Exploratory plasma proteomic analysis in a randomized crossover trial of aspirin among healthy men and women

Xiaoliang Wang[1,2]*, Ali Shojaie[3], Yuzheng Zhang[2], David Shelley[2], Paul D. Lampe[2], Lisa Levy[2], Ulrike Peters[1,2], John D. Potter[1,2], Emily White[1,2], Johanna W. Lampe[1,2]

1 Department of Epidemiology, University of Washington, Seattle, Washington, United States of America, **2** Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington, United States of America, **3** Department of Biostatistics, University of Washington, Seattle, Washington, United States of America

* xwang23@fredhutch.org

Abstract

Long-term use of aspirin is associated with lower risk of colorectal cancer and other cancers; however, the mechanism of chemopreventive effect of aspirin is not fully understood. Animal studies suggest that COX-2, NFκB signaling and Wnt/β-catenin pathways may play a role, but no clinical trials have systematically evaluated the biological response to aspirin in healthy humans. Using a high-density antibody array, we assessed the difference in plasma protein levels after 60 days of regular dose aspirin (325 mg/day) compared to placebo in a randomized double-blinded crossover trial of 44 healthy non-smoking men and women, aged 21–45 years. The plasma proteome was analyzed on an antibody microarray with ~3,300 full-length antibodies, printed in triplicate. Moderated paired t-tests were performed on individual antibodies, and gene-set analyses were performed based on KEGG and GO pathways. Among the 3,000 antibodies analyzed, statistically significant differences in plasma protein levels were observed for nine antibodies after adjusting for false discoveries (FDR adjusted p-value<0.1). The most significant protein was succinate dehydrogenase subunit C (SDHC), a key enzyme complex of the mitochondrial tricarboxylic acid (TCA) cycle. The other statistically significant proteins (NR2F1, MSI1, MYH1, FOXO1, KHDRBS3, NFKBIE, LYZ and IKZF1) are involved in multiple pathways, including DNA base-pair repair, inflammation and oncogenic pathways. None of the 258 KEGG and 1,139 GO pathways was found to be statistically significant after FDR adjustment. This study suggests several chemopreventive mechanisms of aspirin in humans, which have previously been reported to play a role in anti- or pro-carcinogenesis in cell systems; however, larger, confirmatory studies are needed.

Introduction

Low-dose and regular-strength aspirin use is consistently observed to be associated with reduced long-term risk of colorectal cancer (CRC) risk of adenomatous polyps, pre-cancerous lesions that increase risk of CRC [1, 2]. Benefit increases with duration of aspirin use and is associated with 34% reduction in 20-year CRC risk [1, 3]. Evidence consistently suggests that aspirin plays a role at an early stage or even before tumorigenesis [4]. Therefore, studies of aspirin involving healthy individuals may help elucidate biological responses related to the chemopreventive effects of aspirin.

The presumed main mechanism by which aspirin lowers adenomatous polyps and CRC risk is by reducing inflammatory mediators through the inhibition of cyclooxygenase-2 (COX-2) activity [5, 6] and subsequent formation of prostaglandin E2 (PGE2) [7]. Aspirin has also been shown to inhibit the oncogenic Wnt/β-catenin pathway [8] and the extracellular-signal-regulated kinase (ERK) signaling pathway [9] in colon cancer cell lines. Indirect support for these pathways from human studies comes from nested case-control studies which suggest that interactions between the use of non-steroidal anti-inflammatory drugs (NSAIDs) and polymorphisms in oncogenes in the Wnt/β-catenin signaling pathway [10] and NFκB-signaling pathway [11] modify CRC risk [12]. Recently, a genome-wide investigation of gene-environment interactions reported that the association of NSAIDs with CRC risk differed according to genetic variation at 2 SNPs [13]; these are related to genes involved in activation of the PI3K signaling pathway. Other pathways related to transcription factors, cell proliferation and apoptosis have also been suggested [14].

No human intervention trials have yet systematically explored proteomic profiling of aspirin use among human subjects. A randomized controlled trial of diclofenac among overweight individuals identified a group of inflammation-related modulators [15]; another trial suggested a variety of pathways, including cytokine activity pathways, in response to glucosamine and chondroitin supplementation [16]. These studies support the utility of proteomic profiling in characterizing responses to drugs or supplements with pleiotropic effects. We created a high density antibody array containing >3,200 different antibodies to ~2,100 different proteins that we use to interrogate plasma or other biological samples for cellular activation status including proteins involved in apoptosis, proliferation, angiogenesis, immune cell activity/infiltration, and metabolism, etc. Many of the antibodies are to secreted proteins such as cytokines and growth factors including 21 proteins with insulin in their names. We have used these arrays to find biomarkers of ovarian [17, 18], breast [19], pancreas [20, 21] and colon [22] cancer and used the values derived to find pathways important in obesity [23], supplement usage [16], anti-apoptotic cell survival signaling pathways [22], and incisional hernia [24]. The objective of this study was to explore potential mechanisms relevant to the effects of aspirin through proteomic analysis in healthy participants in a randomized trial of aspirin, with a focus on proteins that are related to cancer development.

Materials and methods

Study design

The Aspirin and the Biology of the Colon (ABC) study was a randomized, double-blinded, placebo-controlled, crossover trial [25]. During each intervention period, participants took 325 mg aspirin or a visually identical placebo orally each day for 60 days, with a 3-month washout period between the treatment periods. Study activities, including participant interviews and blood draws, were conducted at the Fred Hutchinson Cancer Research Center (Fred Hutch) Prevention Center Research Clinic. The study procedures were approved by the Fred Hutch

Institutional Review Board; informed, written consent was obtained from all participants prior to participation in the study.

