INTRODUCTION

Three human adenosine diphosphate (ADP) receptors have been cloned: P2Y1, P2Y12 and P2Y13 [1–4]. On platelets, P2Y1 and P2Y12 mediate ADP-induced platelet activation and aggregation [5]. In red blood cells, activation of P2Y13 by the adenosine triphosphate (ATP) metabolite ADP activates a negative feedback loop that inhibits ATP release from erythrocytes [6]. Rare mutations in the P2Y12 gene that disrupt P2Y12 receptor function result in compromised ADP-induced platelet activation and increased bleeding times [1,7,8].

The clinical importance of the P2Y12 receptor as a mediator of platelet activation has become evident in several large-scale clinical studies and inhibition of the P2Y12 receptor with clopidogrel is one of the cornerstones in treatment and prevention of acute coronary syndromes [9]. Even greater P2Y12 inhibition by prasugrel was recently shown to be even more effective in preventing ischemic events than the standard regimen of clopidogrel [10].

A group of single nucleotide polymorphisms (SNPs) in the P2Y12 gene, forming the so called P2Y12 H2 haplotype [11], have been associated with increased platelet responsiveness to ADP and increased risk of peripheral arterial disease (PAD) [11–13]. Recently, Cavallari et al showed an association of the P2Y12 H2 haplotype with coronary artery disease (CAD) [14]. It has also been proposed that this haplotype may account for variations in response to clopidogrel. However, several studies have failed to confirm any association between platelet function and the H2 haplotype [15–17].

The group of polymorphisms that make up the P2Y12 H2 haplotype are all synonymous polymorphisms that do not change the amino acid sequence of the P2Y12 protein, and no mechanistic explanation to the reported increased platelet reactivity to ADP associated with this haplotype has been presented [11]. However, a non-synonymous polymorphism, Met-158-Thr, in the neighboring P2Y13 gene, located only 8 kb away from P2Y12, could be in linkage disequilibrium with the P2Y12 H2 haplotype. We hypothesized that the P2Y13 Met-158-Thr polymorphism of the P2Y13 receptor could account for the reported effects of the P2Y12 H2 haplotype since the receptors share the same ligand, ADP. The P2Y13 receptor has been found on red blood cells and inflammatory cells, both cell types known to interact with platelets [4,6]. The first objective of this study was to examine possible linkage disequilibrium (LD) between the P2Y12 H2 haplotype and the P2Y13 Met-158-Thr polymorphism. After showing that this was the case, we aimed at investigating if the P2Y13 Met-158-Thr polymorphism is associated with acute myocardial infarction (AMI) or diabetes mellitus, two diseases strongly associated with peripheral arterial disease [18,19]. Our hypothesis that the P2Y12 H2 haplotype and SNPs in LD with the P2Y12 H2 haplotype would be associated with AMI was strengthened further by a report linking the P2Y12 H2 haplotype with coronary artery disease (CAD) [14], since myocardial infarction is the major complication of CAD [20]. In order to do so, the Met-158-Thr polymorphism was genotyped in more than 10,000 individuals divided in three study populations: two sub-populations of the Malmo Diet and Cancer study (an AMI case-control population and a diabetes case-control population) and a control subpopulation from the study of early onset AMIs with family history of AMI.

Background and Aims. The aims of this study were to investigate (1) if P2Y13 polymorphisms defining the P2Y12 H2 allele are associated with any other SNPs that may explain the previously reported association with increased ADP induced platelet activation and association with peripheral arterial disease and coronary artery disease and (2) if such variants are associated with acute myocardial infarction (AMI) or classical risk factors for AMI. Methods and Results. The P2Y13 Met-158-Thr polymorphism was found to be in linkage disequilibrium (LD) with the P2Y12 H2 haplotype (all examined SNPs: D’ = 1.0, r2 = 0.936–1.0), defining a novel P2Y12 H2/P2Y13 Thr-158 haplotype. Genotyping of an AMI case control population (n = 1244 cases, 2488 controls) revealed no association of the P2Y13 Thr-158 allele with AMI (OR = 0.96, 95% C.I. 0.82–1.12, P = 0.63). Also, no differences between the genotype frequencies of P2Y13 Met-158-Met and Met-158-Thr/Thr-158-Thr were seen in AMI case-control subpopulations (early onset AMI OR = 1.06, 95% C.I. 0.85–1.31, P = 0.62); family history of AMI (OR = 0.98, 95% C.I. 0.78–1.22, P = 0.83) nor in early onset AMIs with family history of AMI (OR = 1.0, 95% C.I. 0.74–1.36, P = 1.0). Genotyping of the P2Y13 Met-158-Thr polymorphism in a population based sample (n = 6055) revealed no association with cardiovascular risk factors. In addition, the P2Y13 Met-158-Thr polymorphism was genotyped in a diabetes case-control population, and associations were found neither with DM nor with any examined DM risk factors. Conclusion Genotyping. The P2Y13 Met-158-Thr polymorphism is in tight LD with the P2Y12 locus but is not associated with AMI or classical cardiovascular risk factors.
a large population with cardiovascular risk factor data [21,22]) and a diabetes mellitus case-control population with data on several DM risk factors [23].

