Age-related loss of CpG methylation in the tumor necrosis factor promoter

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List of abbreviations: LPS lipopolysaccharide; RA rheumatoid arthritis; PMR polymyalgia rheumatica; CI confidence interval; MDM monocyte-derived macrophages, PBL peripheral blood leucocytes, 5-aza-CdR 5-aza-deoxycytidine.

Abstract

Background: Dysregulated production of TNF has been implicated in the pathogenesis and severity of inflammatory rheumatic diseases, many of which show age-related increased incidence. Ageing is also associated with changes in the immune system including higher systemic levels of pro-inflammatory cytokines. Methylation of DNA is an important regulator of gene expression and changes with age.

Objective: In this study we investigated whether the DNA methylation status of the TNF promoter changed with age in peripheral blood leukocytes and macrophages.

Methods and results: Using pyrosequencing assays we detected age-related demethylation of CpG motifs (-304, -245 and -239) in the TNF promoter in human peripheral blood cells from 312 healthy controls (0.8% per decade, confidence interval (CI)=0.44%-1.13%, $p=1x10^{-5}$) and primary monocyte-derived macrophages (MDM) from a separate population of 78 healthy controls (1.4% per decade, CI=0.79%-2.13%, $p=7x10^{-5}$). Methylation a TNF promoter fragment (-345 to +154) resulted in 78% reduction of reporter gene activity compared with the unmethylated promoter construct.

Conclusions: These data suggest a potential role of accrued changes in DNA methylation in the development of age-related inflammatory diseases, such as rheumatoid arthritis and polymyalgia rheumatica, in which TNF is a pivotal mediator.

1. Introduction

The cytokine TNF is a potent immunomodulating and pro-inflammatory mediator with a wide range of activities [1]. The main cellular source of TNF is the macrophage although significant quantities are produced by other sources such as T and B cells. Systemic levels of TNF are correlated with outcome from severe infections including malaria [2] and meningococcal disease [3], whilst the therapeutic efficacy of TNF inhibitors for the treatment of polymyalgia rheumatica (PMR) [4] and rheumatoid arthritis (RA) [5] confirms its central role in these conditions.

The production of TNF is primarily controlled at the transcriptional and post-transcriptional levels [6]. In response to lipopolysaccharide (LPS) stimulation of macrophages, TNF transcription increases 3-fold, TNF mRNA levels increase 50 to 100-fold and protein production increases approximately 10,000-fold [1]. Sequences within the 1,100bp stretch of DNA between the 3' end of the adjacent lymphotoxin-α gene and the first exon of the TNF gene are central in the control of transcription [7, 8]. Production of TNF shows stable inter-individual variation [9] and alleles of the TNF promoter have been correlated with levels of gene expression [10, 11].

Many aspects of the both the adaptive and innate immune systems change with increasing age leading to a state termed 'inflammageing' [12]. In the adaptive system the ability to generate high affinity antibodies after immunization is reduced perhaps as result of reduced levels of CD8+ CD28- cells [13], in addition to the ratio of naïve to memory T cells alters [14]. Age-related changes in the innate immune system include higher systemic levels of pro-inflammatory cytokines and most studies have

shown higher LPS-induced production of pro-inflammatory cytokines by macrophages from older individuals [15, 16]. The expression of Toll-like receptor TLR 1 by macrophages from healthy older individuals (>65 yrs old) is 39% less than levels from your subjects (21-30 yrs old) with resultant decreased production of pro-inflammatory cytokines following TLR1/2 activation [17].

Although recent studies have challenged the belief that global DNA methylation decreases with age [18, 19], recent evidence suggests that immune genes may be particularly prone to age-related change in DNA methylation [18]. The expression of killer Ig-like receptors on T cells is known to increase with age and *KIR2DL4* promoter methylation is lower in T cells isolated from 70-80 year old donors compared with those from 20-40 year old individuals [20].

We hypothesized that age-related changes in the methylation signature of the *TNF* promoter could contribute to the higher expression of this cytokine in older individuals. To explore this possibility we developed pyrosequencing assays to determine the methylation of CpG motifs around the transcriptional start site (-349 to -78). We report age-related reduced methylation in both peripheral blood leukocytes (PBL) and monocyte-derived macrophages (MDM) from separate healthy control populations. The role of DNA methylation in regulating TNF gene expression was determined a reporter gene assay. Our data suggests that lower DNA methylation in the *TNF* promoter may contribute to both the age-related increased production of this cytokine and the higher incidence of inflammatory diseases.