Study participants

Details of the study and the study population have been described previously [25, 26] and are summarized in S1 Fig. Briefly, healthy men and women, aged 21 to 45 years, were recruited from participants from the greater Seattle area who completed a cross-sectional study of diet and aspirin metabolism between June 2003 and March 2007 [27]. Individuals were excluded if they had: a medical history of gastrointestinal, hepatic, or renal disorders; family history of familial adenomatous polyposis or Lynch syndrome; known intolerance to aspirin or other NSAIDs; weight change greater than 4.5 kg within the past year; current use of prescription medication (including oral contraceptives) or over-the-counter medications; alcohol intake >2 drinks/day; were pregnant or lactating; or were planning to move out of the greater Seattle area within the 12 months of the study period. Given that an aim of the parent study was to determine whether genetic variation in *UGT1A6* influenced response to aspirin [25], participants who met these criteria were further selected based on *UGT1A6* genotypes (rs2070959 and rs1105879) so that all subjects with a *2/*2 genotype and sex-matched participants with a *1/*1 genotype were invited to participate. Additionally, two participants with a *2/*4 genotype were also included and randomized. Clinical measurements were also assessed and participants with abnormal laboratory values were excluded from participation. A total of 55 healthy men and women were recruited into the trial, randomly assigned, blocked on sex and *UGT1A6* genotype, to the order of receiving aspirin or placebo. Forty-four participants completed both intervention periods. The reasons participants dropped out were not related to either intervention or placebo period.

Data collection

Demographics and medical history were obtained through questionnaires at the time of recruitment, including age, ethnicity, previous smoking habits, dietary supplement use, alcohol intake, history of weight change, and general health. Twelve-hour fasting morning blood samples were drawn on day -5 and day 55 of the first intervention period, and days 1 and 55 of the second intervention period. The pre-intervention blood samples were tested for liver and kidney function, and post-intervention blood samples were used for research purposes. Blood samples were collected in EDTA-containing vacutainer tubes; plasma was aliquoted into cryovials and stored at -80˚C until analysis.

Proteomics analyses

Plasma samples were analyzed on a customized antibody array populated with ~3,300 full-length antibodies, printed in triplicate on a single microarray according to published methods [20–24, 28]. Briefly, each sample (200 μg) was combined with the same amount of a Cy3 labeled "reference" pool (from 5 healthy men and 5 women) of albumin and IgG-depleted plasma, placed on the array and Cy3 and Cy5 signals determined. The log2-transformed Cy5/Cy3 ratio, noted as M value, determined the relative concentration of protein compared to reference. For quality-control purposes, triplicate antibodies with coefficients of variation >10% were removed, and experimental variation was normalized using within-array print-tip loess normalization and between-array quartile normalization [29]. The median for each antibody was taken from triplicates as the summary measure.

The two samples from the same person collected at the termination of the two periods (aspirin and placebo) were analyzed in the same batch with the order of treatment periods

randomized. Sex and genotypes were randomly distributed across batches. Additional possible batch effects were checked by principal component analysis (data not shown).

Previous analyses showed that coefficients of variation, for triplicates, for >85% antibodies on the array were less than 10% [18, 28, 30, 31]. Intra-class correlation (R_1) for triplicates was also used to evaluate the reliability of triplicates in this study [32]. 92.5% of the antibodies had at least moderate correlation among triplicates ($R_1 > 0.5$), and 83.5% antibodies had strong correlations among triplicates ($R_1 > 0.7$), suggesting reliable measurements of plasma protein levels. Antibodies were also highly correlated between quality-control duplicate samples that were blinded to the lab analysist.

Statistical analysis

Antibodies with more than 30% missing values across the arrays were excluded from further analysis. Remaining missing data were imputed using the local least squares imputation method, which replaces a target protein that has missing values with a linear combination of 10 similar proteins, chosen by k-nearest neighbors based on Pearson correlation coefficients [33]. Of ~3,300 antibodies on the array, 3,000 proteins were available for statistical analyses and complete data were available on all 44 participants after imputation. Moderated paired t-tests [34] were performed for individual proteins to determine statistically significant differences between aspirin and placebo treatments. Adjustment for potential confounders, including batch effect, sample positions, and covariate effects of sex and *UGT1A6* genotype was carried out using a mixed linear regression model. The proteins were then ranked on the basis of p-values adjusted by Benjamini-Hochberg false discovery rate (FDR) correction, at a significance level of 0.1 [34]. Both the moderated t-tests and FDR corrections were performed using the R LIMMA package [29].

Pathway analyses were also carried out [35], using gene sets in Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Gene Ontology (GO). KEGG gene sets were obtained via REST server to KEGG, and GO gene sets were obtained from MSigDB (http://www.broadinstitute.org/gsea/msigdb/index.jsp). Simulation analyses were performed to compare several gene-set analytical tools [35–37]. The Significance Analysis of Functional Categories (SAFE) framework [35] outperformed the other methods in relation to sensitivity and specificity, and was used in further analysis. Gene sets with fewer than 3 genes were excluded from the analysis. The enriched gene sets were ranked on the basis of adjusted p-values, with an FDR significance level of 0.1.

Results

The demographic characteristics of the 44 study participants are summarized in Table 1. The study population was predominantly Caucasian, and approximately half of the participants were overweight or obese (BMI$\geq$25 kg/m^2).

