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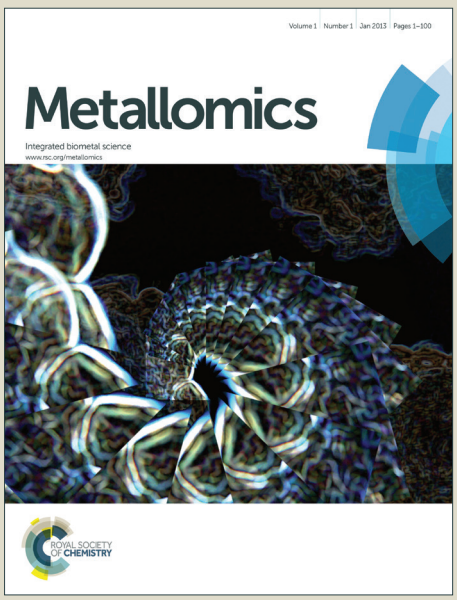

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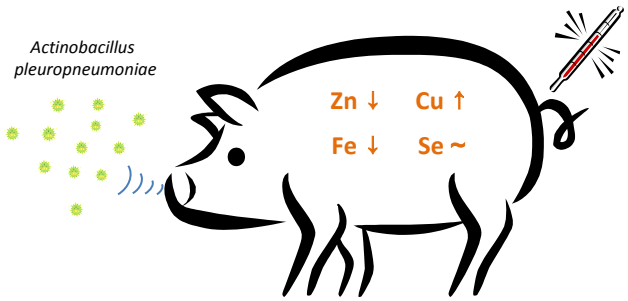

Experimental bacterial lung infection affects trace elements in blood and liver tissue.

Acute and subacute response of iron, zinc, copper and selenium in pigs experimentally infected with *Actinobacillus pleuropneumoniae*

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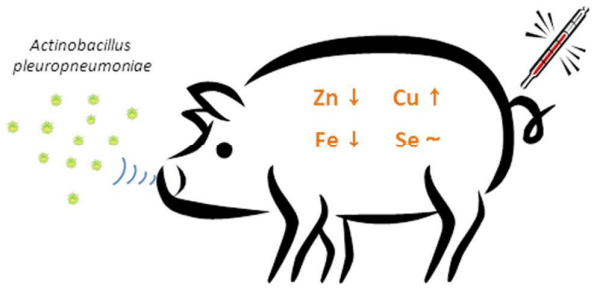
Contents entry



Abstract

This study was performed to characterise the response of iron (Fe), zinc (Zn), copper (Cu) and selenium (Se) in bacterial-induced porcine acute phase reaction (APR). Twenty piglets were challenged by aerosolic infection with *Actinobacillus pleuropneumoniae* (*A.pp.)* serotype 2, ten piglets serving as controls. Blood sampling was done initially and at day 4 and 21 after infection, collection of liver tissue was done at day 21 (autopsy).

A.pp.-infection caused fever and respiratory symptoms. APR at day 4 after infection was marked by an increase in total white blood cells, granulocytes, monocytes in whole blood samples and an increase in globulin/albumin ratio (G/A), α2-globulins, C-reactive protein, haptoglobin, ceruloplasmin (Cp), Cu and Se in serum. Concurrently, there was a decrease in haemoglobin (Hb) and packed cell volume (PCV) in whole blood as well as a decrease in albumin, transferrin, total iron binding capacity and Fe in serum and Zn in plasma. The subacute stage at day 21 was characterised by progressively increased concentrations of lymphocytes, G/A, ß-globulins and γ-globulins reflecting the specific immune reaction. Hb and PCV showed further decreases, all other parameters returned to the initial concentrations. Glutathione peroxidase activity in plasma and liver tissue remained unaffected by *A.pp.*-infection. The liver concentration (day 21) of Zn was found to be higher, that of Se was lower in the *A.pp.*-group, whereas hepatic concentrations of Cu and Fe were not affected by *A.pp.*-infection.

In summary, the acute and subacute stages of *A.pp.*-infection were accurately characterised by the APR-related parameters. Se was only marginally affected by the *A.pp.*-infection. The elevated plasma Cu concentration may be a side effect of the transient hepatic induction of Cp synthesis. Zn responded, being distinctly reduced in plasma and probably having been sequestered in the liver tissue. Reduction in serum Fe can be regarded as an unspecific defence mechanism in *A.pp*-infection to withdraw Fe from bacterial acquisition systems.

Introduction

The model of experimentally induced porcine pleuropleumonia by aerosolic infection with the Gram-negative bacterium *Actinobacillus pleuropneumoniae* (*A.pp.*) was previously used to study biomarkers [1] or serological, bacteriological or immunological issues [2, 3]. General clinical outcomes of the infection are fever, increasing respiratory rate, coughing, dyspnoea and general distress (reviewed by Bosse et al.[4]). The pathophysiological pathway accompanying the clinical outcome is the acute phase response (APR), which is prompted by specific pathogens, leading to the production of a large array of mediators, e.g. cytokines and chemokines. In case of virulent Gram-negative bacteria, the invading pathogens must be held in check by the innate immune system until a specific immune response is mounted as reviewed by Heumann and Roger [5]. Lipopolysaccharide (LPS), a compound in the outer membrane of Gram-negative bacteria, is a major virulence factor leading to the rapid activation of an intracellular signalling pathway as also seen in *A.pp*-infection [6]. The serum acute phase proteins C-reactive protein (CRP) and haptoglobin (Hp) are commonly known to reflect APR following bacterial infections in pigs [7-9].

Generally, trace elements such as copper (Cu), iron (Fe), zinc (Zn), and selenium (Se) were also suspected to be involved in the APR [10, 11], most likely in coherence with known binding proteins in serum. Early studies in human hepatoma cells already suggested that interleukine (IL)-6 directly stimulates hepatic ceruloplasmin (Cp) biosynthesis [12], rendering this Cu-binding protein [13] to be a positive acute phase reactant. Intestinal Fe absorption and Fe storage in macrophages were shown to be regulated by hepcidin [14]. The innate immune response seems to lead to hepcidin induction via cytokines causing a decrease in Fe concentration in serum [15-17] to withdraw Fe from bacterial acquisition. *A.pp.* itself was found to acquire Fe from transferrin (Tf) required for multiplication and persistence of *A.pp.* in the porcine respiratory tract [18]. A decrease in Zn concentration in serum in the APR was suggested previously to be caused by an up-regulation of a Zn-transporter (Zip14) expression induced by

IL-6, playing a role in the mechanism responsible for hypozincemia that accompanies the APR [11, 19]. Previous studies in mice showed that key factors of hepatic selenoprotein biosynthesis were impaired in APR accompanied by a decrease in serum Se concentration [20]. Experimental *A.pp.*-infection is a well-established animal model reflecting the interaction of pathogen and host immune system involving the whole body under natural conditions. Data about trace elements and related parameters in experimental acute, subacute or chronic bacterial infectious stages are rare. Therefore, the data presented as follows may help to add to the extent of knowledge in this field.