**MATERIALS AND METHODS**

**Malmö diet and cancer population (MDC)**

The study population is made up of 28098 randomly selected men (born 1923–1945) and women (born 1923–1950) living in the Swedish city of Malmö (population 250 000). Overall participation rate in the study was 41%.

A baseline examination was performed between 1991–1996, including assessment of dietary habits, a questionnaire on socioeconomic, demographic and lifestyle factors, heredity, medication and previous and current diseases. Blood samples were taken and DNA, lymphocytes, granulocytes, erythrocytes and plasma/serum were stored in a biological bank [21,22].

**AMI Case control population**

On 31 December 2000 the study population was matched with the Swedish National Board of Health and Welfare’s National Patient Registry and Cause of Death Registry. AMI cases (first AMI) were identified using the diagnosis criteria defined by the International Classification of Diseases, 9th and 10th and Revision, Clinical Modification (ICD 9 and 10; ICD 9 codes 410 in the Swedish Patient Registry or 410-414 in the Swedish Cause of death Registry; ICD 10 codes I21 in the Swedish Patient Registry and I21-I25 in the Swedish Cause of Death Registry.

Two gender- and age (±1 year)-matched AMI-free controls from the MDC population were assigned to each AMI case, resulting in a case-control material consisting of 1244 AMI cases and 2488 AMI-free controls. The myocardial infarction group was subdivided into early onset AMI (<62.8 years at first AMI event) and late onset AMIs (LO, >62.8 years at first AMI event). Family history AMIs (FH, n=611) were defined as AMI cases where at least one blood related first degree family member had suffered an AMI, and non familial AMIs (n=633) as cases without any first degree family history of AMI. 319 cases had at least one blood related first degree family member that had suffered an AMI, and non familial AMIs were defined as AMI cases where at least one blood related first degree family member had suffered an AMI, and non familial AMIs (n=633) as cases without any first degree family history of AMI. 319 cases had at least one blood related family member also suffered a myocardial infarction and early onset AMI with family history of AMI. Data of known cardiovascular risk factors was collected in the cardiovascular group only. *subgroups of all AMI cases.

**Cardiovascular group population**

Of the MDC, 6103 individuals were randomly selected into a “Cardiovascular cohort” (MDC-CV), a sample thus being representative of MDC, in whom cardiovascular risk factors were measured, including systolic blood pressure, smoking status and anthropometric data and, in the majority (n=5540), fasting plasma analyses of glucose, lipids and C-reactive protein (CRP). DNA for genotyping was obtained from 6055 of the 6101 selected individuals.

**Diabetes mellitus case-control population**

The diabetes mellitus case control material has been described elsewhere [23]. Briefly, all study subjects originate from the Botnia region in Western Finland and the Helsinki area and age- and gender matched controls were assigned to all type 2 diabetes cases. The case group is composed of 307 unrelated randomly selected individuals with type 2 diabetes (146 males and 161 females, mean age 61 (55–67) years, mean BMI 28.7 (26.0–31.7)). The control group consisted of 307 unrelated individuals with confirmed normal oral glucose tolerance and without a family history of diabetes (146 males and 161 females, mean age 60 (53–67) years, BMI 26.4 (24.1–29.2)).