2. Materials and Methods

2.1 Blood sample collection.

Peripheral blood samples were collected from 312 healthy controls. as part of a large study of rheumatoid arthritis, all subjects were 18 years of age or older with no history of inflammatory joint disease [21]. The MDM were collected from 78 healthy students and staff in the Sheffield Medical School and were isolated from 100 ml of peripheral blood by density gradient centrifugation using standard techniques as previously described [22]. Cells were cultured for 7 days in flat-bottomed 24-well plates (1 x 10^6 cells/well), in 1ml RPMI 1640 medium/well (Invitrogen Ltd, Paisley, UK) supplemented with 2mM L-glutamine and 10% foetal calf serum (FCS), at 37ºC in 95% air, 5% CO_2 prior to DNA extraction. Approval for both cellular collections was obtained from the South Sheffield Research Ethics Committee and informed consent was obtained from all donors. DNA was extracted using sodium perchlorate and chloroform.

2.2 Bisulphite pyrosequencing.

Prior to sequencing DNA was incubated with sodium bisulphite using the EpiTect® kit (Qiagen, Crawley, West Sussex) according to the manufacturers protocol. Forward and biotin labelled reverse primers were designed against bisulphite-modified sequences and sequencing primers were designed using the Pyrosequencing Assay Design (Biotage, Uppsala, Sweden). Amplification reactions were performed in 1 x PCR reaction buffer and 200µM of each dNTP, 0.2µM forward and reverse primers (Table 1) and 1U Qiagen HotStarTaq. An initial incubation of 95ºC for 15 minutes was followed by 50 cycles of 1 minute at 94ºC, 1 minute at 55ºC and 1 minute at 72ºC

with a final elongation step of 72ºC for 10 minutes. The PCR product was bound to streptavidin-sepharose beads (2µl) (GE Healthcare, Uppsala, Sweden), washed sequentially in 70% ethanol, 0.2M sodium hydroxide and wash buffer (10mM Tris-Acetate, pH 7.6) and the single stranded DNA was eluted into the sequencing reaction containing 0.3µM sequencing primer (Table 1) in 20mM Tris-Acetate, 2mM Mg-Acetate pH 7.6. The sequencing reaction was incubated at 80ºC for 5 minutes followed by pyrosequencing on the PyroMark MD (Biotage).

2.3 Reporter gene assays

A *TNF* promoter fragment (-345 to +154) was amplified using high fidelity DNA polymerase (Phusion, New England Biolabs, Ipswich, MA) with forward and reverse primers containing *BglII* and *HindIII* restriction sites respectively (Table 1). The fragment was cloned into a pCR2.1 vector using TA overhangs and verified by sequencing. The promoter fragment was excised, using *BglII* and *HindIII,* and cloned into the multiple cloning site of the CpG-less vector pCpG-basic kindly provided by Michael Rehli and Maja Klug. The construct was methylated by incubation for 2 hours at 37ºC in the presence or absence of 2.5U *Sss*1 (New England Biolabs) with 160µM S-adenosylmethionine. Methylation was assessed by pyrosequencing and was >90%. Methylated and unmethylated constructs were purified using Qiaquick columns (Qiagen) and were used to transfect HeLa cells using Superfect transfection reagent (Qiagen). As a control for transfection efficiency the promoter fragment of the murine EF1 alpha gene was amplified by PCR and subcloned into pGL4-Renilla Luciferase (Promega) reporter and was used as an internal control in the luciferase assays (a kind gift of Dr David Wylie), 24 hours post-transfection cells were stimulated for 6 hours with or without 5ng/ml IL-1β. Luciferase and Renilla activities

were measured using the Dual-Luciferase Reporter System (Promega) according to the manufacturer's protocol.

