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Abbreviations: ALOX5AP, 5-lipoxygenase activating protein; LT, leukotriene; MI, myocardial infarction; SNP, single nucleotide polymorphism.

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ABSTRACT

Leukotrienes are implicated in the pathogenesis of coronary artery disease. Recently two haplotypes (Hap A and Hap B) in the gene encoding arachidonate 5-lipoxygenase activating protein (ALOX5AP), the main regulator of 5-lipoxygenase, have been associated with a doubling of the risk of myocardial infarction. Studies have also shown that treatment with a leukotriene inhibitor reduces biomarkers for coronary risk in patients carrying HapA, raising the possibility of developing genotype-specific therapy. In this study, we examined whether carriage of HapA or HapB is associated with increased leukotriene B_4 (LTB_4) production in healthy subjects. Age and gender-matched healthy HapA carriers (n = 21), HapB carriers (n = 20) and nonA/nonB carriers (n = 18), with no reported history of cardiovascular disease, were recruited following DNA screening of 1268 subjects from a population based study. Blood neutrophils were isolated and LTB_4 production measured in response to stimulation with 1μM of the calcium ionophore A23187. There was no difference in the mean level for LTB_4 production in the three groups (nonA/nonB: 24.9 ± 8.3 ng/10^6 cells; HapA: 22.2 ± 11.9 ng/10^6 cells; HapB: 19.8 ± 4.8 ng/10^6, p = 0.14). The findings indicate that if either the HapA or the HapB haplotype of ALOX5AP indeed increase cardiovascular risk, then the mechanism is not simply due to a systematically observable effect of the haplotype on LTB_4 production in response to stimulation. The results suggest that knowledge of a patient's haplotype may not provide useful information on the likely clinical response to ALOX5AP inhibitors.

INTRODUCTION

Myocardial infarction (MI) is a complex disease resulting from the interaction of environmental and genetic factors. In the majority of subjects coronary atherosclerosis, an inflammatory process, provides the substrate for the condition and MI develops when the atherosclerosis is complicated by plaque fissuring and thrombosis [1]. Leukotrienes (LTs) are inflammatory mediators derived from arachidonic acid by the 5-lipoxygenase pathway [2], which has been recently implicated in the pathogenesis of atherosclerosis [3-4]. In this pathway arachidonic acid is converted to leukotriene A_4 (LTA_4) by the enzyme 5-lipoxygenase, when activated by arachidonate 5-lipoxygenase activating protein (ALOX5AP). LTA_4 is further metabolised to leukotriene B_4 (LTB_4) by LTA_4 hydrolase or to leukotriene C_4 (LTC_4) by LTC_4 synthase [2]. In response to inflammatory and immune stimuli LTs are secreted into the extracellular space [5] and subsequently bind to G-protein coupled cell surface receptors on their target cells [6-9].

The ALOX5AP gene is located on chromosome 13q12.3 and is 28.9 kilobases in length encoding 162 amino acids in 5 exons [10, 11]. Using a combination of linkage and association analysis, Helgadottir et al. [12] recently identified ALOX5AP as a susceptibility gene for MI. A haplotype, HapA, defined by four single nucleotide polymorphisms (SNPs) spanning ALOX5AP, was found to confer an approximately two-fold greater risk of MI in an Icelandic population [12]. A different four SNP haplotype, HapB, within ALOX5AP was found to confer a similarly greater risk of MI in British Northern European Caucasians, recruited from Leicester and Sheffield [12].

The biological mechanism(s) linking HapA and HapB to risk of MI has not been elucidated, although Helgadottir et al. [12] reported that when LTB_4 production from calcium ionophore stimulated blood neutrophils was compared in MI cases and healthy controls male MI cases carrying HapA (n=10), produced the highest amounts of LTB_4 when compared to controls. Male MI cases without HapA (n= 18) also produced more LTB_4 than controls. Although LTB_4 production was greater in male MI cases carrying HapA than male MI cases without HapA, the difference in LTB_4 production between the two groups was not statistically significant [12]. Therefore, whether HapA is indeed associated with increased LTB_4 production and whether HapB, the other disease-associated haplotype, also affects LTB_4 production remains unclear. In the present study we therefore examined whether healthy subjects carrying HapA

or HapB have increased neutrophil LTB_4 production compared to healthy subjects with neither HapA or HapB (nonA/nonB carriers).

