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ARTICLE

Differential haplotypic expression of the interleukin-18 gene

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Interleukin 18 (IL-18) is suspected to play an important role in atherosclerosis and plaque vulnerability. We had previously shown that haplotypes combining two *IL18* gene polymorphisms in complete linkage disequilibrium, C-105T (rs360717) in 5′-untranslated region (UTR) and A + 183G (rs5744292) in 3′-UTR, were related to IL-18 circulating levels and cardiovascular outcome, the $C^{-105}G^{+183}$ haplotype being associated with lower IL-18 levels and lower cardiovascular risk. This study was aimed at investigating the functional role of the two polymorphisms and their haplotypes on *IL18* expression levels. Allelic imbalance experiments conducted in 24 and 20 subjects heterozygous for the C-105T and the A + 183G polymorphisms did not detect any difference when subjects were considered as a whole (-0.009 ± 0.044, $P = 0.85$ and $+0.114 \pm 0.082$, $P = 0.18$, respectively). However, when splitting individuals according to their haplo-genotype, the haplotype $C^{-105}G^{+183}$ was associated with a lower expression level than $C^{-105}A^{+183}$ (-0.287 ± 0.076, $P = 0.005$), but did not differ from $T^{-105}A^{+183}$ (-0.138 ± 0.083, $P = 0.13$). The lower expression associated with $C^{-105}G^{+183}$ was confirmed by real-time reverse transcription-PCR. Transfection of different haplotypic versions of the 3′-UTR did not show any difference in the expression of an upstream reporter gene. A 10-h study of the mRNA degradation kinetics by allelic imbalance with the A + 183G polymorphism did not show any differential allelic degradation. In conclusion, the haplotype associated with lower IL-18 circulating concentrations and a lower cardiovascular risk was consistently associated with decreased *IL18* expression levels, although the exact functional mechanisms remain to be elucidated.
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Introduction

In the search for candidate biomarkers to be used as predictive tools in clinical medicine, interleukin (IL)-18, also known as interferon-γ-inducing factor, has attracted a lot of attention in the past years. This molecule is a pleiotropic cytokine expressed mainly in Kupffer cells and activated macrophages and belonging to the family of IL-1. It has been demonstrated to play a crucial role in both innate and acquired immunity.[1] Given its participation in inflammatory processes, the role of IL-18 has been investigated actively in various pathologies including cancers, digestive inflammatory diseases, asthma, HIV infection, type II diabetes, rheumatoid arthritis, etc.[2] A new role of IL-18 in the homeostasis of energy intake and insulin sensitivity was recently highlighted.[3]

Evidence from animal and experimental models has accumulated for a crucial role of this molecule in cardiovascular diseases.[4] A number of studies using apolipoprotein E knockout mouse models have shown that deficiency or overexpression of IL-18 modulate

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atherosclerosis progression and plaque stability.[5–8] Inhibition of IL-18 has been shown to stimulate ischemia-induced neovascularization[9] and to inhibit neointimal formation after vascular injury.[10] Expression of IL-18 has been detected in human atherosclerotic plaques and associated with plaque progression and plaque vulnerability.[11,12] Finally, epidemiological studies have shown that plasma levels of IL-18 were predictive of future cardiovascular events both in healthy subjects and coronary patients,[13,14] although these findings were recently debated.[15]

We and others have identified several common polymorphisms in the potentially functional regions of the *IL18* gene, including the coding sequence, exon/intron junctions, promoter and untranslated regions (UTRs).[16,17] In a previous analysis of the prospective Athero*Gene* cohort of patients with coronary artery disease (CAD), we reported that the genetic variability of the *IL18* gene was related to various cardiovascular phenotypes, including baseline IL-18 serum levels and future cardiovascular mortality.[17] More specifically, haplotype analyses pointed towards a potential role of two polymorphisms located in 5′- and 3′-UTR, respectively, C-105T (rs360717) and A + 183G (rs5744292). However, it was unknown whether these two variants had a functional role or whether they were only markers of unidentified functional polymorphisms. In this study, we explored the potential impact of these two polymorphisms and their haplotypes on *IL18* expression levels in lymphoblastoid cell lines (LCLs) using different functional experimental approaches.

Materials and methods

The Athero*Gene* cohort

The Athero*Gene* cohort is a prospective cohort including 1288 German patients with an angiographically proven CAD. Patients were followed up for a median period of 5.9 (maximum 7.6) years and 142 cardiovascular deaths were reported. Serum IL-18 levels were measured at baseline and five *IL18* gene polymorphisms were genotyped. Detailed description of the study has been provided in a previous report.[17]

RNA preparation and cDNA synthesis

In all functional experiments described below, *IL18* expression levels were measured in Epstein–Barr virus-transformed LCLs derived from subjects of the ECTIM study (Etude Cas-Témoin sur l'Infarctus du Myocarde).[18] The selection of subjects for the different experiments was made according to their *IL18* genotypes. cDNA was synthesized from RNA extracted from LCLs using the RNeasy Mini kit (Qiagen, Courtaboeuf, France), including the RNase-free DNase step to remove any trace of potentially contaminating genomic DNA (see Supplementary information).

