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## **Extended Report**

### **The autoimmune-associated genetic variant PTPN22 R620W enhances neutrophil activation and function in rheumatoid arthritis patients and healthy individuals**

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## Abstract

**Objectives:** A genetic variant of the leukocyte phosphatase PTPN22 (R620W) is strongly associated with autoimmune diseases including rheumatoid arthritis (RA). Functional studies on the variant have focussed on lymphocytes, but it is most highly expressed in neutrophils. We have investigated the effects of the variant on neutrophil function in health and in patients with RA.

**Methods:** Healthy individuals and RA patients were genotyped for PTPN22 (R620W) and neutrophils isolated from peripheral blood. Neutrophil adhesion and migration across inflamed endothelium were measured. Calcium ( $\text{Ca}^{2+}$ ) release and reactive oxygen species (ROS) production in response to fMLP stimulation were also assessed.

**Results:** Expression of R620W enhanced neutrophil migration through cytokine activated endothelium (non-R620W=24%, R620W=45% migrating cells,  $p<0.001$ ). Following fMLP stimulation, neutrophils that were heterozygous and homozygous for R620W released significantly more  $\text{Ca}^{2+}$  when compared to non-R620W neutrophils, both in healthy individuals and RA patients. fMLP stimulation, after TNF- $\alpha$  priming, provoked more ROS from neutrophils heterozygous for R620W in RA patients (non-R620W vs. R620W = ~1.75-fold increase) and healthy individuals (non-R620W vs. R620W = 4-fold increase) and this increase was statistically significant in healthy individuals ( $p<0.001$ ) but not in RA patients ( $p<0.25$ ).

**Conclusions:** Expression of PTPN22 (R620W) enhanced neutrophil effector functions in health and RA, with migration,  $\text{Ca}^{2+}$  release and production of ROS increased. Neutrophils are found in large numbers in the RA joint and this hyperactivity of R620W cells may directly contribute to the joint damage, as well as to the initiation and perpetuation of the chronic immune mediated inflammatory processes driving the disease.

## **Key words**

Lyp, PTPN22, neutrophil, rheumatoid arthritis

## 1. Introduction

The aetiology of rheumatoid arthritis (RA) is complex but a strong contribution is made by polymorphisms in a number of genes, many of which are involved in the regulation of the immune response [1]. One such gene is PTPN22 in which a single nucleotide polymorphism (SNP) known as R620W, is a prominent risk factor for RA and other autoimmune diseases [2]. The consequences of R620W expression have been widely studied in lymphocytes [3-5] where the polymorphism alters signalling through the B and T cell receptors. Thus R620W may contribute to the survival of a broader repertoire of lymphocytes, some of which may be autoreactive [6 7]. The PTPN22 gene has also been suggested to control other lymphocyte functions since in its absence in mice, regulatory T cells are increased [8] and their adhesion and function are enhanced [9].

The protein product of the PTPN22 gene is a protein tyrosine phosphatase (Lyp) whose role is to counter the effect of tyrosine kinases and regulate signalling pathways by removing phosphorylated tyrosine residues from proteins [10]. To date, many of the studies on Lyp have been conducted in lymphocytes. However, Lyp is most highly expressed by neutrophils [11] where little is currently known about its function. Altered levels of tyrosine phosphorylation have been observed in neutrophils isolated from RA patients [12] and such cells exhibit resistance to cell death and enhanced effector functions [13]. Furthermore, circulating neutrophils isolated from RA patients have been shown to release more calcium ( $\text{Ca}^{2+}$ ) in response to stimulation when compared to individuals with non-rheumatic joint conditions [14]. While the genotype of the patients in these studies was not reported, these data suggest that abnormal neutrophil signalling and function is a specific feature of RA neutrophils.

Quantitatively neutrophils are a major component of the joint infiltrate since up to 90% of leukocytes in the RA joint are neutrophils [15]. This suggests they make a significant contribution to the inflammatory processes driving joint destruction and in some animal

models of arthritis initiation of disease is completely prevented in the absence of neutrophils [16]. Many of the mechanisms used by neutrophils to combat infection also cause significant collateral tissue damage when neutrophil activation is enhanced or occurs inappropriately. For example, the activation-dependent release of proteases and reactive oxygen species (ROS) produce significant damage to joint components including lipids and proteins [17 18]. The trigger for aberrant neutrophil activation is not clear, but results from changes in neutrophil intracellular signalling. In the context of neutrophil intracellular signalling, cytosolic  $\text{Ca}^{2+}$  increase and release are important mediators of other neutrophil functions such as NADPH oxidase activation and adhesion to endothelium. Additionally  $\text{Ca}^{2+}$  signalling processes are highly regulated by a number of proteins, one of which could be Lyp. Given the fact Lyp is abundantly expressed in neutrophils, we investigated the effects of the genetic Lyp variant R620W on neutrophil function, to determine whether this genetic variant contributes to the altered activation and function of neutrophils in RA.

## **2. Materials and Methods**

All reagents were purchased from Sigma Aldrich (United Kingdom), unless otherwise stated.

### **2.1 Experimental procedures**

#### **2.1.1 Subjects**

Patients with RA (508) were recruited from outpatient clinics at University Hospital Birmingham NHS Foundation Trust and Russell's Hall Hospital (Dudley), alongside healthy volunteers (107). All samples were obtained with informed consent and with approval from the Edgbaston Research Ethics Committee (12/WM/0077). All RA patients satisfied the 1987 American College of Rheumatology (ACR) criteria for RA.