METHODS

Subjects

To identify healthy subjects carrying different variants of the ALOX5AP gene, genotypes were determined in 1268 Northern European Caucasians, from 317 nuclear families, recruited in the Genetic Regulation of Arterial Pressure of Humans in the Community (GRAPHIC) study. The GRAPHIC study is a population based study of representative nuclear families (both parents aged 40-60 years and two children aged 18-40 years), recruited from general practices in Leicestershire, to investigate the impact of candidate gene polymorphisms on blood pressure and other cardiovascular traits. Details of recruitment have been described previously [13]. Subjects enrolled in the present study were from the parental generation of the GRAPHIC cohort (data from the offspring generation was only used to help assign haplotypes). None of the subjects recruited for the study reported a history of cardiovascular disease. Subjects with inflammatory conditions, inter-current infection, or known malignancy were excluded. Subjects in the three groups, i.e. carriers of at least one copy of HapA (HapA group), carriers of at least one copy of HapB (HapB group) and subjects with neither HapA nor HapB (nonA/nonB group) were age and sex matched. All the subjects enrolled provided written informed consent and the study was approved by the Leicestershire Research Ethics Committee.

Genotyping

To identify HapA carriers, HapB carriers and nonA/nonB carriers, the 1268 subject were genotyped for the 7 SNPs that define HapA and HapB [Table 1] using an ABI prism 7900HT Sequence Detection System instrument using SDS v2.1 software (Applied Biosystems, Foster City, California, USA). Allelic discrimination was achieved using fluorogenic 5' nuclease activity TaqMan® MGB probe-based assays [12]. Further details of the assays are available from the authors.

Measurement of ionophore-stimulated neutrophil LTB_4 production

Neutrophils were isolated from 12mls of peripheral venous blood collected in EDTA vacutainers. Subjects were seen in the morning in a fasting state and blood samples were stored at room temperature for no longer than 90 min, before being processed. Smokers were

asked to abstain from smoking on the morning of blood donation. Discontinuous density gradient centrifugation, modified from the technique of English and Andersen [14], was used to isolate neutrophils. Briefly 3mls of Histopaque density 1.077g/ml (Sigma, Poole, UK) was layered over 3mls of Histopaque density 1.119g/ml (Sigma), in each of two 15 ml conical bottom polypropylene tubes. 6mls of anticoagulated whole blood was layered over the Histopaque density 1.077g/ml in each tube. This was centrifuged immediately at 700 g for 30 min in swing-out buckets, at 22^{o}C. The neutrophil layer obtained was aspirated and transferred to a clean 15ml conical bottom polypropylene tube. The neutrophils were washed with HEPES buffered saline (150 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l $MgSO_4.7H_2O$ 10 mmol/l HEPES (Sigma) pH 7.4) and were centrifuged at 280 g for 8min at 22 ^{o}C, to pellet neutrophils. The wash step was repeated and the pellet resuspended in Dulbecco's Modified Eagle Medium (DMEM) with GLUTAMAX™ (Fisher Scientific, Loughborough, UK) at 37^{o}C. White cell count in the resuspended pellet was measured with a Beckman Coulter counter and was adjusted to 2 x 10^6 cells ml^{-1}.

Neutrophils (2 x 10^5) were pre-incubated in a 37^{o}C water bath, for 5 min, in the presence of the cyclooxygenase inhibitor indomethacin (Fluka, Gillingham, UK) at a final concentration 10 μg/ml. Neutrophils were incubated for a further 20 min, in the presence or absence of calcium ionophore A23187 (Sigma) at a final concentration 1 μmol/l. Each reaction was quenched by adding 200 μl ice-cold DMEM, followed by centrifugation at 13000 g, at 4^{o}C, for 5 min, to pellet cellular debris. Supernatants were aspirated and stored as aliquots at -80^{o}C, until analysed for LTB_4 levels. The experimental assays were performed in duplicate.

Quantification of LTB_4 levels

LTB_4 concentrations in cell culture supernatants were quantified with LTB_4 enzyme immunoassay (R&D Systems, Abingdon, UK). The principle of this assay has been described previously [12]. Absorbance was read at 405 nm with a Bio-Tek® Elχ800™ absorbance microplate reader (Bio-Tek Instruments, Winooski, Vermont, USA).

