

Monitoring of the course of sepsis in hematooncological patients by extrapituitary prolactin expression in peripheral blood monocytes

Pavlina Cejkova^{1,2}, Vera Chroma¹, Marie Cerna², Marketa Markova³, Josef Marek⁴, Zdenka Lacinova⁴, Martin Haluzik⁴

¹ Department of Anthropology and Human Genetics, Faculty of Science, Charles University in Prague, Czech Republic

² Department of General Biology and Genetics, Third Faculty of Medicine, Charles University in Prague, Czech Republic

³ Institute of Hematology and Blood Transfusion, Prague, Czech Republic

⁴ Third Department of Medicine, General University Hospital and First Medical Faculty, Charles University in Prague, Czech Republic

Corresponding author

RNDr. Pavlina Cejkova, Ph.D.

Department of Anthropology and Human Genetics

Faculty of Science

Charles University in Prague

Vinicna 7, 123 42 Prague 2

Czech Republic

Tel.: +420 221 951 622

Fax: +420 221 951 618

E-mail: pavlina.cejkova@lf3.cuni.cz

Short title

Monocytic PRL, TLR2 and TLR4 in sepsis

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Summary

Our study explored the role of extrapituitary prolactin (PRL) and toll-like receptors (TLR)2 and TLR4 in defense reaction of immune system to bacterial infection. Forty-two patients diagnosed with sepsis were recruited and blood samples were withdrawn after patients' admission to hospital, after the end of acute phase of sepsis and after the sepsis has been resolved, respectively. Seventeen patients died of sepsis; thus, only one sample collected just before death could be processed. PRL and TLR2/4 mRNA levels were measured in CD14+ blood monocytes by QPCR and *PRL* -1149 G/T SNP genotyped. The TLRs mRNA expression was markedly elevated in all patients groups in comparison to healthy controls mRNA levels; the highest upregulation of monocytic TLR2 in sepsis (16.4 times, $P < 0.0001$) was detected in patients who did not survive septic complications. PRL mRNA expression in monocytes from non-survivors tended to be lower (4.5 fold decrease, $P = \text{NS}$) compared to control levels and it was 6.2 times reduced compared to PRL mRNA expression in second blood sample from survivors ($P < 0.05$). The *PRL* -1149 G/T SNP had no effect on PRL mRNA response during sepsis. Our data suggest that increased prolactin mRNA expression in monocytes is associated with better outcome and improved survival rate in sepsis with no apparent effect of *PRL* -1149 G/T SNP on monocytic prolactin response.

Keywords

Prolactin, Toll-like receptors, mRNA, monocytes, sepsis

Introduction

Activation of innate immunity includes the production of the pattern recognition receptors (PRR) such as toll-like receptors (TLR) 2 and 4 and is considered an initial event in the immune defense response against exogenous pathogens (reviewed in Medzhitov 2001). In peripheral blood monocytes/macrophages, the activation of PRR may trigger the release of IL-1, IL-6 and TNF α and stimulate NF κ B and JNK kinase signaling cascades followed by pro-inflammatory cytokine transcription (Salomao *et al.* 2008).

Sepsis, the systemic inflammatory response to severe infection frequently accompanied by multiple organ failure (MOF), is responsible for significant morbidity and mortality rate in critically ill patients. In addition to immune response, sepsis elicits a very reproducible pattern of pituitary hormone secretion, with plasma adrenocorticotrophic hormone (ACTH) and prolactin (PRL) increasing within a few minutes following the insult (Sternberg 2006). The subsequent rapid activation of adrenal steroid release is a key event in the organism's first line of defense during severe illness. TLR2 and TLR4 receptors expressed in cells of human adrenal cortex (Bornstein *et al.* 2004) and TLR ability to influence glucocorticoid production during sepsis propose direct effect of adrenal glands on host defense (Bornstein *et al.* 2006). The picture of bidirectional crosstalk between neuroendocrine and immune systems is completed by the fact that pituitary cells express PRR and can directly recognize and respond to fungal pathogen-associated molecular patterns (PAMPs). This stimulates pituitary cell TLR4 and CD14 gene expression, increases IL-6 production and PRL secretion (Breuel *et al.* 2004).

