Non-Steroidal Anti-Inflammatory Drug Use and Genomic DNA Methylation in Blood

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Abstract

Background
Non-steroidal anti-inflammatory drug (NSAID) use is associated with decreased risk of some cancers. NSAID use modulates the epigenetic profile of normal colonic epithelium and may reduce risk of colon cancer through this pathway; however, the effect of NSAID use on the DNA methylation profile of other tissues including whole blood has not yet been examined.

Findings
Using the Sister Study cohort, we examined the association between NSAID usage and whole genome methylation patterns in blood DNA. Blood DNA methylation status across 27,589 CpG sites was evaluated for 871 women using the Illumina Infinium HumanMethylation27 Beadchip, and in a non-overlapping replication sample of 187 women at 485,512 CpG sites using the Infinium HumanMethylation450 Beadchip. We identified a number of CpG sites that were differentially methylated in regular, long-term users of NSAIDs in the discovery group, but none of these sites were statistically significant in our replication group.

Conclusions
We found no replicable methylation differences in blood related to NSAID usage. If NSAID use does effect blood DNA methylation patterns, differences are likely small.

Background
Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used agents which reduce inflammation by inhibiting the cyclooxygenase pathway’s production of various prostaglandins from arachidonic acid. This pathway is a key part of the inflammatory response. Epidemiologic
studies indicate that long-term NSAID usage is associated with decreased risk of certain cancers including including colorectal cancer, gastric cancer, and potentially breast cancer [1–4]. The biological mechanisms of chemoprevention related to this drug class remain unclear.

In animal models of colorectal cancer development, chronic inflammation is linked to accumulation of DNA methylation changes over time; this may affect epigenetic control of gene expression [5, 6]. These observed methylation changes may occur in the process of repairing damage caused by inflammation; in vitro studies have indicated that inducing oxidative stress and DNA damage in human cancer cell lines results in up-regulation and recruitment of DNA methyltransferases and other gene-silencing proteins to CpG islands [7, 8]. In some human cell lines, endogenously produced prostaglandins from the COX-2 pathway directly increased expression of DNA methyltransferases and influenced methylation of promoter regions of genes involved in epigenetic regulation [9, 10].

Long-term NSAID usage, which reduces systemic inflammation, is associated with differential methylation of certain genes in gastric and colon mucosa [11, 12]. These NSAID-linked methylation differences have only been investigated in a limited number of tissues; differences associated with long-term NSAID use have not been examined on the genomic level in blood. The inflammatory pathway targeted by NSAIDs downregulates the expression of enzymes responsible for maintenance of DNA methylation [13], giving rise to the hypothesis that long-term NSAID use may protect against aberrant genomic methylation changes associated with cancer.

Methods

Study population and data sources

We utilized data from the Sister Study, a prospective cohort of 50,884 women who had a sister with breast cancer but who did not have breast cancer themselves at enrollment [14]. All women provided detailed medication history at enrollment via a computer-assisted telephone interview. As NSAIDs are often used sporadically, we focused on women who used NSAIDs regularly. Women were defined as regular users if they reported taking an NSAID drug 3 or more times a week for at least 3 months. Regular NSAID users provided information on the types of NSAIDs taken and the frequency and duration of their use.

We examined the relationship between methylation and NSAID exposure using two methylation datasets within the Sister Study. The “discovery set” was comprised of 871 women with methylation data available on 27,589 CpG (cytosine-phosphate-guanine) sites from a nested case-cohort study that was designed to identify blood-based DNA methylation markers associated with breast cancer. A second, smaller “replication set” was comprised of 187 women with methylation array data on 485,512 CpG sites from a nested case-control study of diethylstilbestrol (DES) exposure. Study populations and details of methods for methylation data have been previously described [14, 15].