Among the 3,000 proteins tested, nine were statistically different between the aspirin and placebo periods after FDR correction (adjusted p-value<0.1) (Table 2), including energy convertors, hormone receptors, transcriptional factors, and RNA- and DNA-binding proteins. Among these nine proteins, six (MYH1, FOXO1, KHDRBS3, NFKBIE, LYZ and IKZF1) had a higher expression level on aspirin than placebo, whereas the other three (SDHC, NR2F1 and MSI1) had a lower expression level on aspirin than placebo. The most significant protein was succinate dehydrogenase subunit C (SDHC), with an average 34% lower expression level on aspirin than placebo (p-value = 4.47×10^{-05}; FDR-adjusted p-value = 0.06). The next most significant was myosin-1 (MYH1). The expression level of MYH1 on aspirin treatment was 62% higher on average than that on placebo (p-value = 6.83×10^{-05}; FDR-adjusted p-value = 0.06).

Table 1. Demographic characteristics of 44 participants.

Characteristics	N (%)
Age, Mean(SD)	30.43 (5.97)
Sex	
Male	20 (45.5)
Female	24 (54.5)
BMI, kg/m^2	
Normal (<25)	23 (52.3)
Overweight or obese ($\geq$25)	21 (47.7)
Race/Ethnicity	
Caucasian	33 (75.0)
African-American	1 (2.3)
Asian	5 (11.4)
Other	5 (11.4)

https://doi.org/10.1371/journal.pone.0178444.t001

The largest decrease was observed in a nuclear hormone receptor NR2F1 with 60% lower expression level on aspirin than placebo. NF-κB inhibitor epsilon (NFKBIE) had one of the largest increases: the expression level was 63% higher on aspirin than placebo. Because the study was conducted as a randomized crossover study, each participant served as their own

Table 2. Proteins that differed significantly between aspirin and placebo periods with false discovery rate (FDR) <0.10.

Symbol	Function[a]	Missing %[b]	Average expression[c]		Effect size[d]	Fold change[e]	p-value[f]	Adjusted p-value[g]
			aspirin	placebo				
SDHC	Conservative effector of mitochondrial Krebs cycle and respiratory chain	20.5	-0.259	-0.112	-0.594	0.662	4.47×10^{-05}	0.058
MYH1	Energy convertor in base excision repair (BER) pathway	18.2	-0.262	-0.454	0.698	1.622	6.83×10^{-05}	0.058
NR2F1	Nuclear hormone receptor and transcriptional regulator	11.4	-0.371	-0.265	-0.755	0.592	6.94×10^{-05}	0.058
FOXO1	Transcription factor in carbohydrates metabolism and Akt-mTOR signaling pathway	17.0	1.003	0.845	0.520	1.434	7.72×10^{-05}	0.058
KHDRBS3	RNA-binding protein regulating pre-mRNA splicing, signaling and cell cycle control	27.3	0.096	-0.028	0.400	1.320	2.07×10^{-04}	0.087
NFKBIE	NF-κB inhibitor epsilon	23.9	0.530	0.374	0.709	1.634	2.15×10^{-04}	0.087
LYZ	Lysozyme, antimicrobial enzyme	26.1	0.004	-0.147	0.689	1.612	2.29×10^{-04}	0.087
MSI1	RNA-binding protein, posttranscriptional regulator of proliferative activity	26.1	-0.351	-0.176	-0.487	0.714	2.32×10^{-04}	0.087
IKZF1	Transcription factor of zinc-finger DNA-binding and lymphocyte differentiation regulator	4.5	-0.310	-0.426	0.542	1.456	2.67×10^{-04}	0.089

[a] Information pertaining to encoded protein function was derived from PubMed Gene unless otherwise noted.

[b] Missing% is the proportion of samples with missing values on this protein among all 88 samples.

[c] Average expression level was presented in median M values for each protein.

[d] Effect size was the mean difference of M values between two treatment periods standardized by standard deviation of average expression in placebo period.

[e] Fold-change was the standardized ratio between median M values of aspirin and placebo treatment. A fold change >1 indicated greater antibody expression after aspirin treatment compared to placebo; a fold change <1 indicated lower expression after aspirin treatment.

[f] P-values were obtained from mixed linear regression model, adjusted for batch effect, sample position, gender and genotype.

[g] P-values were adjusted for false discovery rate using Benjamini-Horchberg procedure.

https://doi.org/10.1371/journal.pone.0178444.t002

control; predictably, adjusting for batch effects, sample positions, order of aspirin and placebo period, and other covariates did not change test results.

Analyses of differences in individual protein expression levels were also undertaken stratified by sex (S1 Table). For the nine proteins that were statistically different in the overall analysis, the direction of effect (increased or decreased) was consistent across men and women; however, none of the within-group changes in protein levels was statistically significant after FDR correction, probably due to reduced sample size in subgroup analyses.

In gene-set analyses using the SAFE framework, a total of 257 KEGG pathways and 1139 GO pathways were tested. Among them, 21 KEGG and 63 GO pathways were statistically significantly different on aspirin than placebo (p-value<0.05); however, none of these gene categories reached statistical significance after FDR correction at significance level of 0.1 (S2 Table).

Discussion

In this placebo-controlled randomized crossover trial among healthy individuals, plasma levels of nine of the total of ~3,000 antibodies were significantly different between 60-day regular-dose aspirin and placebo, after adjustment for FDR at 0.1. Among these nine proteins, six had higher expression levels, and three were lower on aspirin than placebo. These proteins play important roles in various pathways, including the mitochondrial Kreb's cycle, DNA base-pair repair, and inflammation. However, when correcting for multiple comparisons, we did not identify overall pathways that were statistically significantly different between aspirin and placebo.