**Extent of H2 haplotype linkage disequilibrium and genotyping of case-control and cardiovascular group populations**

Using HapMap and the Human Genome assembly build 36.2, SNPs in or within 1000 base pairs (bp) upstream of the known genes located in the 3q24-25 region (P2Y12 locus) were identified. By means of DNA sequencing using BigDye v. 3.1 (Applied Biosystems, CA, USA) in 20 individuals, selected SNPs were probed for linkage disequilibrium with the known P2Y12 H2 haplotype SNPs using one of the P2Y12 H2 SNPs, rs2046934, as a marker of the P2Y12 H2 haplotype [11]. Single nucleotide polymorphisms (SNPs) that displayed high degrees of LD with the P2Y12 H2 haplotype SNPs were selected for genotyping in a randomly selected sub-population of the DM case-control population (n=293) using TaqMan or Sequenom and a haplotype map was constructed using the Haplovew software [24].

Genotyping of the AMI (n=3732) and DM (n=614) case control populations and the cardiovascular group population (n=6055) was performed using Sequenom (Sequenom Inc., CA, USA) or TaqMan ABI 7900 according to the manufacturers’ instructions. Two different persons who were unaware of the phenotypic status of the study participants read all genotypes. For genotyping primers and probes, see table 2.

**Statistical analysis**

In the AMI case control population, conditional logistic regression was used to calculate odds ratios and p values. The Cardiovascular
group population was subjected to ANOVA and t-tests for continuous normally distributed variables, in case of non-normality Kruskal-Wallis test or Mann-Whitney test was used. Chi-2 test was used to test for significant differences in dichotomous variables. In the DM case control population, variables were log transformed for normal distribution. P-values were calculated using the GLM-ANCOVA using sex and age as covariates. Adjustment for multiple testing was not done. Statistical analyses were performed with SPSS.

Power calculations

For myocardial infarction Accepting a significance level of 0.05, 1244 AMI cases and 2488 controls have a power of 95% to detect a genotype relative risk of 1.20 for the P2Y13 Met-158-Thr polymorphism. Thus, it is unlikely that our result is a false negative finding.

For diabetes Accepting a significance level of 0.05, 307 diabetes cases and 307 controls have a power of 35% to detect a genotype relative risk of 1.20 for the P2Y13 Met-158-Thr polymorphism. Accepting a significance level of 0.05, 532 diabetes cases and 5522 controls in the cardiovascular group population have a power of 79% to detect a genotype relative risk of 1.20 for the P2Y13 Met-158-Thr polymorphism. By analyzing both these two diabetes materials, it is unlikely that our result is a false negative finding. Power calculations were performed using the program CaTS [25].

RESULTS

Extent of P2Y12 H2 haplotype linkage disequilibrium

The pilot linkage disequilibrium analysis of SNPs in the P2Y12 locus revealed three SNPs (rs1466684, rs38211667, rs11922647) that showed signs of LD with the known P2Y12 H2 SNPs. Genotyping in a larger population (n = 295) confirmed complete LD of the three SNPs with the P2Y12 H2 haplotype SNPs (D’ = 1.0, r² = 0.936–1.0, figure 1) [11]. Two of the three SNPs were located in or in close proximity to the P2Y12 gene: rs38211667 in the non-coding region of P2Y12 exon 2 and rs11922647 within 1000 bp of transcription start of transcript variant 2 of the P2Y12 gene. The third SNP (rs1466684) was found to be a non-synonymous SNP of the P2Y13 gene, causing a Met-Thr amino acid substitution at position 158 of the P2Y13 receptor. The complete LD of the P2Y12 H2 haplotype with the P2Y13 Thr-158 variant thus defines a novel P2Y12 H2/P2Y13 Thr-158 haplotype. The P2Y13 Met-158-Thr polymorphism was selected as reference SNP for the P2Y12 H2/P2Y13 Thr-158 haplotype in subsequent genotyping.

Genotyping of the P2Y12 H2/P2Y13 Thr-158 haplotype in the AMI and DM case control populations

In the AMI case control population, containing 3273 individuals, 92.8% of those eligible for the case–control study were genotyped successfully (table 3), representing 1134 pairs in total (n = 1134 cases and one to two control subjects matching every case (n = 2139). Genotype frequencies were in accordance with Hardy–Weinberg Equilibrium.

No association of the P2Y13 Met-158-Thr polymorphism (Thr-158-Met and Thr-158-Thr vs. Met-158-Met) was found with AMI (OR = 0.96, 95% C.I. 0.82–1.12, P = 0.63). Also, no differences were seen in the AMI case subpopulations (EO OR = 1.06, 95% C.I. 0.85–1.31, P = 0.62; FH OR = 0.98, 95% C.I. 0.78–1.22, P = 0.83; EO+FH OR = 1.0, 95% C.I. 0.74–1.36, P = 1.0).