Results

General condition

Before infection, clinical examination of all 30 piglets revealed no pathological clinical signs. At day 4 after infection, the *A.pp.*-infected piglets (APP) showed various signs of an acute *A.pp.*-infection such as coughing, increased breathing rate with pathological breathing noise, an increased body temperature up to 41.4 °C, apathy and symptoms of gastrointestinal disorders such as vomiting or diarrhoea. The controls did not show any clinical signs of sickness. At day 21 after infection in the subacute stage of disease, infected piglets showed sporadic coughing, an increased breathing rate and reduced activity. Body weight and breast circumference increased significantly over time in both groups without differences between the controls and APP at day 4 and 21 after infection: Mean body weight (standard deviation, SD) in the controls/APP group was 7.3 (1.1) kg / 7.4 (1.7) kg initially, 8.3 (1.3) kg /8.3 (1.7) kg at day 4 after infection and 13.9 (2.4) kg / 12.3 (2.9) kg at day 21 after infection, respectively. Mean (SD) breast circumference in the controls/the APP group before infection was 43.0 (2.9) cm and 42.5 (1.9) cm, at day 4 and 21 they were 43.2 (2.0) cm / 42.9 (2.7) cm and 50.8 (2.9) cm/ 48.8 (3.8) cm, respectively. The mean (SD) initial body temperature was

39.1 (0.3) °C and 38.6 (0.6) °C in controls and APP piglets. The APP piglets showed a significantly higher mean body temperature at day 4 after infection (40.1 (0.6) °C), and only occasionally elevated body temperature at day 21 after infection (39.1 (2.3) °C) compared to the initial level. The mean body temperature of the controls remained unaffected at day 4 (39.0 (0.4) °C) and day 21 (39.2 (0.3) °C).

Haematology

The results of haematological examination of whole blood are given in Table 1. Metamyelocytes, myelocytes, eosinophilic granulocytes, basophilic granulocytes and normoblasts were analysed as well, but detected only occasionally in very small amounts (data not shown). Comparing the haematological results before infection with reference limits for weaned piglets [21] there were only very slight deviations: The mean corpuscular haemoglobin concentration (MCHC) was slightly above the upper reference limit (range 321-334 g/l) and the lymphocytes were slightly below the lower reference limit (range 5.5-19.5 x 10^9/l). There were no significant differences between the mean haematological values of the controls and the APP group before infection.

Post-challenge variation in haematological parameters at day 4 were significantly lower concentrations of haemoglobin (Hb) and packed cell volume (PCV) in APP piglets compared to the controls as well as higher concentrations of total white blood cells (WBC), segmented neutrophilic granulocytes, banded neutrophilic granulocytes and monocytes in APP piglets. At day 21 after infection, Hb and PCV showed a further decrease in APP piglets, whereas the total WBC, banded neutrophilic granulocytes and monocytes decreased to levels close to those of the controls. Segmented neutrophilic granulocytes were still higher in APP piglets compared to the controls at day 21. Analysis of variance for the effects of "time" in repeated measurements revealed various time effects in all haematological parameters except banded and segmented neutrophilic granulocytes in the controls. However, only the mean

concentration of segmented neutrophilic granulocytes (13.96 x 10^9/l) in the APP piglets at day 4 after infection were above the upper reference limit for weaned piglets [21].

Serum protein fractions

The results of the examination of serum protein fractions are given in Table 2. Pre-challenge values of total protein (TP) and α2-globulin were slightly lower in the APP piglets compared to the controls, all concentrations being within the reference ranges for weaned piglets.

TP concentration was unaffected by the *A.pp.*-infection. However, a decrease over time was seen in the controls. Albumin (ALB) concentration decreased in both groups over time, but to a higher extent in the *A.pp.*-infected group. An obvious effect of the *A.pp.*-infection was the increasing globulin/albumin (G/A) quotient corresponding to a distinct increase in the α2-globulin fraction at day 4 and still elevated levels at day 21. The ß- and γ-globulin fractions increased until the end of the study in APP piglets, only showing significant group differences between the APP piglets and the controls at day 21.

APR-related proteins

The APR- related parameters in serum of *A.pp* -infected piglets and controls at the three sampling times are shown in Table 3. Significant increases in CRP, Hp and Cp concentrations were seen in the APP group at day 4, whereas there was a decrease in Tf and total iron binding capacity (TIBC). All parameters returned to base levels at day 21. The results of the analysis of 'time effects' in repeated measurements in APP piglets were in accordance with the group differences of the respective APR-related parameter. Effects of 'time' in the controls were seen only occasionally, there being a slight increase in Cp at day 4 compared to the initial and final values.

Trace elements in serum (Se, Cu, Fe) and plasma (Zn)

The results of the trace element analysis in serum/plasma are shown in Table 4. The mean concentration of Fe in serum and Zn in plasma was significantly lower in the APP piglets at day 4 after challenge compared to the controls. At day 21 after infection these effects were not evident anymore. Mean Cu concentration in serum was significantly higher in the APP piglets at day 4 after infection, decreasing thereafter to levels lower than the initial concentration. In the controls, there was a trend towards lower Cu concentrations during the study. The mean Se concentration in the APP piglets was higher at day 4 ($p<0.045$) compared to the controls. However, the controls showed an elevated Se concentration in serum at day 21 compared to the base levels before infection.

Glutathione peroxidase in plasma and liver tissue

The results of the glutathione peroxidase (GPx) activity in plasma and liver tissue are given in Table 5. There were no differences in the GPx activity in plasma before and after infection (day 4 and 21) when comparing the APP piglets and controls. Both groups showed increased GPx3 activities over time as demonstrated by evaluation of repeated measurements. Moreover, group differences in hepatic GPx1 activity at day 21were not evident.

Trace elements in liver tissue

The mean Fe and mean Cu concentrations in liver tissue were unaffected when comparing the APP piglets and the controls. The mean Se concentration was significantly lower and Zn concentration was higher in the APP group compared to the controls (Table 6).