Genotyping

Genotyping was performed using the 5′ nuclease assay with MGB TaqMan probes (Applied Biosystems, Courtaboeuf, France) or dual-labeled oligonucleotides (Sigma Genosys, UK) (Supplementary information). Fluorescence was measured with an ABI PRISM 7000 sequence detection system (Applied Biosystems). Primer and probe sequences as well as amplification conditions for genotyping can be found at our website GeneCanvas (http://www.genecanvas.org).

Allelic imbalance assays

These experiments were successively conducted in 24 subjects heterozygous for the C-105T single-nucleotide polymorphism (SNP) and 20 subjects heterozygous for the A + 183G SNP. For each heterozygous subject, one-tenth of reverse transcription (RT) reaction (from 5 μg of total RNA) and 100 ng of genomic DNA were placed in two different wells of a same plate. The plate containing cDNA and genomic DNA from all subjects heterozygous for the SNP under study was subjected to a similar reaction as for genotyping. When necessary, amplification primers were modified to allow the amplification of a strictly exonic fragment that would give a similar amplification on genomic DNA and cDNA. Samples were submitted to three different RT-PCR (only two for the A + 183G SNP) and the resulting cDNAs were amplified in duplicates. Genomic DNA samples of both types of homozygotes were included as controls. For each SNP under study, the regression line equation predicted from the genomic DNA samples was calculated and for each cDNA sample, the deviation from this theoretical line was assessed, that is, the cDNA coordinate on the y axis was compared to the expected value calculated from the regression equation for the given coordinate on the x axis. For all individuals, the log (ratio) of the observed to the expected value was averaged over the 6 (4) repetitions. The mean of these values was then compared by a t-test to the expected value of 0 under the null hypothesis of no allelic imbalance. A positive value of the mean log(ratio) would indicate an overexpression of the allele plotted on the y axis, whereas a negative value would indicate an overexpression of the allele plotted on the x axis.

Estimation of the components of variance indicated that 71% of the variability of the log(ratios) for the C-105T SNP was due to interindividual differences and 29% to intraindividual differences among the replicated measurements. For the A + 183G SNP, these estimates were 78 and 22%, respectively.

Real-time RT-PCR assays

IL18 mRNA expression levels were compared between individuals homozygous for the three haplotypes generated by the C-105T and A + 183G SNPs by real-time PCR quantification (Supplementary information). mRNA expression data were normalized to the GAPDH

housekeeping gene content. Eleven homozygotes for the $C^{-105}G^{+183}$ haplotype, 13 homozygotes for the $T^{-105}A^{+183}$ haplotype and 13 homozygotes for the $C^{-105}A^{+183}$ haplotype were compared by *t*-tests.

Cloning and transfection

Constructs carrying an *IL18* 3′-UTR fragment under different haplotypic combinations of the A+183G and T+533C SNPs were inserted downstream a reporter luciferase gene and transfected in various cell types, including HepG2, COS 7 and DLD-1 cells (Supplementary information).

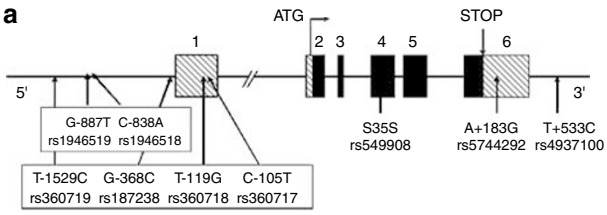
mRNA stability kinetics

LCLs from seven subjects heterozygous for the A+183G SNP were selected. Aliquots of one million cells were prepared and actinomycin D (Boehringer, Mannheim, Germany) was added at the final concentration of 5 μg/ml. Cells were harvested after 0, 1, 2, 4, 6, 8 and 10 h of actinomycin treatment and pellets were frozen until further use. RNA was later extracted for allelic imbalance assessment. The cDNAs ratio of one allele to the other was calculated for each time point and normalized to the ratio at time 0. For each time point, values were measured in triplicates and the mean value was plotted. Intraindividual differences among the replicated measurements varied from 2 to 22% according to time.