Contraregulatory role of prolactin against activation of adrenocortical system has been recognized for a long time. PRL may act directly on adrenal gland to drive corticosterone secretion (Glasow *et al.* 1996) and, PRL receptor expression in cells of adrenal cortex in several species has been discovered (Glasow *et al.* 1998; Glasow *et al.* 1996). Relative PRL

insufficiency was documented in more than 50% cases of neonatal sepsis, indicating thus the significance of immunostimulatory role of prolactin in critical illness (Felmet *et al.* 2005).

Prolactin is a plurifunctional hormone produced by pituitary gland and a number of extrapituitary tissues including immune cells T-lymphocytes, monocytes and macrophages. PRL is known to enhance monocyte, T- and B-lymphocyte immune responses under normal conditions and to have a beneficial effects on cell-mediated immunity (Chavez-Rueda *et al.* 2005; Matalka 2003; Dimitrov *et al.* 2004; Lahat *et al.* 1993). It also stimulates IFN- γ and many other Th1-type cytokines, has comitogenic activity and prevents immune cells from apoptosis.

In this pilot study we tested the hypothesis that prolactin produced by peripheral blood monocytes is involved in the initiation and course of sepsis in hematooncological patients with potentially salutary effects on the septic patients' outcome. Furthermore, we explored the significance of TLR2 and TLR4 expression in circulating monocytes in the outcome of septic patients. To this end, we measured mRNA expression of PRL, TLR2 and TLR4 in circulating monocytes throughout the course of sepsis and tested the potential significance *PRL* -1149 G/T SNP in prolactin mRNA expression response.

Methods

Subjects

For the pilot study 42 hematooncological patients diagnosed with severe infectious event characterized by septicemia and/or multiple organ failure and hospitalized at the Institute of Hematology and Blood Transfusion in Prague, Czech Republic were recruited (age 47.14 ± 2.20 years (mean \pm SEM); 29 male, 13 female). All included patients were treated with broad spectrum antimicrobial regimens (both anti-bacterial and anti-fungal), following the internal guidelines for antimicrobial treatment, modified according to culture results. In 25 patients, collection of their blood samples was performed three times: The first sample (A) was gained after patients' admission to the hospital; the second sample (B) after the end of acute phase, characterized by temperature normalization below 38°C , CRP decrease below 100 mg/l, circulatory stabilization with no need of pressure support and no further organ involvement progression; the third sample (C) when the episode has been completely resolved, with no infectious symptoms, usually at the time of patients discharge from the hospital. The other 17 patients participating in our study did not survive septic complications and therefore, only one sample collected prior to death could be used for the study (non-survivors, N-S).

Absolute leukocyte counts (ALC) were recorded as measured by a routine hospital laboratory complete blood count. Leukopenia was defined as $\text{ALC} < 4.1 \times 10^9/\text{l}$ blood. For determination of infectious etiology routine cultures were performed, together with galactomannan antigen assessment in mold infection. Four groups of patients were fixed: Gram-positive (40.5%), Gram-negative (21.4%), mold (9.5%), further not specified (28.6%).

In total, 27 patients (77.2%) underwent transplantation therapy. Leukocyte numbers in specimens collected after transplantation were in average twice as high as in comparison to specimens prior or without transplantation ($P < 0.05$). Nevertheless, since the observed

increase of ALC and the transplantation therapy *per se* did not influence the prolactin gene expression in monocytes (data not shown), we did not further include the transplantation yes/no information into our analyses.

Control group comprised 40 healthy individuals, voluntary blood donors from the Transfusion Unit of the Faculty Hospital Kralovske Vinohrady (TU FHKV), Prague, Czech Republic (age 41.13 ± 1.77 years (mean \pm SEM); 28 male, 12 female). Health of the control individuals and his/her eligibility for blood donation was judged according to the current TU Standard Operating Procedure: Minimum 2 weeks after complete clinical recovery from infectious disease (if not indicated otherwise) required; allergic persons were not eligible for blood donation during acute phase of allergic reaction or if they suffered from intermediate or severe not stabilized allergic disorder. One blood taking from each healthy subject together with his/her past history was conditioned by subjects acquainted approval. In this control group no clinical tests were performed, only DNA typing and mRNA analysis were carried out.