Discussion

General remarks

The APR is part of the innate immune system, which is triggered by different stimuli including trauma, infection, stress, neoplasia, and inflammation. The physiological purpose is suspected to optimise the early host response against the pathological challenge [22].

An increase in several cytokines, such as IL-6, IL-8, IL-10 and IL-12, following *A.pp.*-infection was indicated to be one major cellular response in lung tissue [23, 24]. This was supported by the detection of an up-regulation of immunity activating genes and other pro-inflammatory mediators of the innate immune response in inflamed lung tissue as well as the differential expression of genes encoding acute phase reactants in liver tissue of *A.pp.*-infected pigs [25, 26]. Even though we did not analyse cytokines in our study, the clinical course of the disease of our piglets showed the clinical consequence of the cytokine action: The clinical signs clearly indicated progressing lung impairment in the challenged animals. There was an initial period with fever (day 4 after infection) followed by a subacute stage with normal body temperature (day 21). However, the equal increase in body weight and breast circumference in both groups underlines the temporary moderate consequences for the body, most likely due to the operative immune reaction terminating the systemical harm of the bacterial lung infection.

Haematology

The elevated counts of total WBC due to preferentially increased neutrophilic granulocytes at day 4 represent a typical reaction found a few days after a bacterial infection. Specifically, the increase in banded neutrophilic granulocytes, a so called ‘left-shift’, is often seen in this period of infection as validated recently for men [27] and was also found in *A.pp.*- infected pigs [28]. Transcriptional profiling of lung tissue from *A.pp.*-infected pigs published recently [26]

indicated an activation of the granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulating stem cells to become granulocytes and monocytes explaining our haematological findings in the respective cells.

Bacterial infections as well as other pathogens were found to cause a so called 'anaemia of chronic disease' (ACD). An inflammation-related hepcidin synthesis leads to a diversion of Fe from the circulation into the reticulo-endothelial system. The resulting Fe limitation for erythropoiesis is paramount for the development of ACD as reviewed in detail by [29]. In our infected piglets, the concentrations in Hb and PCV were lower at day 4 compared to the controls, but with accompanying time effects also in the controls. However, all results were within the reference ranges for weaned piglets [21] indicating that these findings should be interpreted with care. The effects at day 4 may have been caused by the infection, but, we certainly did not observe typical ACD, which is also supported by the non-affected MCHC. That is accountable for the observation that ACD is caused by limited availability of Fe for erythropoiesis [30] being more and more overt over time due to the regeneration of the erythrocytic pool within weeks. It might be interesting to investigate whether a chronic *A.pp.*-infection causes ACD 70-100 days after infection.

Serum protein fractions

The concentration of TP in serum, although decreasing slightly in the controls, remained stable in the APP piglets over time. Among serum protein fractions (Table 2), elevation of G/A ratio by 50% is the most evident finding, this parameter therefore being recommended as being useful for indicating an APR in pigs. The decrease in ALB in APR was attributed to a diminished ALB synthesis in the liver [31], substantiated by a decrease in ALB transcripts as shown by microarray technique recently [25]. However, the functional reason for this decrease is not yet clear. Although there was a significant difference in serum ALB concentration

between the APP piglets and the controls at day 4 after infection, we found a trend towards lower ALB levels in the controls over time as well. The reason for this remains unclear.

The increase in the α2-globulin fraction was contemporaneous to the decrease in ALB concentration at day 4 in APP piglets. Hp belongs to the α2-globulin fraction [22] and may be partly responsible for this increase as an early response in the acute phase of *A.pp.*-infection (see also Table 3).

The β-globulin fraction includes Tf and CRP [22], but these parameters change in contradicting direction in APR, as seen at day 4 after infection, leading to a compensation effect for the total concentration of the ß-globulin fraction. However, there was an increase in this fraction over time in APP piglets as well as obvious differences between groups at day 21. As there were no differences between groups for Tf and CRP at day 21, this effect in the subacute stage must have been caused by other proteins. The γ-globulin fraction primarily comprises IgG [22], reflecting a specific immune response. The production of the specific antibodies takes several days showing differences between the APP-group and controls at the later stage of the disease at day 21. The increase in the γ-globulins exclusively in the APP-group was expected and intended by the study design and underlines the effectiveness of the experimental infection.

APR-related parameter

CRP and Hp are commonly used parameters to reveal APR in *A.pp.*- infected pigs 2-5 day post infection [9, 32]. As expected, both parameters were significantly higher in APP piglets at day 4 after infection, decreasing to initial or even lower concentrations (Table 3) until day 21. This finding was attributed to specific changes in hepatic gene expression found 24 hours post-challenge in *A.pp.*-infected pigs showing an increase of transcripts of CRP and Hp [25].

The metabolic function of Hp is to bind Hb from lysed erythrocytes counteracting the deleterious effects of free Hb in plasma by binding and relaying Hb for clearance via

endocytosis by macrophages [33]. Although others [1] found Hp less discriminative for evaluating the status of acutely *A.pp.*-infected piglets, our results differentiate clearly between the APP piglets and controls at day 4 after infection assessing this parameter as helpful to characterise this stage of an *A.pp.*-infection.

Cu and Cp

Cp, firstly isolated in 1948 as Cu binding protein [13] containing more than 95% of the Cu found in plasma [34], has rarely been analysed in pigs with bacterial lung infections before. As shown for other acute phase reactants, *A.pp.*-infected pigs also showed an increase in transcripts of Cp in the liver tissue [25]. The increase following *A.pp.*-infection in animals of our study was higher compared to elevations observed in non-infectious APR induced experimentally via turpentine injection in pigs [35]. However, the time course was quite similar when comparing both studies showing a post-interventional increase with a maximum around day 4-5 and a decrease until day 20. Interestingly, the Cu concentration in APP piglets increased (day 4) and decreased (day 21) simultaneously to Cp concentration in serum as also shown in other studies [35] , whereas the slight increase in Cp at day 4 in the controls did not correspond to the serum Cu concentration. Cp is synthesised in hepatocytes (apo-Cp) and secreted into the plasma following the incorporation of six atoms of Cu (holo-Cp). This Cu-competent conformation seems to be essential for building a functional holoprotein entering the hepatic secretory pathway [36]. Early studies already showed that hepatic Cp synthesis is induced by cytokines in APR [12]. Therefore, the moderate induction of Cp synthesis in APR of the APP piglets may elevate plasma Cu concentration as a transient side effect, nevertheless not affecting the long-term hepatic Cu concentration (Table 5).

