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TITLE: ASPIRED-XT: ASPirin Intervention for the REDuction of Colorectal Cancer Risk - EXTension

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NCI-Supplied Agent(s): N/A

Other Agent(s): Aspirin, commercial, various manufacturers

This study is an extension study of NCT02394769. This study design was determined to be exempt from IND Requirements per 21 CFR 312.2(b). Notice of formal exemption received 11/12/14.

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SCHEMA

ASPIRED-XT: ASPIrin Intervention for the REDuction of Colorectal Cancer Risk - EXTension

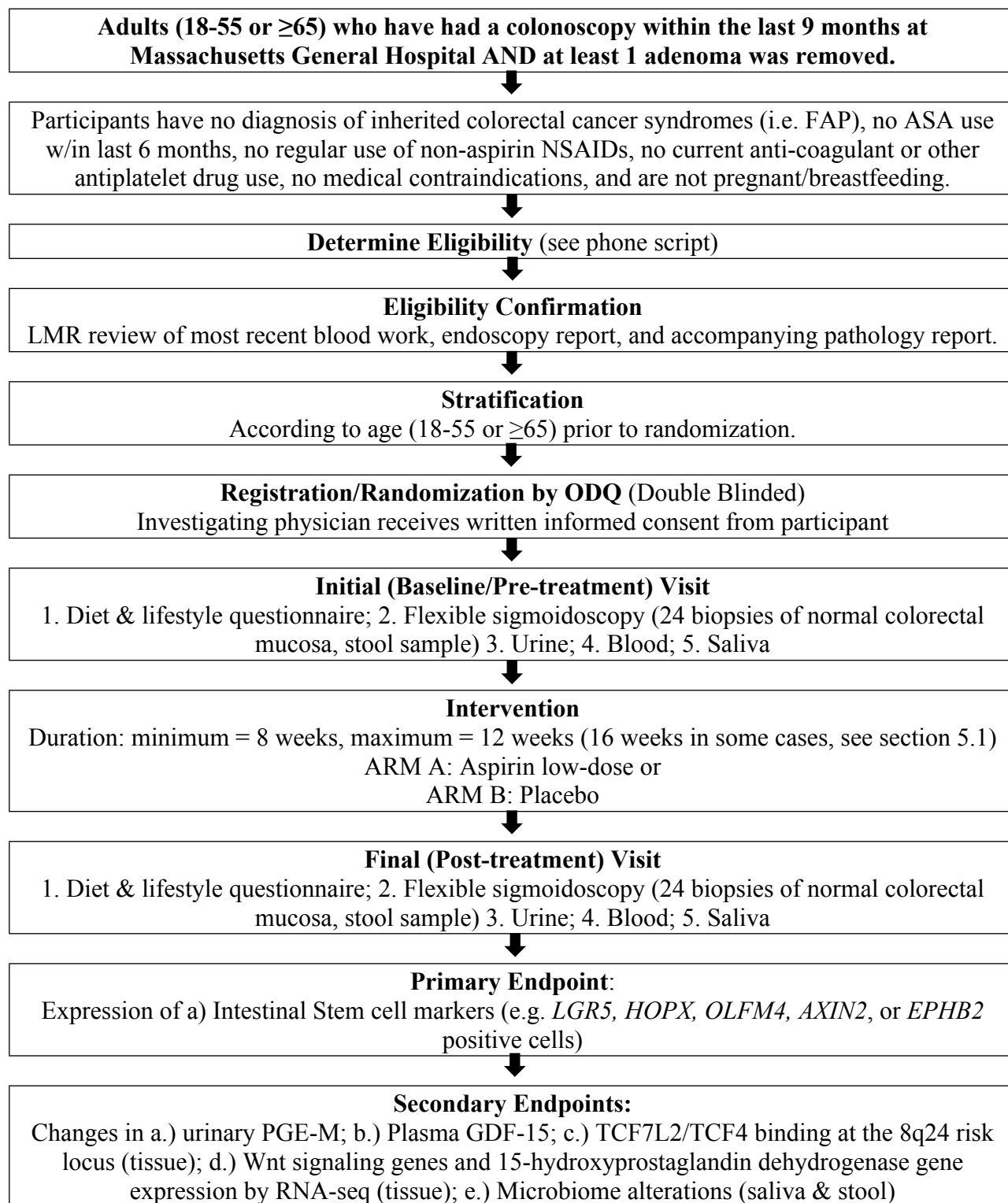


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OBJECTIVES

1.1 Study Design

As a direct extension of the successful ASPIRED trial (NCT02394769; DFHCC Protocol # 14-496), within the gastroenterology practice of Massachusetts General Hospital (MGH), we will conduct a prospective, double-blind, placebo-controlled, randomized clinical trial to measure the effects of daily low-dose (81 mg/day) aspirin on tissue, urine, plasma, and stool biomarkers associated with colorectal cancer with a focus on the effect of age.

1.2 Primary Objectives

To determine the effect of age and aspirin intervention on colon intestinal stem cell (cISC) populations by measuring expression and function, via single cell (sc)RNA-seq, of cISC markers (*LGR5*, *HOPX*, *OLFM4*, *AXIN2*, or *EPHB2*) in colon epithelial samples.

1.3 Secondary Objectives

To measure the effect of low-dose aspirin treatment, according to age, on the following colorectal cancer-associated biomarkers

- Urinary prostaglandin metabolites (PGE-M).
- Inflammatory transcriptional signatures (scRNA-seq).
- Plasma macrophage inhibitory cytokine-1 (GDF-15), an inflammatory biomarker;
- TCF7L2/TCF4 binding at the 8q24 colorectal cancer risk locus in colonic epithelium;
- Wnt signaling genes (i.e. β -catenin, *AXIN-2* and *MYC*) and 15-hydroxyprostaglandin dehydrogenase (*15-PGDH*) gene expression as measured by RNA-seq on sorted colonic epithelial cell populations.
- Bacterial populations and products associated with colorectal cancer in saliva and stool (metagenomics).

BACKGROUND

2.1 Study Disease(s)

Colorectal cancer, or cancer of the large intestine, is currently the second most common cause of cancer death in the United States. In 2020, an estimated 147,950 individuals will be diagnosed and 53,200 people will die from colorectal cancer¹. Age is strongly associated with colorectal cancer incidence, with more than a third of all colorectal cancer diagnosed in the population aged 80 years or older. The death rates are higher in black populations and lowest in Asian individuals. Men have a slightly higher lifetime probability of receiving a colorectal cancer diagnosis (4.4%) compared to women (4.1%).¹

Nationally, incidence rates have declined rapidly since 2000. This is thought to be related to increased access to colorectal cancer screening. Yet, the 5-year survival rate for the second most deadly cancer in the United States is only 64%,¹ necessitating further advances in chemoprevention

strategies to reduce overall colorectal cancer burden.

The USPSTF currently has reported that the evidence supporting a preventative effect for aspirin in adults over the age of 70 is insufficient and in need of further study.^{2,3} Although CRC incidence rises with age in humans and mouse models, the intersection of biological pathways underlying aging and cancer remains unclear. The ASPREE results⁴ highlight a potential lack of chemoprotective benefit associated with low-dose aspirin when that intervention is initiated later in life suggesting age-context specific biology that influences low-dose aspirin sensitivity.

2.2 IND Agent

N/A; Exempt.

This study will investigate the effects of low dose aspirin. We received formal exemption status from the FDA, through the Division of Oncology Products 2 (DOP2), regarding our investigational use of aspirin towards these biomarker outcomes. In consultation with Monica L. Hughes of DOP2, the DOP2 has provided guidance that the study of aspirin in this capacity meets the five exemption criteria as laid out by 21 CFR 312.2:

- The study is not intended to support FDA approval of a new indication or a significant change in the product labeling.
- The study is not intended to support a significant change in the advertising for the product.
- The investigation does not involve a route of administration or dosage level or use in a patient population or other factor that significantly increases the risk (or decreases the acceptability of the risks) associated with the use of the drug product.
- The study is conducted in compliance with institutional review board (IRB) and informed consent regulations set forth in parts 56 and 50 (21 CFR parts 56 and 50)
- The study is conducted in compliance with § 312.7 (promotion and charging for investigational drugs)

2.3 Other Agent(s)

Aspirin

Aspirin (also known as Acetylsalicylic Acid, ASA, or Salicylic Acid Acetate) is an analgesic, antipyretic (fever-reducing), antirheumatic, central nervous system agent, and platelet aggregation inhibitor drug. It is highly lipid soluble and slightly soluble in water. It is a more potent inhibitor of prostaglandin synthesis and platelet aggregation than other salicylic acid derivatives. At low (81 mg/day) doses, aspirin affects platelet aggregation by irreversibly inhibiting prostaglandin cyclooxygenase (COX) preventing the formation of thromboxane A₂, a platelet aggregating factor. At standard doses, aspirin also has an anti-inflammatory effect due to inhibition of inflammatory mediators via COX inhibition in peripheral tissues.

2.4 Rationale

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the US¹. Several prospective studies and RCT's have shown that aspirin reduces colorectal neoplasia.⁵⁻¹⁴ In 2016, the U.S. Preventive Services Task Force (USPSTF) recommended low-dose aspirin for prevention of CVD and CRC in 50-59 yr old adults with $\geq 10\%$ 10-year CVD risk.^{2,15} However, the effects of age on CRC and relevance to aspirin chemoprevention are uncertain. Among individuals over age 70, the USPSTF was “unable to assess the balance of benefits and harms of initiating [low-dose aspirin]”. In 2018, the results of Aspirin to Prevent Events in the Elderly (ASPREE), a binational U.S. / Australia NIA/NCI-sponsored placebo-controlled RCT of 100mg of enteric-coated aspirin in 19,114 adults age 70+ (Whites) and 65+ (U.S. minorities) were released. Low-dose aspirin was associated with no difference in risk of incident cancer in the ASPREE study⁴, supporting the hypothesis that low-dose aspirin may have distinct impacts on CRC incidence based on age of initiation.

Our working model is that the effect of low-dose aspirin on the colon may differ in older individuals due to age-related changes in intestinal stem cell (ISC) number and function secondary to a process of higher basal inflammatory tone, a.k.a. “inflammaging”. A broadly accepted concept is that CRC is driven by oncogenic mutations in Lgr5⁺ ISCs in mice and humans. Recent data support that aspirin-like NSAIDs preferentially eliminate premalignant Lgr5⁺ ISCs.^{16,17} However, we showed that aged mice (20-22 months [mos]), as compared to young adult mice (2-3 mos), have fewer, less regenerative small intestinal Lgr5⁺ ISCs (sISCs),^{18,19} which are also less tumorigenic in an *Apc* tumor suppressor model.¹⁸ Nonetheless, like humans, aged mice spontaneously develop a greater number of tumors, indicating that non-Lgr5⁺ cells are also the origin of intestinal cancers in aged mice and that these cells are less sensitive to low-dose aspirin effects. Low-dose aspirin is well known to modulate prostaglandin (PG) levels, including prostaglandin (PG)E₂. PGE₂ impacts ISC function through its receptor Ptger4 and this signaling can drive ISCs into a fetal-like state (Hox⁺) that is mediated by Hippo/Yap signaling.^{20,21} Thus, in the setting of inflammaging, elevated PGE₂ may irreversibly compromise the colon (cISC) pool leading to compensatory functions within select cISCs that will promote tumorigenesis. Through recent completion of our ASPIrin Intervention for REDuction of CRC risk (ASPIRED; NCT02394769; DFHCC Protocol # 14-496) double-blind, placebo-controlled RCT, we demonstrated that modulation of PG tone and inhibition of PG synthesis is a central function of aspirin's chemoprotective mode of action.³⁵ Our central hypothesis is that aging and age-related processes, such as inflammaging, promote a decrease in the cISC pool (Lgr5⁺ cISCs) that is normally sensitive to aspirin chemoprevention. We propose that initiation of LDA earlier in life protects against this age-related, inflammation-associated, and/or PGE₂-mediated damage to the cISC pool. In contrast, with advancing age there may be a “point-of-no-return” in which initiation of LDA is no longer able to protect against age-related changes in the cISC pool. Thus, a more comprehensive understanding of the differences in the colon epithelium of younger (≤ 55 yrs) versus older (≥ 65 yrs) patients is necessary to define populations that will most likely benefit from low-dose aspirin intervention.