DNA extraction and PRL -1149 G/T SNP genotyping

Genomic DNA from peripheral blood samples collected into EDTA vacutainers was isolated by QiaAmp DNA Mini Kit spin columns (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions.

PRL extrapituitary SNP -1149 G/T genotyping was performed by PCR-RFLP method with originally designed oligonucleotides as described elsewhere (Fojtikova *et al.* 2007). After PCR amplification of 137 bp long fragment, XapI digestion of the amplified DNA fragment was carried out at 37°C for 2 hours and stopped by heat inactivation at 80°C for 20 minutes. The digested and undigested products were visualized using ethidium bromide staining after electrophoresis on a 4% agarose gel.

Monocyte isolation and RNA extraction

Peripheral blood monocytes were separated by CD14 positive immunomagnetic beads (Dyna, Invitrogen, Carlsbad, Ca, USA) following the recommended protocol. Separated monocytes were subjected to RNA isolation by RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) with minor modification. Total amount and purity of RNA were determined by spectrophotometry.

Quantitative real-time PCR (QPCR)

For testing *PRL*, *TLR2* and *TLR4* gene expression in monocytes, ABI 7000 SDS real-time PCR instrument and TaqMan-based gene expression Assays on Demand (Applied Biosystems, Foster City, California, USA) were used: Hs00168730_m1 (*PRL*), Hs00152932_m1 (*TLR2*), Hs00152939_m1 (*TLR4*). As an internal control served human *PGK1* (Phosphoglycerate Kinase-1) gene. The 2^{-dCt} method was applied for relative quantification and detected mRNA values were depicted in arbitrary units (AU); in order to present acquired data uncluttered and with better resolution, values in *PRL* assay were multiplied by 10^6 and in *TLR2* and *TLR4* assays by 10^3 .

Functionality and reliability of the *PRL* assay has been validated by using decidua cells as a positive control, and human HepG2 cell line that served as a negative control.

Statistical analysis

For statistical evaluation Epi Info software (Version 3.3 October 2004, Atlanta, Georgia) and GraphPadPrism software were used. To test for normality we used D'Agostino-Pearson normality test. Data are presented as median with 25% and 75% percentiles, respectively. Data involving two groups were compared using Mann-Whitney rank-sum test

for unpaired values or Wilcoxon test for paired observations; data involving three or more groups were compared by Kruskal-Wallis test or Friedman test for repeated measures. Significance was accepted at $P < 0.05$. Allele and genotype frequencies were determined by direct counting. Statistical differences in allele or genotype distributions were analyzed by either the χ^2 or the Fischer's exact probability test. Significance was defined using a Bonferroni corrected P value lower than 0.05.

Results

Effect of -1149 G/T SNP on physiological levels PRL mRNA in healthy controls

PRL mRNA levels were compared among each genotype group in healthy controls (N = 40). As shown in Figure 1, PRL mRNA expression in GG genotype (median 227.8 AU, 59.8 – 345.8) tended to be higher than in GT (median 113.2 AU, 93.4 – 174.5) and than in TT genotypes (median 104.8 AU, 100 – 285.5), respectively. However, the observed differences did not reach statistical significance (P = NS).

PRL mRNA expression in peripheral blood monocytes derived from all tested groups

Although the physiological mRNA levels of the extrapituitary PRL are in general very low (median 117.1 AU, 93.5 – 227; Figure 2), we observed a tendency to decreased expression in non-survivors (median 25.8 AU, 6.5 – 66.3; P = NS). On the contrary, PRL mRNA expression in sepsis survivors was comparable to that of healthy controls in sample A collected at the time of diagnosis (median 108.5 AU, 46.9 – 1011), in sample B (end of acute phase of sepsis) with a slight tendency to increase (median 160 AU, 41.5 – 439; P = NS), as well as in sample C (after resolution of sepsis) (median 88.1 AU, 13.5 – 352.6; P = NS) with mild decreasing tendency. However, we could observe statistically significant difference of 6.2 magnitude in median of PRL mRNA levels between non-survived patients with the lowest PRL mRNA production and median of the highest PRL mRNA expression in sample B of the survived patients (P < 0.05, Figure 2).