Tf, TIBC and Fe

The acute period of *A.pp.*-infection at day 4 after infection was also accompanied by reduced Tf and Fe concentration in serum, exclusively found in the APP piglets. Moreover, TIBC was significantly lower in the infected animals at day 4 (Table 3). Serum Tf binds Fe ions and normally remains at one-third saturation. Recently published studies revealed hepcidin to be a key factor in Fe homeostasis inhibiting Fe entry into the plasma compartment from the three main sources of Fe: Dietary absorption in the duodenum, the release of recycled Fe from senescent erythrocytes via macrophages and the release of stored Fe from hepatocytes (reviewed by Ganz and Nemeth [30]). Inflammation-associated hepcidin up-regulation seems to be stimulated by cytokines (IL-6, TGF-1, IL-22) and was suggested to be responsible for reductions in serum Fe during the acute phase of both, systemic fungal (C. albicans) and viral (Influenza A), infections [17]. Bacteria have evolved different Fe acquisition systems, e.g. the secretion of siderophores, small molecules with high affinity for Fe, or surface receptors specific for Fe-containing host proteins. *A.pp.* likewise possesses Tf binding proteins which are considered to be a virulence factor [37]. Therefore, the reduction in serum Fe as a part of the APR seems to be a very beneficial reaction in innate immune defence during *A.pp.*-infection. Long-term effects on Fe concentration in liver tissue were not found in our study at necropsy (subacute stage, day 21), underlining the short-term character of the Fe response during the APR only.

Zn

Zn serves functions in catalysis or structural stabilisation of more than 300 enzymes and is a component of an even higher number of additional metalloproteins as well as having important functions as a regulator of immunity (reviewed by [38]). The observation of decreasing serum Zn concentrations in APR seen in *A.pp.*-infected piglets has also been described by others [1, 28], but without reporting on liver Zn concentrations. Recent data

suggested adverse consequences of Zn deficiency enhancing the spread of infection and NF-κB activation in vivo in response to polymicrobial sepsis, leading to enhanced inflammation and lung injury [39]. However, our experimental design implied a sufficient concentration of Zn in food. The Zn concentration in serum and liver of the controls were within reference ranges [21, 40] for pigs. The reason for a decrease in Zn in APR is not fully understood, but there were several different explanatory approaches discussed as reviewed by [38]: (i): Zn ions (similar to Fe) are sequestered from the pathogens to deprive them of essential nutrients, impairing their growth. (ii): Macrophages would increase the concentration of Zn ions intracellularly to intoxicate phagocytised microorganisms. (iii): Reduced Zn^{2+} levels result in a shift of leukopoiesis towards the generation of myeloid cells, and reduced extracellular Zn^{2+} may be a signal promoting monocyte differentiation. Two other hypotheses focus on the roles of Zn^{2+} in the liver. In this organ Zn^{2+} is required for protein synthesis, and the redistribution of Zn^{2+} could reflect the altered metabolic requirements of the liver during the APR serving a role in energy metabolism rather than synthesis of acute phase proteins. Alternatively, Zn^{2+} in combination with metallothionein has been shown to prevent hepatotoxicity, and Zn^{2+}/ metallothionein accumulation may be required for liver protection against oxidative stress that occurs during inflammation [38].

Whether one or a combination of biological reasons discussed above are responsible, the distinctly higher Zn concentration in the liver tissue of our *A.pp.*-infected piglets at day 21 after infection reflects the sequestration of Zn markedly. Interestingly, the Zn concentration in serum at day 21 was found at levels equal to the pre-challenged state and the lower Zn concentration was transient and obvious in the APR at day 4 only. Despite the variations in serum Zn concentration in the controls, the APR-related effect on Zn is also supported by the fact of a distinct reduction in variation within the APP piglets reflected by a low SD at day 4 after infection.

Se

Research on Se-dependent effects has increased markedly during the last decades. Several studies explored the function of selenoproteins in the immune system [41] and critical illness/sepsis [42]. However, Se response in ordinary APR has been rarely studied before and research has focussed mostly on short-term effects within 1-3 days. Maehira and colleagues [10] reported a significant decrease in Se in plasma and liver following artificial LPS challenge in rats. Later, these effects were further investigated by Renko and colleagues [20], suggesting that hepatic Se metabolism becomes progressively disturbed during the LPS-induced APR and negatively affects serum Se status by insufficient biosynthesis of selenoprotein P, thereby interrupting the regular Se metabolism and Se transport. Recently, hypoxia was found to downregulate selenoprotein expression in a cell culture model [43].

The first determination of serum Se concentration after challenge in our study was at day 4 resulting in higher Se concentration in serum of the *A.pp.*-infected piglets compared to the controls. Although high CRP und Hp levels clearly indicate APR in our *A.pp.*-infected piglets at day 4, the serum Se concentrations were contradictory to the studies cited above looking for early APR. The model of LPS-induced APR is often used for experimental studies. However, we do not know if the results are transferrable to APR in bacterial lung infection. Possibly, the APR-effect in serum Se concentration is transient and short-dated to APR induction as shown in APR induction by minor elective surgery in men [44]. Moreover, there might be some unknown procedures affecting Se metabolism in subsequent APR which might be interesting to determine in further studies. As also seen for several other parameters in our study, the differences in serum Se concentration between groups were no longer evident at day 21 after infection. The Se concentration in food and liver tissue were adequate and within the reference ranges for pigs [45]. However, the mean concentration of Se in liver tissue was significantly lower at day 21 in the *A.pp.*-infected piglets. LPS injection in rodents also was found to cause a strong decline in hepatic Se concentrations in sufficient Se-supplemented

mice [46] and rats [10]. Recent findings on hypoxia-dependent shifted pattern of selenoprotein synthesis in hepatocytes [43] were postulated to explain the declining Se status during infections. Our results suggest that this might be a persistent effect remaining for at least three weeks in bacterial-caused pleuro-pneumonia. The hepatic GPx1 activity analysed at day 21 was unaffected by *A.pp.*-infection. Plasma GPx3 activity also showed no differences between groups at the three different times of sampling rendering these selenoproteins less or unimpaired in *A.pp.*-induced APR. Previous investigations [20] also demonstrated no effects of LPS-induced APR on GPx transscripts within three days after challenge. However, the plasma GPx activity of our experimental animals showed obvious time effects in both groups. Precise reasons for this finding are hard to explain but it might be an effect of the age of the piglets.