2.5 Correlative Studies Background

Aspirin, established to reduce the risk of cardiovascular disease events in the general population and among high-risk groups,²² has emerged as the agent with the most consistently observed chemopreventive effect on cancer, particularly of the colorectum.^{5-14,23,24} Prospective studies (including several by our group)^{5,7,9,25-27} and randomized clinical trials of polyp recurrence,^{10,11} hereditary colorectal cancer syndromes,^{13,28} cardiovascular disease prevention,¹² and cancer as a pre-specified outcome,¹⁴ have shown that aspirin reduces incidence of colorectal neoplasia. Recently, these benefits have been extended to other cancers in 8 randomized clinical trials.²⁹ Taken together with studies (including those by our group)⁷ showing benefits of aspirin on overall, cancer-specific, and cardiovascular-specific mortality, there is now convincing evidence that aspirin may prevent the two leading causes of death in the U.S., thereby widening its population-wide impact.³⁰ A systematic review estimated that aspirin significantly reduced all-cause mortality by 6%, major cardiovascular events by 10%, and cancer death by 18%.³¹ Further work by our group and others also supports activity of aspirin against metastases and death after cancer diagnosis.^{27,32-34} We recently completed the ASPIrin Intervention for the REDuction of Colorectal Cancer Risk (ASPIRED) randomized clinical trial. The clinical intervention in a population at high risk of recurrent CRC demonstrated both low-dose (81 mg) and high-dose (325 mg) aspirin significantly reduce urinary PGE-M concentrations, a biomarker for CRC risk.³⁵

Despite these benefits, aspirin is associated with hemorrhage, particularly gastrointestinal bleeding for which risk is associated with increasing age as well as dose and duration of aspirin use.³⁶⁻³⁹ The field is now at a critical juncture to translate these findings into the clinic by defining the populations who will most benefit from aspirin chemoprevention. In light of recent findings regarding aspirin's null association with incident CRC in older individuals (≥ 65), there is a crucial gap in knowledge regarding optimal patient populations for aspirin chemoprevention. Thus, mechanistic work is needed to elucidate fundamental physiological differences in the colon epithelium between older and younger populations, and the potential differential role of aspirin for chemoprevention in aging cohorts to determine if there are biomarkers which may help tailor aspirin recommendations among an older population.

PARTICIPANT SELECTION

Patients that meet the eligibility criteria will be identified through investigators during their routine clinical practice, supplemented by a periodic query of the MGH endoscopy (Provation) and pathology database. Patients also may be identified using Natural Language Processing clinical software that queries medical records. Dr. Andrew Chan on behalf of the entirety of the Gastroenterology Practice (as approved by the Chief of Gastroenterology, Dr. Wolfram Goessling – see attached letter) will sign letters on behalf of the Division of Gastroenterology providers. We plan to recruit patients who have undergone a colonoscopy within the last nine months who are seen by an MGH gastroenterologist either in the ambulatory clinic or for an endoscopic procedure. In some cases, these physicians may not be members of the study team. However, non-study physicians will not perform study-related procedures.

Appropriate patients will be contacted by a letter (attached recruitment letter). If the colonoscopy was performed at MGH, after an adenoma is resected from a patient at MGH, all gastroenterologists routinely contact their patients by mail with the results of their pathology. The

recruitment letter will be included with the pathology results when possible, or it will be sent separately after the pathology report. If the colonoscopy and polypectomy was performed at another hospital, the MGH gastroenterologist will confirm the prior diagnosis of an adenoma through review of pathology reports. Dr. Andrew Chan will sign the letter. If no response is received to this recruitment letter within one week (5 business days) a follow-up phone call or email will be placed by a research assistant/study coordinator (phone script/sample email attached). In some cases, the treating gastroenterologist may approach the patient following the colonoscopy that identified a polyp if he/she feels the patient would be a good candidate for the study. In these cases, the patients may not receive a recruitment letter as the primary method of contact. Instead, they will be directly contacted by phone or email (see call script/sample email) from a member of the research team prior to mailing the letter (if requested). Potential participants will have additional opportunities to ask any questions by contacting an investigator or a trained research coordinator. If the participant is interested in participating, eligibility will be assessed by the study coordinator (see script) and confirmed by the investigator through additional check of the patients' medical record (see "Investigator Representation for Review of Protected Health Information Preparatory to Research" form). If eligibility is confirmed, the patient will be scheduled for their initial study visit and registered with ODQ. The informed consent form will be mailed to the patient prior to the initial visit so that he or she can read it in detail, if requested by the participant. Eligible participants will provide written informed consent at the baseline visit prior to participation in the study. The consenting process will be performed by the clinical investigator who is responsible for patient care.

3.1 Eligibility Criteria

- 3.1.1 Participants must have undergone screening or surveillance colonoscopy with removal of at least one adenoma within the last 9 months.
- 3.1.2 Age greater than or equal to 18 years and less than 55 years or greater than or equal to 65 years at the time of enrollment

This study will only include adult participants because colorectal carcinogenesis in children is more likely to be related to a cancer predisposition syndrome with distinct biological mechanisms compared with sporadic colorectal cancer in adults.

- 3.1.3 Not currently taking aspirin (any dose) within the last 6 months.
- 3.1.4 The effects of aspirin on the developing human fetus are unknown. For this reason, women of child-bearing potential must agree to use adequate contraception (hormonal or barrier method of birth control; abstinence) prior to study entry and for the duration of study participation. Should a woman become pregnant or suspect she is pregnant while she is participating in this study, she should inform her treating physician immediately.
- 3.1.5 Ability to understand and the willingness to sign a written informed consent document.

3.2 Exclusion Criteria

- 3.2.1 Use of any non-aspirin non-steroidal anti-inflammatory drug (NSAID) at any dose at least three times a week during the two months prior to randomization.
- 3.2.2 Diagnosis of inflammatory bowel disease, liver disease with evidence of clinical decompensation or chronic kidney disease (grade 3-4), bleeding diathesis.
- 3.2.3 Any prior diagnosis of invasive (i.e. excluding intramucosal cancer) gastrointestinal cancer (including esophageal, small intestine, colon, pancreatic), or any diagnosis of other cancers (with the exception of non-melanoma skin) in which there has been any active treatment within the last three years.
- 3.2.4 Participants who are receiving any other investigational agents.
- 3.2.5 History of allergic reactions attributed to compounds of similar chemical or biologic composition to aspirin.
- 3.2.6 Known diagnosis of Familial Adenomatous Polyposis (FAP) or Hereditary Non-Polyposis Colorectal Cancer (HNPCC, Lynch Syndrome).
- 3.2.7 Any adenoma that was not completely removed during previous colonoscopy.
- 3.2.8 History of aspirin intolerance, bleeding diathesis, peptic ulcer or gastrointestinal bleed requiring hospitalization, endoscopic complications, or contraindication to colonoscopy.
- 3.2.9 Inability or unwillingness to abstain from non-protocol use of aspirin or NSAIDs or to provide blood, urine, or stool samples or colon biopsies during the study.
- 3.2.10 Uncontrolled intercurrent illness including, but not limited to, ongoing or active infection, symptomatic congestive heart failure, unstable angina pectoris, cardiac arrhythmia, or psychiatric illness/social situations that would limit compliance with study requirements
- 3.2.11 Pregnant or breastfeeding.

Pregnant women are excluded from this study because aspirin is an FDA Category D agent with the potential for teratogenic or abortifacient effects. Because there is an unknown but potential risk for adverse events in nursing infants secondary to treatment of the mother with aspirin, breastfeeding should be discontinued if the mother is treated with aspirin.
- 3.2.12 Participant must be able to swallow pills.
- 3.2.13 Participant is taking any anticoagulant agent (e.g. warfarin) or antiplatelet agent (e.g. clopidogrel).

3.3 Inclusion of Women and Minorities

Both men and women of all races and ethnic groups are eligible for this trial.

4 REGISTRATION AND RANDOMIZATION PROCEDURES

4.1 General Guidelines for DF/HCC Institutions

Institutions will register eligible participants in the Clinical Trials Management System (CTMS) OnCore. Registrations must occur prior to the initiation of any protocol-specific therapy or intervention. Any participant not registered to the protocol before protocol-specific therapy or intervention begins will be considered ineligible and registration will be denied.

An investigator will confirm eligibility criteria and a member of the study team will complete the protocol-specific eligibility checklist.

The eligibility checklist(s) and all pages of the consent form(s) will be emailed to the ODQ at atqact@partners.org. The ODQ will (a) review the eligibility checklist, (b) register the participant on the protocol, and (c) randomize the participant.

Randomization can only occur during ODQ business hours (8:30am - 5pm Eastern Time, Monday through Friday excluding holidays).

Participants will be randomized to blinded treatment arms (either placebo or low-dose aspirin) according to the blinded randomization schedule and provide the treatment assignment code to the Research Pharmacy.

An email confirmation of the registration and/or randomization will be sent to the study coordinator(s) from the registering site, treating investigator and registering person immediately following the registration and/or randomization.

Following registration, participants may begin protocol-specific therapy and/or intervention. Issues that would cause treatment delays should be discussed with the Principal Investigator (PI) of the registering site. If the subject does not receive protocol therapy following registration, the subject must be taken off study in the CTMS (OnCore) with an appropriate date and reason entered.

4.2 Registration Process for DF/HCC Institutions

Applicable DF/HCC policy (REGIST-101) must be followed.

4.3 General Guidelines for Other Investigative Sites

N/A

4.4 Registration Process for Other Investigative Sites

N/A

5. TREATMENT PLAN

5.1 Treatment Regimen

Participants who are eligible to participate in the study will be stratified by age (≥ 18 and < 55 or ≥ 65) and then randomized to a blinded treatment group by ODQ. The investigator will contact the MGH Research Pharmacy for drug dispensation. The MGH Research Pharmacy will provide blinded aspirin capsules at 81 mg or placebo in blinded capsules and assign treatment. The assigned dosage will not change over the course of the study. The first dose of the study medication will be given to patients after the initial flexible sigmoidoscopy (start of randomization). Participants will be expected to take one capsule orally at the blinded dose, once daily, until the return for their final visit (minimum 8 weeks [or 56 days], maximum 12 weeks [or 84 days]). The final visit, 8-12 weeks from the baseline visit, will be scheduled during the baseline visit. On the rare occasion that a participant is unable to come in for their final visit between the 8-12 week window or their study visit is unavoidably moved due to availability of a provider and clinical room or technical core facilities, they will be offered an option to extend their treatment by up to an additional 4 weeks. If the participant agrees, the final visit will be rescheduled within 13-16 weeks from the baseline visit. Offering this option will allow the study team to retain participants and allow them to complete participation without inconveniencing them unnecessarily. The MGH Research Pharmacy will assign the participant one bottle of aspirin or placebo containing 84 blinded capsules (12 weeks, one capsule/day) and participants will return any unused capsules and the bottle to the study staff at their final visit. For participants with extended treatment windows, based on their randomization assignment, the MGH pharmacy will dispense an additional bottle containing a 4-week supply of the study drug to the study staff. The study team will ship the additional medication before the original supply runs out to minimize disruption in treatment and participants will return both bottles on the final visit. The bottle will be labeled with their participant ID. The participant ID number must be recorded on the drug accountability form held by the Research Pharmacy and in the participants case report form (CRF). The MGH Research Pharmacy will maintain a dispensation form for the study for cross-validation of the randomization at the end of the study. These numbers are unique to each participant and must not be re-assigned. Remaining capsules will be counted as a measure of compliance, the number recorded and then the remaining capsules will be immediately destroyed. Weekly calls will be used to monitor adherence and adverse events. These weekly interactions will follow the attached call script with verbal responses will be recorded directly by study staff in our REDCap database. NOTE: These direct entries will serve as the source documentation for these interactions, unless other source documentation is provided.

Patients who initiate an NSAID or aspirin during the study will be withdrawn and an exit visit performed. Reported adverse events and potential risks are described in Section 7. No investigational or commercial agents or therapies other than those described below may be administered. The key to the unblinded treatment codes will be maintained by ODQ. All personnel involved with the clinical conduct of the study will remain blinded until all participants have completed the intervention period and all biospecimens have been collected (post-treatment/final visit).

The study will utilize the InForm EDC database to capture all data related to eligibility, enrollment demographics, protocol compliance, and potential toxicities or adverse events to facilitate review of these features by the IRB. Any additional study-related data will be maintained digitally in the REDCap-based study database. REDCap will be used to store additional data from the study including additional endoscopy/pathology data, medical history information, sample collection metadata related to future analyses, that is not otherwise captured in the InForm database or related to performance of the study. Both databases will remain unfrozen until the last participant is taken “off-study” and is no longer in long-term follow-up.

As these participants are healthy subjects, many interactions with participants while on study occur virtually (i.e. not in a clinical encounter) and thus source documentation within the electronic medical record may be limited. All data collected as a part of this study and entered directly into REDCap that is not otherwise captured in the medical record or other source documentation (i.e. handwritten notes, paper clinical research forms) will be considered source documentation, including notes entered directly into REDCap databases capturing phone interactions with participants (e.g. weekly compliance calls or initial participant self-reported changes to their health to be reported to and reviewed by the principal investigator) or notes related to sample collection or processing not captured elsewhere on paper (e.g. time of collection, issues with sample collection, notes related to processing). Any data captured for the first time on clinical research forms or coordinator notes not otherwise captured in REDCap or the electronic medical record, will be maintained in paper form and organized in participant entries in binders in the laboratory of the PI, this includes but is not limited to email or other documentation of phone conversations between study staff and the participants. The laboratory uses a Freezerworks based system to accession and log all samples stored for downstream analysis. This electronic register will provide the most up-to-date log of samples collected on the study and current status and location of all samples collected or used to date and will remain unlocked.