#### **2.1.2 Genotyping**

PB was collected in vacutainers containing EDTA. DNA was extracted from whole blood using the QuickGene 810 system (Fujifilm, Japan), as described previously [19]. A Roche LightCycler 480 II system (Roche Diagnostics Ltd. UK) identified the single nucleotide polymorphism in PTPN22 (rs2476601) by real-time PCR and melting curve analysis using primers and probes used previously [20]

#### **2.1.3 Isolation of neutrophils**

PB was obtained in vacutainers containing EDTA. 2% dextran was added (1ml/6ml of blood) and the blood left to settle for 30 minutes to separate red blood cells from the buffy coat. Percoll gradients (GE Healthcare, UK) were set up by adding 5ml of 56% Percoll to a 15ml tube, and a Pasteur pipette was used to add 2.5ml of 80% Percoll underneath. The buffy coat was added to Percoll gradients and centrifuged at  $190 \times g_{av}$  for 25 minutes with no brake. The neutrophil layer was removed from the gradient using a Pasteur pipette. Cells were washed twice and resuspended at  $1.0 \times 10^6$ /ml in pre-warmed RPMI 1640 medium

supplemented with 10% heat inactivated foetal calf serum (HIFCS) and 1% glutamine penicillin streptomycin (GPS) (complete culture medium), before use in experiments. The average purity and viability of neutrophils isolated using this method was 96% and 95% respectively.

#### **2.1.4 Adhesion and migration**

Adhesion and transmigration were assessed by direct microscopic observation as previously described [21 22]. Briefly, human umbilical endothelial cells (HUVEC) were isolated from umbilical cords and cultured. Tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ; 0.05 and 5ng/ml; R&D Systems, Abingdon, UK) was added to confluent monolayers for 4 hours before the adhesion assay. After washing the HUVEC, neutrophils ( $1 \times 10^6$ /ml in M199BSA) were added for 6 minutes and then non-adherent neutrophils were removed. Digitised images of the endothelial surface were made using phase contrast microscopy at 37°C at 2 and 9 minutes from the end of the settling period and analysed offline using Image-Pro Plus software (DataCell Ltd, Finchampstead, UK) as previously described [21]. Adhesion was determined as the percentage of neutrophils that adhered by counting all adherent cells per image, then calculating the number of cells per well and dividing this number by the total number of neutrophils added. Transmigration was calculated as a percentage of the adherent cells that appeared phase dark (i.e. underneath the endothelial monolayer). Migration velocities ( $\mu\text{m}/\text{minute}$ ) of phase-dark neutrophils underneath the HUVEC were determined the average distance moved by the centre of the cell per minute.

#### **2.1.5 Calcium signalling**

Neutrophils ( $2.0 \times 10^6$ /ml in RPMI 1640) were incubated with 1 $\mu\text{M}$  Indo-1 AM ester (Invitrogen, USA) for 40 minutes at 37°C, before washing twice in Ca<sup>2+</sup> containing HBSS at 400 $\times g_{av}$  for 6 minutes. Cells ( $1.5 \times 10^6$ ) were placed at 37°C for 10 minutes prior to stimulation with 50 $\mu\text{M}$  fMLP. Samples were placed in a LS50B spectrofluorimeter (Perkin Elmer, UK) and maintained in suspension at 37°C using a magnetic stirrer. Data were collected using FL



Winlab Version 2.1 software with a dual-emission experimental protocol for Indo-1 AM loaded cells.

### **2.1.6 Reactive oxygen species production**

Neutrophils ( $1.0 \times 10^6$ /ml in RPMI 1640) were primed with 5ng/ml TNF- $\alpha$  (R&D Systems, Abingdon, UK) for 15 minutes at 37°C and then treated with 10 $\mu$ M dihydrorhodamine (DHR) for 5 minutes at 37°C. Samples were stimulated with 1 $\mu$ M fMLP for 5 minutes at 37°C, washed in ice-cold PBS with centrifugation at  $400 \times g_{av}$  for 6 minutes and fixed with 2% paraformaldehyde in PBS overnight at 4°C. Fixed cells were washed as before, resuspended in PBS and ROS production determined by DHR fluorescence detected using a CyAn ADP flow cytometer (Beckman Coulter, UK).

### **2.1.7 Statistics**

Data shown are mean  $\pm$ SEM. Mann-Whitney U was used for comparison of groups for continuous variables, and Fisher's exact test with a two-tailed p value for the comparison of categorical variables. For paired data, Wilcoxon matched-pairs signed rank tests were performed. For data comparing more than three groups, analysis was carried out using a two-way ANOVA followed by Bonferonni post-test. P values of  $<0.05$  were considered to be statistically significant.

### 3. Results

To allow us to test the hypothesis that expression of PTPN22 R620W alters neutrophil function, a large cohort of healthy individuals and RA patients were recruited and genotyped for the R620W SNP. The R620W variant was found to be highly expressed in both of the populations assessed (Table 1). The frequency of heterozygotes (AG) among the RA population was slightly decreased when compared to previous work [20] (23% vs. 30%); however the frequency of homozygotes (AA) was the same (2%). Given the paucity of homozygotes we felt it was most appropriate to focus functional investigations on the cells heterozygous (AG) for the variant allele. To do this neutrophils were isolated from healthy individuals (n=16) and RA patients (n=24) closely matched for age and sex. Importantly, there were no significant differences in patient demographics such as disease duration, drug treatments or levels of inflammation, when groups were compared according to PTPN22 genotype (Table 2).

#### **PTPN22 R620W increases neutrophil migration across inflamed endothelium**

Initially we were interested to determine whether R620W altered the ability of neutrophils to be recruited to sites of inflammation. To address this we observed the behaviour (adhesion and migration) of neutrophils isolated from healthy individuals control (GG) and heterozygous (AG) for R620W on endothelial cells (EC) stimulated with TNF- $\alpha$ , as previously described [22]. Neutrophil adhesion increased in a TNF- $\alpha$  dose dependent manner, which was not affected by the PTPN22 genotype (Figure 1A). We observed low levels of migration on resting EC and those stimulated with low dose (0.05ng/ml) TNF- $\alpha$  for both GG and AG expressing neutrophils (Figure 1B). Significantly more AG neutrophils transmigrated through EC treated with high dose TNF- $\alpha$  (5ng/ml) at 2 minutes when compared to neutrophils isolated from GG individuals (GG=24 $\pm$ 4% and AG=44 $\pm$ 7% neutrophils migrated, defined as phase dark cells;  $p$ <0.001, Figure 1B-D). These differences were lost at 9 minutes, with similar levels of migration observed at this later time point (GG=38 $\pm$ 5% and AG=43 $\pm$ 9% neutrophils migrated, defined as phase dark cells). These data suggest that AG neutrophils

are able to migrate through inflamed endothelium more rapidly when compared to their GG counterparts. However, once the cells were underneath the endothelium, neutrophils migrated at similar velocities regardless of PTPN22 genotype (GG=8.1±1.1µm/minute and AG=7.9±0.8µm/minute).