Statistical Analysis

Data are presented as mean ± standard deviation, unless otherwise stated. Departure from Hardy-Weinberg equilibrium for each SNP was tested using the χ^2 test. To determine haplotypes we used the haplotype analysis option in 'Merlin' (Multi-point Engine for Rapid Likelihood INference) [15]. Subjects whose haplotype phase was uncertain were excluded

from the study. Characteristics between the three groups (HapA, HapB and nonA/nonB) were compared by one-way analysis of variance (ANOVA) or the χ^2 test, for continuous and categorical data respectively. Overall association between haplotype and LTB_4 production was tested by the non-parametric Kruskal-Wallis test and comparison between specific groups was carried out using the Mann Whitney U test. Tests for association between LTB_4 production and other parameters (age, gender, smoking status, BMI and total cholesterol level) were carried out using simple linear regression. To assess for inter-assay variability, a coefficient of variation was determined by the following equation: coefficient of variation = ((standard deviation ÷ mean) x 100) [16]. A p-value < 0.05 or less was regarded as significant. All analyses were performed using Stata version 8.2 (Stata Corp.), Excel or GraphPad PRISM software.

RESULTS

Subjects

The flow of subjects through the study is summarised in Figure 1. Of the 634 parental generation subjects genotyped, haplotype phase could not be determined in 63 (10%). Of the remainder, 24.3% subjects carried HapA and 9.3% subjects HapB. On the basis of age and gender matching, 148 subjects were sent letters inviting them to participate in the study and 59 subjects were recruited: 21 HapA carriers, 20 HapB carriers and 18 nonA/nonB carriers. The characteristics of the three haplotype groups are shown in Table 2. The three groups were well matched for all variables**,** apart from a slight difference in mean ambulatory 24 hour diastolic blood pressure. All subjects had a normal white blood cell count.

Effect of ALOX5AP haplotype on LTB_4 Production

LTB_4 production in non-stimulated cells was negligible (range: 0.01 – 0.03 ng/10^6 cells). The net stimulated production of LTB_4 for subjects in the different haplotype groups are shown in Figure 2. There was no difference in the mean level for LTB_4 production in the three groups (nonA/nonB: 24.9 ± 8.3 ng/10^6 cells; HapA: 22.2 ± 11.9 ng/10^6 cells; HapB: 19.8 ± 4.8 ng/10^6, p = 0.14). Stimulated LTB_4 production in one HapA subject was 68.3 ng/10^6 cells and inconsistent with the rest of the data set (Figure 2). When this outlier was excluded, the distribution of LTB_4 production between the haplotype groups remained non-significant (p = 0.11).

There was no association between LTB_4 production and age ($p = 0.94$) (although the age-range was small), gender ($p = 0.93$), smoking status ($p = 0.10$), BMI ($p = 0.87$), or total cholesterol level ($p = 0.21$).

To assess the variability of the assay, the intra-individual coefficient of variation was measured from readings of samples obtained from the same subject on three separate occasions, separated by three weekly intervals. This gave a coefficient of variation of less than 16%.

DISCUSSION

Both descriptive as well as interventional findings suggest an important role for the 5-lipoxygenase pathway in atherosclerosis. 5-lipoxygenase expression increases with evolution of atherosclerotic plaques [4]. In subjects undergoing carotid endarterectomy, LTB_4 level is higher in plaque homogenates from symptomatic compared with asymptomatic subjects suggesting that the pathway may be involved in promoting plaque instability [17]. Antagonism of LTB_4 receptors in hyperlipidaemic mice reduces lipid accumulation and monocyte infiltration in atherosclerotic lesions when compared to hyperlipidaemic control mice [18]. Finally, genetic knockout of the 5-lipoxygenase gene in atherosclerosis prone mice produces a marked reduction in atheroma formation [3]. It has been proposed that LTB_4 induced activation of leukocytes leads to release of lysosomal enzymes, such as myeloperoxidase and the generation of reactive oxygen species that have been associated with propagation and acute complication of atherosclerosis [18].