Experimental

The examinations were part of a comprehensive study looking primarily for imaging techniques (infrared thermography) to optimise the clinical diagnostic methods in experimental *A.pp.*-infection [47]. Pre-selection criteria for the infected group used for the biochemical examinations reported here were as follows: Living until day 21 after infection, positive post-mortal testing for *A.pp.*-infection by cultural examination of lung tissue and exposure of typical clinical signs of lung infection during the study in the infected group. Finally, the samples from 30 animals (20 *A.pp.*-infected pigs and 10 controls) were used for the examination described below.

The study was approved by the Ethical Commission of the Lower Saxony State Office for Consumer Protection and Food Safety (AZ 33.9-42502-12/0835). Precautions aimed at avoiding unnecessary suffering were taken at all stages of the experiment.

Animals

A detailed description of the basic experimental procedure is given in [47]. In brief, the clinically healthy male castrated pigs (German Landrace) were four weeks old. The mean initial body weight (bw) was 7.3 kg (SD 1.1) and 7.4 kg (SD 1.7) in the *A.pp.*-infected group and the controls, respectively. The animals were bred and raised in a closed herd with a high health status, routinely tested negative for *A.pp.*-infection, Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), toxigenic *Pasteurella multocida* as well as endo- and ectoparasites. Initially, all piglets were checked for antibodies against *A.pp* using an Apx-II-Enzyme-linked immunosorbant assay [48].

Animals were housed and cared for under standardised conditions according to [49]and [50]. In a time-period of one week the animals adapted to the new housing conditions. Twice a day commercial feed was supplied with the exception of the examination days and the day of infection, when pigs were only fed in the evening. The pigs were fed in groups. The calculated food ration per animal increased from 450g/day (day 0) to 625 g/day (day 21). The analysis of trace elements in food was done by graphite (Se) and flame (Cu, Fe, Zn) atomic absorption spectrometry, respectively after wet digestion according to national standardised chemical analysis of animal feed [51]. The food analysis for trace elements resulted in the following mean concentrations (wet weight, ww): 152 mg Cu/kg ww, 123 mg Zn/ kg ww, 299 mg Fe/kg ww, 0.45 mg Se/kg ww. The animals had free access to water (tap water quality).

The pigs were divided into two groups: Controls (n=10) and experimentally *A.pp.*-infected pigs (APP, n=20). General inspection of all animals was done daily. Before infection (day 0) and at days 4 and 21 after infection, all pigs were weighed, the breast circumference was measured and the rectal temperature was recorded. Moreover, a clinical examination was carried out, assessing the general appearance as well as specific respiratory signs.

At day 21, all pigs were euthanised by intravenous injection of 60 mg pentobarbital/kg bw (Euthadorm®, CP-Pharma, Burgdorf, Germany) and were sent to necropsy for general inspection and tissue collection from organs.

Experimental infection

All pigs were experimentally infected as previously described [52, 53]. In brief, pigs were exposed in groups of five animals for 10 minutes to the infectious aerosol in an aerosol chamber. A diluted bacterial culture was nebulised resulting in approximately 1 x 10^2 colony forming units of an *A.pp* serotype 2 strain per litre aerosol. For the mock challenge of controls 13 ml of a 154 mM sterile NaCl solution (B. Braun Melsungen AG, Melsungen Germany) was nebulised in the aerosol chamber.

General quality management

Deionised ultrapure water produced by reverse osmosis (R020 Reverse Osmose, Werner, Leverkusen, Germany) and subsequent desalination (Easy pure®, Werner, Leverkusen, Germany) was used for all analyses. All chemicals used were of highest quality and designated for laboratory analysis. Trace element analyses in serum as well as electrophoretic analyses of serum proteins were certified annually by an external quality control programme offered by the Society for Advancement of Quality Assurance in Medical Laboratories (Instand eV, Duesseldorf, Germany). Data from the internal laboratory quality control are given in Table 7.

Blood analysis

Pre-challenge blood samples were taken from all pigs by percutaneous puncture of the vena cava cranialis to analyse the initial status before infection for all investigated parameters. Thereafter, blood samples were taken from all pigs at day 4 and day 21 of the study.

Ethylenediaminetetraacetic acid (EDTA) and sodium heparin were used as anticoagulants for haematology and biochemistry in plasma, respectively. Serum was collected in tubes without additives. EDTA anticoagulated blood was used to analyse PCV, Hb concentration, WBC count as well as WBC differential (Haematology analyser, Celltag alpha, Nihon Kohden, Kleinmachnow, Germany). The WBC differential was double-checked microscopically by blood smear evaluation using Pappenheim panoptic staining. The MCHC was calculated (Hb/PCV). Serum was analysed for TP and ALB using the biuret method (L+T, Eberhard Lehmann GmbH, Berlin, Germany) and the bromcresol green method (L+T, Eberhard Lehmann GmbH, Berlin, Germany, respectively. The G/A ratio was calculated. α1-Globulin, α2-globulin, ß-globulin and γ-globulin were determined by serum electrophoresis (Elphoscan Mini Plus, Sarstedt, Nürmbrecht, Germany).

Cu, Fe and Se were analysed in serum. Zn was analysed in plasma due to the following reasons: (i) The amount of serum available for analysis was limited; (ii) Zn is very sensitive even to slight haemolysis [54] which is generally higher in serum due to the clotting procedure. Therefore, Zn was analysed in plasma continuously.

Zn (plasma) and Cu (serum) were analysed by flame atomic absorption spectrometry using a Cu/Zn multi-element lamp and air/acetylene gas (FLAAS, SOLAAR M, Thermo Fisher Scientific, Karlsruhe, Germany). A three/four point calibration for Zn/Cu was carried out, respectively. In order to adapt to the matrix, sodium chloride (0.9 %, Sodium chloride Suprapur®, Merck, Darmstadt, Germany) was used to prepare the calibration standards (Copper standard solution CertiPUR® / Zinc standard solution CertiPUR®, Merck, Darmstadt, Germany). Samples were diluted 1:10 with deionized ultrapure water. Se was analysed in serum by graphite furnace atomic absorption spectrometry (GFAAS) using Zeeman background correction, argon pure gas, omega cuvettes, a stabilised-temperature-platform-furnace technique (SOLAAR M, Thermo Fisher Scientific, Karlsruhe, Germany) and a four point calibration with matrix adaption using certified standards (Selenium standard solution

CertiPUR®, Merck, Darmstadt, Germany). The samples were diluted 1:5 with a palladium matrix modifier (ingredients: 2% Palladium matrix modifier for graphite furnace AAS Merck, Darmstadt, Germany; 0.2 % Triton® X-100, Fluka Chemie GmbH, Switzerland; 0.2 % Antifoam B Emulsion, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Fe was analysed in serum using a colorimetric test (FerroZine®, Labor + Technik, Berlin, Germany).