Neither the participant nor the study physician will know which of the two treatments (low dose of aspirin or placebo) the participant is receiving. Blinded capsules will be provided by the MGH Research Pharmacy to maintain blinding. The unblinded randomization schedule will not be disclosed to the investigator or any personnel involved in the conduct of the study before the final participant completes the intervention period except as described below. The study statistician will transmit an unblinded randomization schedule directly to the MGH Research Pharmacy prior to study activation.

The investigator or treating physician may unblind a participant’s treatment assignment only in the case of an emergency, when knowledge of the study treatment is essential for the appropriate clinical management or welfare of the participant. Whenever possible, the investigator must first discuss options with the Medical Safety Monitor before unblinding the participant’s treatment assignment. If this is impractical, the investigator must notify the study physician, as soon as possible, but without revealing the treatment assignment of the unblinded participant, unless that information is important for the safety of participants currently in the study. The date and reason for the unblinding must be recorded in the appropriate CRF. The data safety monitor may unblind the treatment assignment for any participant with a serious adverse event (SAE). If the SAE requires that an expedited regulatory report be sent to one or more regulatory agencies, a copy of

the report, identifying the participant's treatment assignment, may be sent to clinical investigators in accordance with local regulations.

If the blind is broken by the investigator, the participant will be permanently discontinued from the study and an Early Termination assessment will be completed. If unblinding is necessary, study staff should contact ODQ to request unblinding for a participant.

5.2 Pre-Treatment Criteria

N/A

5.3 Agent Administration

5.3.1 Aspirin

Blinded aspirin or placebo capsules should be taken orally once every 24 hours. The daily dose will contain 81 mg of aspirin. The capsules should not be crushed, chewed or dissolved. The dose should be taken with food and a full glass of water. If a daily dose is missed or vomited, the dose should be skipped and reported to study staff. A dose is considered missed if more than 24 hours has elapsed since the prior dose.

5.3.2 Other Agent(s)

N/A

5.3.3 Other Modality(ies) or Procedures

N/A

5.3.4 Investigational Imaging Agent Administration

N/A

5.4 General Concomitant Medication and Supportive Care Guidelines

During the study, participants may not consume any other non-steroidal anti-inflammatory drug (NSAID), anticoagulant (e.g. warfarin) or antiplatelet agent (e.g. clopidogrel). To assist participants with maintaining study compliance by informing patients which drugs are classified as NSAIDs, an informative index card ("NSAID List Reference") outlining generic and brand names of common NSAIDs will be given with the capsules at the baseline visit with instructions for the participants to call the study staff if there are any questions. In adults, aspirin is contraindicated for use in individuals with NSAID hypersensitivity. A case report form will capture the concurrent use of all other drugs, over-the-counter medications, or herbal supplements. A list of potential drug-drug interactions is listed in **Appendix B**.

5.5 Criteria for Taking a Participant Off Protocol Therapy

Duration of study drug administration will be at minimum 8 weeks and no more than 12 weeks from the initial visit. For participants that remain on treatment for up to an additional 4 weeks, duration of study drug administration will be extended to no more than 16 weeks from the initial visit. Duration of administration will equal the number of days between initial and final visit. Study drug administration will continue until the final visit or until one of the following criteria applies:

- Intercurrent illness that prevents further administration of study drug
- Unacceptable adverse event(s)
- Participant demonstrates an inability or unwillingness to comply with the oral medication regimen and/or compliance requirements
- Participant decides to withdraw from the protocol therapy
- General or specific changes in the participant's condition render the participant unacceptable for further treatment in the judgment of the treating investigator
- Participant self-administers any additional non-study aspirin or NSAID.
- Participant begins treatment with an oral anticoagulant agent

Participants will be removed from the protocol therapy when any of these criteria apply. The reason for removal from protocol therapy, and the date the participant was removed, must be documented in the case report form (CRF). Alternative care options will be discussed with the participant.

When a participant is removed from protocol therapy and/or is off of the study, the participant's status must be updated in OnCore in accordance with [REGIST-OP-1](#).

In the event of unusual or life-threatening complications, treating investigators must immediately notify the Overall PI, Andrew T. Chan at Partners pager 31100.

5.6 Duration of Follow Up

Participants will be monitored closely until they complete the study. Participants who have completed the study will be those that have returned for the final visit and returned all necessary study materials including unused capsules, pill bottle and questionnaires. Participants removed from protocol therapy for unacceptable adverse event(s) will be followed until resolution or stabilization of the adverse event. We may contact participants routinely by phone (1-2 times annually) for a maximum of 10 years to follow up on additional information including any continued aspirin use and results of any follow-up colonoscopies. Periodically, we may examine their LMR to determine diagnosis of any new digestive diseases or alterations in aspirin use. In the event of an adverse event (AE, see section 7), we will follow-up with the participant within

one month after the AE has resolved.

5.7 Criteria for Taking a Participant Off Study

Participants will be removed from study when any of the following criteria apply:

- Lost to follow-up
- Participant consumes a non-study aspirin or NSAID.
- Participant demonstrates an inability or unwillingness to comply with the oral medication regimen and/or compliance requirements
- Participant begins therapy with an oral anticoagulant or anti-platelet agent
- Withdrawal of consent for data submission
- Death

The reason for taking a participant off study, and the date the participant was removed, must be documented in the case report form (CRF). In addition, the study team will ensure the participant's status is updated in OnCore in accordance with [REGIST-OP-1](#).

DOSING DELAYS/DOSE MODIFICATIONS

There are no dosing delays or modifications.

ADVERSE EVENTS: LIST AND REPORTING REQUIREMENTS

Adverse event (AE) monitoring and reporting is a routine part of every clinical trial. The following list of reported and/or potential AEs (Section 7.1) and the characteristics of an observed AE (Section 7.2) will determine whether the event requires expedited reporting **in addition** to routine reporting.

7.1 Expected Toxicities

According to Micromedex, salicylic acid is widely distributed to all tissues and bodily fluids. Highest concentrations are observed in the plasma, liver, renal cortex, heart and lungs. Early signs of salicylic overdose (salicylism) include tinnitus (ringing of the ears) and occur at plasma concentrations of 200 ug/mL with plasma concentrations over 300 ug/mL being clearly toxic. Severe toxic effects are observed at 400 ug/mL. Death may be expected at a single lethal dose of 30 g. Daily doses of either 81 mg/day or 325 mg/day yield plasma concentrations much below these levels, with 325 mg/day peak serum concentrations not exceeding 150 ug/mL. Of note, aspirin at these doses among the target population with an identical intervention time resulted in no significant adverse events in the ASPIRED trial of 180 individuals, including 39 individuals age 65 or older.

7.1.1 Adverse Events List

7.1.1.1 Adverse Event List(s) for aspirin

As with all drugs which may affect hemostasis, bleeding is associated with aspirin. Hemorrhage

may occur at virtually any site. Risk is dependent on multiple variables including dosage, concurrent use of multiple agents that alter hemostasis, and patient susceptibility. Many adverse effects of aspirin are dose related, and are rare at low dosages. Other serious reactions are idiosyncratic, related to allergy or individual sensitivity. Accurate estimation of frequencies is not possible.

The following adverse events have been reported for aspirin as listed in Micromedex:

- Cardiovascular: Cardiac arrhythmia, edema, hypotension, tachycardia
- Central nervous system: Agitation, cerebral edema, coma, confusion, dizziness, fatigue, headache, hyperthermia, insomnia, lethargy, nervousness, Reye's syndrome
- Dermatologic: Skin Rash, urticaria
- Endocrine & metabolic: Acidosis, dehydration, hyperglycemia, hyperkalemia, hyponatremia (buffered forms), hypoglycemia (children)
- Gastrointestinal: Gastrointestinal ulcer (6% to 31%), duodenal ulcer, dyspepsia, epigastric distress, gastritis, gastrointestinal erosion, heartburn, nausea, stomach pain, vomiting
- Genitourinary: Postpartum hemorrhage, prolonged gestation, prolonged labor, proteinuria, stillborn infant
- Hematologic & oncologic: Anemia, blood coagulation disorder, disseminated intravascular coagulation, hemolytic anemia, hemorrhage, iron deficiency anemia, prolonged prothrombin time, thrombocytopenia
- Hepatic: Hepatitis (reversible), hepatotoxicity, increased serum transaminases
- Hypersensitivity: Anaphylaxis, angioedema
- Neuromuscular & skeletal: Acetabular bone destruction, rhabdomyolysis, weakness
- Otic: Hearing loss, tinnitus
- Renal: Increased blood urea nitrogen, increased serum creatinine, interstitial nephritis, renal failure (including cases caused by rhabdomyolysis), renal insufficiency, renal papillary necrosis
- Respiratory: Asthma, bronchospasm, dyspnea, hyperventilation, laryngeal edema, noncardiogenic pulmonary edema, respiratory alkalosis, tachypnea
- Miscellaneous: Low birth weight
- Postmarketing and/or case reports: Anorectal stenosis (suppository), atrial fibrillation (toxicity), cardiac conduction disturbance (toxicity), cerebral infarction (ischemic), cholestatic jaundice, colitis, colonic ulceration, coronary artery vasospasm, delirium, esophageal obstruction, esophagitis (with esophageal ulcer), hematoma (esophageal), oral mucosa ulcer (aspirin-containing chewing gum), periorbital edema, rhinosinusitis

7.2 Adverse Event Characteristics

- **CTCAE term (AE description) and grade:** The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 5.0 will be utilized for AE reporting. All appropriate treatment areas should have access to a copy of the CTCAE version 5.0. A copy of the CTCAE version 5.0 can be downloaded from the CTEP web site http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm.
- **For expedited reporting purposes only:**
 - AEs for the agent(s) that are listed above should be reported only if the adverse event varies in nature, intensity or frequency from the expected toxicity information which is provided.
 - Other AEs for the protocol that do not require expedited reporting are outlined in the next section (Expedited Adverse Event Reporting) under the sub-heading of Protocol-Specific Expedited Adverse Event Reporting Exclusions.
- **Attribution** of the AE:
 - Definite – The AE *is clearly related* to the study treatment.
 - Probable – The AE *is likely related* to the study treatment.
 - Possible – The AE *may be related* to the study treatment.
 - Unlikely – The AE *is doubtfully related* to the study treatment.
 - Unrelated – The AE *is clearly NOT related* to the study treatment.

7.3 Adverse Event Reporting

- 7.3.1 In the event of an unanticipated problem or life-threatening complications treating investigators must immediately notify the PI.
- 7.3.2 Investigators **must** report to the PI any adverse event (AE) that occurs after the initial dose of study treatment, during treatment, or within 30 days of the last dose of treatment on the local institutional SAE form.
- 7.3.3 Adverse Event Reporting Guidelines

All participating sites will report AEs to the Sponsor-Investigator per DF/HCC requirements, and the IRB of record for each site as applicable per IRB policies. The table below indicates which events must be reported to the DF/HCC Sponsor-Investigator.

Attribution	DF/HCC Reportable Adverse Events(AEs)				
	Gr. 2 & 3 AE Expected	Gr. 2 & 3 AE Unexpected	Gr. 4 AE Expected	Gr. 4 AE Unexpected	Gr. 5 AE Expected or Unexpected
Unrelated	Not required	Not required	5 calendar days [#]	5 calendar days	24 hours [*]

Unlikely					
Possible Probable Definite	Not required	5 calendar days	5 calendar days [#]	5 calendar days	24 hours*
# If listed in protocol as expected and not requiring expedited reporting, event does not need to be reported.					
* For participants enrolled and actively participating in the study or for AEs occurring within 30 days of the last intervention, events must be reported within <u>1 business day</u> of learning of the event.					

7.3.4 Protocol-Specific Adverse Event Reporting Exclusions

For this protocol only, the AEs/grades listed below do not require expedited reporting to the Overall PI or the DF/HCC IRB. However, they still must be recorded through the routine reporting mechanism (i.e. case report forms).

CTCAE SOC	Adverse Event	Grade	Hospitalization/ Prolongation of Hospitalization	Attribution	Comments
N/A					

7.4 Reporting to Hospital Risk Management

Participating investigators will report to their local Risk Management office any participant safety reports, sentinel events or unanticipated problems that require reporting per institutional policy.

7.5 Routine Adverse Event Reporting

All Adverse Events **must** be reported in routine study data submissions to the PI on the toxicity case report forms. **AEs reported through expedited processes (e.g., reported to the IRB, FDA, etc.) must also be reported in routine study data submissions.**

PHARMACEUTICAL INFORMATION

A list of the adverse events and potential risks associated with the investigational agent administered in this study can be found in Section 7.1.

8.1 Aspirin

8.1.1 Description

The systematic (IUPAC) name for aspirin is 2-acetoxybenzoic acid. It is also known as acetylsalicylic acid (ASA) and is a salicylate drug. It is a part of the group of medications called nonsteroidal anti-inflammatory drugs (NSAIDs). The molecular formula is C₉H₈O₄ and the molecular weight is 180.16.