The protein with the most significant difference between treatments was SDHC; plasma levels were lower after aspirin treatment. SDHC is a subunit of succinate dehydrogenase (SDH), a key enzyme complex of the mitochondrial tricarboxylic acid (TCA) cycle, which oxidizes succinate to fumarate [38]. As one part of SDH (also called complex II), it also facilitates transfer of electrons to coenzyme Q (ubiquinone) [39]. Aspirin has been shown to interfere with mitochondrial function [40], as well as inhibit the activity of SDH in rats [41]. Further, repeated mild inhibition of oxidative phosphorylation via inhibition of SDH protects against the decrease in ATP that usually accompanies severe hypoxia and thus can act as neuroprotection [42]. Treatment with aspirin has also been shown *in vivo* to slow down the decline of intracellular ATP by this mechanism of inhibiting SDH [43] and therefore to protect against hypoxia, a common hallmark of tumors that promotes metabolic adaptations and angiogenesis [44]. Furthermore, accumulation of succinate, due to reduced efficiency of SDHC, results in the stabilization of HIF1-α, the degradation of which is promoted by the oncometabolite (R)-2-hydroxyglutarate [45]. Metabolomic analysis in the present study has shown that plasma concentrations of 2-hydroxyglutarate decreased after aspirin treatment in both men and women (p = 0.005) [26]. It is relevant that plasma concentration of HIF1-α was lower after aspirin treatment among carriers of wild *UGT1A6*1/*1* genotype (Data not shown; p = 6.2×10^{-05}; adjusted p-value = 0.186), but not among carriers of *UGT1A6*2/*2* genotype, suggesting that the genotypes of *UGT1A6*, which encodes a UDP-glucuronosyltransferase that participates in glucuronidation of aspirin [46], may modulate the effect of aspirin on downstream metabolic functions. In summary, results from our proteomic analyses, as well as those from metabolomic analyses support a possible additional mechanism for aspirin in cancer prevention.

The expression level of MYH1 was also statistically significantly higher by 62% on aspirin than placebo. As a member of the human homologue of the base excision repair (BER) gene, MYH1 is a DNA glycosylase that removes adenine mispaired with 8-hydroxyguanine from

DNA and protects against oxidative DNA damage [47, 48]. Inherited variants in *MYH* that cause reduced enzyme function have been associated with significantly increased risk of familial and sporadic CRC in observational studies [49–52]. In addition, the prevalence of low-frequency microsatellite instability (MSI) has been found to be higher among *MYH* mutation carriers [51, 53], suggesting possible interaction between the BER and MSI pathways. However, most previous observational studies assessed the association between germline mutations of *MYH* and CRC risk, whereas our study directly measured the plasma level of MYH1. Our findings suggest that environmental factors, such as aspirin, may also have an effect on enzyme levels, regardless of genetic background.

Similarly, NFKBIE was 63% higher on average on aspirin than placebo. NFKBIE is involved in the NF-κB signaling pathway. After cellular stimulation, NFKBIE is highly induced to bind the NF-κB dimer, and provides negative feedback regulation that inhibits NF-κB DNA-binding activity and prevents its nuclear accumulation [54]. As the NF-κB pathway plays an important role in chronic inflammation and tumor promotion, reduction of NF-κB activity is critical in inhibiting the production of pro-inflammatory cytokines [55, 56]. In an observational study among 315 chronic lymphocytic leukemia (CLL) patients, targeted deep sequencing of 18 core complex genes within the NF-κB pathway found that the most frequently mutated genes was *NFKBIE*; further screening revealed that truncated NFKBIE predominated in patients with poor prognosis [57]. Similarly, exome sequencing has also suggested that NFKBIE was highly mutated among melanoma patients [58]. Aspirin has been found to inhibit IκB kinase (IKK) β, which inhibits NF-κB inhibitors by phosphorylation [59]. In a nested case-control study, polymorphisms in IκBKβ were associated with lower CRC risk and the association was stronger among current NSAID users [11]. Findings from our study are among the first in humans to suggest a biologic interaction between aspirin and NFKBIE, thus providing further support for the likelihood that the NF-κB signaling pathway is involved in one of the mechanisms of action of aspirin.

Among the other significant proteins, aspirin treatment was associated with 29% lower level of Musashi1 (MSI1), a neural RNA-binding protein. A previous study among colon cancer patients has shown that overexpression of MSI1 in colon cancer lesions, compared to paired normal colonic mucosa, was associated with poorer metastasis-free survival and poorer overall survival [60]. A variety of other potential mechanisms have also been suggested by our findings, including those involving NR2F1 as a nuclear hormone receptor, Forkhead Box O1 (FOXO1) in Akt-mTOR signaling pathway, and KHDRBS3 in RNA-binding and cell-cycle control. Several of these have limited evidence for a relationship with aspirin in humans, probably due to the fact that most previous human studies have focused on candidate genes, proteins, or pathways. Randomized controlled trials of aspirin conducted among patients with cardiovascular disease found statistically significant reductions in circulating concentrations of high-sensitivity C-reactive protein (CRP), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and thromboxane B2 (TXB2) [61–63].

