxia inducible genes with enrichment of 55 from 175 genes annotated in GO:0001666. We found a mitochondrial NADH dehydrogenase 1 alpha subcomplex 4-like 2 gene (NDUFA4L2) downregulated in unstimulated RAO PBMCs, but upregulated upon stimulation with HDE (Fig 6). NDUFA4L2 is a target gene for hypoxia-inducible factor 1 (HIF-1) [78]. Little is known about the physiological function of the
NDUFA4L2 protein, but it limits reactive oxygen species production in cells under hypoxic conditions [78]. The expression of the HIF-1 alpha subunit gene (HIF1A) was shown to be upregulated in lung cells of horses with RAO. The HIF1A expression level was correlated with the severity of the disease [79] in agreement with the higher expression of HIF-1 in asthmatic bronchial biopsies after antigen challenge [80].

From 392 human HIF-1 target genes identified by [81–83] and obtained via the PAZAR database [84] we identified a substantial proportion (n = 53; 14%) with significantly different expression levels between RAO and CTL in our data. Most of these DE genes (n = 32; 60%) showed an RAO-dependent expression difference only upon stimulation with HDE (S1 Table).

Hypoxia occurs during bronchial asthma attacks [85–87], but a hypoxic microenvironment is characteristic for inflamed tissues in general [88,89]. Furthermore, it has been shown that HIF-1 potentiates allergic inflammation in airways even without true hypoxia [90–92]. During a hypoxic response, the HIF-1α molecule is stabilized and assembles with HIF-1β to form the HIF-1 complex. The activated HIF-1 acts on the hypoxia responsible elements (HRE) in the promoters of target genes and regulates their expression [93]. The HIF-1α subunit is also stabilized in a hypoxia-independent manner that involves proteins of the NF-κB, TNF-α, or TGFβ1 signalling cascades [94–96]. Moreover, HIF-1α expression was also shown to be increased in a time- and dose-dependent manner by LPS in a macrophage-derived cell line [97]. In our study, each stimulating agent investigated (LPS, RCA and HDE) significantly increased (in comparison to unstimulated cells) the expression level of the HIF1A gene and NFKB2 encoding a subunit of the NF-κB transcription factor, which activates cellular signalling pathways via dynamic modulation of cytokines, chemokines and other signalling molecules, and is an important signalling element of the immune response in asthma [98]. However, the RAO condition did not show any significant impact on the HIF1A or NFKB2 expression.

The importance of the HIF-1 signalling pathway in the asthmatic airways lies partially in the fact that it activates many genes implicated in tissue remodelling. Airway remodelling is a characteristic feature of asthma and it involves increased mucus production, fibrosis, thickening of epithelial and smooth muscle layers, and angiogenesis [99]. Many of these processes are also observed in airway remodelling of equine RAO [11]. The resulting obstructed and hyper-responsive airways disrupt the proper functioning of the lungs [99].

**Anti-apoptosis in RAO**

We speculate that the differential CXCL13 expression is unlikely to represent a primary dysregulation of gene expression in RAO, but rather occurs as a secondary effect following an abnormal response to allergens, e.g. monocyte/macrophage activation possibly driven by anti-apoptotic properties of IER3 or CDC20. Activated macrophages can be killed by the Vγ1 subset of γδ T cells in order to maintain homeostasis after infection. This process is mediated by a Fas-FasL interaction [100,101]. Delay in the apoptosis of neutrophils derived from bronchoalveolar lavage fluid (BALF) of the RAO horses has already been described [102]. Moreover, a significant decrease in the ratio of early apoptotic cells in the neutrophil population in the BALF of RAO horses has been recently shown [103]. The authors of these studies suggested a role for the granulocyte/macrophage colony stimulating factor (GM-CSF) in the delayed apoptosis. Our data possibly indicate that this decrease in the apoptosis rate might be additionally mediated by the higher expression of the anti-apoptotic IER3 gene in RAO horses. The IER3 gene is a known inhibitor of apoptosis induced by FAS or tumour necrosis factor (TNF)-α, and is regulated by NF-κB [104]. Schott et al. showed that IER3 protects murine macrophages from LPS-induced cell death [105]. However, IER3 was upregulated in unstimulated RAO cells, whereas CDC20 was significantly upregulated and involved in the most significant pathway
upon all stimulations (Figs 3 and 5, S2 Table). CDC20 is highly expressed in various types of human tumours [106,107] and studies have shown depleting endogenous CDC20 leads to induction of apoptosis [108,109]. The CDC20 binds to APC and promotes degradation of a proapoptotic protein, Bim [110].

The imbalance in macrophage homeostasis may trigger abnormal, allergic, immune responses. However, the role of CDC20 in regards to macrophage homeostasis and RAO/asthma needs further investigation. Furthermore, clarifying the role of monocyte/macrophage activation in RAO may require investigations of these DE genes in the lung compartment of affected horses, since PBMC stimulation likely has limitations in this regard. In a murine model of asthma for instance, resident alveolar macrophages were shown to suppress inflammatory cytokine levels and eosinophil numbers. In contrast, recruited monocytes (which may correspond to the PBMC population) promoted allergic lung inflammation [111]. This suggests that future studies should investigate both the systemic (e.g. in PBMCs) and local (e.g. in resident macrophages and dendritic cells) responses in the RAO model of asthma.