In this context the finding by Helgadottir et al., [12] starting from an initially an unbiased genome-wide linkage analysis approach, that specific haplotypes of the gene encoding for ALOX5AP, the main regulator of the activity of 5-lipoxygenase, are associated with increased risk of MI has raised considerable interest in the role of this gene in explaining some of the genetic susceptibility to coronary artery disease and MI. HapA was also associated with risk of stroke in the Icelandic cohort studied by Helgadottir et al. [12]. Other studies have subsequently also investigated the association between ALOX5AP variants and cardiovascular diseases. HapA was associated with a 36% greater risk of stroke in a Scottish cohort [19]. In a German case-control study [20], HapA was not itself associated with risk of stroke but one of the single markers (SG13S114 in Table 1) constituting this haplotype was significantly associated. The authors suggested that differences in allele and haplotype

frequencies may explain the discrepant results. In a study in Japanese subjects, the frequencies of HapA and HapB were too low to examine for an association but haplotypes constructed from two other SNPs (A162C and T8733A) did influence risk [21].

The study by Helgadottir et al. [12] suggested that carriage of HapA may be associated with a greater production of leukotrienes when the system is stimulated. This could provide an explanation for the association of the haplotype with risk of MI. Indeed, on the basis of this, a Phase II clinical trial assessed the effect of three doses of an ALOX5AP inhibitor (DG-031) on biomarkers of cardiovascular risk in MI subjects carrying HapA and reported a significant dose-dependent suppression of biomarkers of cardiovascular risk [22].

In the present study, we found no evidence for increased LTB_4 production by blood neutrophils when stimulated with the calcium ionophore A23187 in healthy subjects carrying either HapA or HapB of ALOX5AP compared with nonA/nonB carriers. A strength of our study is that we investigated healthy subjects where any genuine genotype-specific effects should be easier to detect without the effects of confounding by disease or treatment. Furthermore, our subjects were selected from the general population (Figure 1) making any selection bias unlikely. The protocol used for stimulating the neutrophils was very similar to that of Helgadottir et al. [12] making it unlikely that methodological differences masked any effects of the genotype in our study. Indeed, in pilot studies we compared LTB_4 production for three different doses of A23187 (0.5, 1 and 2μmol/l) and three different incubation periods, (10, 20 and 30 minutes) in a series of subjects and independently established that 1μM and 20 minutes incubation, which are the same conditions used by Helgadottir et al. [12], were the optimal conditions (unpublished data). A negative finding raises questions about the power of a study. For a functional study with selection on the basis of genotype our study is moderately large and indeed larger than the initial study by Helgadottir et al. [12]. A post-hoc power calculation showed that given the sample sizes, means and standard deviations, the study had 90% power for an alpha of 0.05, to detect a 32% difference in LTB4 production between either the HapA or HapB group compared with the the nonA/nonB group. Thus, the study had reasonable power to detect an effect likely to be of physiological relevance.

Our findings have a number of implications. They indicate that if either the HapA or the HapB haplotype of ALOX5AP genuinely increase cardiovascular risk, then the mechanism is

not simply due to a systematically observable effect of the haplotype on LTB_4 production in response to stimulation. The other important implication of our finding is in relation to use of ALOX5AP inhibitors as therapeutic targets for treating atherosclerotic diseases [5,22]. Although the study by Hakonarson et al. [22] showed that treatment with DG-031 reduced biomarkers of cardiovascular risk in subjects carrying HapA, our results raise the question as to whether the effect is quantitatively or qualitatively genotype-specific or likely to be observed to a similar degree in all subjects. This could have important bearing on the future clinical utility of this class of drugs and the design of outcome trials.

Neutrophils are a major source of leukotrienes and a convenient cell to study. However, it remains possible that the ALOX5AP haplotypes affect leukotriene production in other cell types involved in atherosclerosis, perhaps in the vessel wall. Furthermore, it may be interesting to measure other relevant products of the 5-lipoxygenase pathway, such as LTC_4 (a potent vasoconstrictor of coronary arteries [23]), or *in vivo* LT production, by quantifying urinary concentration of LTB_4 metabolites and cysteinyl leukotriene metabolites [24] to elucidate whether or not these vary by genotype. Finally, we studied healthy subjects and it is possible that the ALOX5AP haploytypes associated with risk of MI affect leukotriene production differently in the presence of cardiovascular risk factors or disease. These limitations of our study need to be borne in mind.