2.4 Statistical Analyses

Because of the clear differences in the methylation levels at the 5' and proximal CpGs we analysed the two regions separately. Within each region we treated the CpGs as repeated measures of methylation in the same individual. All analyses were performed using a mixed effects model in which we included the intra-individual correlations between the methylation levels at the CpG sites. When considering the change in methylation with age, this variable was entered into the model as a continuous variable and gender as a two-level factor variable. Treating the two regions separately meant that the model assumptions of normally distributed error terms were met. There were missing values in the data but only individuals with all CpGs missing in a region were excluded. Generalized likelihood ratio tests are preferentially used in fixed effects models for comparing random effects structures for a given fixed effects structure, however they can be anti-conservative in assessing fixed effects within a mixed effects model; so we use the more reliable Wald tests. Confidence intervals were calculated in the same way using the estimated standard errors and t-distribution assumptions.

3. Results

3.1 Age-related loss of DNA methylation of CpG motifs in the TNF promoter region.

The pyrosequencing assays determined the methlyation of the *TNF* promoter (-349 to -78) containing 7 CpG motifs (Figure 1). The initial study was performed on PBL from 312 healthy controls (age range 20-84 years). Relative methylation differed within the promoter fragment; the three 5' CpG motifs (-304, -245 and -239) were highly methylated (>80%) whereas the proximal motifs (-170, -164, -162 and -147) were predominantly unmethylated (<20%). Using the mixed effects statistical model we tested whether the methylation pattern changed with age; it deceased at the three 5' CpGs by 0.8% per decade (CI=0.44%-1.13%, $p=1x10^{-5}$) with a smaller but statistically significant fall in the proximal motifs (p=0.03) (Fig. 2A & B respectively).

We then performed a similar study on MDM obtained from a separate population of 78 healthy controls. Methylation of the 5' CpGs (>80%) was high compared with that of the 3' motifs (<20%). In this less heterogenous cell population methylation of the 5' motifs decreased by 1.4% per decade (CI=0.79%-2.13%, $p=7x10^{-5}$) (Fig. 2C), however methylation of the 3' motifs did not change with age (Fig. 2D).

Genomic DNA methylation was assessed using a LINE-1 methylation assay and did not change with age in either PBL or MDM (Fig. 2E & F).

3.2 Gender-related differences in DNA methylation

Global methylation was significantly lower in MDM from female donors compared with males, although the absolute difference was relatively modest (81.1% v 82.1% respectively, p=0.004), and was not different in PBLs (p=0.08) (Table 2).

3.3 Effects of methylation of a TNF reporter gene construct

To determine the transcriptional effect of TNF promoter methylation, the HeLa cell line was transfected with methylated or unmethylated TNFprom-PCpGL vectors, and following incubation with 5 ng/ml interleukin-1β, reporter activity was determined. Methylation resulted in a 78% reduction in reporter gene expression (CI=33-128%, p<0.001) compared with the unmethylated promoter (Fig. 3B). Although the assay examined the effects of methylation of all of the CpGs motifs in the TNF promoter fragment rather than the three 5' motifs this data suggests an important role of DNA methylation in regulating TNF transcription.

4. Discussion

In humans, ageing is associated with significant changes in the innate immune system including impairment of neutrophil and macrophage phagocytic activity, increased production of pro-inflammatory cytokines and reduced antibacterial defence [23]. Our data revealing age-related reduction of DNA methylation of the TNF promoter suggest that age-related loss of the epigenetic signature of this cytokine may contribute to inflammaging and contributing to the pathogenesis of late onset rheumatic diseases such as PMR and RA in which this cytokine has been shown to a pivotal mediator of chronic inflammation [24, 25].

Genome wide association studies have resulted in significant advances in our understanding of the genetic basis of common multifactorial diseases, however the heritability of many of these conditions is relatively modest implying significant non-genetic or environmental contributions. Autoimmune diseases, such as rheumatoid arthritis and type 1 diabetes, affect approximately 4% of the population of Western Europe and the United States and have features suggestive of an epigenetic effect including discordance in monozygotic twins, relatively late onset (commonly in the 4^{th} or 5^{th} decade), female predominance and parent-of-origin effects [26]. In many of these conditions TNF acts as a pivotal mediator of inflammation and tissue damage; our data suggests that age-related DNA *TNF* promoter demethylation could contribute to the late peak in incidence of these conditions [27]. The relationship between gender and methylation status is controversial; lower genomic levels have been reported in peripheral blood white cells of healthy females [28], however another study examined mean methylation in a range of organs and primary cell types did not

detect significant gender-related differences [19]. Levels of DNA methylation are lower in peripheral blood T cells from patients with either rheumatoid arthritis or systemic lupus erythematosus compared with healthy controls [29], both of which occur more frequently in females, although whether this is a primary abnormality or is secondary to the disease or its treatment is not known.