Future Perspectives

More than 15% of the DE genes identified in this study did not have an associated HGNC symbol, many horse genes have not been characterized yet, and most gene ontologies come from human, mouse or rat data. Thus, the pathway analysis of the equine data is incomplete and must be interpreted with caution.

However, the DE genes identified can serve as a valuable source of potential therapeutic targets in the treatment of equine RAO and human asthma. The HIF-1α subunit has been already proposed as a therapeutic target in asthma [112] and effectiveness of HIF-1 inhibitors in reducing the symptoms of allergic rhinitis has been reported in mouse models [113]. Also, CXCL13, which was significantly upregulated in RAO samples upon stimulation with LPS/HDE, has been recently proposed as a target gene in asthma treatment based on experiments performed in a mouse asthma model [114]. It also seems to play a key role in other immunologic diseases, e.g. autoimmune myasthenia gravis and multiple sclerosis [115,116]. Up to date, the role of CXCL13 has not been investigated in well phenotyped asthmatic patients, and our study provides the first information on CXCL13 expression differences in a natural model of asthma.

Interestingly, CDC20 has not yet been considered as a putative therapeutic target in asthma. Studies with a CDC20 inhibitor may reveal more information on its role in RAO/asthma. CDC20 may also be considered as a putative biomarker for RAO, since it was upregulated in RAO-samples upon each of the stimulations. In addition, studies on the function of NDUFA4L gene and its role in asthma could also reveal new insights in our understanding of alterations in the immune response or the tissue remodelling.

To further investigate the pathogenesis and mechanism of the immune response in RAO-affected horses and address the potential roles of the genes identified in the present study, global gene expression should be investigated in lung tissues of affected horses and controls, where the main immune response is taking place, at least for some of the DE genes identified in this study, such as the HIF-1 and NF-κB target genes.

However, the results for the stimulated PBMCs described here suggest that in addition to the local inflammation in the lung tissue, a systemic response (or at least a response from cells, which may have come from the lung, but are circulating in peripheral blood) plays a role [23,117–120]. This aspect should be further explored in PBMCs from RAO-affected horses during a natural allergen challenge provoking clinical exacerbation.

The results presented in this study also provide valuable information for future genetic studies aimed at finding expression quantitative trait loci (eQTLs) associated with the differentially
expressed genes. These putative eQTLs could possibly contain causative variants influencing the host susceptibility to the disease and are potential genetic markers that might help breeders to reduce the occurrence of RAO.

Conclusions

Our study revealed that a natural model of asthma, equine RAO, leads to differences in PBMC gene expression. Many of the DE genes are involved in cell cycle regulation, e.g. CDC20. CXCL13 showed the strongest difference between RAO and control horses and might play an important role in RAO. This gene might be upregulated in monocytes/macrophages and/or the Th17 lymphocyte subset. Possible sources of this upregulation are: 1) increased ratio of activated to non-activated immune cells due to an increased expression level of apoptosis suppressor gene, CDC20; and/or 2) the hypoxic environment and upregulation of HIF1A in the lung.

Supporting Information

S1 Fig. Multidimensional scaling of the samples. All samples were plotted across three first principal components and coloured according to (A) stimulating factor, (B) sequencing flow cell, (C) horse ID number. Panels (D-J) show subsets of samples plotted across first two principal components: (D) samples stimulated with hay dust extract labelled by horse ID number; (E) unstimulated samples and samples stimulated with RCA_1; (F) unstimulated samples and samples stimulated with RCA_4; (G) samples stimulated with RCA_1 and RCA_4; (H) unstimulated samples and samples stimulated with RCA_1 or RCA_4; (I) unstimulated samples and samples stimulated with HDE_6, HDE_9, or HDE_12.

S2 Fig. Family dependence of differentially expressed genes between RAO and control samples. For each of the two horse families and upon each stimulation (no stimulating factor (mock), lipopolysaccharides (LPS), recombinant cyathostomin antigen (RCA), and hay dust extract (HDE)) the tests for differential expression (DE) were performed. The bars represent the number of DE genes in each of the families that were significantly different between families indicating an influence of the genetic background of the horses (**Fisher’s exact test; p-value ≤ 0.001). Common DE genes identified in both families were coloured in yellow.

S3 Fig. Legend for GeneGo pathway map. The legend explains all pathway objects and interaction used in the map in Fig 3.

S1 File. R code used for the differential expression analysis.

S1 Table. Results of differential expression analysis – pairwise comparisons. Significantly (with false discovery rate FDR ≤ 0.05) differentially expressed genes between RAO and control horses for each stimulating factor and cohort are listed with log2 fold changes and FDRs.