### **PTPN22 R620W increases Ca<sup>2+</sup> release by neutrophils in response to fMLP stimulation**

To assess the effect of R620W on overall signalling through surface receptors, N-formyl-Met-Leu-Phe (fMLP) was added to mimic the presence of formylated peptides derived from bacteria which would be present during an infection. Formyl peptide receptor (FPR) engagement releases Ca<sup>2+</sup> [23], and thus this was measured to give an indication of the overall signal induced by FPR activation. Neutrophils isolated from healthy individuals heterozygous (AG) and homozygous (AA) for R620W released significantly more Ca<sup>2+</sup> in response to fMLP when compared to age and sex matched individuals without R620W (GG=0.24±0.02 I1R, AG=0.28±0.02 I1R and AA=0.43 I1R, P<0.05, Figure 2 A+C). Similar elevated Ca<sup>2+</sup> release were also observed in RA patients with the R620W variant (Figure 2B+C). In fact, Ca<sup>2+</sup> release was significantly augmented in neutrophils from RA patients when compared to healthy individuals, in a genotype dependent manner: GG<AG<AA (GG=0.39±0.04 I1R, AG=0.44±0.02 I1R and AA=0.56 I1R, Figure 2 B+C).

### **PTPN22 R620W increases reactive oxygen species production by neutrophils following priming with TNF-α**

Given that R620W was shown to increase Ca<sup>2+</sup> release, we examined whether other Ca<sup>2+</sup> dependent functions would also be affected. In order to investigate this, the ability of neutrophils to produce ROS in response to TNF-α priming and fMLP stimulation were tested. Unstimulated neutrophils from healthy subjects with GG or AG R620W genotype produced very little ROS at the basal level (GG=69±13, AG=155±66, n=8, Figure 3 A+B). Although ROS production was increased with priming, no differences were observed between the GG and AG genotypes (TNF-α: GG=110±31 vs. AG=274±131, or fMLP: GG=86±18,

AG=200±95, n=8). In response to priming with TNF- $\alpha$  and then fMLP, AG neutrophils produced 4-fold more ROS when compared to age, gender-matched GG neutrophils (Figure 3C). Neutrophils from RA patients produced little ROS at baseline (GG=173±54, AG=87±28, n=4, Figure 4). Similar to the healthy individuals, ROS production was significantly elevated following treatment with TNF- $\alpha$  and then fMLP, with AG neutrophils producing ~1.75 fold more than GG neutrophils (Figure 4).

## 4. Discussion

The importance of neutrophils in the pathogenesis of RA is becoming increasingly recognised [13] and in some animal models of arthritis their absence completely prevents the initiation of disease [16]. Increased oxidised lipids and proteins are present in RA joints, which may result from enhanced neutrophil activity [17–18]. In particular, release of proteases and ROS have been implicated in joint damage via direct oxidation of joint components or oxidation of immune cell surface proteins, leading to impaired suppressor cell activity and defective immunoregulation [24]. In this study, we investigated a genetic variant (R620W) associated with increased risk of RA, to determine if this contributed to altered neutrophil function. We have found that expression of R620W enhances the function of neutrophils from healthy donors, as characterised by increased trans-endothelial migration, increased  $\text{Ca}^{2+}$  release, and increased production of ROS. The results with cells for RA patients were less clear since, although there was strong trend for enhanced responses in R620W neutrophils, this did not reach statistical significance. The greater variability in RA patient neutrophil responses may be a consequence of the complex nature and heterogeneity of the disease, and such factors as age and smoking. Given that R620W is only 1 of the 101 identified RA risk loci [25], patients are likely to express a number of these which may also influence the cellular response. In our RA cohort there were some smokers which leads to depletion of the antioxidant glutathione [26] and we have shown that the PTP activity of Lyp is susceptible to oxidation [27]. We have also shown that, in CD4 T cells from the elderly, the PTP CD45 is oxidatively inactivated [28] and so it may well be that the oxidation of neutrophil Lyp as a result of smoking or ageing may have confounded our results with the RA neutrophils. However, perhaps the greatest potential source of variability in the patients would be the drugs they were taking. Neutrophil function is particularly influenced by a broad range of therapeutics including glucocorticoids [29], non-steroidals [30] and disease modifying drugs [31] and so eliminating all drug effects in these cells from these patients with established disease would be ethically impossible.

Nevertheless the results overall indicate that Lyp plays an important role in the activation and responses of neutrophils. The role of Lyp in T cell receptor (TCR) signalling is better characterised, where it acts as a negative regulator mainly via dephosphorylating activating tyrosine residues of Src family kinases [32]. Neutrophils express a number of PTPs including CD45, CD148 and Lyp [11 32 33]. CD45 and CD148 have been reported to regulate neutrophil migration [34], whereas a role for Lyp has not yet been identified. T cells expressing R620W release less  $Ca^{2+}$  when activated by CD3 stimulation [5], suggesting R620W results in hypo-responsive T cells. We have found the opposite in neutrophils, since R620W cells were hyper-responsive to stimulation (Figure 2). These contrasting observations suggest the effects of R620W differ depending upon the leukocyte subset, and highlights the importance of studying innate and adaptive immune cells in parallel.