Early studies of genomic DNA methylation reported it to decrease with age [29, 30], however recent evidence indicates that this relationship is more complex. In monozygotic twins the patterns of global and gene specific DNA methylation is very similar in younger twins however older twins (age>50 years) have significant differences especially if they that had spent less time together in earlier life [31]. A recent study reported positive correlations between CpG island methylation and ageing, however methylation of CpG motifs not in CpG islands was negatively correlated with ageing [32]. The complexity of age-related DNA methylation alterations was further highlighted in a family-based study that reported no overall change during an 11 years interval however there was a significant heritable component of this trait [18].

The potential mechanism of age-related demethylation of a single locus is not clear; the cellular activity of DNA methyltranserase 1 decreases with age but this is likely to lead to a global loss in methylation which we did not observe [33]. Another potential mechanism is related to the effects of chronic age-related low grade inflammation leading to the production of reactive oxidative molecules with resultant conversion of 5-methylcytosine to 5-hydroxymethylcytosine [34]. This modification interfers with the activities of DNA methyltransferase 1 with failure to methylate target cytosines

[34], however once again this mechanism would also be expected to result in a genomic rather than a gene specific change in methylation. The conversion of 5-methylcytosine to 5-hydroxymethylcytosine is regulated by members of the TET protein family [35], however little is known of the effects of ageing on TET.

The role of DNA methylation in cancer has been convincingly demonstrated, however its role in determining risk or outcome of inflammatory diseases is much less clear. This is partly due to the technical challenges in performing large-scale assays of DNA methylation that are sufficiently sensitive to detect relatively modest differences such as those found in our study. Our findings suggest that assessment of the methlyation status of TNF could have utility as a biomarker of susceptibility to, or severity of, the many inflammatory and infectious diseases in which TNF is a pivotal mediator. The clinical use of anti-TNF agents has revolutionized the treatment of RA and Crohn's disease and TNF promoter variants have been correlated with response [36]. As macrophages are the primary cellular source of TNF our data suggest that the epigenetic signature of the TNF promoter could be a further marker of response to these agents. A further potential clinical impact is therapeutic modulation of the epigenetic signature of patients with chronic inflammatory diseases, aimed at returning dysregulated gene expression to a homeostatic setting. The change in the epigenetic signature with age is likely to be significantly determined by environmental exposures, including diet, tobacco smoke, alcohol and previous illnesses and therapies [31].

4.1 Study limitations

There are several limitations to our study. The change in methylation of the TNF promoter in PBL could be secondary to an age-related change in the cellular composition of PBL however MDM represent a more homogenous cellular type. Furthermore the biological consequences of relatively modest changes in *TNF* promoter methylation on TNF expression is not clear. Although the reporter gene experiments underlined the potential importance of promoter methylation on expression the assay determined the effects of methylation of the whole promoter fragment rather than individual or clusters of CpGs.

4.2 Conclusions

In summary we report age-related loss of methylation of *TNF* CpG motifs in both peripheral blood leucocytes and macrophages and demonstrate a significant effect of DNA methylation on gene expression. Although the changes were relatively modest it could be that similar changes in the genes encoding other key regulators of inflammation could have synergistic effects leading to larger biological consequences leading to the development of age-related inflammatory rheumatic diseases such as RA and PMR. The recent advances in ultra high throughput DNA sequencing technology will facilitate studies to determine the role of epigenetics in the development of inflammageing.

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Table 1. Oligonucleotide sequences for pyrosequencing, TNF fragment amplification for cloning and TNF mRNA quantitative PCR.