Statistical Analysis

To examine the association between NSAID usage and DNA methylation, we used robust linear regression modeling to identify differential methylation of CpG sites for defined categories of NSAID usage compared to women who reported no regular lifetime use. The false discovery rate (FDR) was set at q<0.05 to correct for multiple testing; the correction method has been previously described [14, 16]. CpG sites passing the false discovery rate in the discovery set were then tested in the replication set for association between NSAID use and methylation. An association was considered replicated if the association p-value fell below a Bonferroni-corrected p-value (p ≤ 0.05 divided by the number of CpGs tested for replication). Data pre-
processing, normalization methods and quality control measures are detailed in the supplementa-
ry material of our previously published papers [14, 16]. To summarize briefly, methyla-
tion intensity values were background-corrected using the Robust Multichip Average (RMA)
method [17] and quantile-normalized across arrays. Methylation array plates included controls
with known methylation levels to assess precision of measurement and duplicate samples to
assess reproducibility of results within the assay. Each methylation array included probes to
assess bisulfite conversion efficiency and negative control probes to measure background fluo-
rescent intensity. Samples with poor bisulfite conversion efficiency (< 3,800) or having >5% of
probes with unreliable measures (detection P > 0.05) were excluded. The methylation outcome
was calculated using fluorescence intensities for unmethylated (U) and methylated (M) alleles
as M/(M+U+100), which is a continuous variable ranging from 0 (completely unmethylated)
to 1 (100% methylated) based on the ratio. In both data sets a nonspecific filtering step was
applied to filter out the 20% CpGs with the smallest interquartile range (IQR) of methylation
values before association analysis. CpG probes with single nucleotide polymorphisms (SNPs)
present at target sites (428 CpGs from the HumanMethylation27 BeadChip and 20,869 CpGs
from the HumanMethylation450 BeadChip) were excluded from the analysis. In the 27K data
set we tested 21,659 probes and in the 450K data set we tested 369,120 probes.

To examine the association between long-term NSAID usage and DNA methylation, we
used robust linear regression modeling to identify differential methylation of CpG sites for
each defined strata of NSAID usage compared to a participant group with the lowest reported
lifetime NSAID usage. Singular value decomposition (SVD) analysis of the raw dataset revealed
that the top principal components derived from the methylation beta value matrix were highly
correlated with plate, bisulfite conversion intensities and age. We adjusted for these factors
together with breast cancer status in all association analyses. All association tests were also
adjusted for the proportions of different types of white blood cells estimated using a method
described by Houseman et al. [18, 19]

We conducted the robust linear regression modeling analyses separately in the breast can-
cer-free subcohort as well as in the entire study group with adjustment for breast cancer case
status. We examined the effect of different durations of NSAID usage on methylation, as well
as NSAID class, dosage and frequency of use. NSAID use and methylation analyses were con-
ducted using SAS 9.3 (SAS Institute, Cary, NC, USA) and R (http://www.r-project.org.). Power
calculations were conducted with Stata 13.1 (Statacorp, College Station, TX, USA).

Ethics Statement

Informed written consent was obtained from all participants prior to participation. The study
was approved by the Institutional Review Boards of the National Institute of Environmental
Health Sciences (NIEHS), National Institutes of Health, and the Copernicus Group (http://
www.cgor.com/irb-services/).

Results

All included participants were white, non-hispanic, and either had developed invasive breast
cancer or were cancer free at the time of methylation analysis. 490 women reported ever regu-
larly using NSAIDs in the discovery set of 871 participants (Table 1). Usage rates did not differ
 between cases and non-cases (56.4% and 56.2%). In our replication set, 54% reported regular
NSAID use. Across cases and controls, users reported a mean regular lifetime NSAID use of
approximately 7.7 years. Regular users reported a mean of 27.9 “pill-years” of use, which was
calculated as the number of NSAID pills taken per week multiplied by the number of years that
The dose was taken. 329 women (37.8%) reported that they had taken an NSAID drug daily for at least 3 months within the past 12 months.

In an analysis limited to women who did not develop breast cancer, no CpGs were differentially methylated between regular NSAID users and non-users, or when limited to NSAID users with recent daily use of the drugs. When examining years of NSAID use and pill-years of NSAID use as continuous exposures, we found 8 CpGs associated with pill-years of use that passed the FDR in the discovery set. However, only one of these CpGs reached \( p < 0.05 \) in the replication set and failed to pass the Bonferroni correction.