Certified reference materials (CRM) at two levels were used for quality control in trace element analysis (Se: ClinChek® Control, Level 1 & 2, Recipe, Munich, Germany; Cu: Seronorm™, Human & Human High, Invicon, Munich, Germany; Zn and Fe: Human Assayed Multi Sera, Level 2 & 3, Randox, UK). Additionally, internal quality control was extended by using a porcine pool sample.

TIBC represents the blood's capacity to bind Fe to serum proteins, predominantly to Tf. TIBC was measured (i) by adding supraphysiological amounts of Fe to saturate the free binding site of Tf, (ii) removal of unbound excess Fe by absorption onto solid magnesium carbonate and (iii) finally determining Fe that is dissociated from Tf at acidic pH as reviewed by Kasvosve and Delanghe [55] using a commercial test kit (Labor + Technik, Berlin, Germany). Tf was calculated from TIBC according to the manufacturer's instructions. Serum CRP was determined by ELISA (Phase Porcine CRP Assay, Tridelta Development Ltd, Maynooth, Ireland). Hp was analysed in serum using a colorimetric method (Tridelta Phase Haptoglobin Assay, Tridelta Development Ltd, Maynooth, Ireland). Serum Cp was measured photometrically using N,N-Dimethyl-p-P-phenylenediamine-di-hydrochloride as a substrate in sodium acetate buffer referring to the method described by Martinez-Subiela and colleagues [56]. Plasma GPx (GPx3) was analysed according to Paglia and Valentine [57] and normalised to g of protein concentration.

Fe, Zn, Cu and Se in liver tissue

The liver tissue of the animals was collected at necropsy (day 21 after infection). The samples were weighed in small tubes, shock-frozen in liquid nitrogen and stored at -80°C until analysis. Trace element concentrations were determined by GFAAS (Se) and FLAAS (Fe, Cu, Zn) (SOLAAR M, Thermo Fisher Scientific, Karlsruhe, Germany) after sample digestion using 4ml HNO_3 (Nitric Acid 65% Suprapur®, Merck, Darmstadt, Germany) and 1ml H_2O_2 (Hydrogen peroxide 30% Suprapur®, Merck, Darmstadt, Germany) in polytetrafluorethylene vessels (Teflon®) in a microwave digestion unit (Start, MLS GmbH, Leutkirch, Germany). The equipment contains an automatic temperature control system which allows continuous monitoring and control (±1°C) of internal temperature within a standard reference vessel.

Sample dilution for Se measuring was done by dilution (1:5) with a palladium matrix modifier as described for Se analysis in serum. Sample dilution for Cu, Zn and Fe was done using deionised ultrapure water (1:20). The calibrations standards were prepared using commercial standard solutions (CertiPUR®, Merck, Darmstadt, Germany) and nitric acid (Nitric Acid 65% Suprapur®, Merck, Darmstadt, Germany). All concentrations were adjusted to tissue wet weight (ww). Internal quality control was realised by using an internal standard (aliquots of liver tissue, shock-frozen in liquid nitrogen and stored at -80°C until analysis).

Cytosolic GPx activity in liver tissue

For enzymatic analysis of cytosolic GPx activity in liver tissue, 1:25 (w/v) crude homogenates were prepared in ice-cold HEPES-buffer in a glass tube on ice using a Potter-Elvehjem-homogeniser with four intervals (15 sec) of homogenisation and 4 intervals (15 sec) of specimen sedimentation in between. The homogenate was centrifuged at 17000xg for 10 min at 4°C. The supernatant was collected in a new tube, shock-frozen in liquid nitrogen and stored at -80°C until analysis.

GPx activity was analysed according to Paglia and Valentine [57]. Enzyme activities were normalised to g of protein concentration in the homogenate. Protein concentration was determined using the Bradford method [58].

Statistics

All calculations were carried out using SAS 9.3 (SAS Institute Inc., Cary, North Carolina, USA). The normality of distribution was tested by the Shapiro-Wilk test. Data were analysed by ANOVA for repeated measurements. Moreover, differences in concentrations of parameters analysed in blood over time within groups after challenge were tested by the t-test for repeated measurements or Wilcoxon signed-rank-test for repeated measurements, respectively. Differences between controls and APP before infection and at days 4 and 21 after infection were evaluated using the Student`s t-test. If data had no normality of distribution, the Wilcoxon two-sample test was used. Data are presented in mean and SD or median and interquartile range (IQR) depending on normal or non-normal distribution of data, respectively.

Conclusion

In summary, the model of *A.pp.*-infection proved advantageous for evaluating trace element response showing a distinct impact and the individual effects of bacteria-induced APR on Fe, Zn, Cu and Se metabolism. Acute *A.pp.*-infection caused a significant but transient decrease in Fe, Tf and TIBC in serum not affecting the long-term hepatic Fe concentration of the animals. Most likely, this hepcidin-regulated procedure exists to reduce Fe availability for the pathogen. The transient elevation of plasma Cu concentration may predominately be a side effect of moderate induction of liver Cp synthesis in APR of the *A.pp.*-infected pigs not affecting the long-term hepatic Cu concentration of the animals. The Zn concentration in

serum at day 21 was found at levels equal to the pre-challenged state and the lower Zn concentration in APP piglets was transient and obvious in the APR at day 4 only. Notwithstanding the multilateral discussion of several underlying mechanisms in the literature [38], distinctly higher Zn concentration in the liver tissue of our *A.pp.*-infected piglets at day 21 markedly reflect the sequestration of Zn into this organ. Serum and liver Se concentrations in *A.pp.*-induced APR showed higher serum Se concentrations at day 4 and lower liver Se concentrations at day 21 in the *A.pp.*-infected animals compared to controls. Therefore, the reactions of these parameters were contradictory to findings in rodents 24 hours after being challenged with LPS. Due to the fact that current knowledge of the influence in subacute and even chronic periods of APR initiated by bacteria is still very limited, this observation requires further investigation. However, the selenoproteins GPx1 and GPx3 in liver and plasma remained unaffected.

Finally, an additional fact should be mentioned: Frequently, the onset of a lung infection on pig farms initiates diagnostic blood sampling of the diseased animals. As shown in our study, there are several APR-related changes in trace element concentrations in blood and liver samples. These samples are unsuitable for assessing the current nutritional trace element status of the herd and may cause misleading interpretations.