Compared to previous human studies of aspirin or other NSAIDs, our study has the strength that our antibody microarray has a high coverage of the proteome (2,100 proteins from 3,000 antibodies), which allowed for more complex proteomic profiling of the impact of aspirin. Most of the proteins that differed significantly between aspirin and placebo treatment are involved in various pathways associated with carcinogenesis, illustrating the potential range of biologic effects of aspirin *in vivo*. Secondly, most of the previous studies were focused on mechanisms inhibited by aspirin in tumor cells or in patients with a focus on tumor progression; ours is among the first to directly evaluate the effects of aspirin among healthy individuals. Therefore, we had the opportunity to identify, agnostically, proteins and mechanisms that are promoted by aspirin treatment; this, nonetheless, remains relevant to understanding

how aspirin prevents tumor progression. In addition, the crossover design allowed participants to serve as their own control, which minimized unmeasured inter-individual variability. Because the samples from the same participant were randomly ordered and analyzed within the same batch, additional adjustment for batch effects and sample positions did not change the results. There was also a 3-month wash-out period between treatments (longer than the usual 60-day periods); this minimizes carry-over effects.

There are also some limitations. First, our analysis was primarily designed to examine the signaling effects of aspirin and has no specific hypothesis or protein to validate. Subsequent steps would involve testing on another set of subjects who took aspirin or placebo but these samples are not available to us at this time. Second, the duration of treatment was two months, and this may not characterize the long-term effects of aspirin use in cancer prevention. Thirdly, our sample size was relatively small and lacked substantial as plasma protein levels were not the main outcome of the intervention. Effect sizes were also small which may reflect the fact that all the participants were generally healthy and therefore would have had relatively low expression of pro-inflammatory proteins; this, in turn would leave less room for change with aspirin intervention. Furthermore, the expression levels of proteins are context-specific: our findings using plasma may differ from those in other tissues, such as colon mucosa. Six of the nine proteins typically are found in the nucleus, so their presence in plasma might result from cellular leakage/damage or exosome formation. In support of the latter, aspirin has been reported to affect the content of platelet-derived exosomes [64]. Lastly, we used a liberal significance level of 0.1 for FDR adjustment. Although a higher threshold was used to identify more possible candidates, it might also lead to more false positive results. Therefore, further investigations of these identified proteins are needed.

To our knowledge, this is the first randomized trial to systematically evaluate the effect of aspirin on plasma protein profiles in healthy men and women. We identified several proteins that differed significantly between aspirin and placebo, some of which have been previously reported as playing a role in inflammation and carcinogenesis. The involvement of various biologic pathways suggests that the chemopreventive mechanisms of aspirin in humans are complex. Larger, confirmatory studies with a longer period of aspirin exposure are needed.

Supporting information

S1 Data File. Participant information.
(XLSX)

S2 Data File. Microarray data.
(XLSX)

S1 Fig. Flow chart of participant enrollment and study design.
(DOCX)

S1 Table. Plasma level differences in the expression of the nine significant proteins, stratified by sex.
(DOCX)

S2 Table. List of gene sets with unadjusted p-values <0.05.
(DOCX)

Author Contributions

Conceptualization: XW JP EW JL.

Data curation: XW YZ DS PL LL.

Formal analysis: XW AS YZ.

Funding acquisition: JP EW JL.

Investigation: DS PL LL JL.

Methodology: XW AS YZ.

Project administration: LL.

Resources: DS LL.

Software: XW AS YZ.

Supervision: PL EW JL UP.

Validation: XW YZ.

Visualization: XW.

Writing – original draft: XW.

Writing – review & editing: XW AS YZ PL LL UP JP EW JL.

References

1. Algra AM, Rothwell PM. Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomised trials. Lancet Oncol 2012; 13:518–27. https://doi.org/10.1016/S1470-2045(12)70112-2 PMID: 22440112

2. Murff HJ, Shrubsole MJ, Chen Z, Smalley WE, Chen H, Shyr Y, et al. Nonsteroidal anti-inflammatory drug use and risk of adenomatous and hyperplastic polyps. Cancer Prev Res (Phila) 2011; 4:1799–807.

3. Rothwell PM, Wilson M, Elwin CE, Norrving B, Algra A, Warlow CP, et al. Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. Lancet 2010; 376:1741–50. https://doi.org/10.1016/S0140-6736(10)61543-7 PMID: 20970847

4. Chubak J, Kamineni A, Buist DSM, Anderson ML, Whitlock EP. Edtion ed. Aspirin Use for the Prevention of Colorectal Cancer: An Updated Systematic Evidence Review for the US Preventive Services Task Force. Rockville (MD), 2015.

5. Thun MJ, Henley SJ, Patrono C. Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues. J Natl Cancer Inst 2002; 94:252–66. PMID: 11854387

6. Gupta RA, Dubois RN. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat Rev Cancer 2001; 1:11–21. https://doi.org/10.1038/35094017 PMID: 11900248

7. Ulrich CM, Bigler J, Potter JD. Non-steroidal anti-inflammatory drugs for cancer prevention: promise, perils and pharmacogenetics. Nat Rev Cancer 2006; 6:130–40. https://doi.org/10.1038/nrc1801 PMID: 16491072

8. Bos CL, Kodach LL, van den Brink GR, Diks SH, van Santen MM, Richel DJ, et al. Effect of aspirin on the Wnt/beta-catenin pathway is mediated via protein phosphatase 2A. Oncogene 2006; 25:6447–56. https://doi.org/10.1038/sj.onc.1209658 PMID: 16878161