Results

Brief recall of the effects of the *IL18* gene polymorphisms in the Athero*Gene* Study

Our previous molecular screening of the *IL18* gene had identified nine common polymorphisms generating six frequent haplotypes in Europeans, which could be tagged by five SNPs (Figure 1).[17] These haplotypes were found significantly associated with circulating IL-18 levels in 1288 CAD patients ($P=0.002$). By comparison to the most frequent haplotype, the highest difference was observed with the sole haplotype carrying the G^{+183} allele, which was associated with a decrease in IL-18 levels ($P<10^{-3}$). However, another haplotype exhibited a borderline association ($P=0.056$), and this haplotype was the only one carrying the T^{-105} allele. The C-105T and A+183G polymorphisms were in complete linkage disequilibrium (LD), the T^{-105} allele being always associated with the A^{+183} allele. When restricting the haplotype analysis to these two SNPs, the association became even stronger (Figure 2, $P=0.0002$ for the global association). By comparison to haplotype $C^{-105}A^{+183}$, haplotypes $C^{-105}G^{+183}$ and $T^{-105}A^{+183}$ were each associated with a decrease in IL-18 levels ($P<10^{-4}$ and $P=0.006$, respectively), but did not differ one from each other ($P=0.12$). The same haplotypes were consistently associated with a lower cardiovascular mortality during the follow-up



b

G-887T	C-105T	S35S(A/C)	A+183G	T+533C	Frequency
G	C	A	A	C	0.306
G	C	A	G	T	0.247
G	C	C	A	T	0.027
T	C	A	A	T	0.103
T	C	A	A	C	0.021
T	T	C	A	T	0.286

Figure 1 Common polymorphisms and haplotypes of the *IL18* gene. (**a**) Schematic representation of the *IL18* gene. Hatched boxes indicate UTRs, black boxes coding exons. Groups of boxed polymorphisms are in nearly complete concordance. (**b**) Haplotypes defined by the five tag SNPs and their frequencies in the Athero*Gene* study including subjects of European origin.

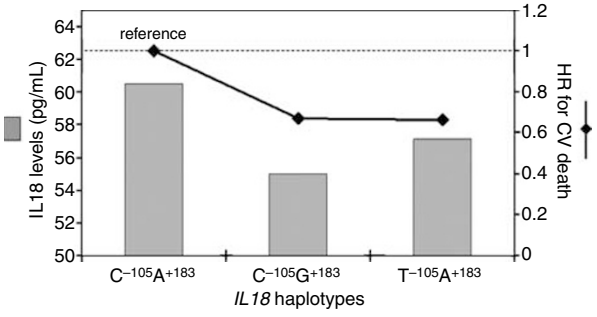


Figure 2 Mean effects of *IL18* haplotypes on baseline serum IL-18 levels (grey bars) and hazard ratios (HRs) for future cardiovascular (CV) death (black diamonds) in the prospective Athero*Gene* cohort.

(Figure 2, $P=0.048$ for the global association, $P=0.031$ and 0.021 for the effects of haplotypes $C^{-105}G^{+183}$ and $T^{-105}A^{+183}$ by reference to $C^{-105}A^{+183}$). We then focused our functional analyses on these two SNPs.

Allelic imbalance assays

The C-105T and A+183G polymorphisms are located in potentially regulatory regions (5′- and 3′-UTR, respectively) and might then affect the level of gene expression. Differential allelic expression of the *IL18* gene was investigated by the method of allelic imbalance.[19] This approach compares, in individuals heterozygous for a given transcribed polymorphism, the ratio of cDNAs of each allele to that of genomic DNAs (constitutively equal to 1:1). In such individuals, both alleles exert their influence in the presence of the same genetic background

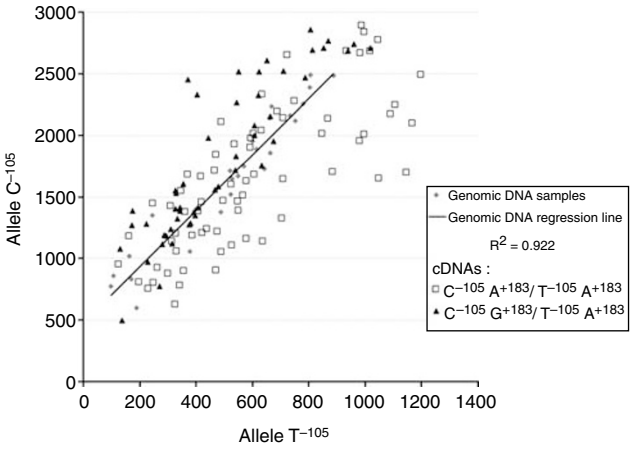


Figure 3 Allelic imbalance experiment in cDNAs of individuals heterozygous for the *IL18* C-105T SNP. Coordinates on the *x* and *y* axes indicate the fluorescence signals associated with each allele. The regression line is predicted from the genomic DNA samples (grey diamonds). It corresponds to a perfect equimolar ratio between genomic DNA of each allele. The R^2 value indicates the fitness of the prediction to the genomic DNA values. The cDNA samples from the same individuals are plotted on the graph. cDNA samples are split according to their genotype at the A+183G SNP, leading to two distinct haplo-genotypes (outlined circles and black triangles).

and environmental/experimental conditions, and therefore confounding effects are limited. The two aforementioned SNPs were successively investigated by this method.

cDNA and genomic DNA samples from 24 LCLs heterozygous for the C-105T SNP were amplified in the presence of TaqMan probes specific for each allele, and fluorescence signals associated with each allele were plotted (Figure 3). The regression line equation predicted from the genomic DNA samples was associated with an R^2 of 92%, compared with 100% for a perfect equimolar ratio between the two alleles. The cDNA samples exhibited a greater variability than the genomic DNA samples, but they were equally distributed on each side of the regression line, showing no higher expression of an allele over the other (mean of the log(ratios) $\pm$ SE: -0.009 ± 0.044, $P = 0.85$).