TLR2 mRNA expression in peripheral blood monocytes derived from all tested groups

Both TLR2 and TLR4 mRNA expression in all studied patient groups showed steep increase compared to mRNA levels in controls (Figure 3 and 4). Interestingly, median TLR2 mRNA levels in survivors was markedly higher in comparison to healthy individuals but not as much

as in non-survivors: The highest TLR2 mRNA expression was detected in non-survivors (median 166.1 AU, 104.8 – 352.1; 16.4 fold increase compared to control subjects, $P < 0.0001$), followed by stepwise decrease from sample A of survived patients (median 100.8 AU, 42.1 – 138; 10 fold increase in comparison to controls, $P < 0.0001$) through sample B (median 86.6 AU, 63.4 – 124.6; 8.5 fold increase when compared to controls, $P < 0.0001$) down to sample C (median 65.6 AU, 40.7 – 120.1; 6.5 fold increase in comparison to control group, $P < 0.0001$, Figure 3).

TLR4 mRNA expression in peripheral blood monocytes derived from all tested groups

Except for samples derived from non-survived patients, the median TLR4 mRNA expression was in general higher in all studied groups than that of TLR2 (Figure 4). Healthy control subjects (median 13.9 AU, 10.7 – 19.8) showed 11.75 times lower mRNA expression than detected in non-survived patients (median 163.8, 88.4 – 279.6; $P < 0.0001$). After an initial increase in sample A in patients who survived septic event (median 150.7 AU, 94.1 – 267; 10.8 fold higher compared to control subjects, $P < 0.0001$) we observed further increase in sample B (median 176.8 AU, 162.7 – 264.4; 12.7 fold higher compared to control subjects, $P < 0.0001$) followed by final decrease below the A sample level in sample C (median 119.9 AU, 87.8 – 216.2; 8.6 fold higher compared to control individuals, $P < 0.0001$).

Pathogen type and its effect on TLR2 and TLR4 mRNA expression

Based on the pathogen type detected in patients' hemoculture we split patients into four groups: G+ bacteria, G- bacteria, mold, not specified. Nevertheless, we were not able to find an association between any type of pathogen and level of TLR2 and TLR4 mRNA (data not shown).

Discussion

Our study was designed to evaluate the role of extrapituitary prolactin produced by peripheral blood monocytes of hematooncological patients with sepsis and to determine the impact of innate immune mechanisms represented mainly by action of Toll-like receptors on the immunological processes accompanying sepsis. Furthermore, we tested the potential significance of *PRL* -1149 G/T SNP on prolactin mRNA expression response to sepsis.

Previously published study carried out on Jurkat cells demonstrated that the -1149 G allele has changed consensual sequence and significantly increased promoter activity compared to the -1149 T allele (Stevens *et al.* 2001). In line with this, we expected highest *PRL* mRNA levels associated with G allele and GG genotype. In our study, G allele in homozygous constitution tended to increase expression of *PRL* gene (2 fold increase in comparison to TT homozygotes) but the difference did not reach statistical significance, most probably owing to relatively small sample size. Further, we focused on potential effect of the *PRL* -1149 G/T SNP on *PRL* mRNA expression in three consecutive blood drawings in septic patients but we did not detect any specific pattern (data not shown). Taken together, our data do not support the impact of *PRL* -1149 G/T genotype on the course of prolactin production by monocytes during sepsis.

It is important to note that similarly to primary human lymphocytes and other extrapituitary sites, PRL release from peripheral blood monocytes under physiological conditions is very low, compared to its pituitary production. These findings suggest specific regulatory mechanisms and different mode of action of extrapituitary prolactin from prolactin produced by anterior pituitary and also its different activity at the systemic level. It is conceivable that extrapituitary PRL acts rather locally than systemically (in lymph nodes, spleen or organs infiltrated by immune cells etc.) and thus, small amounts of immune cell-

derived PRL may achieve high concentrations in the adjacent micro-environment (Montgomery 2001).