9. Pan MR, Chang HC, Hung WC. Non-steroidal anti-inflammatory drugs suppress the ERK signaling pathway via block of Ras/c-Raf interaction and activation of MAP kinase phosphatases. Cell Signal 2008; 20:1134–41. https://doi.org/10.1016/j.cellsig.2008.02.004 PMID: 18374541

10. Nan H, Morikawa T, Suuriniemi M, Imamura Y, Werner L, Kuchiba A, et al. Aspirin use, 8q24 single nucleotide polymorphism rs6983267, and colorectal cancer according to CTNNB1 alterations. J Natl Cancer Inst 2013; 105:1852–61. https://doi.org/10.1093/jnci/djt331 PMID: 24317174

11. Seufert BL, Poole EM, Whitton J, Xiao L, Makar KW, Campbell PT, et al. IkappaBKbeta and NFkappaB1, NSAID use and risk of colorectal cancer in the Colon Cancer Family Registry. Carcinogenesis 2013; 34:79–85. https://doi.org/10.1093/carcin/bgs296 PMID: 23002237

12. Andersen V, Christensen J, Overvad K, Tjonneland A, Vogel U. Polymorphisms in NFkB, PXR, LXR and risk of colorectal cancer in a prospective study of Danes. BMC Cancer 2010; 10:484. https://doi.org/10.1186/1471-2407-10-484 PMID: 20836841

13. Nan H, Hutter CM, Lin Y, Jacobs EJ, Ulrich CM, White E, et al. Association of aspirin and NSAID use with risk of colorectal cancer according to genetic variants. JAMA 2015; 313:1133–42. https://doi.org/10.1001/jama.2015.1815 PMID: 25781442

14. Andersen V, Vogel U. Systematic review: interactions between aspirin, and other nonsteroidal anti-inflammatory drugs, and polymorphisms in relation to colorectal cancer. Aliment Pharmacol Ther 2014; 40:147–59. https://doi.org/10.1111/apt.12807 PMID: 24889212

15. van Erk MJ, Wopereis S, Rubingh C, van Vliet T, Verheij E, Cnubben NH, et al. Insight in modulation of inflammation in response to diclofenac intervention: a human intervention study. BMC Med Genomics 2010; 3:5. https://doi.org/10.1186/1755-8794-3-5 PMID: 20178593

16. Navarro S W E, Kantor ED, Zhang Y, Rho J, Song X, Milne GL, Lampe PD, Lampe JW. Randomized trial of glucosamine and chondroitin supplementation on inflammation and oxidative stress biomarkers and plasma proteomics profiles in healthy humans. PLoS One 2015; 10:e0117534. https://doi.org/10.1371/journal.pone.0117534 PMID: 25719429

17. Ramirez AB, Loch CM, Zhang Y, Liu Y, Wang X, Wayner EA, et al. Use of a single-chain antibody library for ovarian cancer biomarker discovery. Mol Cell Proteomics 2010; 9:1449–60. https://doi.org/10.1074/mcp.M900496-MCP200 PMID: 20467042

18. Loch CM, Ramirez AB, Liu Y, Sather CL, Delrow JJ, Scholler N, et al. Use of high density antibody arrays to validate and discover cancer serum biomarkers. Mol Oncol 2007; 1:313–20. https://doi.org/10.1016/j.molonc.2007.08.004 PMID: 19383305

19. Buas MF, Rho JH, Chai X, Zhang Y, Lampe PD, Li CI. Candidate early detection protein biomarkers for ER+/PR+ invasive ductal breast carcinoma identified using pre-clinical plasma from the WHI observational study. Breast Cancer Res Treat 2015; 153:445–54. https://doi.org/10.1007/s10549-015-3554-5 PMID: 26319120

20. Mirus JE, Zhang Y, Li CI, Lokshin AE, Prentice RL, Hingorani SR, et al. Cross-species antibody microarray interrogation identifies a 3-protein panel of plasma biomarkers for early diagnosis of pancreas cancer. Clin Cancer Res 2015; 21:1764–71. https://doi.org/10.1158/1078-0432.CCR-13-3474 PMID: 25589628

21. Mirus JE, Zhang Y, Hollingsworth MA, Solan JL, Lampe PD, Hingorani SR. Spatiotemporal proteomic analyses during pancreas cancer progression identifies serine/threonine stress kinase 4 (STK4) as a novel candidate biomarker for early stage disease. Mol Cell Proteomics 2014; 13:3484–96. https://doi.org/10.1074/mcp.M113.036517 PMID: 25225358

22. Rho JH, Ladd JJ, Li CI, Potter JD, Zhang Y, Shelley D, et al. Protein and glycomic plasma markers for early detection of adenoma and colon cancer. Gut 2016: https://doi.org/10.1136/gutjnl-2016-312794 PMID: 27821646

23. Garrison CB, Lastwika KJ, Zhang Y, Li CI, Lampe PD. Proteomic Analysis, Immune Dysregulation, and Pathway Interconnections with Obesity. J Proteome Res 2017; 16:274–87. https://doi.org/10.1021/acs.jproteome.6b00611 PMID: 27769113

24. Bohm J, Pianka F, Stuttgen N, Rho J, Gigic B, Zhang Y, et al. Discovery of novel plasma proteins as biomarkers for the development of incisional hernias after midline incision in patients with colorectal cancer: The ColoCare study. Surgery 2017; 161:808–17. https://doi.org/10.1016/j.surg.2016.08.025 PMID: 27745870