The signalling pathways mediating TNF- $\alpha$  priming of neutrophils are not well known, however it could be that Lyp is a regulator of these events. TNF- $\alpha$  has been shown to increase intracellular  $Ca^{2+}$  in neutrophils, which is associated with increased co-localisation of gp91<sup>PHOX</sup>, a component of the NADPH oxidase enzyme necessary for ROS production [35]. It has been proposed that TNF- $\alpha$  moves the cytosolic sub units (p40<sup>PHOX</sup> p47<sup>PHOX</sup>, and p67<sup>PHOX</sup>) of the NADPH oxidase closer to the cell membrane prior to stimulation [36]. High levels of TNF- $\alpha$  are present in RA joints, and neutrophils from these sites display increased p47<sup>PHOX</sup> phosphorylation and ROS production [37], highlighting increased priming and activity of neutrophils. There are a number of phosphorylation sites present in the structure of these subunits, which regulate their location and activation status [38-40]. To date, only serine and threonine phosphorylation sites have been documented in neutrophils, but a study in ECs has shown that p47<sup>PHOX</sup> can be subject to tyrosine phosphorylation [40]. Therefore, it is possible that Lyp could dephosphorylate p47<sup>PHOX</sup> at this site, and a change in Lyp function could alter activity of the NADPH oxidase.

$Ca^{2+}$  signalling is regulated by a number of proteins, including Vav, a guanine exchange factor (GEF) which acts as an adaptor molecule [41]. Substrate trapping experiments using

T cells found that Vav is a substrate of Lyp [42], which may also be the case in neutrophils. Vav is also a mediator of  $\beta 2$  integrin-dependent functions including migration [43], suggesting changes to Vav phosphorylation could mediate changes in neutrophil function induced by R620W. Another potential target for Lyp is phospholipase C (PLC) gamma2, important for Fc-receptor and integrin-mediated neutrophil functions [44]. PLC gamma2 is activated by phosphorylation downstream of Src-family kinases [44] and Lyp could be involved in dephosphorylation of PLC gamma2 to reduce neutrophil activation. Lyp is known to target Src-family kinases upstream of PLC gamma1 in T cells [32], which may also be similar in neutrophils. Signalling via PLC gamma2 in neutrophils could be of importance in RA, as mice lacking PLC gamma2 are protected from the development of arthritis [44].

Our results show that the presence of R620W has no effect on neutrophil adhesion, but results in more rapid transendothelial migration (Figure 1), which is dependent upon ligation of ICAM-1 by  $\beta 2$  integrin's, and triggered by an increase in intracellular  $Ca^{2+}$  [45]. These events mediate activation of RHO GTPase and p38 MAPK, which contract the EC, allowing neutrophils to migrate [46]. Lyp could regulate these signalling proteins similarly to CD45 and CD148, which have been shown to regulate PI3K, pERK and Src-family kinases needed for migratory responses [34].

In conclusion, our studies suggest that neutrophils expressing R620W are more intrinsically active and more susceptible to priming by TNF- $\alpha$ . Given the success of RA treatments which target TNF- $\alpha$ , our work suggests an additional mechanism by which anti-TNF treatment is effective is by reducing neutrophil priming and activation in patients with this genetic variant. This suggests that stratification of patients by PTPN22 genotype could be beneficial, and may help to choose the most appropriate treatment for patients positive for this genetic mutation. In particular, this work identifies a genetic mechanism by which over-activation of neutrophils could contribute to increased ROS release and subsequent damage in RA joints. Lastly, this study highlights that the effects of R620W are not restricted to lymphocytes, where previous studies have mainly been focussed.

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## **Contributors**

RB, KAK and JPS genotyped patients and healthy individuals. RB, KAK and HMM performed the experiments. GDK and CDB recruited and diagnosed patients and acquired the clinical data. SPY conceived the project and acquired the funding. SPY and RB wrote the first draft of the paper and all authors contributed to analysis of the data and drafting of the manuscript.

**Competing Interests.** None.



## Figure legends

**Figure 1 Heterozygosity for PTPN22 R620W increased neutrophil migration across inflamed endothelium.** HUVEC were treated in the presence or absence of TNF- $\alpha$  (0, 0.05, 5ng/ml) for 4 hours. Neutrophils were isolated from healthy individuals control (GG) and heterozygous (AG) for PTPN22 R620W and allowed to settle on the HUVEC monolayer for 6 minutes, after which non-adherent cells were removed by washing and neutrophil behaviour was analysed by phase contrast microscopy. **(A)** Neutrophil adhesion assessed at 2 minutes expressed as a percentage of total cells added. ANOVA shows a significant effect of cytokine treatment, but not PTPN22 genotype, on neutrophil adhesion,  $P < 0.0001$ . **(B)** Neutrophil transmigration was assessed at 2 and 9 minutes and expressed as a percentage of adherent cells that had migrated. Figure shows the percentage of neutrophils transmigrated after 2 minutes. ANOVA shows a significant effect of cytokine treatment ( $P < 0.0001$ ) and PTPN22 genotype ( $P < 0.05$ ) on neutrophil transendothelial migration. Representative micrographs of the behaviour of neutrophils isolated from **(C)** GG or **(D)** AG individuals on HUVEC treated with 5ng/ml TNF. Round phase bright cells are defined as those cells adherent to the surface of the endothelium. Dark, distorted phase dark cells are defined as cells that have migrated and are underneath the endothelium. Data are mean  $\pm$  SEM from 6 (GG) and 7 (AG) independent experiments. \*\*\* =  $P < 0.001$  by Bonferonni post-test compared with control (GG) neutrophils.