Among the 24 individuals heterozygous for the C-105T SNP, nine were heterozygous and 15 were homozygous at the A+183G locus. Given the complete LD between the C-105T and A+183G SNPs, haplo-genotypes could be inferred without ambiguity for both types of individuals. The former group had the haplo-genotype $C^{-105}A^{+183}/T^{-105}A^{+183}$ (Figure 3, outlined squares) and the latter group the haplo-genotype $C^{-105}G^{+183}/T^{-105}A^{+183}$ (Figure 3, solid triangles). The outlined squares spread on the two sides of the regression line, indicating no difference of expression between haplotypes $C^{-105}A^{+183}$ and $T^{-105}A^{+183}$ (mean $\pm$ SE: -0.079 ± 0.059, $P = 0.20$). There was a trend for the solid triangles to be situated above the line, but the difference was statistically borderline (mean $\pm$ SE: $+0.108 \pm 0.047$, $P = 0.05$).

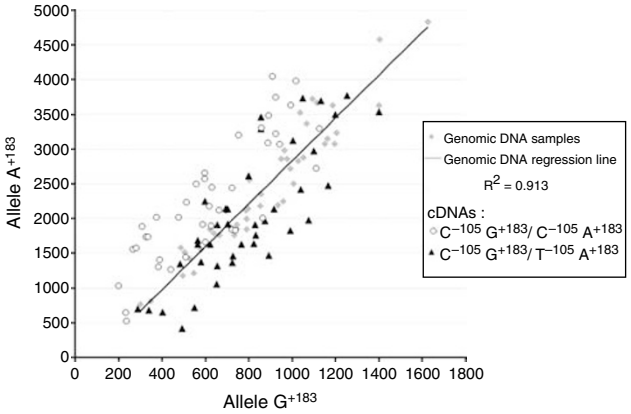


Figure 4 Allelic imbalance experiment in cDNAs of individuals heterozygous for the *IL18* A+183G SNP (see legend to Figure 3 for explanations).

The same experiment was performed in 20 LCLs heterozygous for the A+183G SNP. Again, there was no difference when comparing the expression of allele A^{+183} to that of allele G^{+183} as a whole (Figure 4, mean $\pm$ SE: $+0.114 \pm 0.082$, $P = 0.18$). However, when splitting alleles into haplotypes according to the C-105T SNP, the $C^{-105}A^{+183}$ haplotype was overexpressed compared to the $C^{-105}G^{+183}$ haplotype, as shown by the outlined circles tending to cluster above the regression line (10 subjects, mean $\pm$ SE: $+0.287 \pm 0.076$, $P = 0.005$). By contrast, the $C^{-105}G^{+183}$ and the $T^{-105}A^{+183}$ did not differ from each other, as shown by the solid triangles spreading on both sides of the line (10 subjects, mean $\pm$ SE: -0.138 ± 0.083, $P = 0.13$). Note that the solid triangles in Figures 3 and 4 represent cDNAs from the same individuals and look like mirror images.

In summary of these two experiments, haplotype $C^{-105}A^{+183}$ appeared to be associated with a higher expression level of the *IL18* messenger than haplotype $C^{-105}G^{+183}$, whereas this latter did not substantially differ from haplotype $T^{-105}A^{+183}$. These results paralleled those shown in Figure 2 of the association of haplotypes with circulating IL-18 levels and cardiovascular mortality.

The synonymous Ser35Ser (A/C) SNP, located in exon 4, is in almost complete association with the C-105T ($D' = +0.94$), and all 24 samples heterozygous for the C-105T SNP happened to be also heterozygous for the Ser35Ser SNP. As a reproducibility control, a similar allelic imbalance experiment conducted with probes for the Ser35Ser SNP showed results that were virtually superimposable on those obtained with the C-105T SNP (data not shown), providing a validation of the allelic imbalance results.