S2 Table. Results of differential expression analysis – factorial effects. Genes significantly (false discovery rate FDR ≤ 0.05) regulated by overall RAO-effect with log2 fold changes for baseline RAO and every RAO-interaction effect studied.
S3 Table. Gene enrichment analysis with GeneGo and GeneCodis. For each stimulating factor a separate analysis was performed using the list of significant DE genes between RAO and control horses (FDR < 0.05). The file includes 5 sheets representing results for: 1) pathway maps; 2) GO processes; 3) process networks; 4) diseases (by biomarkers); and 5) KEGG pathways. (XLSX)

Acknowledgments

The authors would like to thank all participating horse owners and veterinarians for their support of this study. We thank Muriel Fragnière, Ismaël Padioleau, the Genomics Platform at the University of Geneva Medical Center, and the Next Generation Sequencing Platform of the University of Bern for performing sequencing experiments and the Vital-IT high-performance computing center of the Swiss Institute of Bioinformatics for performing computationally intensive tasks (http://www.vital-it.ch/).

Author Contributions

Conceived and designed the experiments: VG TL ETD EM. Performed the experiments: SL EH JK-R MD AP. Analyzed the data: AP VJ. Wrote the paper: AP VJ TL VG EM.

References

1. Ansotegui I, Arruda LKP, Badellino HA, Baena-Cagnani CE, Bahna SL, Baldacci S, et al. White Book on Allergy: Update 2013. Pawankar R, Holgate ST, Canonica GW, Lockey RF, Blaiss MS, editors. Milwaukee: World Allergy Organization (WAO); 2013.

2. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. Nat Med. 2012; 18: 716–725. doi: 10.1038/nm.2678 PMID: 22561835

3. Duffy DL, Martin NG, Battistutta D, Hopper JL, Mathews JD. Genetics of asthma and hay fever in Australian twins. Am Rev Respir Dis. 1990; 142: 1351–1358. doi: 10.1164/ajrccm/142.6_Pt_1.1351 PMID: 2252253

4. Edfors-Lubs M-L. Allergy in 7000 twin pairs. Allergy. 1971; 26: 249–285. doi: 10.1111/j.1398-9995.1971.tb01300.x

5. Strina A, Barreto M, Cooper P, Rodrigues L. Risk factors for non-atopic asthma/wheeze in children and adolescents: a systematic review. Emerg Themes Epidemiol. 2014; 11: 5. doi: 10.1186/1742-7622-11-5 PMID: 2496333

6. Temesi G, Virág V, Hadadi É, Ungvári I, Fodor LE, Bikov A, et al. Novel genes in Human Asthma Based on a Mouse Model of Allergic Airway Inflammation and Human Investigations. Allergy Asthma Immunol Res. 2014; 6: 496–503. doi: 10.1168/ajrccm/142.6_Pt_1.1351 PMID: 25374748

7. Nials AT, Uddin S. Mouse models of allergic asthma: acute and chronic allergen challenge. Dis Model Mech. 2008; 1: 213–220. doi: 10.1242/dmm.000323 PMID: 19093027

8. Mullane K, Williams M. Animal models of asthma: reprise or reboot? Biochem Pharmacol. 2014; 87: 131–9. doi: 10.1016/j.bcp.2013.06.026 PMID: 23831953

9. Moran G, Folch H. Recurrent airway obstruction in horses—An allergic inflammation: A review. Vet Med (Praha). 2011; 56: 1–13.

10. Bründler P, Frey CF, Gottstein B, Nussbaumer P, Neuhaus S, Gerber V. Lower shedding of strongylid eggs by Warmblood horses with recurrent airway obstruction compared to unrelated healthy horses. Vet J. 2011; 190: e12–5. doi: 10.1016/j.tvjl.2010.12.029 PMID: 21315626

11. Leclere M, Lavoie-Lamoureux A, Lavoie J-P. Heaves, an asthma-like disease of horses. Respirology. 2011; 16: 1027–46. doi: 10.1111/j.1440-1843.2011.02033.x PMID: 21824219

12. Obel NJ, Schmitterlöw CG. The Action of Histamine and other Drugs on the Bronchial Tone in Horses suffering from Alveolar Emphysema (Heaves). Acta Pharmacol Toxicol (Copenh). 1948; 4: 71–80. doi: 10.1111/j.1600-0773.1948.tb03489.x

13. Neuhaus S, Bruender P, Frey CF, Gottstein B, Doerr MG, Gerber V. Increased parasite resistance and recurrent airway obstruction in horses of a high-prevalence family. J Vet Intern Med. 2010; 24: 407–13. doi: 10.1111/j.1939-1676.2009.0465.x PMID: 20102498
14. Swinburne JE, Bogle H, Klukowska-Rötzler J, Drögemüller M, Leeb T, Temperton E, et al. A whole-genome scan for recurrent airway obstruction in Warmblood sport horses indicates two positional candidate regions. Mamm Genome. 2009; 20: 504–15. doi: 10.1007/s00335-009-9214-5 PMID: 19760324