Gene	Plasmid construct	Primer sequence
TNF	Forward	5' AGATCTCCAAAAGAAATGGAGGCAAT 3'
	Reverse	5' AAGCTTGAAGCCGTGGGTCAGTATGT 3'
	Pyrosequencing	
	Forward	5' TTGGTTTTTAAAAGAAATGGAGGT 3'
	Reverse	5' Biotin ATAATAAACCCTACACCTTCTATCT 3'
	Sequencing	5' GGTTTTGAGGGGTATG 3'
CpG	Forward	5' TGGGGAAGGGGTTTAGTTTTTAG 3'
-304	Reverse	5' Biotin TCAAAAATACCCCTCACACTC 3'
	Sequencing	5' TAGTGGTTTAGAAGATTTTT 3'
	Forward	5' AATTTTTTTGGTGGAGAAATTTATG 3'
-245, -239	Reverse	5' Biotin CCTACACACAAATCAATCAATAACC 3'
	Sequencing	5' GGGTTTTGTATTTTTTGTTT 3'
	Forward	5' TTTTTGAGTTAGGTGTGGGATATA 3'
-170, -164,	Reverse	5' Biotin AAAAATCAAAAAATTCCCTTTC 3'
-162, -147	Sequencing	5' AGTTAGGTGTGGGATATAGT 3'
LINE-1	Forward	5' TTTTTGAGTTAGGTGTGGGATATA 3'
	Reverse	5' Biotin AAAAATCAAAAAATTCCCTTTC 3'
	Sequencing	5' AGTTAGGTGTGGGATATAGT 3'

Table 2. The methylation status of the TNF promoter 5' CpG motifs (-304, -245 and -239) and global methylation in PBCs and MDMs derived from males and females.

Cells	Gene		Mean Methylation	P
Macrophages	TNF	Males	84.1	0.343
		Females	83.2	
	LINE1	Males	82.1	0.004**
		Females	81.1	
PBC	TNF	Males	80.2	0.788
		Females	80.3	
	LINE1	Males	82.4	0.08
		Females	81.6	

Legends to figures.

Figure 1. Structure of the TNF promoter including transcription factor consensus sequences, CpG motifs and single nucleotide polymorphisms.

Figure 2. Age-related change in DNA methylation of CpG motifs in the TNF promoter region. Pyrosequencing assays were used to quantitate methylation of CpG motifs in the distal (-304, -245 and -239) and proximal regions (-170, -164, -162, -147 and -120) of the TNF promoter in PBLs (A & B respectively) and MDMs (C & D respectively). The percentage change in DNA methylation per decade is given for statistically significant results. Global DNA methylation in PBLs (E) and MDMs (F) was determined using a LINE-1 methylation assay.

Figure 3. Effects of TNF promoter methylation on reporter gene expression. Methylation of the TNF promoter fragment downregulates reporter gene expression. The TNF promoter fragment was cloned upstream of the luciferase reporter gene in the PCpGL vector and unmethylated (hashed bar) or *Sss*1 methylated (solid bar) vectors these were used to transfected HeLa cells. Cells were incubated with or without IL-1β and luciferase activity was measured. Bars represent mean with error bars showing SEM. Analysis was performed using ANOVA (n = 9 from 3 independent experiments).