For analysis of the PRL mRNA expression in sepsis, 25 patients who survived the condition and 17 patients who died of septic complications were compared. Based on the previous results gained on pituitary prolactin (Dostal *et al.* 2003) we expected augmentation of PRL expression early after development of sepsis with subsequent stepwise decrease along with diminution of septic signs. However, data gained from our survivors subgroup suggest a delay in activation of PRL expression in monocytes, at least in some of examined subjects (Figure 2). We hypothesize that the delay in mRNA expression observed in our study may be explained by the precedential choice of utilization of intracellular PRL (IC-PRL) protein from storage vesicles as a prompt source of prolactin. Interestingly and contrary to our hypothesis, Ben Jonathan and colleagues found no evidence for PRL storage granules at extrapituitary sites (Ben Jonathan *et al.* 2008). These authors suggested that the absence of storage granules implies constitutive PRL release rather than the calcium-dependent exocytosis like in lactotrophs. To further dissect this controversy we performed immunohistochemic staining of PBMC using monoclonal antibody against human prolactin (AbD Serotec) and in contrast to aforementioned Ben Jonathans' data we were able to see IC-PRL localized mainly in close vicinity to nucleus and in 25% of cells also in vesicle-like depots in cytoplasm (data not shown).

In 17 patients who died of sepsis, the pattern of PRL mRNA expression was different from sepsis survivors. We observed non-significant tendency towards decrease in PRL mRNA expression compared to healthy subject levels and even 6.2 times lower expression in comparison with the highest PRL mRNA levels detected in the second blood drawing B from survivor subgroup ($P < 0.05$). It has already been demonstrated that prolactin is important for maintaining normal immune response in several pathological states; moreover, PRL ability to

improve survival of animals subjected to sepsis after hemorrhage has been documented (Zellweger *et al.* 1996b; Zellweger *et al.* 1996a). Our finding is in line with results of work by Felmet and colleagues who studied the effect of hypoprolactinemia in children with nosocomial sepsis. Felmet reported that hypoprolactinemia aggravated septic condition and that PRL inhibited stress-induced apoptosis of lymphocytes, confirming the significance of PRL anti-apoptotic effect (Felmet *et al.* 2005).

Since our data revealed tendency to the lowest PRL mRNA levels in monocytes from patients who died of sepsis, we found it interesting to discover any potential correlation between mortality in sepsis and *PRL* -1149 G/T genotypes. Nevertheless, there was no effect of studied SNP on patients' survival (data not shown). Despite the known impact of *PRL* -1149 alleles on *PRL* transcription in immune cells (Stevens *et al.* 2001) our data did not show any effect of this polymorphism on PRL mRNA levels.

The innate immune system, particularly Toll-like receptors provide the first line of defense against invading bacteria and other pathogens (for review, see Knapp 2010). Monocytes are the key cellular component of the innate immunity with high expression of Toll-like receptors in response to bacterial infection and sepsis and are of great importance in immune system defense against various pathogens (Haveman *et al.* 1999; Jiang *et al.* 2005). Here we show, similarly with previously published results (Tsujiimoto *et al.* 2004; Armstrong *et al.* 2004), that both TLR2 and TLR4 mRNA monocytic expression was upregulated in all groups of patients in comparison to the basal state in healthy subjects. This finding mirrors the ongoing inflammation and the immune system response against infection. We observed higher increase in TLR2 mRNA expression in non-survivor compared to survivor group suggesting inappropriate response of non-survivals immune system before death. In survivals group, gradual decrease in *TLR* genes mRNA expression along with diminution of clinical signs of sepsis was observed. Intriguingly, even in sample C withdrawn after resolution of

septic episode and confirmed clinical improvement, neither TLR2 nor TLR4 mRNA levels reached the basal levels comparable to those in healthy controls.