In the diabetes mellitus (DM) case control study 576 individuals (93.8%) were genotyped successfully. No associations of the P2Y13 polymorphism (Thr-158-Met and Thr-158-Thr vs. Met-158-Met) were found with diabetes mellitus or any examined DM risk factor (table 4).

| SNP_ID | SNP location | Forward | Reverse | Mass Extension/Probes |
|--------|--------------|---------|---------|----------------------|
| rs1466684 | P2Y13 Met-158-Thr | *5′-ACGTGGATGCTTCATCTGGTGTTGCTC-3′ | *5′-ACGTGGATGCGGTCTTCATCTGGTGTTGCTC-3′ | *5′-ACGTGGATGCTTCATCTGGTGTTGCTC-3′ |
| rs11922647 | P2Y13 intron 1 | *5′-ACGTGGATGCTTCATCTGGTGTTGCTC-3′ | *5′-ACGTGGATGCTTCATCTGGTGTTGCTC-3′ | *5′-ACGTGGATGCTTCATCTGGTGTTGCTC-3′ |
| rs38211667 | P2Y13 untranslated | *5′-ACGTGGATGCTTCATCTGGTGTTGCTC-3′ | *5′-ACGTGGATGCTTCATCTGGTGTTGCTC-3′ | *5′-ACGTGGATGCTTCATCTGGTGTTGCTC-3′ |
| rs2046934 | P2Y13 intron 2 | #5′-TGTAGAATGACATTTGGATCTC-3′ | #5′-TGTAGAATGACATTTGGATCTC-3′ | #5′-TGTAGAATGACATTTGGATCTC-3′ |

Table 2. Primers and probes used for genotyping of high linkage disequilibrium polymorphisms in P2Y12 and one in P2Y13.

Figure 1. Linkage disequilibrium map of the P2Y12 H2/P2Y13 Thr-158 haplotype. All examined SNPs displayed a very high degree of linkage disequilibrium (D’ = 1.0; r² = 0.936–1.0) (see figure for each individual r² value). doi:10.1371/journal.pone.0001462.g001
Table 3. Genotyping of Met-158-Thr in AMI cases and corresponding controls.

|                        | Met-158-Met | Met-158-Thr | Thr-158-Thr | Met-158-Thr+Thr-158-Thr | Total |
|------------------------|-------------|-------------|-------------|-------------------------|-------|
| Control count n (%)    | 1437 (67.2) | 638 (29.8)  | 64 (3)      | 702 (33)                | 2139  |
| AMI count n (%)        | 772 (68.1)  | 332 (29.3)  | 30 (2.6)    | 362 (32)                | 1134  |
| **Early onset AMI**    |             |             |             |                         |       |
| EO Control count n (%) | 715 (66)    | 323 (29.8)  | 31 (2.9)    | 354 (33.1)              | 1069  |
| EO AMI count n (%)     | 368 (64.7)  | 176 (35.3)  | 16 (3.2)    | 192 (34.3)              | 560   |
| **Late onset of AMI**  |             |             |             |                         |       |
| LO Control count n (%) | 722 (67.5)  | 315 (29.4)  | 33 (3.1)    | 348 (33)                | 1070  |
| LO AMI count n (%)     | 404 (70.4)  | 156 (27.2)  | 14 (2.4)    | 170 (30)                | 574   |
| **Family history of AMI** |         |             |             |                         |       |
| FH Control count n(%)  | 723 (68.3)  | 301 (28.4)  | 35 (3.3)    | 336 (32)                | 1059  |
| FH AMI count n (%)     | 387 (69)    | 157 (28)    | 17 (3)      | 174 (31)                | 561   |
| **No family history of AMI** |     |             |             |                         |       |
| NFH Control count n(%) | 714 (66.1)  | 337 (31.2)  | 29 (2.7)    | 366 (34)                | 1080  |
| NFH AMI count n (%)    | 385 (67.2)  | 175 (30.5)  | 13 (2.3)    | 188 (33)                | 573   |
| **Early onset and family history of AMI** | | | | | |
| EO+FH Control count n (%) | 373 (67.6) | 159 (28.8)  | 20 (3.6)    | 179 (32)                | 552   |
| EO+FH AMI count n (%)  | 197 (67.9)  | 87 (30)     | 6 (2.1)     | 93 (32)                 | 290   |