Real-time RT-PCR assays

In an attempt to confirm these results, mRNA levels of subjects homozygous for the three haplotypes were

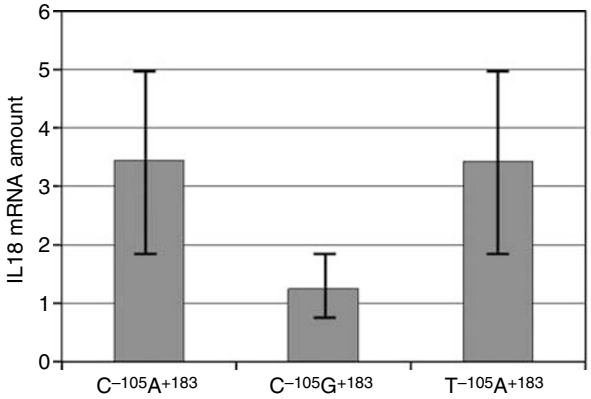


Figure 5 Mean (95% CI) *IL18* mRNA levels in individuals homozygous for the three *IL18* haplotypes generated by the C-105T and A+183G SNPs ($P=0.025$ for the comparison of $C^{-105}G^{+183}$ *vs* $T^{-105}A^{+183}$; $P=0.028$ for $C^{-105}G^{+183}$ *vs* $C^{-105}A^{+183}$).

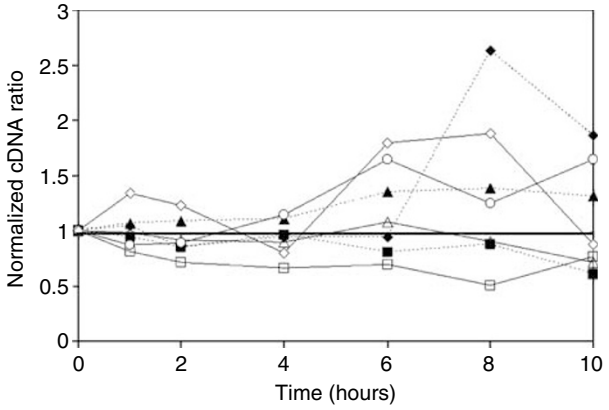


Figure 6 10-h kinetics of the allelic cDNA ratios of individuals heterozygous for the A+183G polymorphism. LCLs were treated with actinomycin D. The cDNA ratios at the different time points were normalized to the ratio at time 0. Solid symbols represent individuals with the haplo-genotype $C^{-105}A^{+183}/C^{-105}G^{+183}$ ($n=3$) and outlined symbols represent individuals with the haplo-genotype $T^{-105}A^{+183}/C^{-105}G^{+183}$ ($n=4$).

quantified by real-time RT-PCR. Individuals homozygous for the $C^{-105}G^{+183}$ haplotype ($n=11$) exhibited the lowest level of expression of IL-18 ($P=0.03$ for the two comparisons of this haplotype to $C^{-105}A^{+183}$ and $T^{-105}A^{+183}$, respectively). By contrast, individuals homozygous for the $T^{-105}A^{+183}$ haplotype ($n=13$) did not differ from those homozygous for the $C^{-105}A^{+183}$ ($n=13$) ($P=0.99$) (Figure 5).

Effect of the *IL18* 3′-UTR on an upstream reporter gene

To directly test a potential effect of the *IL18* 3′-UTR on expression levels, a construct carrying an approximately 800 bp fragment overlapping the A+183G SNP and the other SNP in 3′-UTR (T+533C) was inserted downstream the luciferase gene in three different versions reproducing the three haplotypes generated by these two SNPs. After transfection in various cell lines (COS7, CHO HepG2 and DLD-1 cells), normalized luciferase measurements did not significantly differ between the various haplotypic constructs (data not shown).

IL18 messenger stability

Given that the 3′-UTR is a region known to impact the stability of mRNA, we further investigated whether the A+183G polymorphism might be associated with differences in *IL18* mRNA stability. LCLs from seven individuals heterozygous for the A+183G SNP were incubated for 0, 1, 2, 4, 6, 8 and 10 h with actinomycin D, an inhibitor of transcription. Differential allelic expression was assessed at each time point using the allelic imbalance method. The ratios of allelic cDNAs were normalized to the ratio at time 0 to focus on the variation of ratios over time irrespective of baseline values. In presence of a substitution that affects mRNA stability, one would expect the ratios to progressively deviate from 1 in a given direction, either positive or negative depending on the mRNA degradation of each allele. As shown in Figure 6, the ratios actually tended to depart from 1 as time elapsed, but in both directions with no clear tendency. Splitting individuals according to their genotype at the C-105T (solid and outlined symbols) did not reveal any further tendency of a particular haplotype.

Discussion

This study was aimed at exploring the functional role of *IL18* gene polymorphisms suspected to influence IL-18 circulating levels and cardiovascular risk. Among the common polymorphisms identified by molecular screening of the *IL18* gene, none led to an amino-acid change and then, to a modification of the protein structure, making unlikely a qualitative effect of polymorphisms. Because the two polymorphisms associated with phenotypes were located in regulatory regions, we oriented our functional experiments toward the investigation of gene expression variability.