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Tables

Table 1: Selected haematological parameters in APP piglets and controls at the three times of sampling. Data are given as mean (standard deviation) or median (interquartile range).

					repeated measurement (p-value)		
parameter	**group**	**day 0**	**day 4**	**day 21**	**day 0 - day 4**	**day 0 - day 21**	**day 4 - day 21**
Hb [g/l]	control	137 (9.2)	128 (9.1)	108 (6.2)	n.s.	<0.001	<0.001
	APP	134 (7.7)	113 (10.7) [c]	107 (8.8)	<0.001	<0.001	<0.05
PCV [l/l]	control	0.379 (0.023)	0.365 (0.026)	0.309 (0.015)	n.s.	<0.001	<0.001
	APP	0.373 (0.018)	0.318 (0.030) [c]	0.306 (0.022)	<0.001	<0.001	n.s.
MCHC [g/l]	control	360 (6.53)	351 (7.76)	350 (9.98)	<0.05	<0.05	n.s.
	APP	360 (9.34)	355 (7.88)	351 (10.70)	n.s.	<0.05	n.s.
total WBC [x 10^9/l]	control	10.18 (4.09)	11.71 (2.53)	14.51 (3.25)	n.s.	<0.01	n.s.
	APP	10.34 (5.97)	21.70 (3.69) [c]	16.21 (5.03)	<0.001	<0.001	<0.001
banded granulocytes [x 10^9/l]	control	0.080 (0.000-0.480)	0.075 (0.00-0.280)	0.185 (0.080-0.330)	n.s.	n.s.	n.s.
	APP	0.095 (0.045-0.170)	0.830 (0.30-1.585) [b]	0.110 (0.035-0.185)	<0.01	n.s.	<0.001
segmented granulocytes [x 10^9/l]	control	4.65 (2.24)	6.41 (1.74)	6.41 (1.83)	n.s.	n.s.	n.s.
	APP	5.12 (2.01)	13.90 (2.81) [c]	9.21 (4.41) [a]	<0.001	<0.001	<0.001
lymphocytes [x 10^9/l]	control	4.30 (0.99)	4.86 (1.26)	7.56 (2.15)	n.s.	<0.001	<0.01
	APP	3.66 (0.83)	5.96 (1.45)	6.41 (1.72)	<0.001	<0.001	n.s.
monocytes [x 10^9/l]	control	0.075 (0.05-0.13)	0.225 (0.08-0.54)	0.265 (0.12-0.57)	<0.05	<0.01	n.s.
	APP	0.190 (0.06-0.28)	0.730 (0.62-0.88) [a]	0.300 (0.11-0.37)	<0.001	n.s.	<0.001

Significant differences between groups (controls vs APP): [a] $p<0.05$ [b] $p<0.01$ [c] $p<0.001$ n.s.= not significant

Table 2: Major fractions of serum proteins in APP piglets and controls at the three times of sampling. Data are given as mean (standard deviation)

					repeated measurement (p-value)		
parameter	**group**	**day 0**	**day 4**	**day 21**	**day 0 - day 4**	**day 0 - day 21**	**day 4 - day 21**
TP [g/l]	control	47.3 (2.5)	43.7 (2.7)	40.9 (4.2)	<0.001	<0.001	<0.05
	APP	45.0 (2.4)[a]	45.1 (3.4)	45.9 (4.3)[b]	n.s.	n.s.	n.s.
ALB [g/l]	control	30.6 (1.8)	26.1 (1.4)	22.9 (5.0)	<0.001	<0.001	<0.05
	APP	30.8 (3.5)	21.1 (2.5)[c]	19.8 (4.1)	<0.001	<0.001	n.s.
G/A	control	0.550 (0.053)	0.675 (0.063)	0.832 (0.234)	n.s.	n.s.	n.s.
	APP	0.475 (0.147)	1.146 (0.145)[c]	1.389 (0.425)	<0.001	<0.01	<0.001
α1-globulin [g/l]	control	0.740 (0.096)	0.770 (0.106)	0.720 (0.114)	n.s.	n.s.	n.s.
	APP	0.745 (0.100)	0.775 (0.112)	0.760 (0.143)	n.s.	n.s.	n.s.
α2-globulin [g/l]	control	8.68 (0.90)	8.52 (1.31)	8.94 (1.28)	n.s.	n.s.	n.s.
	APP	7.84 (0.74)[a]	13.1 (1.48)[c]	10.3 (1.16)[b]	<0.001	<0.001	<0.001
β-globulin [g/l]	control	6.32 (0.82)	6.42 (1.03)	6.39 (0.74)	n.s.	n.s.	n.s.
	APP	5.87 (0.90)	7.25 (1.48)	8.11 (1.08)[c]	<0.001	<0.001	<0.05
γ-globulin [g/l]	control	3.55 (0.52)	3.26 (0.49)	3.25 (0.58)	n.s.	n.s.	n.s.
	APP	3.25 (0.99)	3.89 (1.33)	6.67 (2.22)[c]	n.s.	<0.001	<0.001

Significant differences between groups (controls vs APP): [a] $p<0.05$ [b] $p<0.01$ [c] $p<0.001$ n.s.= not significant

Table 3: APR - related parameters in serum of APP piglets and controls at the three times of sampling. Data are given as mean (standard deviation) or median (interquartile range).

					repeated measurement (p - value)		
parameter	**group**	**day 0**	**day 4**	**day 21**	**day 0 - day 4**	**day 0 - day 21**	**day 4 - day 21**
CRP [mg/l]	control	120 (94.4-293)	267 (32.7-352)	90.9 (33.2-113)	n.s.	n.s.	n.s.
	APP	140 (35.5-224)	2321 (1476-4144)[c]	44.4 (22.0-191)	<0.001	n.s.	<0.001
Hp [mg/l]	control	563 (220)	537 (120)	934 (557)	n.s.	n.s.	n.s.
	APP	423 (149)	2811 (750)[c]	593 (395)	<0.001	n.s.	<0.001
Cp [mg/l]	control	1471 (1224-1697)	1612 (1349-2006)	1304 (1132-1483)	<0.01	n.s.	<0.01
	APP	1464 (1342-1831)	2687 (2532-2821)[c]	1402 (1280-1460)	<0.001	<0.05	<0.001
Tf [mg/l]	control	2681 (621)	2184 (598)	2603 (596)	n.s.	n.s.	n.s.
	APP	2503 (719)	1674 (489)[a]	2325 (610)	<0.001	n.s.	<0.001
TIBC [µmol/l]	control	57.8 (50.6-77.1)	47.8 (41.7-62.1)	64.1 (55.5-67.4)	n.s.	n.s.	n.s.
	APP	55.0 (44.2-65.0)	33.9 (29.4-48.2)a	51.9 (42.6-58.5)	<0.001	n.s.	<0.001