Figure 1

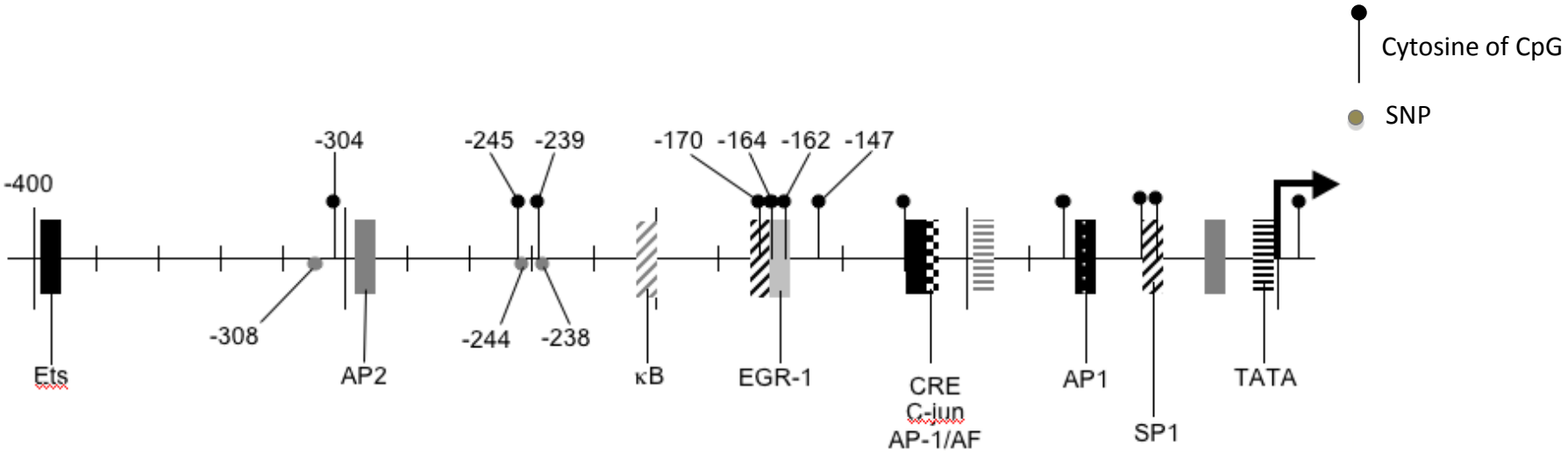

Cytosine of CpG
SNP
-400
-304
-245
-239
-170
-164
-162
-147
-308
-244
-238
Ets
AP2
κB
EGR-1
CRE
C-jun
AP-1/AF
AP1
SP1
TATA

Figure 2.