The first method used for detecting an effect of the SNPs on *IL18* gene expression was the method of allelic imbalance. The term 'allelic imbalance' has been frequently used in cancer to describe the partial or complete disappearance of one allele in heterozygous samples, unmasking the phenomenon of loss of heterozygosity by deletion of genomic fragments.[20] Recently, it has been employed to describe another mechanism, probably very common, which is the difference of abundance of messengers produced by each allele of a given gene in heterozygous samples. The method of allelic imbalance, in which both alleles are assessed in the same unique genetic background and the same environmental/experimental

conditions, has the great advantage of eliminating potential confounding effects as well as noise variability due to uncontrolled external factors. The only requirement is the availability of a transcribed polymorphism allowing the simultaneous comparison of cDNA ratios and genomic DNA ratios. By this method, it could be shown that differential gene expression was very common in the human genome.[21–23] Different techniques have been used for the quantification of two different alleles discriminated by a marker, including mini-sequencing, microarrays, sequencing, mass spectrometry, northern blotting, etc. In this study, we used the TaqMan technology with probes specific of the two polymorphisms under investigation. With this method, we were previously able to show that the gene encoding caspase-1, the enzyme processing the IL-18 inactive precursor to the biologically active cytokine, was differentially expressed.[24] We applied this method successively to the C-105T and A + 183G polymorphisms, but we could not detect any difference of allele expression when heterozygotes for either polymorphism were considered as a whole. However, after splitting heterozygotes for one SNP according to their genotype at the other SNP, we were able to show differential 'haplotypic' expression. The $C^{-105}A^{+183}$ haplotype was associated with a higher *IL18* gene expression than the $C^{-105}G^{+183}$ haplotype, whereas this latter did not significantly differ from the $T^{-105}A^{+183}$ haplotype. By real-time RT-PCR, we could confirm that the $C^{-105}G^{+183}$ haplotype was associated with the lowest mRNA levels. It is important to note that the method of allelic imbalance does not allow the direct study of haplotypes generated by polymorphisms located far apart because the probes cannot cover more than 30 bp. However, we could circumvent this problem thanks to the complete LD between the C-105T and A + 183G polymorphisms, which allowed us to deduce haplotypes from genotypes without ambiguity. This 'indirect' haplotypic approach proved to be very relevant because none of the polymorphisms alone was associated with expression levels and it is only when studying haplotypes that an effect could be detected. This extension of the method of allelic imbalance to 'haplotypic' imbalance can be applied as soon as haplotypes can be deduced without ambiguity, which may be the case for many genes because of the strong LD generally observed within human genes.

Because our initial findings pointed toward the A + 183G polymorphism as having the strongest effect on phenotypes,[17] we explored the effects of the 3′-UTR variability on gene expression. Indeed, the 3′-UTRs of genes are known to contain regulatory elements affecting messenger stability and degradation, nuclear export, translation, etc. Several 3′-UTR polymorphisms have been reported in the literature to have an important impact on gene expression, as for example, a recently reported variant in the 3′-UTR of the sheep myostatin gene that results in the creation of a microRNA target site and the degradation of the myostatin messenger.[25] To directly test the effect of the two SNPs located in the *IL18* 3′-UTR (A + 183G and T + 533C), constructs including their different haplotypic combinations were inserted downstream a classical reporter gene. However, these experiments failed to show any effect of the 3′-UTR variants on gene expression in all cell lines tested. It is possible that this particular genomic fragment (and its variants) has no effect on gene expression when isolated from its usual context or from other gene regions important for regulation by interactive processes. It is also possible that the cellular systems that we used were not adequate to reveal regulatory effects, or that some additional treatments by inductors of inflammation (lipopolysaccharide (LPS), phormol-myristate acetate (PMA), etc.) might be necessary to uncover and enhance a modest effect. These artificial systems can in theory help dissect the mechanisms underlying a functional SNP. However, they can also set up experimental conditions so distant from the *in vivo* conditions that these functional effects can be either misinterpreted or missed.

We more specifically explored the possibility that the A + 183G polymorphism might affect the *IL18* messenger stability by combining allelic imbalance of the A + 183G SNP and transcription inhibition by actinomycin D. This experiment showed that there was an interindividual variability of allelic messenger degradation, as shown by the increasing dispersion of the cDNAs ratios with time, but it failed to demonstrate any specific trend as would be expected if one allele was degraded faster than the other. Splitting individuals according to their genotype at the C-105T SNP did not reveal any further tendency. This 10-h time course was *a priori* sufficient to reveal a change in degradation speed as the half-life of the *IL18* messenger in cord blood and peripheral blood mononuclear cells has been estimated to be around 3–5 h.[26] Some mRNA instability elements have been described in genes from the IL-1 family and two of them have been shown to be present in the human *IL18* gene.[27] However, they have been identified within the coding sequence of the gene, and the SNP closest to such a regulatory site is the synonymous Ser35Ser variant, distant from 6 bp to the estimated motif, in a position unlikely to have a significant effect.