Orally administered immediate release aspirin is well and completely absorbed from the gastrointestinal tract. It is rapidly metabolized in the plasma to salicylic acid and then primarily conjugated in the liver for form salicyluric acid, a phenolic glucuronide, an acyl glucuronide. Elimination follows zero order pharmacokinetics. Salicylic acid and metabolite concentrations excreted in the urine are 10% salicylic acid, 75% salicyluric acid, 10% phenolic glucuronide, and 5% acyl glucuronide. Peak plasma concentrations occur within 1-2 hours of dosing. Half-life for elimination of the parent drug is approximately 20 minutes. For salicylates the half-life is dose-dependent with a standard-dose (300-600 mg) half-life of approximately 3 hours and high-dose (1 g) half-life of approximately 6 hours.

In adults, aspirin is contraindicated for use with ketorolac or ketorolac tromethamine (enhanced gastrointestinal adverse effects such as peptic ulcers, gastrointestinal bleeding, and/or perforation). Aspirin and NSAID use with anti-coagulants (i.e. warfarin or heparin) or selective serotonin reuptake inhibitors (SSRIs; i.e. zimeldine, fluoxetine, paroxetine, nefazodone, citalopram, clovoxamine, escitalopram, flesinoxan, femoxetine) may lead to an increased risk of bleeding. Aspirin use is cautioned in those individuals that have bleeding disorders, consume 3 or more alcoholic drinks per day, are pregnant, or are experiencing gastrointestinal symptoms (peptic ulcer disease), renal failure, or severe hepatic insufficiency.

8.1.2 Form

Aspirin is an odorless, white, needle-like crystalline or powdery substance. Generic aspirin is provided as an oral tablet. For the study, these tablets will be crushed by the MGH research pharmacy into a powder. The powder at the appropriate dose is then placed in an appropriate size gel capsule with lactose filler. In the case of placebo, an identical size capsule filled only with lactose will be used. The capsules will be packaged in a pill bottle containing 84 capsules (12-week daily supply). Participants with extended treatment windows, will receive an additional pill bottle containing 28 capsules (4-week daily supply).

8.1.3 Storage and Stability

Aspirin can be stored at room temperature; protected from moisture. Hydrolysis of aspirin occurs upon exposure to water or moist air, resulting in salicylate and acetate, which possess a vinegar-like odor. Do not use if a strong odor is present.

8.1.4 Compatibility

N/A

8.1.5 Handling

N/A

8.1.6 Availability

Aspirin is commercially available from various manufacturers and will be supplied by the MGH Research Pharmacy free of charge to the participants.

8.1.7 Preparation

For the study, these tablets will be crushed by the MGH research pharmacy into a powder. The powder at the appropriate dose is then placed in an appropriate size gel capsule with lactose filler. In the case of placebo, an identical size capsule filled only with lactose will be used.

8.1.8 Administration

Participants will orally administer one study capsule per day. Each participant will be randomized into a treatment arm (aspirin or placebo)

8.1.9 Ordering

Aspirin will be provided by the MGH Research Pharmacy and paid for with NCI research funding.

8.1.10 Accountability

The MGH Research Pharmacy will maintain careful record of the inventory and disposition of study aspirin using their protocols for drug accountability.

8.1.11 Destruction and Return

Participants will return the pill bottle with any remaining capsules at their final visit to measure daily compliance. Any remaining capsules will be destroyed immediately

BIOMARKER, CORRELATIVE, AND SPECIAL STUDIES

9.1 Laboratory Correlative Studies

9.1.1 Background

As this study is an extension of the original ASPIRED trial, we have designed the correlative

studies to mirror those proposed in ASPIRED to allow for stratification according to participant age. We hypothesize that aspirin, by reducing risk of multiple cancers and cardiovascular disease, has a favorable risk-benefit profile for most individuals and influences several neoplastic pathways which can be exploited as biomarkers of chemopreventive efficacy, however these mechanisms may be differentially effective in the contextual setting of advanced age. The following study will provide age-dependent effects of aspirin treatment on specific biomarkers of colorectal carcinogenesis. In doing so, we aim to provide causality and identify biomarkers for aspirin's overall risk-benefit established by other studies. We and others have put forth considerable effort to determine measurable biomarkers implicated in colorectal carcinogenesis. A discussion of these biomarkers and their significance is provided below:

9.1.1.1 Single cell RNA-sequencing

Although the mechanisms of age-related inflammation (inflammaging) are complex (i.e. Myeloid skewing⁴⁰), prostaglandins and specifically PGE₂ is particularly relevant as a driver of inflammaging in the intestine as 1) one of the major pro-inflammatory factors that is produced constitutively during homeostasis and is elevated during injury/repair;^{41,42} 2) signaling through Ptger4 (EP4 receptor) in the intestinal epithelium promotes tumorigenesis and affects wound associated epithelial (WAE) cell differentiation during repair;^{20,21,43,44} and 3) is a clear direct target of low-dose aspirin.^{45,46} the regulated expression of PGE₂ by control of the rate limiting enzyme Ptg2 occurs in subset of intestinal fibroblasts, though most other cell types can be induced to produce it in settings of chronic damage. An important molecular target of PGE₂ is the Hippo/Yap pathway which in turn drives a fetal-like conversion of cISCs that occurs during wound repair.^{47,48} in young mice with mucosal injury, this process is transient in ISCs. During uncomplicated repair in these young mice, the repertoire of cISCs changes from Lgr5+ to Yap-activated to Hopx+ enriched cells.⁴⁹ after repair, cISCs revert to the adult repertoire where Lgr5+ cISCs dominate.⁴⁹ thus, there is a compelling scientific premise that PGE₂ signaling, via the Yap pathway, in intestinal epithelial cells plays a critical role in directing effects on cISCs and alterations associated with inflammaging, which in turn impacts the effects of age and low-dose aspirin on cISCs. Using scRNA-sequencing we will interrogate colon epithelial cell population changes following low-dose aspirin intervention in younger (≥ 18 and < 55) adults compared to older adults (≥ 65), and in relation to both age groups receiving placebo intervention. We will estimate ISC relative gene expression, function and number (proportion of ISC subtypes relative to differentiated epithelial cell subtypes) where we will focus on epithelial subtypes (EPCAM, KRT8, KRT18, etc.) Enriched for a priori determined markers of ISC markers (LGR5, HOPX, OLFM4, AXIN2, or EPHB2 positive cells). We will also identify differential gene expression within the scRNA-seq data associated with low-dose aspirin intervention and/or age.

9.1.1.2 Urinary PGE-M

We have previously shown that aspirin's influence on colorectal cancer is mediated at least in part through inhibition of COX-2,^{26,27} which catalyzes production of prostaglandin E2 (PGE2), leading to induction of proliferation, migration, and invasiveness, promotion of angiogenesis, resistance to apoptosis, and modulation of cellular and humoral immunity. We have estimated overall prostaglandin tone by accurately measuring its major metabolite, PGE-M (11alpha-hydroxy,9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid), in urine.⁵⁰ This assay is widely accepted as the

“best way to quantify systemic PGE2 production in vivo.”⁵¹ Prediagnostic levels of PGE-M are associated with risk of colorectal cancer and adenoma.⁵²⁻⁵⁴ PGE-M has also been associated with gastric and breast cancer.⁵⁵⁻⁵⁷ In the Nurses’ Health Study (NHS), women in the highest quartile PGE-M had a multivariate odds ratio (OR) of 1.66 (CI, 1.04-2.66) for high-risk adenoma compared to women in the lowest.⁵⁸ Moreover, aspirin/NSAIDs was associated with a significant reduction in adenoma risk among women with high (OR, 0.61; CI, 0.43-0.87) but not low PGE-M (OR 1.05; CI, 0.50-2.19). These results support the potential for PGE-M to define subsets of the population who may obtain differential chemopreventive benefit from aspirin. Moreover, in a study of 10 individuals, two doses of 650 mg of aspirin 14 hours apart reduced PGE-M by 44%.⁵⁹ We recently published the results of the ASPIRED randomized clinical trial that utilized urinary PGE-M as the primary outcome following daily intervention of aspirin at 81 mg/day or 325 mg /day, versus placebo. We demonstrated that aspirin significantly reduced urinary PGE-M concentrations compared to placebo by 15% ($P = 0.018$) and 28% ($P < 0.0001$), respectively.⁶⁰ This reduction is also clinically significant as it corresponds to a predicted 10% reduction in adenoma recurrence in half of the aspirin-treated individuals. Thus, PGE-M may also serve as a biomarker to assess the effectiveness of aspirin in reducing risk of adenoma. Our experience in measuring PGE-M and successful execution of multiple randomized clinical trials, including the aforementioned ASPIRED in addition to a study performed to determine the dose-response relation between oral vitamin D and plasma 25(OH)D supports our ability to pursue a similar randomized clinical trial of aspirin.^{61,62}

9.1.1.3 Plasma GDF-15

The circulating inflammatory cytokine macrophage inhibitory cytokine-1 (MIC-1/GDF-15), also known as growth differentiation factor 15 (GDF-15),⁶³ and placental bone morphogenetic protein (PLAB),⁶⁴ prostate derived factor (PDF),⁶⁵ may be an important mediator in the systemic inflammatory response.⁶⁶ Elevated levels of GDF-15 have been associated with risk of atherosclerosis and arthritis.^{67,68} GDF-15 has also been linked to cancers, including those of the prostate, thyroid, pancreas, and colon,^{69,70} and recurrent adenoma.⁷¹ Experimental evidence suggests that GDF-15, as a member of the human transforming growth factor- β (TGF β 1) superfamily, may play a specific role in carcinogenesis.⁷²⁻⁷⁴ We recently reported that the multivariate relative risk (RR) for colorectal cancer was 1.93 (CI, 1.27–2.94) comparing extreme quintiles of GDF-15 ($p_{trend}=0.004$) and among individuals with high GDF-15, aspirin/NSAIDs are associated with a lower risk of COX-2 positive (multivariate RR=0.60; CI, 0.41-0.88) but not COX-2 negative colorectal cancer (multivariate RR=1.21; CI, 0.71–2.07).⁷⁵ Taken together, these results support the potential for plasma GDF-15 to serve as a biomarker to define subsets of the population who may obtain differential chemopreventive benefit from aspirin. Thus, randomized studies are needed to determine if aspirin (81 mg/d or 325 mg/d) specifically reduces levels of GDF-15. Our experience in measuring GDF-15 and successful execution of a randomized clinical trial of oral vitamin D that assessed the effect of treatment on inflammatory markers supports our ability to pursue a similar randomized clinical trial of aspirin.⁷⁶

9.1.1.4 ChIP-seq in colonic epithelium, Wnt/ β -catenin

Activation of the *Wnt*/ β -catenin signaling pathway plays a critical role in colon tumorigenesis.⁷⁷⁻⁷⁹ β -catenin is a key effector in *Wnt*-signaling since T cell factor (TCF) family members transcribe

their target genes only when bound to β -catenin. Several studies have shown that aspirin may directly suppress *Wnt* signaling through COX-independent pathways.⁸⁰⁻⁸³ In addition, compelling evidence supports a critical interaction between prostaglandin pathways and *Wnt* signaling such that aspirin may inhibit *Wnt* signaling through suppression of COX-mediated synthesis of PGE₂.⁸⁴⁻⁸⁹ Although these experimental data are compelling, human studies in support of an effect of aspirin mediated through *Wnt* are limited. In a case-control study of 76 patients, aspirin or ibuprofen use was associated with decreased nuclear staining of β -catenin and the *Wnt* target gene cyclin D1 in sporadic adenoma.⁸² Consistent with these findings, we found that the benefit of regular aspirin use on colorectal cancer risk was most pronounced in individuals with T alleles of rs6983267,⁹⁰ a 8q24 colorectal cancer susceptibility SNP⁹¹⁻⁹³ that we have shown is associated with impaired β -catenin binding to TCF4 adjacent to *MYC*.⁹⁴ In a murine model, rs6983267 influences *MYC* expression and intestinal tumorigenesis.⁹⁵ We corroborated these results by ChIP-seq showing that aspirin influenced binding of TCF4 in colorectal cancer cell lines heterozygous for rs6983267.⁹⁰ The next important step will be to determine if, *in vivo*, aspirin results in differential binding of TCF4 in regulatory sites adjacent to key cancer-associated genes such as 8q24 within colonic epithelium.