15. Lavoie J-P, Maghni K, Desnoyers M, Taha R, Martin JG. Neutrophilic Airway Inflammation in Horses with Aspergillus fumigatus extract and cyathostomin antigen stimulation on cytokine expression by PBMC in horses with recurrent airway obstruction. Vet Immunol Immunopathol. 2005; 104: 91–101. doi: 10.1016/j.vetimm.2005.07.013 PMID: 15797472

16. Klukowska-Rötzler J, Swinburne JE, Drögemüller M, Leeb T, Temperton E, et al. The interleukin 4 receptor gene and its role in recurrent airway obstruction in Swiss Warmblood horses. Anim Genet. 2012; 43: 450–3. doi: 10.1111/j.1365-2052.2011.02277.x PMID: 22497430

17. Lavoie J-P, Maghni K, Taha R, Martin JG. Neutrophilic Airway Inflammation in Horses with Heaves Is Characterized by a Th2-type Cytokine Profile. Am J Respir Crit Care Med. 2001; 164: 1410–1413. doi: 10.1164/rccm2012091 PMID: 11704587

18. Ramseyer A, Gaillard C, Burger D, Straub R, Jost U, Boog C, et al. Effects of genetic and environmental factors on chronic lower airway disease in horses. J Vet Intern Med. 2007; 21: 149–56. PMID: 17338163

19. Lavoie J-P, Maghni K, Desnoyers M, Taha R, Martin JG. Neutrophilic Airway Inflammation in Horses with Aspergillus fumigatus extract and cyathostomin antigen stimulation on cytokine expression by PBMC in horses with recurrent airway obstruction. Vet Immunol Immunopathol. 2005; 104: 91–101. doi: 10.1016/j.vetimm.2005.07.013 PMID: 15797472

20. Klukowska-Rötzler J, Swinburne JE, Drögemüller M, Leeb T, Janda J, Leeb T, et al. The interleukin 4 receptor gene and its role in recurrent airway obstruction in Swiss Warmblood horses. Anim Genet. 2012; 43: 450–3. doi: 10.1111/j.1365-2052.2011.02277.x PMID: 22497430

21. Horohov DW, Beadle RE, Mouch S, Pourciau SS. Temporal regulation of cytokine mRNA expression in equine recurrent airway obstruction. Vet Immunol Immunopathol. 2005; 108: 237–45. doi: 10.1016/j.vetimm.2005.07.013 PMID: 16998607

22. Cordeau M-E, Joubert P, Dewachi O, Hamid Q, Lavoie J-P. IL-4, IL-5 and IFN-γ mRNA expression in pulmonary lymphocytes in equine heaves. Vet Immunol Immunopathol. 2004; 97: 87–96. doi: 10.1016/j.vetimm.2003.08.015 PMID: 14700540

23. Kleiber C, McGorum BC, Horohov DW, Pirie RS, Zurbriggen A, Straub R. Cytokine profiles of peripheral blood and airway CD4 and CD8 T lymphocytes in horses with recurrent airway obstruction. Vet Immunol Immunopathol. 2005; 104: 91–7. doi: 10.1016/j.vetimm.2004.10.002 PMID: 15661334

24. Jost U, Klukowska-Rötzler J, Leeb T, Swinburne JE, Ramseyer A, Bugno M, et al. A region on equine chromosome 13 is linked to recurrent airway obstruction in horses. Equine Vet J. 2007; 39: 236–41. doi: 10.1111/j.1744-0596.2007.01296.x PMID: 17520975

25. Pirie RS, Dixon PM, McGorum BC. Evaluation of nebulised hay dust suspensions (HDS) for the diagnosis and investigation of heaves. 3. Effect of fractonation of HDS. Equine Vet J. 2002; 34: 343–347. doi: 10.1076/02640220276249892 PMID: 12117105

26. Pirie RS, Collie DDS, Dixon PM, McGorum BC. Inhaled endotoxin and organic dust particulates have synergistic proinflammatory effects in equine heaves (organic dust-induced asthma). Clin Exp Allergy. 2003; 33: 676–83. doi: 10.1046/j.1365-2222.2003.01640.x PMID: 12752698

27. Pirie RS, Dixon PM, McGorum BC. Endotoxin contamination contributes to the pulmonary inflammatory and functional response to Aspergillus fumigatus extract inhalation in heaves horses. Clin Exp Allergy. 2003; 33: 1289–1296. doi: 10.1046/j.1365-2746.2003.01679.x PMID: 12956737

28. Pacholewska A, Drögemüller M, Klukowska-Rötzler J, Lanz S, Hamza E, Dermitzakis ET, et al. The transcriptome of equine peripheral blood mononuclear cells. PLoS One. 2015; doi: 10.1371/journal.pone.0122011

29. Boldman KG, Kriese LA, VanVleck LD, Kachman SD. A Manual for Use of MTDFREML. Washington, DC.: U. S. Department of Agriculture, Agricultural Research Service; 1993.