When women who developed breast cancer during follow-up were included in the discovery set with adjustment for case status, we identified 122 CpG sites that were differentially methylated at \( \text{FDR} < 0.05 \) for ever-users of NSAIDs compared to never-users (Table 2). When we examined these sites in our replication set we found that only 2 of these 122 sites were differentially methylated at \( p < 0.05 \), and both associations failed to pass the Bonferroni correction for testing of 122 sites. When examining years of NSAID use and pill-years of NSAID use as continuous exposures, we found 6 CpGs associated with pill-years of use which passed the FDR in the discovery set but failed to replicate. We identified 48 CpGs in the discovery set that were

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Table 1. Demographic characteristics of women in the discovery set (N = 871).

|                          | All women (N = 871) | Breast cancer-free women (N = 573) | Breast cancer cases (N = 298) |
|--------------------------|---------------------|----------------------------------|-------------------------------|
|                          | N   | (%)   | N   | (%)   | N   | (%)   |
| **BMI**                  |     |       |     |       |     |       |
| <25                      | 371 | (42.6)| 245 | (42.8)| 126 | (42.3)|
| 25–29                    | 226 | (25.9)| 153 | (26.7)| 73  | (24.5)|
| 29–35                    | 159 | (18.3)| 98  | (17.1)| 61  | (20.5)|
| 35+                      | 115 | (13.2)| 77  | (13.4)| 38  | (12.7)|
| **Menopausal Status**    |     |       |     |       |     |       |
| Pre-menopausal           | 328 | (37.7)| 219 | (38.2)| 109 | (36.6)|
| Post-menopausal          | 543 | (62.3)| 354 | (61.8)| 189 | (63.4)|
| **Smoking Status**       |     |       |     |       |     |       |
| Never                    | 458 | (52.6)| 294 | (51.3)| 164 | (55.0)|
| Social                   | 20  | (2.3) | 15  | (2.6) | 5   | (1.7) |
| Past                     | 331 | (38.0)| 222 | (38.7)| 109 | (36.6)|
| Current                  | 62  | (7.1) | 42  | (7.3) | 20  | (6.7) |
| **Ever used an NSAID regularly** |     |       |     |       |     |       |
| No                       | 381 | (43.7)| 251 | (43.8)| 130 | (43.6)|
| Yes                      | 490 | (56.3)| 322 | (56.2)| 168 | (56.4)|
| **Mean age at baseline** |     |       |     |       |     |       |
| Mean (SD)                | 55.1 | (9.1) | 54.6 | (8.9) | 56.1 | (9.2) |
| **Total years of use reported among users** |     |       |     |       |     |       |
| Mean (SD)                | 7.7  | (9.3) | 7.8  | (9.6) | 7.6  | (8.7) |
| **Total pill-years of use reported among all users** |     |       |     |       |     |       |
| Mean (SD)                | 27.9 | (68.7)| 28.2 | (67.7)| 27.2 | (70.6)|
| **Used an NSAID daily in the past 12 months** |     |       |     |       |     |       |
| No                       | 542 | (62.2)| 363 | (63.4)| 179 | (60.1)|
| Yes                      | 329 | (37.8)| 210 | (36.6)| 119 | (39.9)|

*aused an NSAID \( \geq 3 \) times a week for 3 or more months

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associated with daily use of NSAIDs in the past 12 months, but these CpGs did not reach statistical significance in the replication set with the Bonferroni correction. All CpG sites passing the FDR in the discovery set are listed in S1 Table.

Discussion

"Laboratory and observational studies suggest that NSAID use may influence patterns of DNA methylation in various tissues. Gastric tissue from long-term NSAID users have been shown to have differential methylation at selected genes [11], and in vitro NSAID treatment has been shown to alter DNA methylation in human fibroblast and lung cancer cell lines [13, 20].