9.1.1.5 Gene expression in colonic epithelium - 15-PGDH and Wnt signaling

The synthesis of tumor-promoting prostaglandins is regulated not only by COX-2, but also by the PGE₂-catabolizing enzyme hydroxyprostaglandin dehydrogenase 15-(NAD) (15-PGDH), which acts as COX-2's physiological antagonist (**Fig 1**).^{96,97} This function has led to characterization of *15-PGDH* as a tumor suppressor in several human cancers, including colorectal, gastric, breast, prostate and lung.⁹⁸⁻¹⁰⁸ 15-PGDH is highly expressed in normal colon and is ubiquitously downregulated in colorectal cancer.^{99,102,108-111} In a mouse model, knock out of *15-PGDH* (*HGPD*) increased colonic PGE₂, markedly increased colon tumor numbers, and conferred resistance to the anti-tumor effect of the COX-2 inhibitor celecoxib. In a pilot analysis of the APC Trial, low *15-PGDH* expression in normal colon mucosa was associated with lack of response of response to celecoxib for the prevention of recurrent adenomas.¹⁰⁹ We recently extended this finding to aspirin in the Nurse's Health Study and the Health Professionals Follow-up Study.¹¹² Using a validated RT-qPCR assay to quantify *15-PGDH* mRNA expression in normal colonic mucosa,¹¹³ we found that the multivariate hazards ratio associated with aspirin use was 0.49 (CI, 0.34-0.71) among those with high *15-PGDH* within normal colon but 0.90 (CI, 0.63-1.27) among subjects with low expression of *15-PGDH* ($p_{\text{heterogeneity}}=0.02$). These results suggest that the anticancer activity of aspirin in colonic mucosa is dependent on high *15-PGDH* expression, with low levels of *15-PGDH* expression conferring resistance to aspirin's tumor preventive effects. Despite these findings, however, it is unclear if aspirin directly alters *15-PGDH* levels. A prior study showed that β -catenin/TCF4 binds the *15-PGDH* promoter to downregulate *15-PGDH* expression.¹¹⁴ This would suggest that if aspirin treatment functions through inhibition of β -catenin/TCF4 binding, *15-PGDH* expression should also be upregulated, which would lower PGE₂ levels, potentially serving as negative feedback by further weakening β -catenin function. However, in a pilot study of 45 patients, we found that aspirin (325 mg/d) was associated with a 10% increase in colonic *15-PGDH* expression but the sample size was too small to determine statistical significance ($p=.12$).¹¹³ Thus, a larger randomized treatment study is needed to determine if aspirin (81 mg/d or 325 mg/d) specifically inhibits gene expression in colonic cells associated with *Wnt* signaling pathway (*CTNNB1*, *AXIN-2* and *MYC*) and *15-PGDH*.

9.1.1.6 Aspirin, the oral and gut microbiome, and colorectal cancer

Colorectal cancer incidence rates are rapidly rising in less-developed nations as they adopt features of a Western lifestyle such as diet¹¹⁵ and altered microbial^{116,117}. This rise in colorectal cancer incidence¹¹⁸ implicates non-genetic factors such as diet¹¹⁹⁻¹²², gene-environment interactions¹²³, and alterations in the microbiome and associated immune responses¹²⁴⁻¹²⁷. Unlike human genetic risk factors, microbial contributors to colorectal cancer risk and progression are modifiable, making their identification for risk assessment and mitigation particularly critical. There is growing recognition of the association between the oral and gut microbiota and colorectal carcinogenesis. The oral and gut microbiome plays critical roles in epithelial cell proliferation and differentiation, intestinal immunity, nutrient processing and metabolite production, and resistance to infection by pathogenic organisms^{128,129}. Although there have been few detailed studies, specific microbes may be associated with colorectal carcinogenesis¹³⁰⁻¹³⁵. Recent publications have detailed microbial alterations and potential biomarker species involved in colorectal cancer^{136,137}. Thus, it is critical to investigate microbial changes associated with chemopreventive therapeutics. To date, no studies have determined the biomolecular mechanisms by which oral or gut microbial activity may be altered or respond to aspirin treatment implicated in colorectal cancer risk and progression.

9.1.2 Study Design

Using our gastroenterology practice population, we will implement a prospective randomized clinical trial to measure dose-dependent effects of aspirin on urine, saliva, plasma, stool, and tissue biomarkers of colorectal carcinogenesis and aspirin chemopreventive efficacy. At MGH, we will target 160 individuals over a three-year period. Eligible patients will have had a previous colonoscopy within the last 9 months at MGH and had at least one adenoma removed during the previous procedure. Eligible patients must meet all eligibility requirements and none of the exclusion criteria as outlined in Section 3: Eligibility criteria.

9.1.2.1 Prior to the initial visit

No bowel preparation or anesthesia will be necessary for the procedure since the sigmoidoscope will only be advanced to the distal sigmoid colon.

9.1.2.2 Initial (baseline) Visit

At the initial visit, the study physician will obtain written, informed consent for the study as well as a standard clinical consent for a flexible sigmoidoscopy. While waiting for confirmation of registration from Oncore/ODQ, the participants will complete a brief lifestyle and dietary questionnaire with a study coordinator. Following confirmation of registration, patients will undergo measurements of height, weight, waist and hip circumference and provide a blood (40 mL in vacutainers), saliva and urine specimen. If requested, patients will be able to provide urine and saliva samples while waiting for confirmation for the sake of time and participant comfort. In the event that the patient is not able to be registered by ODQ, the questionnaire and any urine, or saliva specimens will be destroyed immediately. A study gastroenterologist will then perform a flexible

sigmoidoscopy, advancing to the level of the distal sigmoid colon. No more than a total of 24 mucosal biopsies will be taken from the rectum and sigmoid and immediately placed in collection tubes. In the MGH GI Unit, we routinely perform endoscopic biopsies regardless of concurrent aspirin use, a practice consistent with recommended guidelines.¹³⁸ A study of the safety of multiple endoscopic biopsies in research subjects from a National Institutes of Health series found that performing large numbers of endoscopic biopsies (mean number = 38.2 ± 15.6 biopsies per procedure) are “well tolerated and appears to have no more than minimal risk without appreciably increasing the risk of otherwise routine endoscopy.”¹³⁹ Furthermore, there are no statistically significant association between risk of complications and the number of biopsies, type of procedure (flexible sigmoidoscopy vs. colonoscopy), colonic location of biopsy, operator, polypectomy, or, importantly, the use of non-steroidal anti-inflammatory drugs¹³⁹. The number of biopsies is also consistent with our existing study protocols, including the preceding ASPIRED trial, and has never been associated with any adverse events (See: “Endoscopy Protocol: Tissue Specific Immunity Against HIV-1”; PI: Kwon, Ragon Institute). We have estimated that 24 biopsies will be necessary to complete the proposed analyses. These biopsies will support derivation of the primary outcome scRNAseq data and all secondary outcome studies. The ChIP-seq experiments proposed require approximately 500,000 epithelial cells for each sample. We estimate that from a single pinch biopsy we will recover approximately 50,000 epithelial cells following cell sorting. The additional biopsies will be required for RNAseq/RT-PCR experiments (i.e. 15-PGDH) as well as for validation experiments based on the results of genomic and metagenomic analyses using targeted sequencing approaches. Due to the small number of cells obtained from pinch biopsies and the input requirements for these assays, we will use tissue culture techniques (i.e. intestinal organoids) to expand cell populations. This will allow us to perform these comprehensive analyses without additional burden to the participants (i.e. increasing tissue yields by using larger or more biopsies). During flexible sigmoidoscopy, stool will be aspirated through the endoscope or retrieved using a Roth net and immediately frozen or stored in a solution to preserve nucleic acids.

Following the visit, questionnaire data will be transferred to the Partners secure REDCap electronic database system.

Participants will be provided with \$200 (US) compensation and free parking for up to 4 hours for this initial visit. The final visit will be scheduled with the patient at his or her convenience.

9.1.2.3 Final Visit

Participants will return for a second and final visit between 8 and 12 weeks from their initial visit. Participants that are unable to come in within this time frame will be offered an option to remain on treatment for up to an additional 4 weeks and reschedule their final visit between 13 and 16 weeks. An abbreviated diet and lifestyle questionnaire will be administered to update information from the baseline questionnaire. Participants will also provide blood, saliva and urine samples and undergo a second flexible sigmoidoscopy procedure with mucosal biopsies. A bowel preparation will not be necessary for the follow-up flexible sigmoidoscopy. Up to 24 mucosal biopsies will be taken, as described for the baseline visit. A stool specimen will also be collected, as previously described. An abbreviated diet and lifestyle questionnaire will be administered to update information from the baseline questionnaire.

Participants will be provided with an additional \$200 (US) compensation and free parking for up to 4 hours for this final visit. Total compensation will equal \$400 (US) for successful completion of the study.

In the unlikely event that clinical space issues or technical core availability leads to study staff having to initiate rescheduling a participant in the extended 12-16 week time frame, participants will be offered an additional \$50 (US) in remuneration at the time of their final visit to account for the added time commitment incurred by the participants. However, if individuals are rescheduled in the 13-16 week time frame due to a participant initiated rescheduling request, no additional remuneration will be offered as the extended time on study is at the request and for the convenience of the participant.

9.1.3 Processing of Biospecimens

Immediately following each flexible sigmoidoscopy, urine specimens will be aliquoted into 1.2 mL aliquots and blood specimens will be processed into whole blood, plasma, and buffy coat aliquots. Stool specimens will be stored in cryovials and immediately frozen. Saliva will be collected in specialized saliva collection tubes, aliquoted, and immediately frozen. Mucosal biopsies will be processed for subsequent endpoints as described below, but briefly, they will be dissociated into single cells for scRNAseq and downstream cell sorting, used for generation of intestinal organoid cell lines, cryopreserved for downstream -omics analyses, or fixed in formalin and paraffin-embedded. Any excess colon tissue will be banked for future studies, including validation of mechanistic insights generated by these translational outcomes. All aliquots of stool, saliva, urine, plasma, buffy coat, and epithelial cells will be frozen at -80°C until analysis. We may access FFPE blocks of polyps/adenomas removed during the participant's qualifying colonoscopy with accompanying pathology reports to correlate our findings with tissue-specific markers in the original adenoma. If during long-term follow-up, participants are diagnosed with recurrent adenomas or colorectal cancer, we may similarly request these materials to correlate baseline findings with these recurrent lesions.

9.1.4 Analysis of scRNA-sequencing (primary endpoint)

9.1.4.1 Collection of Specimens

Mucosal biopsy samples from normal sigmoid colon will be collected at initial and final visit.

9.1.4.2 Handling of Specimens

Tissues will be dissociated into single cells with DNAase and collagenase, filtered, and viability assessed with trypan blue. Protocols based on the Broad Human Cell Atlas Project will be followed for the 10x Genomics Chromium or Honeycomb seqwell system or similar platform according to availability, including presorting and aggregate cell identification using flow cytometry/cell sorting. Libraries will be prepared using Nextera XT DNA Library Preparation Kits (Illumina) by the Harvard Single Cell Core or within Dr. Yilmaz's laboratory and associated

core facilities within the Koch Institute according to their standard protocols and according to availability.¹⁴⁰ At least 30,000 cells will be pooled, multiplexed, and will be run on a single NovaSeq run or capable next generation sequencing platform. Reads will be mapped to the human genome (hg19) and analyzed using standard QC and analytic pipelines. Samples will be processed by members of the Chan/Drew Laboratory listed on the study protocol.

9.1.4.3 Site Performing Study

MGH is performing the study. Members of the Chan/Drew laboratory will interface with the specific internal core facilities available to the study team to perform single cell data generation and analysis.

9.1.5 Analysis of Urinary PGE-M

We will use mass spectroscopy to measure PGE-M (in single batches of pre- and post-treatment urine in the Eicasonoid Core laboratory at Vanderbilt University.^{50,58}

9.1.5.1 Collection of Specimens

Urine samples will be collected at both the initial and final visit.

9.1.5.2 Handling of Specimens

Samples will be placed in a refrigerator within 2 hours of collection and transferred to -80°C within 4 days of collection. Urine will be split into 1.2 mL aliquots in eppendorf tubes prior to freezing. Samples will be stored at -80°C until analysis.

9.1.5.3 Shipping of Specimens

A 1.2 mL aliquot of each urine sample will be shipped on dry ice overnight at the end of the study to the Eicosanoid Core Laboratory at Vanderbilt University at the following address:

Attn: Ginger L. Milne, Ph.D.
Vanderbilt University Medical Center
561 Preston Research Building
Nashville, TN 37232-6602 USA

9.1.5.4 Site Performing Study

MGH is performing the study. Assays on the urine samples will be carried out by the Eicasonoid Core Laboratory at Vanderbilt University

9.1.6 Analysis of blood derivatives

For the GDF-15 endpoint, we will use an ELISA to measure GDF-15 (CV=8%) in pre- and post-

treatment plasma in the core clinical laboratory at Boston Children's Hospital.^{58,75} Alternatively, GDF-15 is measurable using a broad spectrum proteomics platform (e.g. O-link) and may be used in place of this targeted assay. Using methods described in Nan et al, we will genotype all trial participants for rs6983267 using DNA derived from buffy coat. Additional downstream analyses using blood samples, including untargeted metabolomics, proteomics, or other assays, including cell-free DNA or similar assays showing promise for early detection of cancer, may be pursued that will support additional translational research approaches.