**Figure 2 Heterozygosity and homozygosity for PTPN22 R620W increases calcium release by neutrophils.** Neutrophils were isolated from healthy individuals and rheumatoid arthritis patients control (GG), heterozygous (AG) and homozygous (AA) for PTPN22 R620W. Cells were washed and loaded with the Indo-1 AM ester. Cells were washed again in HBSS and transferred to an acrylic cuvette. After a baseline was established, 50 $\mu$ M fMLP was added. **(A)** One representative measure of experiments using neutrophils isolated from healthy individuals, GG (solid line), AG (dashed line) and AA (dotted line). **(B)** One representative measure of experiments using neutrophils isolated from rheumatoid arthritis patients, GG (solid line), AG (dashed line) and AA (dotted line). **(C)** Mean peak rise in Indo-1 AM ratio after stimulation of neutrophils isolated from healthy individuals (HI) and rheumatoid arthritis patients (RA). Results show the mean ( $\pm$ SEM) of six experiments using healthy individuals (GG and AG) and seven experiments using rheumatoid arthritis patients (GG and AG). \*  $P < 0.05$  Wilcoxon matched-pairs signed rank test compared with control (GG) neutrophils.

**Figure 3 Heterozygosity for PTPN22 R620W enhances the production of reactive oxygen species by neutrophils isolated from healthy individuals following priming with TNF- $\alpha$ .**

Neutrophils were isolated from age and sex matched healthy individuals control (GG) and heterozygous (AG) for PTPN22 R620W. Neutrophils were primed with TNF- $\alpha$  for 15 minutes at 37°C. Dihydrorhodamine (DHR) was added to cells for 5 minutes at 37°C and cells were then stimulated with 1 $\mu$ M fMLP for 5 minutes at 37°C. Cells were washed in ice-cold PBS and fixed using 2% paraformaldehyde overnight at 4°C. Reactive oxygen species production was assessed by measuring DHR fluorescence using flow cytometry. **(A)** One representative experiment using control neutrophils (non-shaded) and heterozygous neutrophils (shaded). The numbers shown in the histogram plots refer to the mean fluorescence intensity of DHR measured when control neutrophils (black) and heterozygous neutrophils (grey) were assessed by flow cytometry. **(B)** Average DHR fluorescence in unstimulated (black bars), TNF- $\alpha$  primed (white bars), fMLP stimulated (checked bars), and primed and stimulated neutrophils (striped bars). **(C)** Percentage increase in reactive oxygen species production by neutrophils primed with TNF- $\alpha$  and stimulated with fMLP, when compared to unstimulated neutrophils. Results are expressed as a percentage of control (GG) neutrophils of an age and sex matched individual. Results show the mean ( $\pm$ SEM) of eight separate experiments. \*\* P<0.001 Wilcoxon matched-pairs signed rank test compared with control (GG) neutrophils.

**Figure 4 Heterozygosity for PTPN22 R620W enhances the production of reactive oxygen species by neutrophils isolated from rheumatoid arthritis patients following priming with TNF- $\alpha$ .**

Neutrophils were isolated from rheumatoid arthritis patients, control (GG) and heterozygous (AG) for PTPN22 R620W. Neutrophils were primed with TNF- $\alpha$  for 15 minutes at 37°C. Dihydrorhodamine (DHR) was added to cells for 5 minutes at 37°C and cells were then stimulated with 1 $\mu$ M fMLP for 5 minutes at 37°C. Cells were washed in ice-cold PBS and fixed using 2% paraformaldehyde overnight at 4°C. Reactive oxygen species production was assessed by measuring DHR fluorescence using flow cytometry. **(A)** One representative experiment using control neutrophils (non-shaded) and heterozygous neutrophils (shaded). The numbers shown in the histogram plots refer to the mean fluorescence intensity of DHR measured when control neutrophils (black) and heterozygous neutrophils (grey) were assessed by flow cytometry. **(B)** Average DHR fluorescence in unstimulated (black bars), TNF- $\alpha$  primed (white bars), fMLP stimulated (checked bars), and primed and stimulated

neutrophils (striped bars). **(C)** Percentage increase in reactive oxygen species production by neutrophils primed with TNF- $\alpha$  and stimulated with fMLP, when compared to unstimulated neutrophils. Results show the mean ( $\pm$ SEM) of four separate experiments.

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Table 1 – Frequency of PTPN22 R620W in the cohort of healthy subjects, disease control subjects and rheumatoid arthritis patients

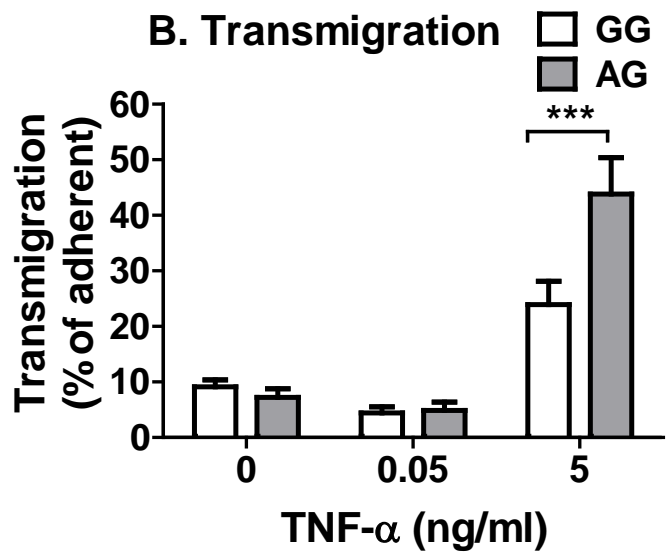
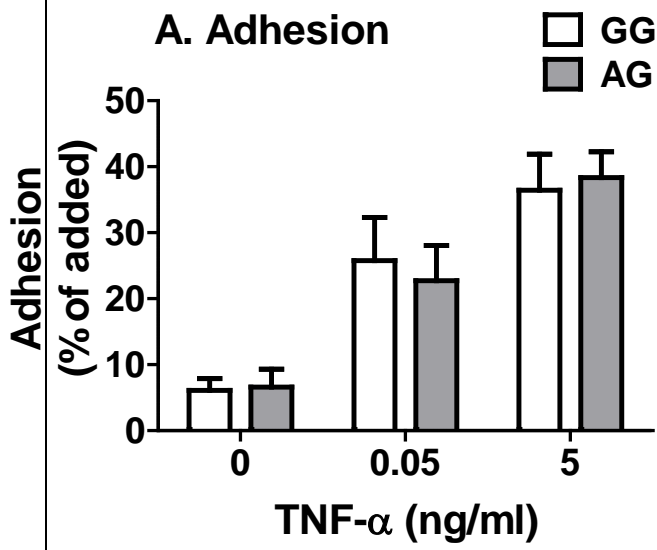
PTPN22 R620W genotype	Healthy individuals (n=107)	Rheumatoid arthritis patients (n=380)	Rheumatoid arthritis patients (n=397)*	Odds ratio for genotype (95% CI) HI vs. RA	p value
Control (GG)	79% (n=85)	75% (n=284)	68% (n=272)	-	-
Heterozygote (AG)	20% (n=21)	23% (n=87)	30% (n=119)	1.31 (0.811-1.882)	0.372
Homozygote (AA)	1% (n=1)	2% (n=9)	2% (n=6)	2.11 (0.187-23.73)	0.615