The AMI case group (n = 1244) contains the early onset (EO), late onset (LO), family history (FH) and no family history (NFH) as well as early onset with family history (EO+FH) subgroups. *Both heterozygous and homozygous P2RY13 Thr-158 carriers.
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Table 4. Genotyping of the P2Y12 H2/P2Y13 Thr-158 haplotype in a diabetes mellitus (DM) case control population (307 DM cases, 307 controls).

|                        | Met-158 [n]# | Thr-158* [n]# | p-value |
|------------------------|-------------|---------------|---------|
| **Controls (n = 289)** |             |               |         |
| BMI (kg/m²)            | 26.4(24.1–29.2) [213] | 26.6(24.2–29.3) [70] | 0.49    |
| WH                     | 0.88(0.82–0.95) [211] | 0.88(0.82–0.96) [70] | 0.64    |
| P-Glu (mmol/L)         | 5.42(5.09–5.80) [214] | 5.50(5.06–5.93) [73] | 0.56    |
| P-Glu [120 min] (mmol/L)| 5.85(4.97–6.70) [191] | 5.82(5.02–6.75) [64] | 0.90    |
| P-INS (mU/L)           | 7.66(4.95–10.22) [210] | 6.55(4.66–9.47) [68] | 0.10    |
| Triglycerids (mmol/L)  | 1.17(0.92–1.67) [198] | 1.32(0.81–1.69) [65] | 0.86    |
| HDL-cholesterol (mmol/L)| 1.36(1.16–1.66) [199] | 1.37(1.18–1.73) [64] | 0.91    |
| HOMA                   | 1.76(1.8–2.53) [207] | 1.68(1.05–2.49) [68] | 0.13    |

| **Diabetes mellitus (n = 287)** | Met-158 [n]# | Thr-158* [n]# | p-value |
|---------------------------------|-------------|---------------|---------|
| Age at onset (years)            | 53(46–59) [201] | 53(48–60) [74] | 0.67    |
| BMI (kg/m²)                     | 28.7(25.9–31.7) [206] | 28.2(25.7–31.2) [78] | 0.53    |
| WH                              | 0.93(0.87–0.99) [202] | 0.94(0.90–1.00) [77] | 0.76    |
| P-Glu (mmol/L)                  | 9.49(7.90–11.75) [207] | 8.87(7.12–11.35) [78] | 0.28    |
| P-Glu [120 min] (mmol/L)        | 15.43(11.67–20.18) [108] | 14.24(12.37–16.84) [37] | 0.16    |
| P-INS (mU/L)                    | 11.95(6.99–21.15) [198] | 11.54(7.87–19.09) [77] | 0.60    |
| Triglycerids (mmol/L)           | 1.57(1.1–2.2) [197] | 1.66(1.21–2.39) [75] | 0.17    |
| HDL-cholesterol (mmol/L)        | 1.19(1.02–1.45) [191] | 1.15(1.01–1.36) [71] | 0.31    |
| HOMA                            | 4.98(3.00–9.20) [197] | 4.70(3.00–9.88) [77] | 0.30    |

*Variables were log-transformed for normal distribution. P-values were calculated using the GLM-ANCOVA using sex and age as covariates. No associations were found with neither DM nor any known DM risk factor. *Both heterozygous and homozygous P2Y12 H2/P2Y13 Thr-158 carriers.

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Table 5. Association of known cardiovascular risk factors with the P2Y12 H2/P2Y13 Thr-158 genotype in the cardiovascular group (CVG).

| Cardiovascular risk factors | Met-158-Thr genotype | Mean ± st dev | P value (two-tailed) |
|----------------------------|----------------------|---------------|---------------------|
| Systolic blood pressure (mm Hg) | Met | 141.6 ± 19.1 | 0.60 |
|                             | Thr | 141.3 ± 19.1 |           |
| Diastolic blood pressure (mm Hg) | Met | 873 ± 9.5 | 0.15 |
|                             | Thr | 869 ± 9.4 |           |
| Body-Mass Index (weight/kg x kg) | Met | 26.0 ± 4.0 | 0.16 |
|                             | Thr | 25.8 ± 4.0 |           |
| Waist (cm)                  | Met | 84.7 ± 12.9 | 0.09 |
|                             | Thr | 84.1 ± 13.0 |           |
| Diabetes mellitus (%)* | Met | 8.8 | 0.94 |
|                             | Thr | 8.9 |           |
| Cholesterol (mmol/l)* | Met | 6.2 ± 1.0 | 0.93 |
|                             | Thr | 6.2 ± 1.1 |           |
| Triglycerides (mmol/l)* | Met | 1.4 ± 0.9 | 0.38 |
|                             | Thr | 1.4 ± 0.8 |           |
| HDL (mmol/l)* | Met | 1.4 ± 0.4 | 0.78 |
|                             | Thr | 1.4 ± 0.4 |           |
| LDL (mmol/l)* | Met | 4.2 ± 1.0 | 0.96 |
|                             | Thr | 4.2 ± 1.0 |           |
| LDL/HDL ratio* | Met | 3.2 ± 1.1 | 0.91 |
|                             | Thr | 3.2 ± 1.2 |           |
| CRP (mg/L)* # | Met | $1.4 (0.7–2.8)$ | 0.83 |
|                             | Thr | $1.4 (0.7–2.9)$ |           |