9.1.6.1 Collection of Specimens

40 mL of whole blood will be collected at both the initial and final visit in blood vial vacutainers.

9.1.6.2 Handling of Specimens

Upon the day of collection, an aliquot of whole blood will be centrifuged into plasma and buffy coat. Plasma samples will be aliquoted into 1.2 mL aliquots and frozen. Buffy coat will be aliquoted into 500 uL aliquots and frozen. Blood, plasma and buffy coat samples will be stored at -80°C until analysis.

9.1.6.3 Shipping of Specimens

An aliquot of sample will be shipped on dry ice overnight at the end of the study to the performing laboratory or sequencing core for completion of the assay. Material transfer agreements will be in place or covered by service contracts. Any excess/unused specimen will be returned to the Chan laboratory.

9.1.7 ChIP-seq Analysis of Colonic Epithelium

Dr. Matthew Freedman at the Dana Farber Cancer Institute will employ a ChIP-seq protocol for TCF4 colonic epithelial cells^{90,141}.

9.1.7.1 Collection of Specimens

Mucosal biopsy samples will be collected at both the initial and final visit at MGH and immediately frozen.

9.1.7.2 Handling of Specimens

Mucosal biopsy specimens will be stored frozen and sent at the end of the study to the Freedman laboratory at DFCI.

9.1.7.3 Shipping of Specimens

Mucosal biopsy specimens will be delivered by courier on dry ice to the Freedman laboratory at DFCI.

9.1.7.4 Site Performing Study

MGH is performing the study. Dr. Matthew Freedman's laboratory at DFCI will perform ChIP-seq analyses through the DFCI core laboratory.

9.1.8 RNA-seq Analysis of Colonic Epithelium

The Chan lab or local core will process bulk cell lysates from aliquots of the epithelial cells for RNA extraction using the RNAeasy micro kit. Total RNA will be converted into a cDNA library using Illumina TruSeq RNA sample preparation kit followed by paired end 50 cycles sequencing on a Illumina HiSeq 2500 platform or similar platform.¹⁴²

9.1.8.1 Collection of Specimens

Mucosal biopsy specimens will be stored frozen and sent at the end of the study for sequencing.

9.1.8.2 Handling of Specimens

RNA/cDNA will be provided to a sequencing core for RNAseq.

9.1.8.3 Shipping of Specimens

Mucosal biopsy specimens or extracted nucleic acids or sequencing libraries will be sent on dry ice to the appropriate sequencing core. Material transfer agreements will be in place or covered by service contracts.

9.1.8.4 Site Performing Study

MGH is performing the study.

9.1.9 Microbiome analysis

We will perform a "multi'omics" analysis (16S sequencing, metagenomics, transcriptomics, see Section 13) of microbial DNA and RNA on pre- and post-treatment stool and saliva samples to examine the biomolecular mechanisms by which oral and gut microbial activity may be altered or respond to aspirin treatment. Stool and saliva samples from all cohorts will be sent, coded, to the Broad Institute for processing and analysis.

9.1.9.1 Collection of Specimens

Stool will be collected using a Roth net or aspirated through the endoscope during flexible sigmoidoscopy at both the initial and final visit. Participants will be asked to spit into a saliva collection tube at both the initial and final visit.

9.1.9.2 Handling of Specimens

An aliquot (~200 mg) of stool will be put in tubes provided by the Broad Institute. The remaining stool will be frozen in a 15 mL conical tube at -80°C. Saliva samples will be collected and stored frozen in collection vials until analysis. The entire saliva sample and the stool aliquot will be sent to the Broad Institute for processing and analysis.

9.1.9.3 Shipping of Specimens

Stool/saliva specimens will be shipped on dry ice overnight at the end of the study to:

Broad Institute
Attn: BSP platform
301 Binney Street Lab 5076
Cambridge, MA 02142
617-714-8952

9.1.9.4 Site Performing Study

MGH is performing the study. The omics assays will be performed by the Broad Institute

9.1.10 *In vitro* assessment of aspirin treatment on epithelial derived colon organoids.

We will perform parallel *in vitro* studies of aspirin treatment on organoid tissue cultures derived from untreated (baseline) and treated (follow-up) pinch biopsy specimens in the Chan, Drew, Yilmaz and Stappenbeck laboratories. By doing so, we aim to validate other *in vitro* models (CRC cell lines) of aspirin chemoprevention and/or innovate beyond these established methods. Tissue culture experiments will include exposure to aspirin and its derivatives, as well as other host environmental factors (e.g. diet-derived factors, alcohol, other medications/supplements, etc.). Genomic, transcriptomic, and proteomic assays will be used to characterize these lines and assess the effects of these exposures on cellular signaling processes. Additionally, genome-editing tools (i.e. CRISPR/Cas9) may be used to manipulate the genomic background of organoid lines to recapitulate genetic environments associated with sensitivity or resistance to aspirin treatment determined by the previously described assays.

9.1.10.1 Collection of Specimens

Pinch biopsies will be collected during the flexible sigmoidoscopy in collection media and immediately dissociated into single crypts. Single crypts will be plated and grown into organoid cultures using methods developed by Miyoshi & Stappenback (*Nature Methods*, 2013). Biopsies may also be preserved in a freezing medium which will allow derivation of organoids in the future, rather than in real time.

9.1.10.2 Handling of Specimens

Up to 4 pinch biopsies will be collected during the flexible sigmoidoscopy in collection media and immediately dissociated into single crypts. Single crypts will be plated and grown into organoid cultures using methods developed by Miyoshi & Stappenback (*Nature Methods*, 2013). After serial passaging and expansion, aliquots of established organoid cultures will be cryogenically frozen in

liquid nitrogen and stored at MGH until needed for *in vitro* experiments. Some of these lines may be sent to established collaborators, in addition to Drs. Yilmaz (MGH/MIT) and Stappenbeck (Cleveland Clinic). To date, we have identified the following collaborators: Dr. Graham Casey (University of Virginia). Any collaborator receiving stocks of cells will be sent organoid lines only after a Materials Transfer Agreement is processed. To preserve our biobank of organoid cell lines and maintain quality control, all collaborators will be instructed to return two cryopreserved vials of organoids after initial expansion at their institution.

9.1.10.3 Shipping of Specimens to Collaborators

Cryovials of organoid cultures may be shipped to the following collaborators:

Casey Lab – University of Virginia
c/o Sarah Plummer
101 Hospital Drive, Davis Wing, RM3275
Charlottesville, VA 22908
434-282-7657

9.1.10.4 Site Performing Study

MGH is performing the study. Samples may be sent to Cleveland Clinic and the University of Virginia

9.2 Laboratory Correlative Studies

N/A

9.3 Special Studies

N/A

STUDY CALENDAR

	Initial Visit	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8	Wk 9	Wk 10	Wk 11	Wk 12	Final Visit ^{b,c}	EDC Timepoints
Aspirin or Placebo Daily		a	a	a	a	a	a	a	a	Taken daily until final visit				Return unused aspirin	
Informed consent	X														
Demographics	X													X	Initial & Final Visit

	Initial Visit	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8	Wk 9	Wk 10	Wk 11	Wk 12	Final Visit ^b	EDC Timepoints
Lifestyle and Diet Questionnaire	X													X	Initial & Final Visit
Height	X													X	Initial Visit
Weight	X													X	Initial Visit
Waist & Hip Circumference	X													X	Initial Visit
Flexible Sigmoidoscopy	X													X	Initial & Final Visit
Tissue Biopsy Specimens Collected	X													X	Initial & Final Visit
Blood Samples	X													X	Initial & Final Visit
Saliva Samples	X													X	Initial & Final Visit
Stool Samples	X													X	Initial & Final Visit
Urine Samples	X													X	Initial & Final Visit
Drug Compliance Calls ^d		X	X	X	X	X	X	X	X	X	X	X	X		Weekly
Adverse event evaluation ^d		X	X	X	X	X	X	X	X	X	X	X	X	X	Weekly

a: Aspirin or Placebo: Arm assigned, post-randomization. *See drug administration.*

b: Final visit will be scheduled during the initial visit and will occur a minimum of 8 weeks and maximum of 12 weeks after the initial visit.

c: In the event that the final visit is rescheduled in the extension 13-16 week period, the study calendar will follow the same weekly schedule as weeks 9-12, respectively until the final visit. The final visit will be performed identically whether this occurs in the 8-12 or 13-16 week time frame.

d: Once weekly, participants will be contacted by phone to monitor adherence to drug administration and check for adverse events.

MEASUREMENT OF EFFECT

11.1 Other Response Parameters

This study only uses laboratory-based endpoints to measure the effect of aspirin treatment as this is a biomarker study. There are no clinically observable metrics (i.e. tumor size) that will be used as a primary endpoint or primary effect measure in this study. The primary efficacy endpoint will be colon tissue ISC populations and functional gene expression. A detailed discussion of this primary laboratory endpoint is included in section 9.

DATA REPORTING / REGULATORY REQUIREMENTS

Adverse event lists, guidelines, and instructions for AE reporting can be found in Section 7.0 (Adverse Events: List and Reporting Requirements).

12.1 Data Reporting

12.1.1 Method

The DF/HCC Office of Data Quality (ODQ) will collect, manage, and perform quality checks on the data for this study.

Note: If your study has been assigned to CDUS-Complete reporting, **all** adverse events (both routine and expedited) that have occurred on the study and meet the mandatory CDUS reporting guidelines must be reported via the monitoring method identified above. If your study has been assigned to CDUS-Abbreviated reporting, no adverse event reporting (routine or expedited) is required to be reported via CDUS.

12.1.2 Responsibility for Data Submission

Investigative sites are responsible for submitting data and/or data forms to the Office of Data Quality (ODQ) in accordance with DF/HCC policies.

12.2 Data Safety Monitoring

The DF/HCC Data and Safety Monitoring Committee (DSMC) will review and monitor toxicity and accrual data from this study. The committee is composed of medical oncologists, research nurses, pharmacists and biostatisticians with direct experience in cancer clinical research. Information that raises any questions about participant safety will be addressed with the Sponsor-Investigator and study team.

The DSMC generally reviews each protocol up to four times a year with the frequency determined by the outcome of previous reviews. Information to be provided to the committee may include: up-to-date participant accrual; current dose level information; DLT information; all grade 2 or higher unexpected adverse events that have been reported across all sites; summary of all deaths occurring within 30 days of intervention for Phase I or II protocols; for gene therapy

protocols, summary of all deaths while being treated and during active follow-up; any response information; audit results, and a summary provided by the study team. Other information (e.g. scans, laboratory values) will be provided upon request.

12.3 Multi-Center Guidelines

N/A

12.4 Collaborative Agreements Language

N/A

STATISTICAL CONSIDERATIONS

This study is a randomized clinical trial that will provide measurements of age-dependent effects of aspirin treatment on specific biomarkers of colorectal carcinogenesis. In doing so, we aim to provide causality for aspirin's overall risk-benefit established by other studies, and further define optimal populations who will benefit from aspirin chemoprevention. We and others have put forth considerable effort to determine measurable biomarkers implicated in colorectal carcinogenesis. A comprehensive discussion of these biomarkers and their significance is provided in Section 9.

We will utilize patient-derived organoids to further interrogate fundamental mechanisms of aspirin chemoprevention, because experimental models may not reflect the true biological anti-cancer activity of aspirin in humans. *In vitro* models often employ aspirin doses that are not achievable *in vivo* and cannot capture the influence of the tissue microenvironment¹⁴³. Animal models are inherently limited in their ability to recapitulate the complexity of human colorectal cancer. Thus, this study will uniquely offer additional *human* evidence of a causal association between aspirin and carcinogenesis and biomarkers that can be used to define populations most likely to benefit.

We will recruit 160 participants, 80 “older (65+)” and 80 “younger” (≥ 18 and < 55), who recently underwent removal of their adenomatous polyps at MGH to conduct a double blind three-arm randomized controlled trial of placebo and low-dose aspirin treatment for 2 months. The rationale for selection of this population is to be 1) consistent with prior randomized clinical trials of aspirin;¹⁰ 2) include patients that are more motivated to participate; 3) include patients with colonic mucosa predisposed to future neoplasia¹⁴⁴; 4) to capture sufficient numbers of patients to elucidate age-dependent effects of low-dose aspirin treatment, because age of initiation has emerged as a potential determinant of aspirin chemopreventive efficacy¹⁴⁵⁻¹⁴⁷. The rationale for selection of our aspirin dose is that this is the most common formulation available in the U.S. for long-term use.

Our pilot data to support the power estimates for the statistical design of this study and the scRNAseq-based primary endpoint were derived using the inDrop platform. As of 2022, this platform is no longer consistently available to study teams. Instead, we will use the 10x Genomics Chromium single-cell system or comparable Honeycomb Seqwell system. We anticipate that this will have no impact on our ability to study our primary endpoint, and in fact, believe the data

generated will be of similar if not higher quality. However, to ensure that the estimated effect sizes remain accurate, an interim analysis will be performed as described below. As a result of this interim analysis, if effect sizes associated with aspirin intervention are lower than anticipated, we may alter the remaining treatment group sizes such that we randomize at least 2 aspirin:1 placebo in each strata. Similarly, if effect sizes are greater than expected we may reduce the overall enrollment target. The protocol will be modified according to the description of the interim analysis below.