\* Genotype frequency of PTPN22 R620W from Toms et al 2011 for comparative purposes [20]. Differences in genotype frequency between healthy individuals and rheumatoid arthritis patients were analysed using Fisher's exact test with a two-tailed p value with additional calculation of odds ratio. There were no significant differences in genotype frequency when comparing healthy individuals and rheumatoid arthritis patients. HI; healthy individuals, RA; rheumatoid arthritis.

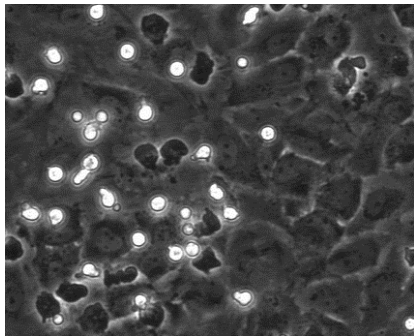
Table 2 – Demographic data of the study participants used for *in vitro* neutrophil functional studies

	Healthy individuals (n=16)			Rheumatoid arthritis patients (n=24)		
	GG (n=8)	AG (n=8)	p value	GG (n=12)	AG (n=12)	p value
PTPN22 R620W genotype						
Sex, female n (%)	5 (63)	5 (63)	1.000	7 (58)	8 (67)	1.000
Age (years) median (IQR)	29 (16)	26 (30)	0.873	71 (25)	65 (15)	0.259
Smoker n (%)	0 (0)	0 (0)	-	3 (25)	6 (50)	0.400
CRP (mg/l) median (IQR)	-	-	-	11 (28)	3 (5)	0.101
RF positive n (%)	-	-	-	8 (67)	9 (75)	1.000
Disease duration ≥10 year's n (%)	-	-	-	10 (83)	5 (42)	0.089
Treatment with Methotrexate n (%)	-	-	-	4 (33)	5 (42)	1.000
Treatment with DMARD's n (%)	-	-	-	6 (67)	11 (92)	0.069
Treatment with steroids n (%)	-	-	-	4 (33)	3 (25)	1.000
Treatment with NSAID's n (%)	-	-	-	8 (100)	7 (88)	1.000

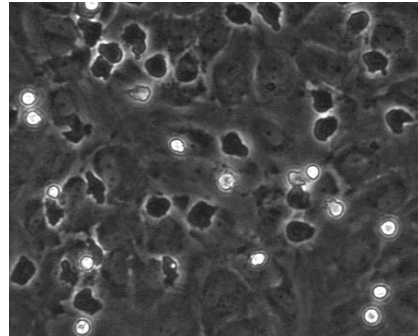
Data were analysed using Mann-Whitney U for comparison of groups for continuous variables, and Fisher's exact test with a two-tailed p value for the comparison of categorical variables. There were no significant differences when groups were compared according to PTPN22 genotype. IQR; interquartile range, CRP; C-reactive protein, RF; Rheumatoid factor, DMARD's; Disease modifying anti-rheumatic drugs, NSAID's; Non-steroidal anti-inflammatory drugs.