In summary, in stimulated blood neutrophils isolated from healthy subjects, we were unable to show any effect on LTB_4 production of two haplotypes of the ALOX5AP gene that have been recently associated with risk of MI. Although the findings do not exclude the possibility that the haplotypes do confer increased cardiovascular risk through some other mechanism, they suggest that knowledge of a patient's haplotype may not provide useful information on the likely clinical response to ALOX5AP inhibitors.

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TABLES AND FIGURES

Table 1 Description of the single nucleotide polymorphisms in the ALOX5AP gene that were genotyped to determine HapA and HapB haplotype status.

Ref SNP ID	deCODE SNP ID	Gene Locaton	Relative Position	SNPs defining HapA	SNPs defining HapB
Rs17222814	SG13S25	Promoter	-10189 G>A	G	-
Rs17216473	SG13S377	Promoter	-5777 G>A	-	A
Rs10507391	SG13S114	Intron 1	2354 T>A	T	A
Rs4769874	SG13S89	Intron 3	16699 C>A	G	-
Rs9551963	SG13S32	Intron 4	22805 C>A	A	-
Rs9315050	SG13S41	Intron 4	26303 A>G	-	A
Rs17222842	SG13S35	3' UTR	30375 G>A	-	G

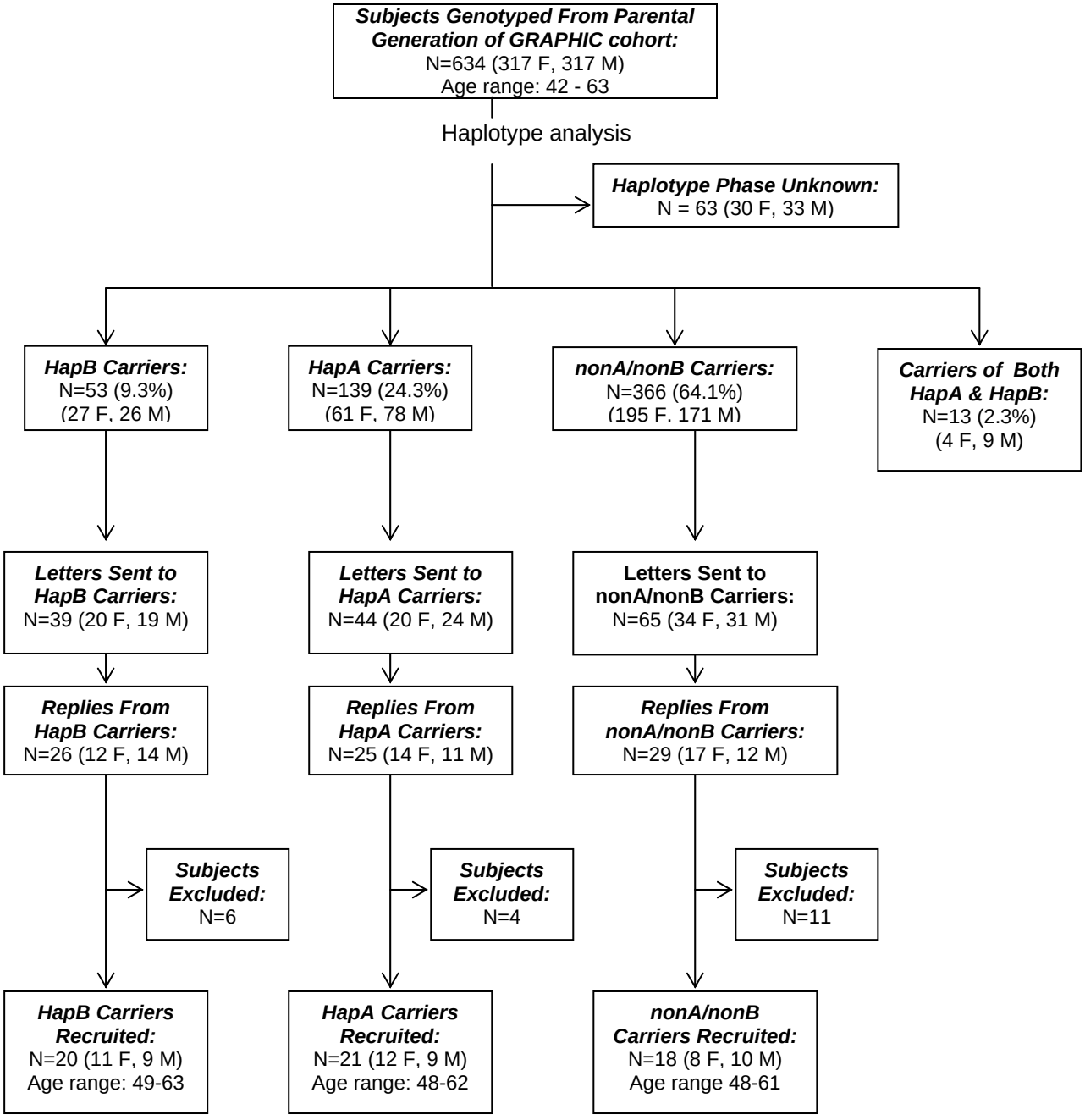
Ref SNP ID, refers to the reference for the SNP in the NCBI database [10]. The decode SNP ID is the original identifier given to the SNP by deCode Genetics and is also available on the NCBI site. The relative position refers to position in base p[airs from the start of the first exon. The minor allele frequency is for Caucasian subjects analysed in the HapMap project (www.hapmap.org).