13.1 Study Design/Endpoints

The primary aim of this study is to estimate the effect of low-dose aspirin on cISC repertoire and functional gene expression. A detailed discussion of the background of the importance of these ISC populations is provided in Section 9.1.1.1. Briefly, we will determine the effect of low-dose aspirin and aging on colon epithelial cells, cISC populations, and functional gene expression changes using scRNA-seq. The rationale for selection of change in epithelial cell repertoire and functional gene expression as the primary endpoint is: 1) our experience with collecting and isolating cells for scRNA-seq; 2) our experience analyzing scRNA-seq data through established pipelines; 3) a widely accepted theory in the field is that colorectal cancer is driven by oncogenic mutations in a subset of colon epithelial cells - the Lgr5+ ISCs. Aging may decrease these Lgr5+ cells in an inflammation-driven mechanism¹⁸⁻²¹; 4) recent data suggests aspirin-like NSAIDs preferentially eliminate premalignant Lgr5+ ISCs^{16,17}.

Additional endpoints will include urinary PGE-M, plasma GDF-15, TCF4 binding in ChIP-seq assays of colonic epithelium, expression of genes associated with *Wnt* signaling (*CTNNB1*, *AXIN2* and *MYC*) and *15-PGDH* in colonic epithelium. Additional effects of aspirin on spectral biomarkers of colorectal carcinogenesis and on the oral/gut microbiome will also be investigated. Our study design for each of these endpoints is comprehensively discussed in Section 9. Statistical considerations relating to these endpoints are provided in the following sections.

13.2 Sample Size, Accrual Rate and Study Duration

Patients will be accrued in two stages with no early stopping rules. We will enroll 20 patients (10 older (≥ 65) and 10 younger (≥ 18 and < 55)) randomized 1:1 to low-dose aspirin or placebo control. Once this interim target enrollment is reached in both strata an interim analysis of the scRNAseq data as described for the primary outcome below will be performed to identify the effect size of aspirin intervention on single cell gene expression in younger and older individuals. Investigators will remain blinded to treatment assignment for these 20 individuals. Enrollment will continue during the interim analysis. A partial dataset consisting of coded identifiers, blinded treatment group, age strata, and single-cell transcript files will be transmitted to the study statistician who has access to the randomization sheets (Molin Wang). Data files will be stripped of study specific identifiers, relabelled with group only identifiers (aspirin or placebo; younger or older), and provided to the bioinformatics team in Thad Stappenbeck's laboratory for analysis. Investigators will not break the new code and will remain blinded to participant identities. Following the interim analysis for post-treatment gene-expression levels, Dr. Wang will perform the power analysis again and advise whether more allocation to the aspirin treatment group is recommended (i.e. aspirin intervention effect sizes are less than expected) with remaining individuals or the target

accrual and overall sample size should be modified.

We anticipate the total sample size to be 160 patients, unless formally modified through amendment to this protocol. We will enroll an additional 70 older (≥ 65) and 70 younger (≥ 18 and < 55) randomized at an allocation to be determined by the interim analysis, but beginning as 1:1 allocation, to low-dose aspirin or placebo control. We expect accrual to be approximately 60 patients per year for 2.5 years. This accrual rate is based upon accruals from the initial phase of the SPIRED trial. Volunteers were reimbursed ~\$400, as described here. Each patient will be followed for at minimum 8 weeks and at maximum 12 weeks. Participants with an extended treatment window will be followed for up to 4 additional weeks, for a maximum of 16 weeks. This total sample size accounts for participant drop out, as endoscopy studies typical experience a dropout rate of approximately 20%.¹⁴⁸.

For our **primary outcome**, comparison at post-treatment of gene-expression levels in low-dose aspirin vs placebo, we utilized the RNASeq power calculation package “RnaSeqSampleSizeData” in R.¹⁴⁹ We assumed that the minimum average read counts among the ISC-related genes in the control group is 5, the maximum dispersion of read counts 0.5, and the ratio of the geometric mean of normalization factors is 1.¹⁴⁹ With these assumptions, we will be able to reject the null hypothesis that the population means of two pairwise comparison groups are equal with power of 0.8 to detect a minimum effect size of fold-change 1.82 using an exact test described by Robinson and Smyth¹⁵⁰ and implemented in the R package, with false discovery rate (FDR) associated with the test set to 0.10. For comparing younger low-dose aspirin group to the remaining three treatment groups (older/younger placebo and older low-dose aspirin user; $n=40$ in each group), we assumed that differences in expression levels pre- and post- treatment follow a normal distribution, and after standardization to S.D. = 1, with our sample size of 40 and 120 in the two groups respectively, we will be able to identify a difference of 0.66 standard deviations between the two groups with 80% power and at an α -threshold of 0.005 to account for multiple comparisons. For our **secondary outcomes**, based on prior studies,^{50,58,59} we assumed an intra-class correlation (ICC) of 0.1, and a scaled S.D. of 1.0 for a single measurement of PGE-M and will have 80% power to detect an interaction for age with low-dose-aspirin’s effects on PGE-M for a difference of 1.2 S.D. in $\Delta_{LDA} - \Delta_{placebo}$ between the older and younger, a conservative estimate of reported effect size.^{58,75} Additional endpoints are considered exploratory. Power can be further improved through combination with the SPIRED cohort where data was similarly measured among 180 individuals.

Our accrual target rates for ethnic and racial minorities will likely be consistent with the rate at which we see these minorities in the gastroenterology practice at MGH and with the prior SPIRED trial. (See below.)

Accrual Targets				
Ethnic Category	Sex/Gender			
	Females		Males	Total
Hispanic or Latino	7	+	7	= 14
Not Hispanic or Latino	73	+	73	= 146
Ethnic Category: Total of all subjects	80	+	80	= 160

Racial Category					
American Indian or Alaskan Native	2	+	2	=	4
Asian	3	+	3	=	6
Black or African American	5	+	5	=	10
Native Hawaiian or other Pacific Islander	2	+	2	=	4
White	68	+	68	=	136
Racial Category: Total of all subjects	80	+	80	=	160

13.3 Stratification Factors

Participants will be stratified in two groups according to age: ≥ 18 and < 55 years old, or ≥ 65 years old. Randomization to either the placebo or low-dose aspirin group will be balanced within each stratum. Efficacy of intervention will be determined within each stratum as well, as our primary hypothesis is to determine age-dependent effects of aspirin.

13.4 Interim Monitoring Plan

An interim analysis will be performed as described in 13.2

13.5 Analysis of Primary Endpoints

13.5.1 Analysis of epithelial scRNA-seq (primary endpoint): Dr. Omer Yilmaz and Curtis Huttenhower will supervise scRNA-seq bioinformatics and biostatistics. We will use a one vs. all approach, where we will assess the difference in the change of ISC marker gene expression post-intervention relative to pre-intervention for younger participants randomized to low-dose aspirin compared to the change in ISC marker gene expression in the remaining three groups: younger adults randomized to placebo, older adults randomized to placebo, and older adults randomized to low-dose aspirin. The scRNA-seq data analysis will span several dimensions: **1)** distinct populations will segregate in the analyzed group of cells with a focus on cISCs by identifying variably expressed genes followed by dimensionality reduction t-Distributed stochastic neighbor embedding (t-SNE) and uniform manifold approximation and projection (UMAP). This will allow us to estimate ISC relative gene expression, function and number (proportion of ISC subtypes relative to differentiated epithelial cell subtypes) where we will focus on epithelial subtypes (*EPCAM*, *KRT8*, *KRT18*, etc.) enriched for *a priori* determined markers of ISCs (*LGR5*, *HOPX*, *OLFM4*, *AXIN2*, or *EPHB2* positive cells); **2)** comparison between pre-treatment samples from younger and older adults will reveal distinct populations that emerge during aging (e.g. shifts towards fetal-like Hopx⁺ cISCs vs. Lgr5⁺ cISCs; enriched for Yap markers); whereas pre- and post-treatment samples can be used to identify distinct populations that emerge post-low-dose aspirin exposure; **3)** comparison of the transcriptomes within each distinct population that arises within each cohort will have a unique molecular and functional signature that will allow us to identify nodes and

signaling modules enriched according to age or low-dose aspirin exposure, both independently and jointly. Within the scRNA-seq data, we will identify differentially expressed genes (DEGs) associated with age and/or low-dose aspirin intervention.¹⁵¹ DEG tests will be performed using MAST.¹⁵² We will focus on PG and YAP/Hippo signaling networks given these are the leading *a priori* pathways that underlie the anti-cancer effect of aspirin.

13.6 Analysis of Secondary Endpoints

- 13.6.1 **Analysis of Urinary PGE-M:** Our primary biostatistician, Dr. Wang, will supervise intent-to-treat analyses comparing the effect of each treatment on 2-month end-of-treatment change in PGE-M compared to the change in the place group, using a two-sample t-test. In secondary analyses, we will use multivariate linear regression models to adjust for other covariates in case there exists imbalances in determinants of change in PGE-M levels between arms. A robust variance estimate will be used to eliminate any normality assumptions for the residuals.
- 13.6.2 **Analysis of Plasma GDF-15:** Our biostatistician, Dr. Wang, will supervise intent-to-treat analyses comparing the effect of each treatment group on 2-month end-of-treatment change in GDF-15 using the two-sample t test. In secondary analyses, we will determine if these changes differ according to baseline urinary PGE-M using a multivariate linear regression analysis including an interaction term of the baseline urinary PGE-M and change in GDF-15. We will also use multivariate linear regression models for the change scores to adjust for other determinants of change in GDF-15 levels in case there are imbalances between the arms
- 13.6.3 **ChIP-seq Analysis of Colonic Epithelium:** Dr. Freedman and Dr. Curtis Huttenhower, our computational biologist (HSPH) will lead analysis of the ChIP-seq data (>60 million reads, 50-bp paired end) using the publicly available Cistrome Analysis Pipeline.⁹⁷ Short-read sequences from ChIP-seq data will be aligned to the reference genome (hg19) using the Burrows-Wheeler Aligner to create BAM files.¹⁵³ BAM files will be uploaded to Cistrome and peak calling will be performed using an automated pipeline implementing the Model-based Analysis of ChIP-seq (MACS2) tool, which will output peak regions, peak summits, fold enrichment, *p* value, and false discovery rate (FDR < 0.01).¹⁵⁴ Biological replicates among cases and controls will be aggregated using the Model-based Meta-analysis of ChIP-seq data (MMChIP-seq) tool to account for batch effects.¹⁵⁵ Normalization and differential peak calling of ChIP-seq data between experimental conditions will be performed using MANorm (threshold $p < 0.05$).¹⁵⁶ Our main analysis will quantify the differential fold enrichment at the TCF4 promoter adjacent to rs6983267 associated with aspirin. In a secondary analysis, we will assess if this binding is particularly enhanced among those with germline GT/TT rs6983267 genotypes and according to baseline PGE-M using cross-product terms for aspirin with genotype or PGE-M level and assess for significance using the Wald test.

- 13.6.4 **Gene Expression Analysis of Colonic Epithelium:** Dr. Freedman and Dr. Huttenhower

will supervise gene expression analysis. RNA-seq sequence data (> 50 million reads) will be mapped to hg19 through use of TopHat2.¹⁵⁷ Cufflinks will be used to assemble the transcriptome, and Cuffdiff will be used to identify differentially expressed genes between cases and controls with a fold change of 1.2 or greater with a p-value <.05.¹⁵⁸ Our main analysis will examine if aspirin results in differential fold changes in expression of the *Wnt* signaling genes (β -catenin, *AXIN-2* and *MYC*) and *15-PGDH*. In secondary analyses, we will determine if these changes differ according to baseline level of urinary PGE-M. To identify additional potential effectors of the *Wnt* signaling pathways regulated by aspirin, we will utilize the Broad Institute's publicly available gene-set enrichment analysis tools (GSEA) that contain regularly updated pathway components. We will confirm our findings for *15-PGDH* using our previously described RT-qPCR protocol in Dr. Sandy Markowitz's laboratory (Case Western Reserve).^{113 112} Briefly, RNA from colonic epithelium will undergo RT-qPCR assays for *15-PGDH* following the MIQE guidelines.¹⁵⁹ Villin (VIL1) and E-Cadherin (CDH1) will be used as the reference gene set for normalization using established software.¹⁶⁰⁻¹⁶² Demonstrating consistent results using RNA-seq with our validated RT-qPCR assay for 15-PGDH reported in prior studies^{109,112,113} will additionally validate our findings and establish a RT-qPCR pipeline to confirm novel targets derived from RNA-seq.