Gaussian distribution was observed for all above risk factors except CRP that showed a natural logarithmic distribution. n = 6055. * n = 5540. † = median, interquartile range. #Thr = Both heterozygous and homozygous P2RY13 Thr-158 carriers.

Genotyping of P2Y13 Met-158-Thr in the cardiovascular group population

5846 individuals (96.5%) of 6055 in the cardiovascular group (CVG) were genotyped successfully. No association was found for the P2Y13 Met-158-Thr polymorphism (Thr-158-Met and Thr-158-Thr vs. Met-158-Met) regarding any of the examined cardiovascular risk factors, including systolic blood pressure, diastolic blood pressure, BMI, waist circ., diabetes, total cholesterol, triglycerides, HDL, LDL, CRP, smoking or alcohol intake (table 5). Furthermore, no stronger association with the above mentioned risk factors was seen in the homozygous P2Y13 Thr-158 group compared to the heterozygous Met-158-Thr carriers (data not shown).

**DISCUSSION**

In this study we show that the P2Y12 H2 haplotype [11] is in complete linkage disequilibrium with the non-synonymous Met-158-Thr polymorphism in the P2Y13 gene, defining a P2Y12 H2/P2Y13 Thr-158 haplotype. Based on the observed LD between the studied P2Y12 and P2Y13 polymorphisms, we assume that all disease and risk factor associations made with the P2Y13 Met-158-Thr polymorphisms are also valid for the P2Y12 H1/H2 haplotypes. We hypothesized that this finding could provide a potential mechanistic explanation to the previously observed clinical associations of the P2Y12 H2 haplotype with CAD, PAD or platelet function [11,12,14], since the receptors share the same ligand. However, no associations of the P2Y13 Met-158-Thr polymorphism with AMI or DM were found in our large material. Indeed, no associations with any of the investigated cardiovascular or diabetes mellitus risk factors were observed. This was unexpected, since strong associations have been reported between CAD, PAD, AMI and diabetes [18–20]. All studies involving the P2Y12 H2 haplotype are listed in table 6.

The concentration of extracellular nucleotides in the blood is tightly regulated by ectonucleotidases on leukocyte and endothelial cells to prevent excessive ADP accumulation and subsequent platelet activation [26]. Red blood cells contain millimolar amounts of ATP and are therefore a major source of nucleotides in the blood [6,27]. This ATP pool could potentially be an important contributor to the regulation of platelet activation. Recently, it was shown that extracellular ADP activates P2Y13 expressed on red blood cells, resulting in a subsequent decreased release of nucleotides from the red blood cells in a classic negative feedback manner [6]. It is possible that this negative feedback loop might be important in the regulation of nucleotide-induced platelet activation in vivo. Thus, a non-synonymous polymorphism leading to a structurally and functionally altered P2Y13 could potentially alter the nucleotide concentrations in the blood stream, thereby affecting platelet activation in vivo. Indeed, in silico analysis using the polymorphism phenotyping prediction software PolyPhen [28] indicated that the P2Y13 Met-158-Thr amino acid substitution could possibly affect the function of the P2Y13 receptor.