Although we hypothesized that methylation differences linked to long-term NSAID use would be detectable in peripheral blood, we found no strong evidence of such an effect. No CpGs were associated with ever regularly using NSAIDs in the discovery set of women who did not subsequently develop cancer. When we expanded the discovery set to include, with adjustment, the women who subsequently developed cancer, we did find 122 CpGs that were associated with NSAIDs. Because both sets of women in the discovery set were distributed randomly to the same set of chips and batches that were adjusted for in the analysis, it seems unlikely that the change in the number of detected CpGs is due to batch, chip, or other technical effects. Nor
was there evidence of insufficient adjustment or residual confounding from the case group: the coefficients of the 122 CpGs remained almost the same in the cancer-free women alone compared to cancer-free plus cases ($R^2 = 0.99$). It is possible that the increased sample size resulted in increased power to detect true association, or that the CpGs detected in the expanded analysis were the result of random effects. Although we cannot formally exclude either possibility, our examination of the replication set failed to provide independent evidence for true association.

Use of a replication set provides a useful means of reducing spurious findings, but failure to replicate does not eliminate the possibility that associations with small effect size may still exist. We used Bonferroni correction for multiple testing in the replication set, and such correction may be overly conservative—yet even at a nominal threshold $p$ value = 0.05, only 2 of the 122 sites were significant (fewer than expected by chance) and only one had the same direction of methylation effect with NSAID use. Although we have used this same replication set to confirm other small effects related to smoking [15] and participant age [21], we cannot exclude the possibility that there might be unconfirmed true positives in the discovery set. With the observed effect size of approximately 1% change in methylation seen in the discovery set, this replication sample of 187 individuals achieves between 16% and 60% power for detection using standard deviations for methylation values ranging from 0.07 to 0.03 respectively. This calculation is based on a two sample t-test with $\alpha = 0.05$, and the range of standard deviations tested reflect the approximate range observed for the ten top CpGs in our comparisons. We note that a study of blood DNA from 88 men found that those taking acetylsalicylic acid had lower methylation at the ATP-binding cassette transporter A1 (ABCA1) gene [22] but we found no evidence for association at the 2 CpGs representing this gene on the 27K array.

It is possible that any effect of NSAID use on DNA methylation might be specific to tissues other than blood. Epigenome-wide studies conducted using DNA from tissues such as gastric epithelium, colon epithelium, and normal breast tissue may be more successful in identifying DNA methylation differences related to NSAID use. However, these tissues are difficult to obtain fresh-frozen in large numbers, which limits potential sample size. Formalin-fixed paraffin embedded (FFPE) tissue samples are more readily available than frozen tissue, and although methods are available for methylation analysis of DNA from FFPE samples, the resulting data are of lesser quality [23].

Strengths of this study include the availability of methylation array data for a large number of women with corresponding detailed NSAID exposure assessment. We were able to estimate lifetime long-term exposures to NSAID drugs including aspirin-containing and non-aspirin containing drugs. This study is substantially larger than prior studies of NSAID use and DNA methylation at candidate genes, and is the first to use epigenome-wide analysis. But we cannot exclude the possibility that small effects, or sites not covered in the 27K array, or in other tissues might still be associated with NSAID use.

**Supporting Information**

**S1 Table. CpG sites passing FDR threshold in discovery set.** CpG sites from the Illumina Infinium HumanMethylation27 beadchip array passing the FDR ($q < 0.05$) in the discovery set of 871 women. No associations replicated with a Bonferroni correction in a second set of 187 women. CpGs marked with an asterisk (’*) replicated at an unadjusted $p$-value of 0.05. (DOCX)

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Author Contributions
Conceived and designed the experiments: LEW SK SH DPS JAT. Performed the experiments: LEW ZX. Analyzed the data: LEW ZX. Contributed reagents/materials/analysis tools: SK SH DPS JAT. Wrote the paper: LEW SK ZX SH DPS JAT.