30. Kehrli D, Jandova V, Fey K, Jahn P, Gerber V. Multiple Hypersensitivities Including Recurrent Airway Obstruction, Insect Bite Hypersensitivity, and Urticaria in 2 Warmblood Horse Populations. J Vet Intern Med. 2014; 29: 320–10. doi: 10.1111/jvim.12473 PMID: 25270534

31. Ramseyer A, Gaillard C, Burger D, Straub R, Jost U, Boog C, et al. Effects of genetic and environmental factors on chronic lower airway disease in horses. J Vet Intern Med. 2007; 21: 149–56. PMID: 17338163

32. Laumen E, Doerr MG, Gerber V. Relationship of horse owner assessed respiratory signs index to characteristics of recurrent airway obstruction in two Warmblood families. Equine Vet J. 2010; 42: 142–148. doi: 10.1111/j.1742-3376.2009.00748.x PMID: 1956250
33. Wade CM, Giulotto E, Sigurdsson S, Zoll M, Gnerre S, Imsland F, et al. Genome Sequence, Comparative Analysis, and Population Genetics of the Domestic Horse. Science (80-.): 2009; 326: 865–867. doi:10.1126/science.1178158
34. Anders S, Pyl PT, Huber W. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics. 2014; 31: 166–169. doi: 10.1093/bioinformatics/btu638 PMID: 25260700
35. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol. 2010; 11: R106. doi: 10.1186/gb-2010-11-10-r106 PMID: 20979621
36. Robinson MD, McCarthy DJ, Smyth GK. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010; 26: 139–40. doi: 10.1093/bioinformatics/btp616 PMID: 19910308
37. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the uniﬁcation of biology. The Gene Ontology Consortium. Nat Genet. 2000; 25: 25–29. doi: 10.1038/75556. Gene PMID: 10802651
38. Nogales-Cadenas R, Carmona-Saez P, Vazquez M, Vicente C, Yang X, Tirado F, et al. GeneCodis: Interpreting gene lists through enrichment analysis and integration of diverse biological information. Nucleic Acids Res. 2009; 37: doi: 10.1093/nar/gkp416
39. Carmona-Saez P, Chagoyen M, Tirado F, Carazo JM, Pascual-Montano A. GENE CODIS: a web-based tool for finding signiﬁcant concurrent annotations in gene lists. Genome Biol. 2007; 8: R3. doi: 10.1186/gb-2007-8-1-r3 PMID: 17204154
40. Tabas-Madrid D, Nogales-Cadenas R, Pascual-Montano A. GeneCodis3: A non-redundant and modular enrichment analysis tool for functional genomics. Nucleic Acids Res. 2012; 40. doi: 10.1093/nar/gks402
41. Kinsella RJ, Kähäri A, Haider S, Zamora J, Proctor G, Spudich G, et al. Ensembl BioMarts: A hub for data retrieval across taxonomic space. Database. 2011; 2011. doi: 10.1093/database/bar030
42. Pu D, Wang W. Toll-like receptor 4 agonist, lipopolysaccharide, increases the expression levels of cytokines and chemokines in human peripheral blood monocellular cells. Exp Ther Med. D.A. Spandidos; 2014; 8: 1914–1918. doi: 10.3892/etm.2014.2025
43. Karnati H, Pasupuleti S, Kandi R, Undi R, Sahu I, Kannaki TR, et al. TLR-4 signalling pathway: MyD88 independent pathway up-regulation in chicken breeds upon LPS treatment. Vet Res Commun. 2015; 39: 73–9. doi: 10.1007/s11259-014-9621-2 PMID: 25417198
44. Turnquist HR, Cardinal J, Macedo C, Rosborough BR, Sumpter TL, Geller DA, et al. mTOR and GSK-3 shape the CD4+ T-cell stimulatory and differentiation capacity of myeloid DCs after exposure to LPS. Blood. 2010; 115: 4758–4769. doi: 10.1182/blood-2009-10-251488 PMID: 20335217
45. Delf S, Kitzmüller C, Steinberger P, Himly M, Jahn-Schmid B, Fischer GF, et al. Differential activation of dendritic cells by toll-like receptors causes diverse differentiation of naïve CD4(+) T cells from allergic patients. Allergy. 2014; doi: 10.1111/all.12501 46. Libermann TA BD. Activation of interleukin-6 gene expression through the NF-κB transcription factor. Mol Cell Biol. 1990; 10: 2327–2334. doi: 10.1128/MCB.10.5.2327Updated PMID:2183031
47. Wang C, Gong B, Bushel PR, Thierry-Mieg J, Thierry-Mieg D, Xu J, et al. The concordance between RNA-seq and microarray data depends on chemical treatment and transcript abundance. Nat Biotechnol. 2014; 32: 926–32. doi: 10.1038/nbt.3001 PMID: 25150839
48. Robinson MD, Oshlack A. A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol. 2010; 11: R25. doi: 10.1186/gb-2010-11-3-r25 PMID: 20196687
49. Bullard JH, Purdom E, Hansen KD, Dudoit S. Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments. BMC Bioinformatics. 2010; 11: 94. doi: 10.1186/1471-2105-11-94 PMID: 20167110
50. Sood A, Petersen H, Blanchette CM, Meek P, Picchi MA, Belinsky SA, et al. Methylation genes in spu- tum among older smokers with asthma. Chest. 2012; 142: 425–431. doi: 10.1378/chest.11-2519 PMID: 22345380
51. Fehr AR, Yu D. Control the host cell cycle: viral regulation of the anaphase-promoting complex. J Virol. 2013; 87: 8818–25. doi: 10.1128/JVI.00888-13 PMID: 23760246
52. Castro A, Bemis C, Vigneron S, Labbé J-C, Lorca T. The anaphase-promoting complex: a key factor in the regulation of cell cycle. Oncogene. 2005; 24: 314–325. doi: 10.1038/sj.onc.1207973 PMID: 15678131
53. Wäsch R, Engelbert D. Anaphase-promoting complex-dependent proteolysis of cell cycle regulators and genomic instability of cancer cells. Oncogene. 2005; 24: 1–10. doi: 10.1038/sj.onc.1208017 PMID: 15637585
54. Nakayama T. Immune-specific production of gamma interferon in human lymphocyte cultures in response to mumps virus. Infect Immun. 1983; 40: 486–492. PMID: 6404827
55. Green JA, Cooperband SR, Kibrick S. Immune specific induction of interferon production in cultures of human blood lymphocytes. Science. 1969; 164: 1415–1417. doi:10.1126/science.164.3886.1415 PMID: 5783715