Significant differences between groups (controls vs APP): [a] p<0.05 [b] p<0.01 [c] p<0.001 n.s.= not significant

Table 4: Concentrations of selected trace elements in serum (Se, Cu, Fe) and plasma (Zn) of APP piglets and controls at the three times of sampling. Data are given as mean (standard deviation) or median (interquartile range).

					repeated measurement (p-value)		
parameter	**group**	**day 0**	**day 4**	**day 21**	**day 0 - day 4**	**day 0 - day 21**	**day 4 - day 21**
Fe [µmol/l]	control	24.0 (7.5)	25.7 (8.0)	16.8 (7.4)	n.s.	<0.05	<0.05
	APP	16.8 (6.8)[a]	6.7 (2.5)[c]	20.7 (10.2)	<0.001	n.s.	<0.001
Zn [µmol/l]	control	12.3 (2.6)	9.3 (1.7)	12.5 (2.4)	<0.05	n.s.	<0.01
	APP	10.9 (1.3)	6.2 (0.8)[c]	11.5 (2.5)	<0.001	n.s.	<0.001
Cu [µmol/l]	control	36.0 (34.1-39.0)	29.3 (26.3-31.2)	25.5 (23.9-27.5)	<0.01	<0.01	n.s.
	APP	35.4 (32.3-37.3)	41.8 (40.0-43.0)[c]	25.8 (22.7-33.3)	<0.001	<0.001	<0.001
Se [µg/l]	control	79.4 (13.5)	80.6 (19.9)	96.5 (8.4)	n.s.	<0.001	<0.001
	APP	83.7 (13.1)	89.3 (10.7)[a]	96.3 (7.6)	<0.01	<0.001	<0.01

Significant differences between groups (controls vs APP): [a] $p<0.05$ [c] $p<0.001$ n.s.= not significant

Table 5: Activity of glutathione peroxidase in plasma (GPx3) and liver tissue (GPx1) of APP piglets and controls at the three times of sampling/ post mortem (liver GPx1). Data are given as mean (standard deviation)

					repeated measurement (p - value)		
parameter	**group**	**day 0**	**day 4**	**day 21**	**day 0 - day 4**	**day 0 - day 21**	**day 4 - day 21**
Plasma GPx3 [U/g protein]	control	55.6 (9.37)	65.4 (9.39)	68.5 (9.46)	<0.05	<0.01	<0.05
	APP	54.9 (11.8)	61.2 (11.3)	61.6 (12.8)	<0.01	<0.01	n.s.
Liver GPx1 [U/g protein]	control	-	-	478 (60.4)	-	-	-
	APP	-	-	533 (135)	-	-	-

Differences between groups (controls vs APP): not significant; - = no data available n.s.= not significant

Table 6: Fe, Zn, Cu and Se concentrations in liver tissue (wet weight) of APP pigs and controls at day 21. Data are given as mean (standard deviation) or median (interquartile range)

parameter	group	day 21
Fe [mg/kg]	control	304 (292-406)
	APP	291 (252-440)
Zn [mg/kg]	control	58.5 (47.4 - 75.9)
	APP	80.1 (67.3 - 85.0) [a]
Cu [mg/kg]	control	52.2 (30.2)
	APP	60.7 (23.0)
Se [mg/kg]	control	0.596 (0.087)
	APP	0.507 (0.090) [a]

Significant differences between groups (controls vs APP): [a] $p<0.05$

Table 7: Data from internal quality control

Parameter	Method	Inter-assay coefficient of variation (%)	Detection limit
Cu/Zn (liver incl. sample digestion)	Flame AAS (SOLAAR M, Thermo Fisher Scientific, Karlsruhe, Germany)	7.7 / 5.6	3 µg/l*
Se (liver incl. sample digestion)	GFAAS (SOLAAR M, Thermo Fisher Scientific, Karlsruhe, Germany)	14.5	3 µg/l*
Fe (liver incl. sample digestion)	Flame AAS (SOLAAR M, Thermo Fisher Scientific, Karlsruhe, Germany)	6.5	9.2 µg/l
Protein (tissue homogenate)	Bradford method (Biorad Laboratories, München, Germany)	3.6	0.2 g/l
Fe (serum)	FerroZine® (L+T, Eberhard Lehmann GmbH, Berlin, Germany)	11.6	0.90 µmol/l
TIBC (serum)	TIBC Test (Precipitating agent, L+T, Eberhard Lehmann GmbH, Berlin, Germany)	n.a.	2.70 µmol/l
Se/Cu(serum) Zn (plasma)	GFAAS/FLAAS (SOLAAR M, Thermo Fisher Scientific, Karlsruhe, Germany)	5.3 / 4.7 4.0	3 µg/l*
Plasma GPx3	Paglia/Valentine method (Randox, Crumlin, UK)	6.4	1 U/l
Liver GPx1	Paglia/Valentine method (Randox, Crumlin, UK)	17.5	1 U/l
TP	Biuret method (L+T, Eberhard Lehmann GmbH, Berlin, Germany)	1.75	2 g/l
ALB	Bromcresol green method (L+T, Eberhard Lehmann GmbH, Berlin, Germany)	3.2	2 g/l
α1-globulin	Electrophoresis, Elphoscan Mini Plus (Sarstedt, Nümbrecht Germany)	16.3	n.a.
α2-globulin	Electrophoresis, Elphoscan Mini Plus (Sarstedt, Nümbrecht Germany)	13.6	n.a.
β-globulin	Electrophoresis, Elphoscan Mini Plus (Sarstedt, Nümbrecht Germany)	10.9	n.a.
γ-globulin	Electrophoresis, Elphoscan Mini Plus (Sarstedt, Nümbrecht Germany)	6.9	n.a.
Hp (serum)	Colorimetric assay (Tridelta Development Ltd, Maynooth, Ireland)	5.7	n.a.
CRP (serum)	Solid phase sandwich Immunoassay (Tridelta Development Ltd, Maynooth, Ireland)	9.7	4.7 mg/l
Cp (serum)	DPD (N,N-Dimethyl-p-P-phenylenediamine-di-hydrochloride) colorimetric method (in-house method)	4.9	3.3 mg/l

All data were evaluated during the routine internal quality control procedure using internal species specific (if available) or commercial reference materials, n.a.: data not available. * Instrumental detection limit.