References
1. Cuzick J, Otto F, Baron JA, Brown PH, Burn J, Greenwald P, et al. Aspirin and non-steroidal anti-inflammatory drugs for cancer prevention: an international consensus statement. Lancet Oncol. 2009; 10(5):501–7. PMID:19410194. doi:10.1016/S1470-2045(09)70035-X
2. Zhao Y-d, Zhu S, Li X-w, Wang F, Hu F-I, Li D-d, et al. Association between NSAIDs use and breast cancer risk: a systematic review and meta-analysis. Breast Cancer Research and Treatment. 2009; 117(1):141–50. doi:10.1007/s10549-008-0228-6 PMID:18979210
3. Brasky TM, Bonner MR, Moysich KB, Ambrosone CB, Nie J, Tao MH, et al. Non-steroidal anti-inflammatory drug (NSAID) use and breast cancer risk in the Western New York Exposures and Breast Cancer (WEB) Study. Cancer Causes Control. 21(9):1503–12. PMID:20499154. doi:10.1007/s10552-010-9579-5
4. Takkouche B, Regueira-Mendez C, Etminan M. Breast cancer and use of nonsteroidal anti-inflammatory drugs: a meta-analysis. J Natl Cancer Inst. 2008; 100(20):1439–47. PMID:18840819. doi:10.1093/jnci/djn324
5. Hahn MA, Hahn T, Lee DH, Esworth RS, Kim BW, Riggs AD, et al. Methylation of polycomb target genes in intestinal cancer is mediated by inflammation. Cancer Res. 2008; 68(24):10280–9. PMID:19074896. doi:10.1158/0008-5472.CAN-08-1957
6. Katsurano M, Niwa T, Yasui Y, Shigematsu Y, Yamashita S, Takeshima H, et al. Early-stage formation of an epigenetic field defect in a mouse colitis model, and non-essential roles of T- and B-cells in DNA methylation induction. Oncogene. 31(3):342–51. PMID:21685942. doi:10.1038/onc.2011.241
7. O'Hagan HeatherÂ A, Wang W, Sen S, DeStefanoÂ Shields C, Lee StellaÂ S, Zhang YangÂ W, et al. Oxidative Damage Targets ComplexesContaining DNA Methyltransferases, SIRT1, and Polycomb Members to Promoter CpG Islands. Cancer Cell. 20(5):606–19. doi:10.1016/j.ccr.2011.09.012 PMID:22094255
8. Oberdoerffer P, Michan S, McVay M, Mostoslavsky R, Vann J, Park S-K, et al. SIRT1 Redistribution on Chromatin Promotes Genomic Stability but Alters Gene Expression during Aging. Cell. 2008; 135(5):907–18. doi:10.1016/j.cell.2008.10.025 PMID:19041753
9. Huang SK, Scruggs AM, Donaghy J, McEachin RC, Fisher AS, Richardson BC, et al. Prostaglandin E2 increases fibroblast gene-specific and global DNA methylation via increased DNA methyltransferase expression. p. 3703–14.
10. Xia D, Wang D, Kim SH, Kato H, DuBois RN. Prostaglandin E2 promotes intestinal tumor growth via DNA methylation. Nat Med. 18(2):224–6. PMID:22270723. doi:10.1038/nm.2608
11. Tahara T, Shibata T, Yamashita H, Nakamura M, Yoshioka D, Okubo M, et al. Chronic nonsteroidal anti-inflammatory drug (NSAID) use suppresses multiple CpG islands hyper methylation (CHM) of tumor suppressor genes in the human gastric mucosa. Cancer Science. 2009; 100(7):1192–7. doi: 10.1111/j.1349-7006.2009.01175.x PMID:19432886
12. Noreen F, Roosli M, Gaj P, Pietrzak J, Weis S, Urfer P, et al. Modulation of age- and cancer-associated DNA methylation change in the healthy colon by aspirin and lifestyle. J Natl Cancer Inst. 2014; 106(7). Epub 2014/06/30. doi:10.1093/jnci/dju161 PMID:24973978; PubMed Central PMCID: PMCPMC4112799.
13. Huang SK, Scruggs AM, Donaghy J, McEachin RC, Fisher AS, Richardson BC, et al. Prostaglandin E (2) increases fibroblast gene-specific and global DNA methylation via increased DNA methyltransferase expression. Faseb J. 26(9):3703–14. PMID:22645246. doi:10.1096/fj.11-203323
14. Xu Z, Bolick SC, DeRoo LA, Weinberg CR, Sandler DP, Taylor JA. Epigenome-wide association study of breast cancer using prospectively collected sister study samples. J Nat Cancer Inst. 2013; 105(10):694–700. Epub 2013/04/13. doi:10.1093/jnci/djt045 PMID:23578854; PubMed Central PMCID: PMCPMC3653821.
15. Harlid S, Xu Z, Panduri V, Sandler DP, Taylor JA. CpG sites associated with cigarette smoking: analysis of epigenome-wide data from the sister study. Environmental health perspectives. 2014; 122 (7):673–8. Epub 2014/04/08. doi: 10.1289/ehp.1307480 PMID: 24704858; PubMed Central PMCID: PMCPMC4080519.