**C. Transmigration of GG neutrophils**



**D. Transmigration of AG neutrophils**





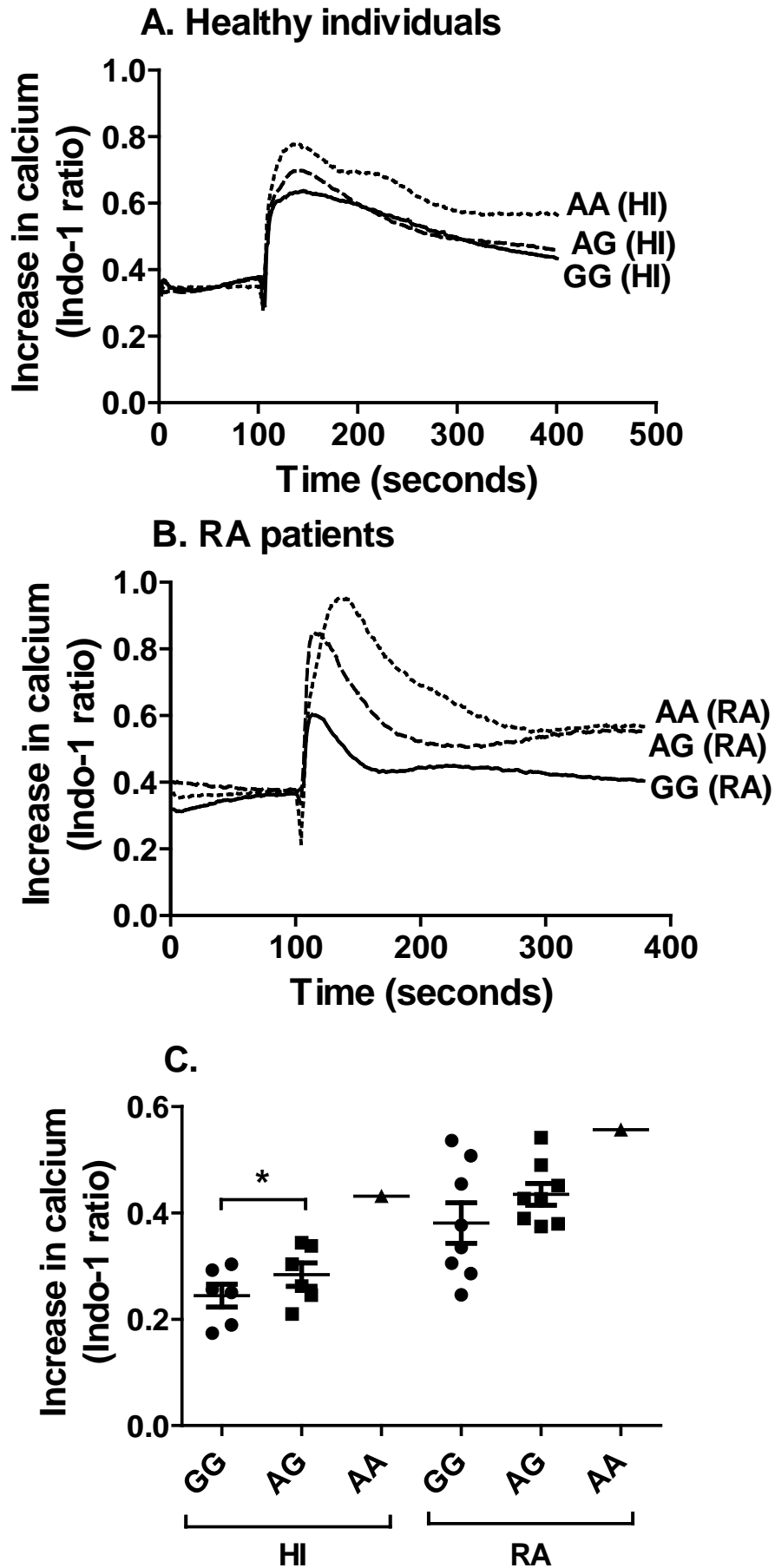


Figure 3

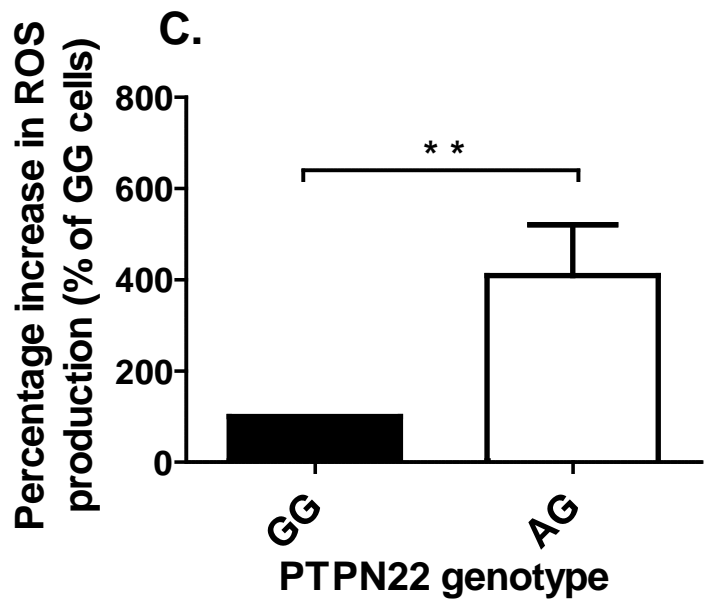