In 2002 Fontana et al reported the P2Y12 H2 haplotype to be associated with a gain of function in terms of ADP induced platelet aggregation. The polymorphisms in the H2 haplotype are either

Table 6. Published studies on the P2Y12 H2 haplotype and platelet ADP response and cardiovascular disease.

| Study author | Study population (n) | Reported outcome |
|--------------|----------------------|-----------------|
| Fontana [11] | 98                   | P2Y12 H2 haplotype is associated with increased ADP-induced platelet aggregation |
| Fontana [11] | 514                  | P2Y12 H2 haplotype is associated with peripheral arterial disease |
| Cavallari [14] | 1378                | The P2Y12 H2 haplotype is associated with coronary artery disease |
| Angiolillo [30] | 119                  | The P2Y12 H2 haplotype does not influence platelet response to clopidogrel |
| Hetherington [31] | 200                | No association of P2Y12 H2 haplotype with ADP-induced platelet aggregation |
| von Beckerath [17] | 416              | P2Y12 gene H2 haplotype is not associated with increased adenosine diphosphate-induced platelet aggregation after initiation of clopidogrel therapy with a high loading dose |
| Schetter [16] | 540                  | No association of P2Y12 H2 haplotype and an increased risk of cardiovascular events in a population with CAD |
| Amisten et al | 10401             | The P2Y12 H2/P2Y13 Thr-158 haplotype is not associated with AMI, cardiovascular risk factors or diabetes |

* doi:10.1371/journal.pone.0001462.t005
located in intronic regions of the gene or were silent, i.e. causing no alterations of the P2Y12 receptor protein. The possibility remained that the polymorphisms could potentially be coupled to mRNA processing or translation events, thereby altering P2Y12 receptor protein expression. However, no such data has been presented.

In a subsequent study, Fontana et al also reported an association between the P2Y12 H2 haplotype and PAD [12]. PAD causes an increased atherosclerotic burden throughout the whole cardiovascular system and patients with PAD have a marked increase in coronary artery disease [29]. The progression of chronic atherosclerotic lesions is mainly driven by an inflammatory reaction, with recruitment of inflammatory cells and subsequent reactive changes in the vessel wall [21]. In acute thrombotic complications of atherosclerosis, such as myocardial infarction, platelets constitute the major role. A gain of inflammatory cells and subsequent reactive changes in the vessel wall might likely be more prominent when studied in a setting were platelet activation is a main pathogenic factor, such as AMI. However, to our surprise, genotyping in several thousand individuals revealed no association with AMI.

The early onset or family history AMI case-control subpopulations are believed to contain a stronger genetic component of AMI. The lack of association also in these populations emphasizes further that the P2Y12 H2/P2Y13 Thr-158 haplotype is not associated with cardiovascular disease.

REFERENCES

1. Hollopeter G, Jaunten HM, Vincent D, Li G, England L, et al. (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 409: 202–207.

2. Ayyanathan K, Webb TE, Sandhu AK, Athwal RS, Barnard EA, et al. (1996) Cloning and chromosomal localization of the human P2Y1 purinoceptor. Biochim Biophys Acta 1218: 783–788.

3. Zhang FL, Luo L, Gustafson E, Lachowicz J, Smith M, et al. (2001) ADP is the cognate ligand for the orphan G protein-coupled receptor SP1199. J Biol Chem 276: 8068–8075.

4. Communi D, Gonzalez NS, Dethieux M, Brezillon S, Lannoy V, et al. (2001) Identification of a novel human ADP receptor coupled to G3i. J Biol Chem 276: 41479–41483.

5. Murugappa S, Kunapuli SP (2006) Role of ADP receptors in platelet function. Front Biosci 11: 1977–1986.

6. Wang L, Olivecrona G, Gotberg M, Olsson ML, Winzell MS, et al. (2005) ADP acting on P2Y13 receptors is a negative feedback pathway for ATP release from human red blood cells. Circ Res 96: 109–116.

7. Cattaneo M, Zighetti ML, Lombardi R, Martinez C, Lecchi A, et al. (2003) Molecular bases of defective signal transduction in the platelet P2Y12 receptor of a patient with congenital bleeding. Proc Natl Acad Sci U S A 100: 1978–1983.

8. Remijn JA, Mj B, Steunk AL, Engle H, et al. (2007) Novel molecular defect in the platelet ADP receptor P2Y12 of a patient with haemorrhagic diathesis. Clin Chem Lab Med 45: 187–189.

9. Bertrand ME, Simoons ML, Fox KA, Wallein LC, Hamm CW, et al. (2002) Management of acute coronary syndromes in patients presenting without persistent ST-segment elevation. Eur Heart J 25: 1809–1840.