Several studies have reported an effect of SNPs located in the *IL18* promoter region on transcriptional activity[16,28–30] or IL-18 production by monocytes.[31] Although there is a large heterogeneity between studies in the size of the fragments cloned, the various constructs, the cell lines and the SNPs studied, the haplotype harboring the T^{-105} allele (differently named according to studies) was repeatedly found associated with a decreased transcriptional activity. This effect was generally more easily detectable when the cell lines were challenged with LPS, PMA or other stimuli. This might explain why we did not detect any effect of the C-105T polymorphism alone. However, the $C^{-105}G^{+183}$

haplotype, which was in this study the haplotype associated with the lowest activity, was ignored in all previous studies that focused only on SNPs in the promoter.

The allelic imbalance experiments showing a lower *IL18* gene expression associated with the $C^{-105}G^{+183}$ haplotype do not necessarily imply that the haplotype is functional by itself. This haplotype may act as a marker of a yet unidentified regulatory polymorphism in LD with it. Our molecular screening covered the regions of the gene *a priori* the most likely to be functional, and with 190 chromosomes screened, it had a power of 99.5% of detecting all polymorphisms with a minor allele frequency >0.01. However, we cannot rule out the possibility that a regulatory polymorphism located in an intron or at a longer distance of the *IL18* gene was missed. *Cis*-acting regulatory elements have already been described to act on gene expression over a very long distance, more than 100 kb,[32] but such interactions might be very difficult to analyze. Another interpretation might be that the haplotype itself is functional, for example by affecting the binding to transcription factors. Although the A+183G SNP is located in 3′-UTR, it is possible that the 5′- and 3′-UTR interact in the regulation of the expression by a folding of the DNA strand. Finally, it must be kept in mind that the C-105T polymorphism belongs to a group of four polymorphisms in nearly complete association. One (T-119G) is located in the close vicinity of C-105T, whereas the two others (G-368C and T-1529C) are further upstream in 5′. It is not possible from our experiments to disentangle the effect of these four SNPs, or even to determine if it is their haplotypic combination which is functionally relevant.

In conclusion, we identified a haplotype that was consistently associated with lower *IL18* expression levels, lower IL-18 circulating concentrations and a lower risk of cardiovascular death. Although the exact functional mechanisms remain to be elucidated, these results strongly reinforce the hypothesis of an etiologic role of IL-18 in atherosclerosis and its complications.