Table 2. Demographics of studied subjects carrying different haplotypes for ALOX5AP

Variable	**HapA (n=21)**	**HapB (n=20)**	**nonA/nonB (n=18)**	**p-value**
Sex (% male)	42.9	45.0	55.6	0.71
Age (years)	55.1 ± 4.2	56.1 ± 4.1	56.9 ± 3.4	0.36
Current Smoker (%)	4.8	5.0	11.1	0.68
BMI ((kg/ m^2)	26.8 ± 4.0	27.0 ± 3.9	27.2 ± 4.5	0.96
Total cholesterol (mmol/L)	5.3 ± 0.9	5.9 ± 1.1	5.4 ± 0.9	0.09
HDL cholesterol (mmol/L)	1.4 ± 0.3	1.5 ± 0.4	1.3 ± 0.3	0.13
Mean ambulatory 24 hour DBP (mmHg)	72.7 ± 5.5	78.4 ± 7.5	76.7 ± 8.8	0.04
Mean ambulatory 24 hour SBP (mmHg)	119.7 ± 8.9	127.4 ± 14.0	122.2 ± 12.4	0.12
Glucose (mmol/L)	5.0 ± 1.1	5.5 ± 1.5	5.0 ± 0.8	0.83
Albulmin (g/L)	43.5 ± 2.6	44.4 ± 2.2	44.1 ± 1.8	0.45
Creatinine (μmol/L)	80.4 ± 14.1	81.8 ± 12.0	83.1 ± 13.0	0.81
Urea (mg/dL)	5.5 ± 1.0	5.3 ± 1.2	5.8 ± 1.5	0.48
Sodium (mmol/L)	138.3 ± 2.2	138.4 ± 2.1	139.3 ± 1.7	0.27
Potassium (mmol/L)	4.7 ± 1.2	4.6 ± 1.1	4.2 ± 0.5	0.35
White blood cell count (x 10^6 cells ml^{-1})	5.7 ± 1.4	5.5 ± 1.2	5.7 ± 1.2	0.83

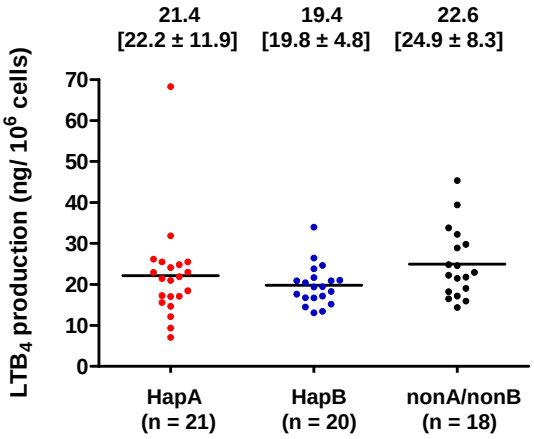
BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure.

Figure 1. Flow chart showing subject flow through the recruitment process and study



F = females, M = males

Figure 2 Dot plot showing distribution of stimulated LTB_4 production by neutrophils from subjects carrying HapA, HapB and nonA,nonB haplotyes of ALOX5AP.



The horizontal bars and the values at the top of the columns are medians. The values below the medians in square brackets are the mean ± standard deviation.