In monocytes, CD14 receptor facilitates TLR2 and TLR4 activation through transferring TLR-specific ligands (Wright *et al.* 1990). However, there is increasing evidence that bacterial peptides are not the only ligands that can activate TLR2 and TLR4. It has been demonstrated that TLR2 and TLR4 are involved also in trauma where Gram-negative or Gram-positive bacteria were not detected in circulation or local organ sites (Lorne *et al.* 2010). This fact may explain our finding of activation of TLR2 and TLR4 mRNA in all septic patients regardless of type of pathogen detected in hemoculture. Interestingly, we did not see a clear relationship between Gram-negative hemoculture and TLR4, or between Gram-positive hemoculture and TLR2 mRNA upregulation, respectively.

Collectively, our data together with previously published observations show 4.5 times lowered PRL mRNA levels in monocytes derived from non-survived septic patients, and statistically significantly lower expression in comparison with the highest PRL mRNA levels detected in monocytes derived from survived patients. The 1.5 fold upregulation of PRL mRNA expression detected in survived patients at the end of acute phase of sepsis when compared to healthy controls reflects an ongoing immune activation of stressed organism. These data confirm the significance of prolactin for better outcome and improved survival rate in sepsis and suggest that extrapituitary prolactin plays an important role in the activation of the innate immune system during bacterial infection. In our study, we were not able to show the involvement of functional *PRL* -1149 G/T SNP and its significance for monocytic prolactin mRNA expression regulation. Our data also indicate that the expression of Toll-like receptors mRNA during sepsis is markedly increased in comparison to mRNA levels in healthy controls, which reflects general response of organism to infection.

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Figure legend:

Fig. 1: Effect of *PRL* -1149 G/T SNP on physiological mRNA expression in peripheral blood monocytes. The horizontal bar represents the group median. The level of mRNA expression (Y axis) is shown in arbitrary units (multiplied by 10^6).

Fig. 2: Comparison of *PRL* mRNA levels of all studied groups. The horizontal bar represents the group median. Controls ~ healthy control subjects; N-S ~ non-survived patients; A, B, C ~ consecutive blood drawings in patients-survivors. AU ~ Arbitrary Units multiplied by 10^6 .

Fig. 3: Comparison of *TLR2* mRNA levels of all studied groups. The horizontal bar represents the group median. Controls ~ healthy control subjects; N-S ~ non-survived patients; A, B, C ~ consecutive blood drawings in patients-survivors. AU ~ Arbitrary Units multiplied by 10^3 .

Fig. 4: Comparison of *TLR4* mRNA levels of all studied groups. The horizontal bar represents the group median. Controls ~ healthy control subjects; N-S ~ non-survived patients; A, B, C ~ consecutive blood drawings in patients-survivors. AU ~ Arbitrary Units multiplied by 10^3 .