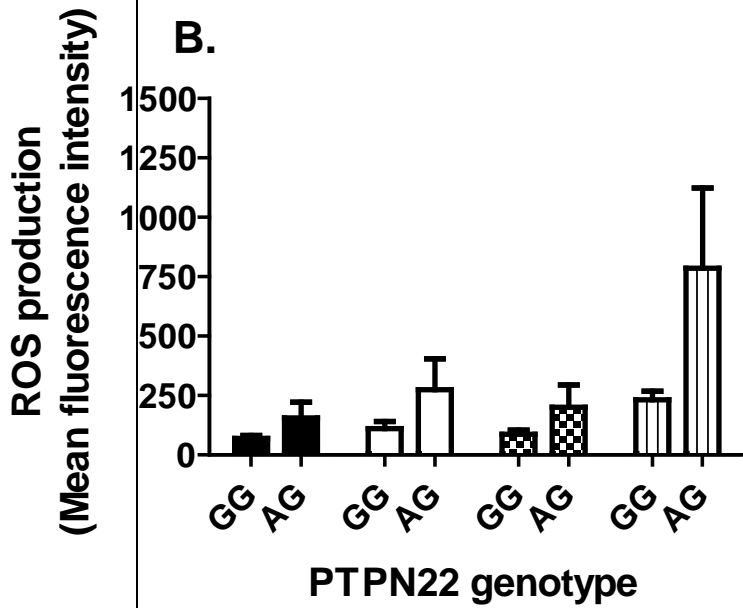
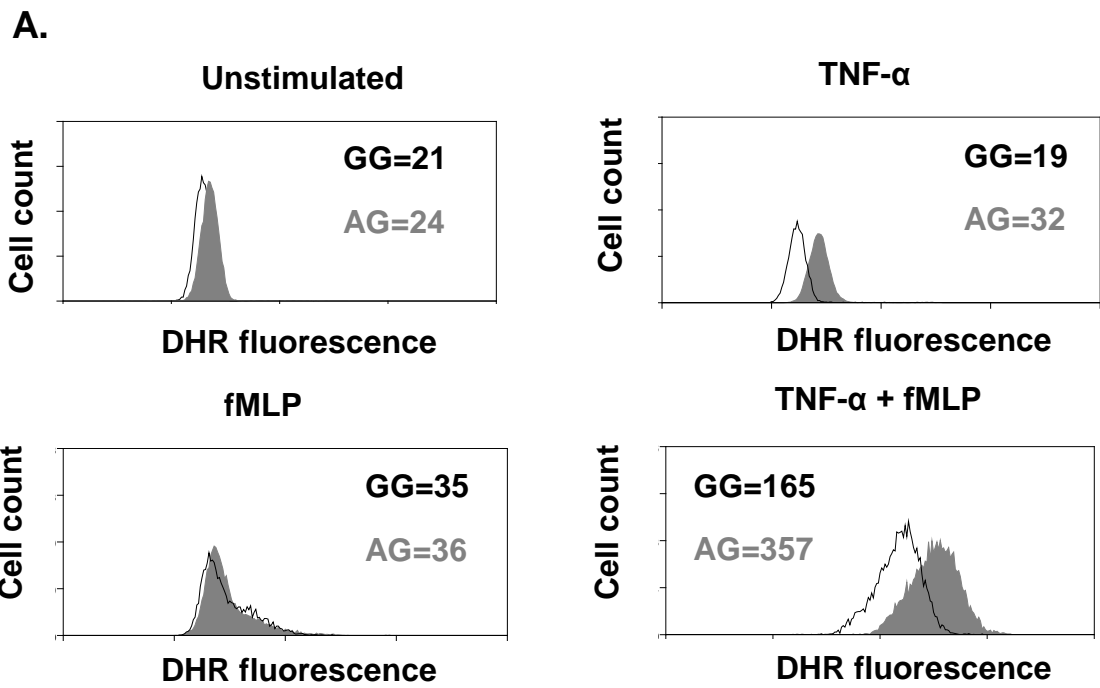
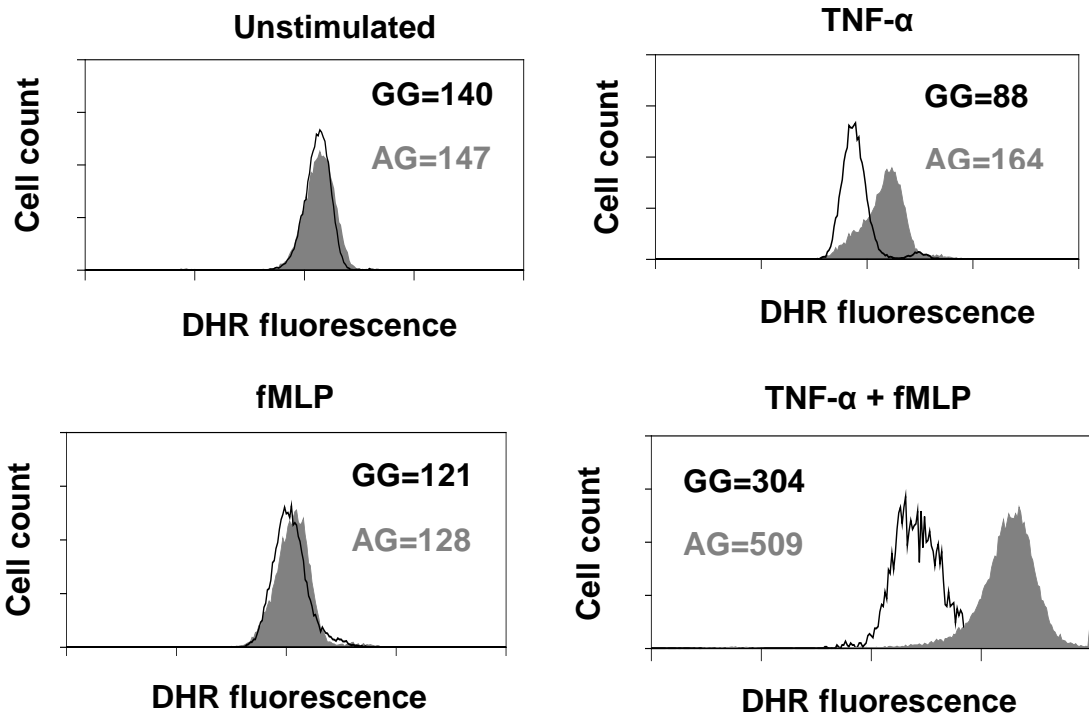
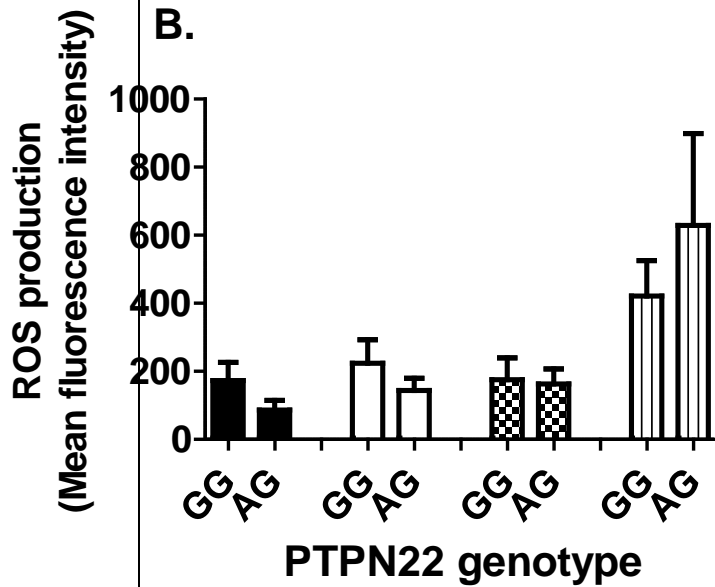


Figure 4

A.



B.



C.