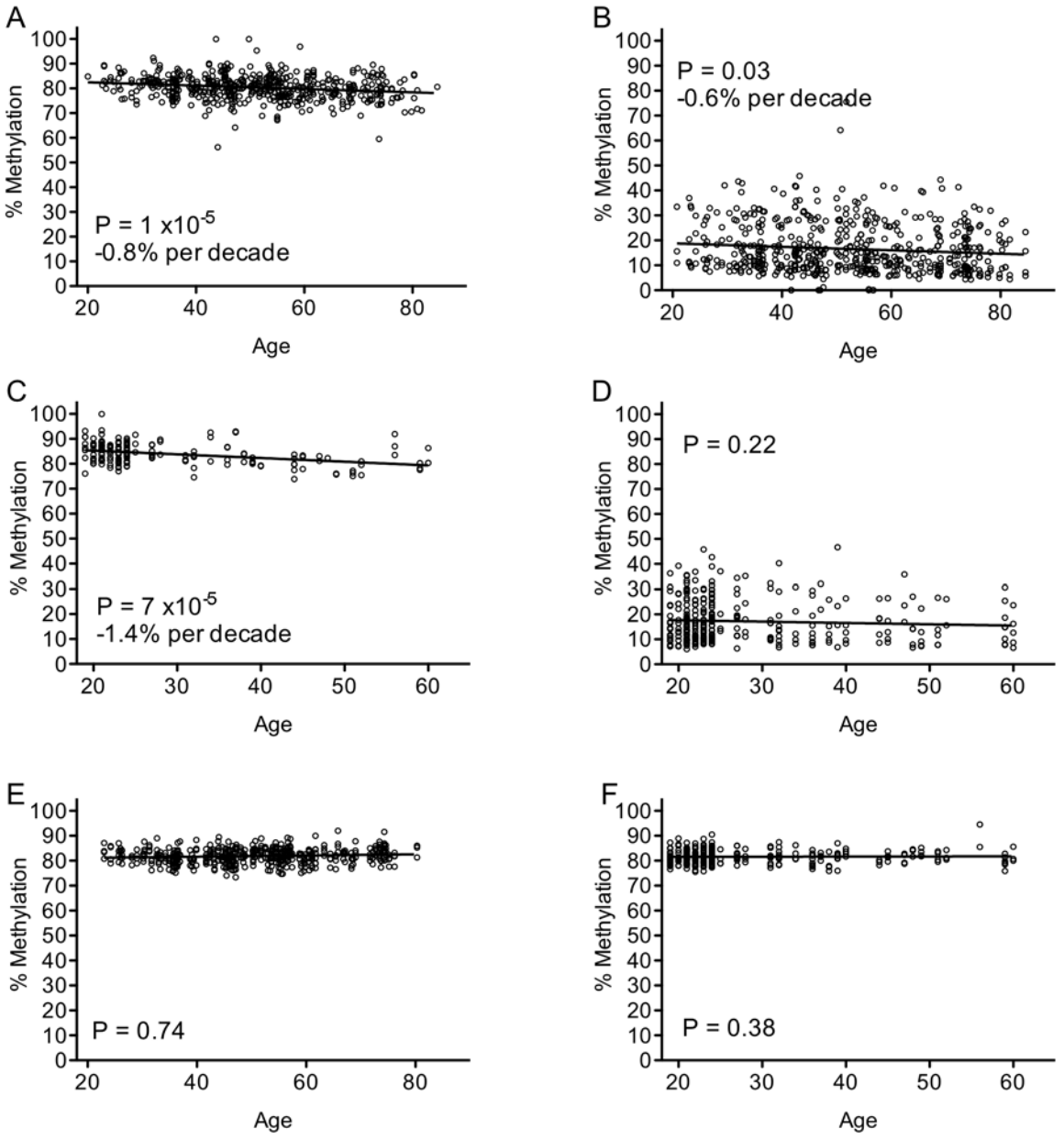
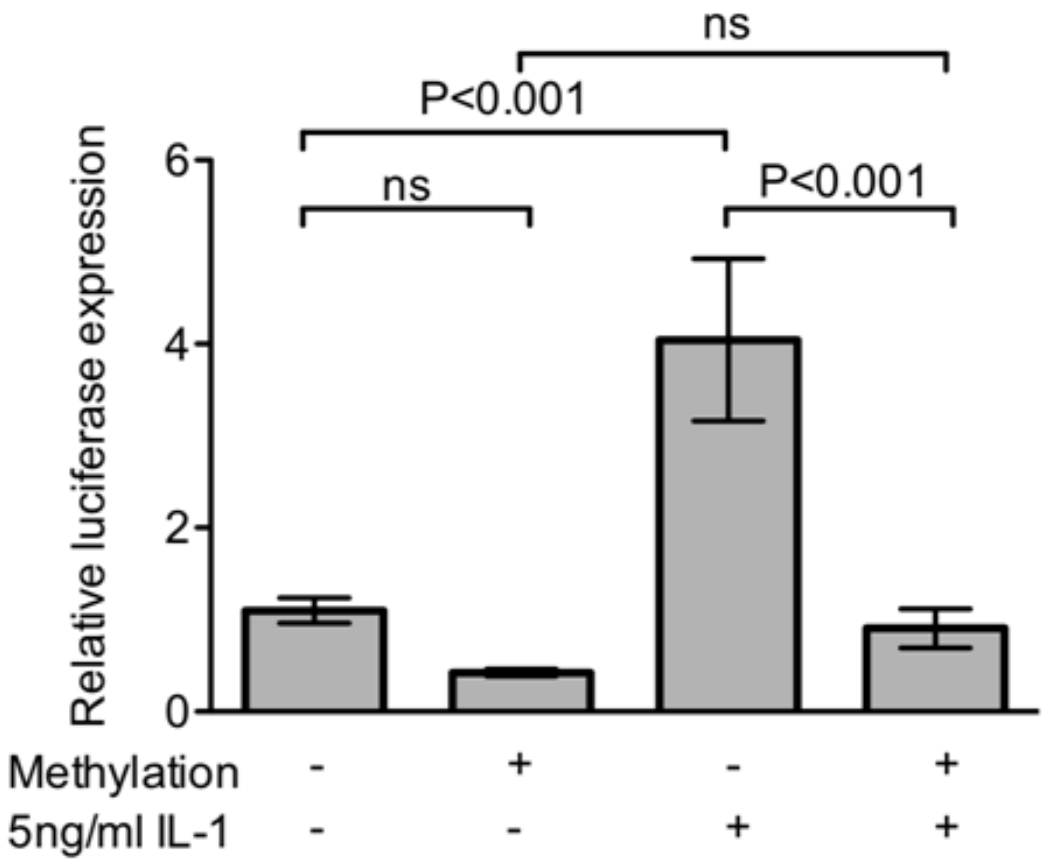

A
% Methylation
Age
P = 1 x10-5
-0.8% per decade
B
P = 0.03
-0.6% per decade
C
P = 7 x10-5
-1.4% per decade
D
P = 0.22
E
P = 0.74
F
P = 0.38

Figure 3.


ns
P<0.001
ns
P<0.001
Relative luciferase expression
6
4
2
0
Methylation - + - +
5ng/ml IL-1 - - + +