Figure 1

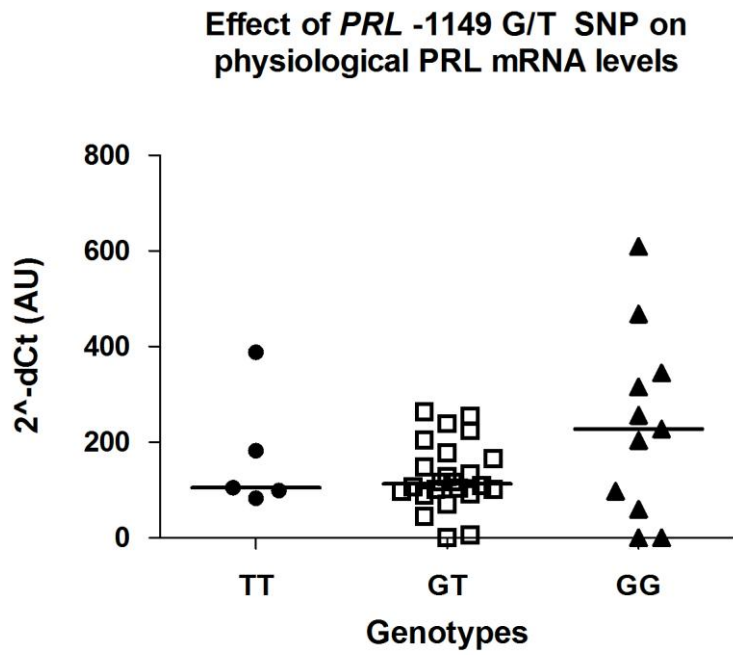


Figure 2

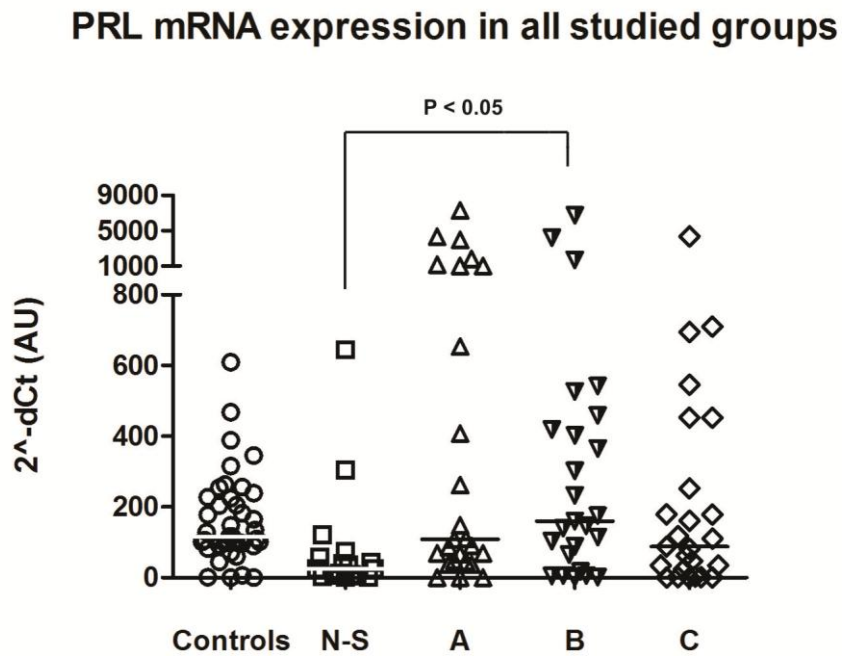


Figure 3

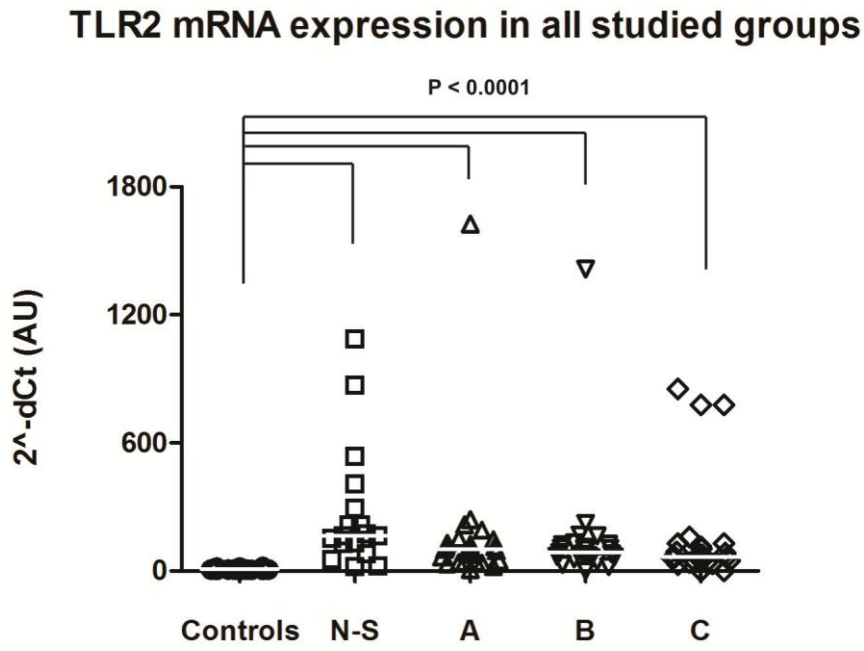


Figure 4