Acknowledgements

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References

1 Dinarello CA: Interleukin-18. *Methods* 1999; **19**: 121–132.
2 McInnes IB, Gracie JA, Leung BP, Wei XQ, Liew FY: Interleukin 18: a pleiotropic participant in chronic inflammation. *Immunol Today* 2000; **21**: 312–315.
3 Netea MG, Joosten LA, Lewis E *et al*: Deficiency of interleukin-18 in mice leads to hyperphagia, obesity and insulin resistance. *Nat Med* 2006; **12**: 650–656.
4 Caligiuri G, Kaveri S, Nicoletti A: When interleukin-18 conducts, the Preludio sounds the same no matter who plays. *Arterioscler Thromb Vasc Biol* 2005; **25**: 655–657.
5 Mallat Z, Corbaz A, Scoazec A *et al*: Interleukin-18/interleukin-18 binding protein signaling modulates atherosclerotic lesion development and stability. *Circ Res* 2001; **89**: E41–E45.
6 Whitman SC, Ravisankar P, Daugherty A: Interleukin-18 enhances atherosclerosis in apolipoprotein E(−/−) mice through release of interferon-gamma. *Circ Res* 2002; **90**: E34–38.
7 Elhage R, Jawien J, Rudling M *et al*: Reduced atherosclerosis in interleukin-18 deficient apolipoprotein E-knockout mice. *Cardiovasc Res* 2003; **59**: 234–240.
8 de Nooijer R, von der Thusen JH, Verkleij CJ *et al*: Overexpression of IL-18 decreases intimal collagen content and promotes a vulnerable plaque phenotype in apolipoprotein-E-deficient mice. *Arterioscler Thromb Vasc Biol* 2004; **24**: 2313–2319.
9 Mallat Z, Silvestre JS, Le Ricousse-Roussanne S *et al*: Interleukin-18/interleukin-18 binding protein signaling modulates ischemia-induced neovascularization in mice hindlimb. *Circ Res* 2002; **91**: 441–448.
10 Maffia P, Grassia G, Di Meglio P *et al*: Neutralization of interleukin-18 inhibits neointimal formation in a rat model of vascular injury. *Circulation* 2006; **114**: 430–437.
11 Mallat Z, Corbaz A, Scoazec A *et al*: Expression of interleukin-18 in human atherosclerotic plaques and relation to plaque instability. *Circulation* 2001; **104**: 1598–1603.
12 Gerdes N, Sukhova GK, Libby P, Reynolds RS, Young JL, Schonbeck U: Expression of interleukin (IL)-18 and functional IL-18 receptor on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for atherogenesis. *J Exp Med* 2002; **195**: 245–257.
13 Blankenberg S, Tiret L, Bickel C *et al*: Interleukin 18 is a strong predictor of cardiovascular death in stable and unstable angina. *Circulation* 2002; **106**: 24–30.
14 Blankenberg S, Luc G, Ducimetière P *et al*: Interleukin-18 and the risk of coronary heart disease in European men: The Prospective Epidemiological Study of Myocardial Infarction (PRIME). *Circulation* 2003; **108**: 2453–2459.
15 Koenig W, Khuseyinova N, Baumert J *et al*: Increased concentrations of C-reactive protein and IL-6 but not IL-18 are independently associated with incident coronary events in middle-aged men and women: results from the MONICA/KORA Augsburg case–cohort study, 1984–2002. *Arterioscler Thromb Vasc Biol* 2006; **26**: 2745–2751.
16 Giedraitis V, He B, Huang WX, Hillert J: Cloning and mutation analysis of the human IL-18 promoter: a possible role of polymorphisms in expression regulation. *J Neuroimmunol* 2001; **112**: 146–152.
17 Tiret L, Godefroy T, Lubos E *et al*: Genetic analysis of the interleukin-18 system highlights the role of the interleukin-18 gene in cardiovascular disease. *Circulation* 2005; **112**: 643–650.
18 Parra HJ, Arveiler D, Evans AE *et al*: A case-control study of lipoprotein particles in two populations at contrasting risk for coronary heart disease. The ECTIM Study. *Arterioscler Thromb* 1992; **12**: 701–707.
19 Liljedahl U, Fredriksson M, Dahlgren A, Syvanen AC: Detecting imbalanced expression of SNP alleles by minisequencing on microarrays. *BMC Biotechnol* 2004; **4**: 24.
20 Yan H, Dobbie Z, Gruber SB *et al*: Small changes in expression affect predisposition to tumorigenesis. *Nat Genet* 2002; **30**: 25–26.
21 Lo HS, Wang Z, Hu Y *et al*: Allelic variation in gene expression is common in the human genome. *Genome Res* 2003; **13**: 1855–1862.
22 Pastinen T, Sladek R, Gurd S *et al*: A survey of genetic and epigenetic variation affecting human gene expression. *Physiol Genomics* 2004; **16**: 184–193.
23 Heighway J, Bowers NL, Smith S, Betticher DC, Koref MF: The use of allelic expression differences to ascertain functional polymorphisms acting in cis: analysis of MMP1 transcripts in normal lung tissue. *Ann Hum Genet* 2005; **69**: 127–133.

24 Blankenberg S, Godefroy T, Poirier O *et al*: Haplotypes of the caspase-1 gene, plasma caspase-1 levels, and cardiovascular risk. *Circ Res* 2006; **99**: 102–108.
25 Clop A, Marcq F, Takeda H *et al*: A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. *Nat Genet* 2006; **38**: 813–818.
26 Satwani P, Ayello J, Ven C *et al*: Immaturity of IL-18 gene expression and protein production in cord blood (CB) versus peripheral blood (PB) mononuclear cells and differential effects in natural killer (NK) cell development and function. *Br J Haematol* 2005; **130**: 284–292.
27 Bufler P, Gamboni-Robertson F, Azam T, Kim SH, Dinarello CA: Interleukin-1 homologues IL-1F7b and IL-18 contain functional mRNA instability elements within the coding region responsive to lipopolysaccharide. *Biochem J* 2004; **381**: 503–510.
28 Zhou Y, Yamaguchi E, Hizawa N, Nishimura M: Roles of functional polymorphisms in the interleukin-18 gene promoter in sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 2005; **22**: 105–113.
29 Liang XH, Cheung W, Heng CK, Wang DY: Reduced transcriptional activity in individuals with IL-18 gene variants detected from functional but not association study. *Biochem Biophys Res Commun* 2005; **338**: 736–741.
30 Sugiura T, Maeno N, Kawaguchi Y *et al*: A promoter haplotype of the interleukin-18 gene is associated with juvenile idiopathic arthritis in the Japanese population. *Arthritis Res Ther* 2006; **8**: R60.
31 Arimitsu J, Hirano T, Higa S *et al*: IL-18 gene polymorphisms affect IL-18 production capability by monocytes. *Biochem Biophys Res Commun* 2006; **342**: 1413–1416.
32 Forton JT, Udalova IA, Campino S, Rockett KA, Hull J, Kwiatkowski DP: Localization of a long-range cis-regulatory element of IL13 by allelic transcript ratio mapping. *Genome Res* 2007; **17**: 82–87.

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