25. Thomas SS, Makar KW, Li L, Zheng Y, Yang P, Levy L, et al. Tissue-specific patterns of gene expression in the epithelium and stroma of normal colon in healthy individuals in an aspirin intervention trial. BMC Med Genet 2015; 16:18. https://doi.org/10.1186/s12881-015-0161-6 PMID: 25927723

26. Liesenfeld DB, Botma A, Habermann N, Toth R, Weigel C, Popanda O, et al. Aspirin Reduces Plasma Concentrations of the Oncometabolite 2-Hydroxyglutarate: Results of a Randomized, Double-Blind, Crossover Trial. Cancer Epidemiol Biomarkers Prev 2016; 25:180–7. https://doi.org/10.1158/1055-9965.EPI-15-0697 PMID: 26585118

27. Navarro SL, Saracino MR, Makar KW, Thomas SS, Li L, Zheng Y, et al. Determinants of aspirin metabolism in healthy men and women: effects of dietary inducers of UDP-glucuronosyltransferases. J Nutrigenet Nutrigenomics 2011; 4:110–8. https://doi.org/10.1159/000327782 PMID: 21625173

28. Rho JH, Lampe PD. High-throughput screening for native autoantigen-autoantibody complexes using antibody microarrays. J Proteome Res 2013; 12:2311–20. https://doi.org/10.1021/pr4001674 PMID: 23541305

29. Smyth GK. Limma: linear models for microarray data. Edtion ed. In: Gentleman R, Carey V, Dudoit S, Irizarry R, Huber I.W, ed. Bioinformatics and Computational Biology Solutions Using R and Bioconductor. New York: Springer, 2005:397–420.

30. Li CI, Mirus JE, Zhang Y, Ramirez AB, Ladd JJ, Prentice RL, et al. Discovery and preliminary confirmation of novel early detection biomarkers for triple-negative breast cancer using preclinical plasma

samples from the Women's Health Initiative observational study. Breast Cancer Res Treat 2012; 135:611–8. PNC3439142. https://doi.org/10.1007/s10549-012-2204-4 PMID: 22903690

31. Ramirez AB, Lampe PD. Discovery and validation of ovarian cancer biomarkers utilizing high density antibody microarrays. Cancer Biomark 2010; 8:293–307. https://doi.org/10.3233/CBM-2011-0215 PMID: 22045360

32. Bartko JJ. The intraclass correlation coefficient as a measure of reliability. Psychol Rep 1966; 19:3–11. https://doi.org/10.2466/pr0.1966.19.1.3 PMID: 5942109

33. Kim H, Golub GH, Park H. Missing value estimation for DNA microarray gene expression data: local least squares imputation. Bioinformatics 2005; 21:187–98. https://doi.org/10.1093/bioinformatics/bth499 PMID: 15333461

34. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004; 3:Article3.

35. Barry WT, Nobel AB, Wright FA. Significance analysis of functional categories in gene expression studies: a structured permutation approach. Bioinformatics 2005; 21:1943–9. https://doi.org/10.1093/bioinformatics/bti260 PMID: 15647293

36. Wu D, Smyth GK. Camera: a competitive gene set test accounting for inter-gene correlation. Nucleic Acids Res 2012; 40:e133. https://doi.org/10.1093/nar/gks461 PMID: 22638577

37. Chen LS, Paul D, Prentice RL, Wang P. A regularized Hotelling's test for pathway analysis in proteomic studies. J Am Stat Assoc 2011; 106:1345–60. https://doi.org/10.1198/jasa.2011.ap10599 PMID: 23997374

38. Desideri E, Vegliante R, Ciriolo MR. Mitochondrial dysfunctions in cancer: genetic defects and oncogenic signaling impinging on TCA cycle activity. Cancer Lett 2015; 356:217–23. https://doi.org/10.1016/j.canlet.2014.02.023 PMID: 24614286

39. Yankovskaya V, Horsefield R, Tornroth S, Luna-Chavez C, Miyoshi H, Leger C, et al. Architecture of succinate dehydrogenase and reactive oxygen species generation. Science 2003; 299:700–4. https://doi.org/10.1126/science.1079605 PMID: 12560550

40. Lutwak-Mann C. The effect of salicylate and cinchophen on enzymes and metabolic processes. Biochem J 1942; 36:706–28. PMID: 16747500

41. Kaplan EH, Kennedy J, Davis J. Effects of salicylate and other benzoates on oxidative enzymes of the tricarboxylic acid cycle in rat tissue homogenates. Arch Biochem Biophys 1954; 51:47–61. PMID: 13181459

42. Riepe MW, Niemi WN, Megow D, Ludolph AC, Carpenter DO. Mitochondrial oxidation in rat hippocampus can be preconditioned by selective chemical inhibition of succinic dehydrogenase. Exp Neurol 1996; 138:15–21. https://doi.org/10.1006/exnr.1996.0042 PMID: 8593890

43. Riepe MW, Kasischke K, Raupach A. Acetylsalicylic acid increases tolerance against hypoxic and chemical hypoxia. Stroke 1997; 28:2006–11. PMID: 9341711

44. Majmundar AJ, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. Mol Cell 2010; 40:294–309. https://doi.org/10.1016/j.molcel.2010.09.022 PMID: 20965423

45. Koivunen P, Lee S, Duncan CG, Lopez G, Lu G, Ramkissoon S, et al. Transformation by the (R)-enantiomer of 2-hydroxyglutarate linked to EGLN activation. Nature 2012; 483:484–8. https://doi.org/10.1038/nature10898 PMID: 22343896