10. Vivost C, Brown F, McCabe CH, Montalescot G, Ruzysz W, et al. (2007) Prasugrel versus Clopidogrel in Patients with Acute Coronary Syndromes. N Engl J Med.

11. Fontana P, Gaussem P, Bickel W, Jeanne C, Lecchi A, et al. (2003) P2Y12 gene H2 haplotype is not associated with increased adenosine diphosphate-induced platelet aggregation after initiation of clopidogrel therapy with a high loading dose. Blood Coagul Fibrinolysis 16: 199–204.

12. Lee DK, Nguyen T, Lynch KR, Cheng R, Vanni WB, et al. (2001) Discovery and mapping of ten novel G protein-coupled receptor genes. Genes 273: 53–94.

13. Criqui MH, Langer RD, Fronk A, Friedson HS, Klauber MR, et al. (1992) Mortality over a period of 10 years in patients with peripheral arterial disease. N Engl J Med 326: 301–306.

14. McCulloch PA (2007) Coronary artery disease. Clin J Am Soc Nephrol 2: 611–616.

15. Amistent S, Melander O, Wiborg B, Berglund G, Erdfange D (2006) Increased risk of acute myocardial infarction and elevated levels of C-reactive protein in patients with the The-67 variant of the ATP receptor P2Y11 in blood. Front J.

16. Manjer J, Carlsson P, Eklund G, Sollberg B, Janson L, et al. (2001) The Malmo Diet and Cancer Study: representativity, cancer incidence and mortality in participants and non-participants. Eur J Cancer Prev 10: 409–419.

17. Orho-Melander M, Blannemark M, Svensson MK, Riderstale M, Lindgren CM, et al. (2002) Variants in the calpain-10 gene predispose to insulin resistance and elevated free fatty acid levels. Diabetes 51: 2630–2694.

18. Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21: 263–265.

19. Skoll AD, Scott LJ, Abecasis GR, Boehnke M (2000) Joint analysis is more efficient than replication-based analysis for two-stage genome-wide association studies. Nat Genet 30: 209–213.

20. Cosade SB, Pearson JD (1989) Metabolism of adenine nucleotides in human red blood cells. Circ Res 96: 189–196.

21. Amistent S, Melander O, Wiborg B, Berglund G, Erdfange D (2006) Increased risk of acute myocardial infarction and elevated levels of C-reactive protein in patients with the The-67 variant of the ATP receptor P2Y11 in blood. Front J.

22. Amistent S, Melander O, Wiborg B, Berglund G, Erdfange D (2006) Increased risk of acute myocardial infarction and elevated levels of C-reactive protein in patients with the The-67 variant of the ATP receptor P2Y11 in blood. Front J.

23. Orho-Melander M, Blannemark M, Svensson MK, Riderstale M, Lindgren CM, et al. (2002) Variants in the calpain-10 gene predispose to insulin resistance and elevated free fatty acid levels. Diabetes 51: 2630–2694.

24. Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21: 263–265.

25. Skoll AD, Scott LJ, Abecasis GR, Boehnke M (2000) Joint analysis is more efficient than replication-based analysis for two-stage genome-wide association studies. Nat Genet 30: 209–213.

26. Cosade SB, Pearson JD (1989) Metabolism of adenine nucleotides in human red blood cells. Circ Res 96: 189–196.

27. Bergfeld GR, Forrester T (1992) Release of ATP from human erythrocytes in response to insulin resistance and elevated free fatty acid levels. Diabetes 51: 2630–2694.

28. Ramensky V, Bork P, Sunyaev S (2002) Human non-synonymous SNPs: server and survey. Nucleic Acids Res 30: 3894–3900.

29. Leng GC, Li AJ, Foskes FG, Whiteman M, DuBard J, et al. (1996) Incidence, natural history and cardiovascular events in symptomatic and asymptomatic peripheral arterial disease in the general population. Int J Epidemiol 25: 1172–1181.

30. Angiolillo DJ, Fernandez-Ontoria A, Bernardos E, Ramires C, Cavallari U, et al. (2005) Lack of association between the P2Y12 receptor gene polymorphism and platelet response to clopidogrel in patients with coronary artery disease. Thromb Res 116: 491–497.

31. Herbstreit ML, Singh RK, Lodwick D, Thompson JR, Goodall AH, et al. (2005) Dimorphism in the P2Y12 ADP receptor gene is associated with increased platelet activation response to ADP. Arterioscler Thromb Vasc Biol 25: 252–257.