16. Harlid S, Xu Z, Panduri V, D’Aloisio AA, DeRoo LA, Sandler DP, et al. In utero exposure to diethylstilbestrol and blood DNA methylation in women ages 40–59 years from the sister study. PLoS One. 2015; 10(3):e0118757. Epub 2015/03/10. doi: 10.1371/journal.pone.0118757 PMID: 25751399; PubMed Central PMCID: PMCPMC4353728.

17. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics (Oxford, England). 2003; 4(2):249–64. Epub 2003/08/20. doi: 10.1093/biostatistics/4.2.249 PMID: 12925520.

18. Houseman EA, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC bioinformatics. 2012; 13:86. Epub 2012/05/10. doi: 10.1186/1471-2105-13-86 PMID: 22568884; PubMed Central PMCID: PMCPMC3532182.

19. Accomando WP, Wiencke JK, Houseman EA, Butler RA, Zheng S, Nelson HH, et al. Decreased NK cells in patients with head and neck cancer determined in archival DNA. Clinical cancer research: an official journal of the American Association for Cancer Research. 2012; 18(22):6147–54. Epub 2012/09/28. doi: 10.1158/1078-0432.ccr-12-1008 PMID: 23014525; PubMed Central PMCID: PMCPMC3500449.

20. Pan MR, Chang HC, Chuang LY, Hung WC. The nonsteroidal anti-inflammatory drug NS398reactivates SPARC expression via promoter demethylation to attenuate invasiveness of lung cancer cells. Exp Biol Med (Maywood). 2008; 233(4):456–62. PMID: 18367635.

21. Xu Z, Taylor JA. Genome-wide age-related DNA methylation changes in blood and other tissues relate to histone modification, expression and cancer. Carcinogenesis. 2014; 35(2):356–64. Epub 2013/11/30. doi: 10.1093/carcin/bgt391 PMID: 24287154; PubMed Central PMCID: PMCPMC3908753.

22. Guay SP, Legare C, Houde AA, Mathieu P, Bosse Y, Bouchard L. Acetylsalicylic acid, aging and coronary artery disease are associated with ABCA1 DNA methylation in men. Clinical epigenetics. 2014; 6 (1):14. Epub 2014/08/06. doi: 10.1186/1868-7083-6-14 PMID: 25093045; PubMed Central PMCID: PMCPMC4120725.

23. Siegel EM, Berglund AE, Riggs BM, Eschrich SA, Putney RM, Ajidahun AO, et al. Expanding epigenomics to archived FFPE tissues: an evaluation of DNA repair methodologies. Cancer Epidemiol Biomarkers Prev. 2014; 23(12):2622–31. Epub 2014/12/05. doi: 10.1158/1055-9965.epi-14-0464 PMID: 25472669; PubMed Central PMCID: PMCPMC4256717.