46. Kuehl GE, Bigler J, Potter JD, Lampe JW. Glucuronidation of the aspirin metabolite salicylic acid by expressed UDP-glucuronosyltransferases and human liver microsomes. Drug Metab Dispos 2006; 34:199–202. https://doi.org/10.1124/dmd.105.005652 PMID: 16258079

47. Slupska MM, Baikalov C, Luther WM, Chiang JH, Wei YF, Miller JH. Cloning and sequencing a human homolog (hMYH) of the Escherichia coli mutY gene whose function is required for the repair of oxidative DNA damage. J Bacteriol 1996; 178:3885–92. PMID: 8682794

48. Hayashi H, Tominaga Y, Hirano S, McKenna AE, Nakabeppu Y, Matsumoto Y. Replication-associated repair of adenine:8-oxoguanine mispairs by MYH. Curr Biol 2002; 12:335–9. PMID: 11864576

49. Al-Tassan N, Chmiel NH, Maynard J, Fleming N, Livingston AL, Williams GT, et al. Inherited variants of MYH associated with somatic G:C—>T:A mutations in colorectal tumors. Nat Genet 2002; 30:227–32. https://doi.org/10.1038/ng828 PMID: 11818965

50. Kambara T, Whitehall VL, Spring KJ, Barker MA, Arnold S, Wynter CV, et al. Role of inherited defects of MYH in the development of sporadic colorectal cancer. Genes Chromosomes Cancer 2004; 40:1–9. https://doi.org/10.1002/gcc.20011 PMID: 15034862

51. Cleary SP, Cotterchio M, Jenkins MA, Kim H, Bristow R, Green R, et al. Germline MutY human homologue mutations and colorectal cancer: a multisite case-control study. Gastroenterology 2009; 136:1251–60. https://doi.org/10.1053/j.gastro.2008.12.050 PMID: 19245865

52. Win AK, Dowty JG, Cleary SP, Kim H, Buchanan DD, Young JP, et al. Risk of colorectal cancer for carriers of mutations in MUTYH, with and without a family history of cancer. Gastroenterology 2014; 146:1208–11 e1–5. https://doi.org/10.1053/j.gastro.2014.01.022 PMID: 24444654

53. Colebatch A, Hitchins M, Williams R, Meagher A, Hawkins NJ, Ward RL. The role of MYH and microsatellite instability in the development of sporadic colorectal cancer. Br J Cancer 2006; 95:1239–43. https://doi.org/10.1038/sj.bjc.6603421 PMID: 17031395

54. Kearns JD, Basak S, Werner SL, Huang CS, Hoffmann A. IkappaBepsilon provides negative feedback to control NF-kappaB oscillations, signaling dynamics, and inflammatory gene expression. J Cell Biol 2006; 173:659–64. https://doi.org/10.1083/jcb.200510155 PMID: 16735576

55. Greten FR, Karin M. The IKK/NF-kappaB activation pathway-a target for prevention and treatment of cancer. Cancer Lett 2004; 206:193–9. https://doi.org/10.1016/j.canlet.2003.08.029 PMID: 15013524

56. Li Q, Withoff S, Verma IM. Inflammation-associated cancer: NF-kappaB is the lynchpin. Trends Immunol 2005; 26:318–25. https://doi.org/10.1016/j.it.2005.04.003 PMID: 15922948

57. Mansouri L, Sutton LA, Ljungstrom V, Bondza S, Arngarden L, Bhoi S, et al. Functional loss of IkappaBepsilon leads to NF-kappaB deregulation in aggressive chronic lymphocytic leukemia. J Exp Med 2015; 212:833–43. https://doi.org/10.1084/jem.20142009 PMID: 25987724

58. Shain AH, Garrido M, Botton T, Talevich E, Yeh I, Sanborn JZ, et al. Exome sequencing of desmoplastic melanoma identifies recurrent NFKBIE promoter mutations and diverse activating mutations in the MAPK pathway. Nat Genet 2015; 47:1194–9. https://doi.org/10.1038/ng.3382 PMID: 26343386

59. Yin MJ, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. Nature 1998; 396:77–80. https://doi.org/10.1038/23948 PMID: 9817203

60. Li D, Peng X, Yan D, Tang H, Huang F, Yang Y, et al. Msi-1 is a predictor of survival and a novel therapeutic target in colon cancer. Ann Surg Oncol 2011; 18:2074–83. https://doi.org/10.1245/s10434-011-1567-9 PMID: 21442350

61. Gao XR, Adhikari CM, Peng LY, Guo XG, Zhai YS, He XY, et al. Efficacy of different doses of aspirin in decreasing blood levels of inflammatory markers in patients with cardiovascular metabolic syndrome. J Pharm Pharmacol 2009; 61:1505–10. https://doi.org/10.1211/jpp/61.11.0010 PMID: 19903376

62. Solheim S, Arnesen H, Eikvar L, Hurlen M, Seljeflot I. Influence of aspirin on inflammatory markers in patients after acute myocardial infarction. Am J Cardiol 2003; 92:843–5. PMID: 14516890

63. Ikonomidis I, Andreotti F, Economou E, Stefanadis C, Toutouzas P, Nihoyannopoulos P. Increased proinflammatory cytokines in patients with chronic stable angina and their reduction by aspirin. Circulation 1999; 100:793–8. PMID: 10458713

64. Goetzl EJ, Goetzl L, Karliner JS, Tang N, Pulliam L. Human plasma platelet-derived exosomes: effects of aspirin. FASEB J 2016; 30:2058–63. https://doi.org/10.1096/fj.201500150R PMID: 26873936