56. Schoenborn J, Wilson C. Regulation of Interferon-γ During Innate and Adaptive Immune Responses. Adv Immunol. 2007; 96: 41–101. doi: 10.1016/S0065-2776(07)96002-2 PMID: 17981204

57. Gerard NP, Gerard C. Complement in allergy and asthma. Curr Opin Immunol. 2002; 14: 705–708. doi: 10.1016/S0952-7915(02)00410-7 PMID: 12413519

58. Lajoie S, Lewkowich IP, Suzuki Y, Clark JR, Dienger K, et al. Complement-mediated regulation of the IL-17A axis is a central genetic determinant of the severity of experimental allergic asthma. Nat Immunol. 2010; 11: 928–935. doi: 10.1038/ni.1926 PMID: 20802484

59. Köhl J, Wills-Karp M. A dual role for complement in allergic asthma. Curr Opin Pharmacol. 2007; 7: 283–289. doi: 10.1016/j.coph.2007.01.005 PMID: 17475559

60. Karp CL, Grupe A, Schadt E, Ewart SL, Keane-Moore M, Cuomo PJ, et al. Identification of complement factor 5 as a susceptibility locus for experimental allergic asthma. Nat Immunol. 2000; 1: 221–226. doi: 10.1038/79759 PMID: 10973279

61. Zhang X, Köhl J. A complex role for complement in allergic asthma. Expert Rev Clin Immunol. 2010; 6: 269–277. doi: 10.1586/eri.10.84 PMID: 20402389

62. Lin H, Lee E, Hestir K, Leo C, Huang M, Bosch E, et al. Discovery of a cytokine and its receptor by functional screening of the extracellular proteome. Science. 2008; 320: 807–811. doi: 10.1126/science.1154370 PMID: 18467591

63. Wang Y, Colonka M. Interkeukin-34, a cytokine crucial for the differentiation and maintenance of tissue resident macrophages and Langerhans cells. Eur J Immunol. 2014; 44: 1575–1581. doi: 10.1002/eji.201344365 PMID: 24737461

64. Masteller EL, Wong BR. Targeting IL-34 in chronic inflammation. Drug Discov Today. 2014; 19: 1212–1216. doi: 10.1016/j.drudis.2014.05.016 PMID: 24906044

65. Bleul CC, Fuhlbrigge RC, Casasnovas JM, Aiuti A, Springer TA. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). J Exp Med. 1996; 184: 1101–1109. doi: 10.1084/jem.184.3.1101 PMID: 9064327

66. Schulz-Knappe P, Mägert HJ, Dewald B, Meyer M, Cetin Y, Kubbies M, et al. HCC-1, a novel chemokine from human plasma. J Exp Med. 1996; 183: 295–299. doi: 10.1084/jem.183.1.295 PMID: 8551235

67. Augsten M, Hägglöf C, Olsson E, Stolz C, Tsagozis P, Levchenko T, et al. CXCL14 is an autocrine growth factor for fibroblasts and acts as a multi-modal stimulator of prostate tumor growth. Proc Natl Acad Sci U S A. 2009; 106: 3414–3419. doi: 10.1073/pnas.0813144106 PMID: 19218429

68. Blain KY, Kwiatkowski W, Zhao Q, La Fleur D, Naik C, Chun T-W, et al. Structural and functional characterization of CC chemokine CCL14. Biochemistry. 2007; 46: 10008–15. doi: 10.1021/bi700936w PMID: 17691823

69. Ansel KM, Ngo VN, Hyman PL, Luther SA, Förster R, Sedgwick JD, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. Nature. 2000; 406: 309–314. doi: 10.1038/35018581 PMID: 10917533

70. Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med. 1989; 170: 2081–2095. doi: 10.1084/jem.170.6.2081 PMID: 2531194

71. Klier J, May A, Fuchs S, Schilling U, Plank C, Winter G, et al. Immunostimulation of bronchoalveolar lavage cells from recurrent airway obstruction-affected horses by different CpG-classes bound to gelatin nanoparticles. Vet Immunol Immunopathol. 2011; 144: 79–87. doi: 10.1016/j.vetimm.2011.07.009 PMID: 21831455

72. Takagi R, Higashi T, Hashimoto K, Nakano K, Mizuno Y, Okazaki Y, et al. B cell chemoattractant CXCL13 is preferentially expressed by human Th17 cell clones. J Immunol. 2008; 181: 186–9. PMID: 18566383
Singh RP, Hasan S, Sharma S, Nagra S, Yamaguchi DT, Wong DTW, et al. Th17 cells in inflammation and autoimmunity. Autoimmun Rev. 2014; 13: 1174–1181. doi: 10.1016/j.autrev.2014.08.019 PMID: 25151974

Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Pagé N, et al. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. J Allergy Clin Immunol. 2001; 108: 430–438. doi: 10.1067/mai.2001.117929 PMID: 11544464

Wang Q, Li H, Yao Y, Xia D, Zhou J. The overexpression of heparin-binding epidermal growth factor decreases oxygen consumption by inhibiting Complex I activity. Biochem J. 2011; 430–438. doi: 10.1126/science.1202526

Ahmad T, Kumar M, Mabalirajan U, Pattnaik B, Aggarwal S, Singh R, et al. Hypoxia Response in Mice. Int Arch Allergy Immunol. 2009; 149: 98–105. doi: 10.1007/s00218-009-9348-7

Van Uden P, Kenneth NS, Rocha S. Regulation of hypoxia-inducible factor-1alpha by NF-kappaB. Biochem J. 2008; 417: 847–854. doi: 10.1042/BJ20080476 PMID: 18393939

Rius J, Guma M, Schachtrup C, Akassoglou K, Zinkemagel AS, Nizet V, et al. NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. Nature. 2008; 453: 807–11. doi: 10.1038/nature06905 PMID: 18432192
96. McMahon S, Charbonneau M, Grandmont S, Richard DE, Dubois CM. Transforming growth factor beta1 induces hypoxia-inducible factor-1 stabilization through selective inhibition of PHD2 expression. J Biol Chem. 2006; 281: 24171–24181. doi: 10.1074/jbc.M604507200 PMID: 16815840

97. Blouin CC, Pagé EL, Soucy GM, Richard DE. Hypoxic gene activation by lipopolysaccharide in macrophages: Implication of hypoxia-inducible factor 1α. Blood. 2004; 103: 1124–1130. doi: 10.1182/blood-2003-07-2427 PMID: 14525767

98. Poynter ME, Cloots R, van Woerkom T, Butnor KJ, Vacek P, Taatjes DJ, et al. NF-κB Activation in Airways Modulates Allergic Inflammation but Not Hyperresponsiveness. J Immunol. 2004; 173: 7003–7009. doi: 10.4049/jimmunol.173.11.7003 PMID: 15557197

99. Tang MLK, Wilson JW, Stewart AG, Royce SG. Airway remodelling in asthma: Current understanding and implications for future therapies. Pharmacol Ther. 2006; 112: 474–488. doi: 10.1016/j.pharmthera.2006.05.001 PMID: 16759709

100. Dalton JE, Pearson J, Scott P, Carding SR. The interaction of gamma delta T cells with activated macrophages is a property of the V gamma 1 subset. J Immunol. 2003; 171: 6488–6494. PMID: 14662848

101. Dalton JE, Howell G, Pearson J, Scott P, Carding SR. Fas-Fas ligand interactions are essential for the binding to and killing of activated macrophages by gamma delta T cells. J Immunol. 2004; 173: 3660–3667. PMID: 15536111

102. Turlej RK, Fiévez L, Sandersen CF, Dogné S, Kirschvink N, Leukef P, et al. Enhanced survival of lung granulocytes in an animal model of asthma: evidence for a role of GM-CSF activated STAT5 signalling pathway. Thorax. 2001; 56: 696–702. doi: 10.1136/thorax.56.9.696 PMID: 11514690

103. Niedzwiedz A, Jaworski Z, Tykalowski B, Smialek M. Neutrophil and macrophage apoptosis in bronchoalveolar lavage fluid from healthy horses and horses with recurrent airway obstruction (RAO). BMC Vet Res. 2014; 10: 29. doi: 10.1186/1746-6148-10-29 PMID: 24460911

104. Wu MX, Ao Z, Prasad K V, Wu R, Schlossman SF. IEX-1L, an apoptosis inhibitor involved in NF-kappaB-mediated cell survival. Science. 1998; 281: 998–1001. doi: 10.1126/science.281.5379.998 PMID: 9703517

105. Schott J, Reiter S, Philipp J, Hanek J, Schäfer H, Stoeckl G. Translational Regulation of Specific mRNAs Controls Feedback Inhibition and Survival during Macrophage Activation. PLoS Genet. 2014; 10. doi:10.1371/journal.pgen.1004368

106. Jiang J, Jedinak A, Sliva D. Ganodermanontriol (GDNT) exerts its effect on growth and invasiveness of breast cancer cells through the down-regulation of CDC20 and uPA. Biochem Biophys Res Commun. 2011; 415: 325–329. doi:10.1016/j.bbrc.2011.10.055 PMID: 22033405

107. Kato T, Daigo Y, Aragaki M, Ishikawa K, Sato M, Kaji M. Overexpression of CDC20 predicts poor prognosis in primary non-small cell lung cancer patients. J Surg Oncol. 2012; 106: 423–430. doi: 10.1002/jso.23109 PMID: 22488197

108. Huang HC, Shi J, Orth JD, Mitchison TJ. Evidence that Mitotic Exit Is a Better Cancer Therapeutic Target Than Spindle Assembly. Cancer Cell. 2009; 16: 347–358. doi: 10.1016/j.ccr.2009.08.020 PMID: 19800579

109. Kidokoro T, Tanikawa C, Furukawa Y, Katagiri T, Nakamura Y, Matsuoka K. CDC20, a potential cancer therapeutic target, is negatively regulated by p53. Oncogene. 2008; 27: 1562–1571. doi: 10.1038/sj.onc.1210799 PMID: 17873905

110. Van L, Tan M, Yang I, Inuzuka H, Dai X, Wu T, et al. APCDCd20 Suppresses Apoptosis through Targeting Bim for Ubiquitination and Destruction. Dev Cell. 2014; 29: 377–391. doi:10.1016/j.devcel.2014.04.022 PMID: 24871945

111. Zaslona Z, Przybranowski S, Wilce K, van Rooijen N, Teitz-Tennenbaum S, Osterholzer JJ, et al. Resident Alveolar Macrophages Suppress, whereas Recruited Monocytes Promote, Allergic Lung Inflammation in Murine Models of Asthma. J Immunol. 2014; 193: 4245–4253. doi: 10.4049/jimmunol.1400580 PMID: 25225663

112. Voekele NF, Spiegel S. Why is effective treatment of asthma so difficult? An integrated systems biology hypothesis of asthma. Immunol Cell Biol. 2009; 87: 601–5. doi: 10.1038/icb.2009.45 PMID: 19546879

113. Mo J-H, Kim J-H, Lim DJ, Kim EH. The role of hypoxia-inducible factor 1α in allergic rhinitis. Am J Rhinol Allergy. 2014; 28: e100–6. doi: 10.2500/aja.2014.28.4025 PMID: 24717944

114. Baay-Guzman GJ, Huerta-Yepez S, Vega MI, Aguilar-Leon D, Campillos M, Blake J, et al. Role of CXCL13 in asthma: novel therapeutic target. Chest. 2012; 141: 886–94. doi: 10.1378/chest.11-0633 PMID: 22016489

115. Festa ED, Hankiewicz K, Kim S, Skurnick J, Wolansky LJ, Cook SD, et al. Serum levels of CXCL13 are elevated in active multiple sclerosis. Mult Scler. 2009; 15: 1271–1279. doi: 10.1177/1352458509107017 PMID: 19805441
116. Meraouna A, Cizeron-Clairac G, Le Panse R, Bismuth J, Truffault F, Tallaksen C, et al. The chemokine CXCL13 is a key molecule in autoimmune myasthenia gravis. Blood. 2006; 108: 432–440. doi: 10.1182/blood-2005-06-2383 PMID: 16543475

117. Hansen S, Baptiste KE, Fjeldborg J, Betancourt A, Horohov DW. A comparison of pro-inflammatory cytokine mRNA expression in equine bronchoalveolar lavage (BAL) and peripheral blood. Vet Immunol Immunopathol. 2014; 158: 238–243. doi: 10.1016/j.vetimm.2014.02.001 PMID: 24603016

118. Lindberg Å, Robinson NE, Näsman-Glaser B, Jensen-Waern M, Lindgren JÅ. Assessment of leukotriene B4 production in leukocytes from horses with recurrent airway obstruction. Am J Vet Res. 2004; 65: 289–295. doi: 10.2460/ajvr.2004.65.289 PMID: 15027674

119. Henríquez C, Perez B, Morales N, Sarmiento J, Carrasco C, Morán G, et al. Participation of T regulatory cells in equine recurrent airway obstruction. Vet Immunol Immunopathol. 2014; 158: 128–134. doi: 10.1016/j.vetimm.2013.12.005 PMID: 24503328

120. Sarmiento J, Perez B, Morales N, Henriquez C, Vidal L, Folch H, et al. Apoptotic effects of tamoxifen on leukocytes from horse peripheral blood and bronchoalveolar lavage fluid. Vet Res Commun. 2013; 37: 333–338. doi: 10.1007/s11259-013-9571-0 PMID: 23846832