13.6.5 Microbiome Analysis: Our collaborators at the Broad Institute will oversee metagenomic and metatranscriptomic profiling of stool and saliva samples. Aspirin use and dose will be associated with microbial operational taxonomic units (OTUs) using the Biobakery3 computational analysis pipeline. We expect to find that study participants taking placebo have decreased oral and gut bacterial diversity and experience perturbations in normal microbiota composition that have been previously correlated with an increased risk for colorectal cancer compared to those receiving aspirin treatment.

13.7 Reporting and Exclusions

Participants who never start protocol therapy or do not return for final flexible sigmoidoscopy and sample collection will be considered inevaluable and will be excluded from all analyses.

13.7.1 Evaluation of Toxicity

N/A

13.7.2 Evaluation of the Primary Efficacy Endpoint

The primary efficacy analysis will be performed on an intention-to-treat basis, with primary endpoints determined for all patients who complete the final flexible sigmoidoscopy and sample collection, regardless of whether the patient complied with study drug use.¹⁶⁷

13.7.3 Evaluation of the Secondary Endpoints

The secondary endpoint analyses will be performed on an intention-to-treat basis, with secondary endpoints determined for all patients who complete the final flexible

sigmoidoscopy and sample collection, regardless of whether the patient complied with study drug use.¹⁶⁷

PUBLICATION PLAN

The results should be made public within 24 months of reaching the end of the study. The end of the study is the time point at which the last data items are to be reported, or after the outcome data are sufficiently mature for analysis, as defined in the section on Sample Size, Accrual Rate and Study Duration. We plan to publish in a peer-reviewed journal; thus, the initial release may be an abstract that meets the requirements of the International Committee of Medical Journal Editors. A full report of the outcomes should be made public no later than three (3) years after the end of the study.

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APPENDIX A

POSSIBLE DRUG-DRUG INTERACTIONS WITH ASPIRIN

ACE Inhibitors: Salicylates may diminish the antihypertensive effect of ACE Inhibitors. They may also diminish other beneficial pharmacodynamic effects desired for the treatment of CHF. The effects are likely dose-related. 100 mg doses aspirin appear to cause no problems, whereas 300 mg doses appear to significantly affect ACE Inhibitor efficacy. *Risk C: Monitor therapy*

Agents with Antiplatelet Properties (e.g., P2Y₁₂ inhibitors, NSAIDs, SSRIs, etc.): May enhance the adverse/toxic effect of Salicylates. Increased risk of bleeding may result. *Risk C: Monitor therapy*

Agents with Antiplatelet Properties (e.g., P2Y₁₂ inhibitors, NSAIDs, SSRIs, etc.): May enhance the antiplatelet effect of other Agents with Antiplatelet Properties. *Risk C: Monitor therapy*

Alendronate: Aspirin may enhance the adverse/toxic effect of Alendronate. Specifically gastrointestinal adverse events. *Risk C: Monitor therapy*

Ammonium Chloride: May increase the serum concentration of Salicylates. *Risk C: Monitor therapy*

Anticoagulants: Agents with Antiplatelet Properties may enhance the anticoagulant effect of Anticoagulants. *Risk C: Monitor therapy*

Anticoagulants: Salicylates may enhance the anticoagulant effect of Anticoagulants. *Risk C: Monitor therapy*

Antidepressants (Tricyclic, Tertiary Amine): May enhance the antiplatelet effect of Aspirin. *Risk C: Monitor therapy*

Apixaban: Agents with Antiplatelet Properties may enhance the adverse/toxic effect of Apixaban. Specifically, the risk for bleeding may be increased. *Risk C: Monitor therapy*

Calcium Channel Blockers (Nondihydropyridine): May enhance the anticoagulant effect of Salicylates. **Exceptions:** Bepridil [Off Market]. *Risk C: Monitor therapy*

Carbonic Anhydrase Inhibitors: Salicylates may enhance the adverse/toxic effect of Carbonic Anhydrase Inhibitors. Salicylate toxicity might be enhanced by this same combination.

Exceptions: Brinzolamide; Dorzolamide. *Risk D: Consider therapy modification*

Carisoprodol: Aspirin may increase serum concentrations of the active metabolite(s) of Carisoprodol. Specifically, Meprobamate concentrations may be increased. Aspirin may decrease the serum concentration of Carisoprodol. *Risk C: Monitor therapy*

Collagenase (Systemic): Agents with Antiplatelet Properties may enhance the adverse/toxic effect of Collagenase (Systemic). Specifically, the risk of injection site bruising and/or bleeding may be increased. *Risk C: Monitor therapy*

Corticosteroids (Systemic): Salicylates may enhance the adverse/toxic effect of Corticosteroids (Systemic). These specifically include gastrointestinal ulceration and bleeding. Corticosteroids (Systemic) may decrease the serum concentration of Salicylates. Withdrawal of corticosteroids may result in salicylate toxicity. *Risk C: Monitor therapy*

Dabigatran Etxilate: Agents with Antiplatelet Properties may enhance the anticoagulant effect of Dabigatran Etxilate. Agents with Antiplatelet Properties may increase the serum concentration of Dabigatran Etxilate. This mechanism applies specifically to clopidogrel. Management: Increase monitoring for signs/symptoms of bleeding. The dabigatran Canadian product monograph specifically recommends avoiding concomitant use with GPIIb/IIIa inhibitors or ticlopidine, or with aspirin used for stroke prevention in atrial fibrillation. *Risk C: Monitor therapy*

Dasatinib: May enhance the anticoagulant effect of Agents with Antiplatelet Properties. *Risk C: Monitor therapy*

Floctafenine: May enhance the adverse/toxic effect of Aspirin. An increased risk of bleeding may be associated with use of this combination. Floctafenine may diminish the cardioprotective effect of Aspirin. *Risk X: Avoid combination*

Ginkgo Biloba: May enhance the anticoagulant effect of Salicylates. Management: Consider alternatives to this combination of agents. Monitor for signs and symptoms of bleeding (especially intracranial bleeding) if salicylates are used in combination with ginkgo biloba. *Risk D: Consider therapy modification*

Glucosamine: May enhance the antiplatelet effect of Agents with Antiplatelet Properties. *Risk C: Monitor therapy*

Heparin: Aspirin may enhance the anticoagulant effect of Heparin. *Risk C: Monitor therapy*

Herbs (Anticoagulant/Antiplatelet Properties) (eg, Alfalfa, Anise, Bilberry): May enhance the adverse/toxic effect of Agents with Antiplatelet Properties. Bleeding may occur. *Risk D: Consider therapy modification*

Herbs (Anticoagulant/Antiplatelet Properties) (eg, Alfalfa, Anise, Bilberry): May enhance the adverse/toxic effect of Salicylates. Bleeding may occur. *Risk D: Consider therapy modification*

Hyaluronidase: Salicylates may diminish the therapeutic effect of Hyaluronidase. Management: Patients receiving salicylates (particularly at larger doses) may not experience the desired clinical response to standard doses of hyaluronidase. Larger doses of hyaluronidase may be required. *Risk D: Consider therapy modification*

Hypoglycemic Agents: Salicylates may enhance the hypoglycemic effect of Hypoglycemic Agents. *Risk C: Monitor therapy*

Ibritumomab: Agents with Antiplatelet Properties may enhance the adverse/toxic effect of Ibritumomab. Both agents may contribute to impaired platelet function and an increased risk of bleeding. *Risk C: Monitor therapy*

Ibrutinib: May enhance the adverse/toxic effect of Agents with Antiplatelet Properties. *Risk C: Monitor therapy*

Influenza Virus Vaccine (Live/Attenuated): May enhance the adverse/toxic effect of Salicylates. Specifically, Reye's syndrome may develop. *Risk X: Avoid combination*

Ketorolac (Nasal): May enhance the adverse/toxic effect of Aspirin. An increased risk of bleeding may be associated with use of this combination. Ketorolac (Nasal) may diminish the cardioprotective effect of Aspirin. *Risk X: Avoid combination*

Ketorolac (Systemic): May enhance the adverse/toxic effect of Aspirin. An increased risk of bleeding may be associated with use of this combination. Ketorolac (Systemic) may diminish the cardioprotective effect of Aspirin. *Risk X: Avoid combination*

Loop Diuretics: Salicylates may diminish the diuretic effect of Loop Diuretics. Loop Diuretics may increase the serum concentration of Salicylates. *Risk C: Monitor therapy*

Methotrexate: Salicylates may increase the serum concentration of Methotrexate. Salicylate doses used for prophylaxis of cardiovascular events are not likely to be of concern. *Risk D: Consider therapy modification*

Multivitamins/Fluoride (with ADE): May enhance the antiplatelet effect of Aspirin. Aspirin may decrease the serum concentration of Multivitamins/Fluoride (with ADE). Specifically, aspirin may decrease the absorption of ascorbic acid. *Risk C: Monitor therapy*

Multivitamins/Minerals (with ADEK, Folate, Iron): May enhance the antiplatelet effect of Aspirin. Aspirin may decrease the serum concentration of Multivitamins/Minerals (with ADEK, Folate, Iron). Specifically, aspirin may decrease absorption of ascorbic acid. *Risk C: Monitor therapy*

Multivitamins/Minerals (with AE, No Iron): May enhance the antiplatelet effect of Aspirin. Aspirin may decrease the serum concentration of Multivitamins/Minerals (with AE, No Iron). Specifically, aspirin may decrease the absorption of ascorbic acid. *Risk C: Monitor therapy*

NSAID (COX-2 Inhibitor): Aspirin may enhance the adverse/toxic effect of NSAID (COX-2 Inhibitor). Management: Concurrent use of aspirin at doses beyond cardioprotective levels is not recommended. While concurrent use of low-dose aspirin with a COX-2 inhibitor is permissible, patients should be monitored closely for signs/symptoms of GI ulceration/bleeding. *Risk D: Consider therapy modification*

NSAID (Nonselective): May enhance the adverse/toxic effect of Salicylates. An increased risk of bleeding may be associated with use of this combination. NSAID (Nonselective) may diminish the cardioprotective effect of Salicylates. Salicylates may decrease the serum concentration of NSAID (Nonselective). *Risk D: Consider therapy modification*

Obinutuzumab: Agents with Antiplatelet Properties may enhance the adverse/toxic effect of Obinutuzumab. Specifically, the risk of serious bleeding-related events may be increased.

Risk C: Monitor therapy

Omacetaxine: Aspirin may enhance the adverse/toxic effect of Omacetaxine. Specifically, the risk for bleeding-related events may be increased. Management: Avoid concurrent use of aspirin with omacetaxine in patients with a platelet count of less than 50,000/uL. *Risk X:*

Avoid combination

Omega-3 Fatty Acids: May enhance the antiplatelet effect of Agents with Antiplatelet Properties.

Risk C: Monitor therapy

Pentosan Polysulfate Sodium: May enhance the adverse/toxic effect of Agents with Antiplatelet Properties. Specifically, the risk of bleeding may be increased by concurrent use of these agents. *Risk C: Monitor therapy*

Pentoxifylline: May enhance the antiplatelet effect of Agents with Antiplatelet Properties. *Risk C: Monitor therapy*

Potassium Acid Phosphate: May increase the serum concentration of Salicylates. *Risk C: Monitor therapy*

PRALAtrexate: Salicylates may increase the serum concentration of PRALAtrexate. Salicylate doses used for prophylaxis of cardiovascular events are unlikely to be of concern. *Risk D: Consider therapy modification*

Probenecid: Salicylates may diminish the therapeutic effect of Probenecid. *Risk C: Monitor therapy*

Prostacyclin Analogues: May enhance the antiplatelet effect of Agents with Antiplatelet Properties. *Risk C: Monitor therapy*

Rivaroxaban: Agents with Antiplatelet Properties may enhance the anticoagulant effect of Rivaroxaban. Management: Avoid concurrent use of antiplatelet medications with rivaroxaban unless the anticipated benefits outweigh the risks of bleeding. *Risk D: Consider therapy modification*

Salicylates: Agents with Antiplatelet Properties may enhance the adverse/toxic effect of Salicylates. Increased risk of bleeding may result. *Risk C: Monitor therapy*

Salicylates: May enhance the anticoagulant effect of other Salicylates. *Risk C: Monitor therapy*

Selective Serotonin Reuptake Inhibitors: May enhance the antiplatelet effect of Aspirin. *Risk C: Monitor therapy*

Serotonin/Norepinephrine Reuptake Inhibitors: May enhance the antiplatelet effect of Aspirin. *Risk C: Monitor therapy*

Thrombolytic Agents: Agents with Antiplatelet Properties may enhance the anticoagulant effect of Thrombolytic Agents. *Risk C: Monitor therapy*

Thrombolytic Agents: Salicylates may enhance the adverse/toxic effect of Thrombolytic Agents. An increased risk of bleeding may occur. *Risk C: Monitor therapy*

Ticagrelor: Aspirin may enhance the antiplatelet effect of Ticagrelor. Aspirin may diminish the therapeutic effect of Ticagrelor. More specifically, the benefits of ticagrelor relative to clopidogrel may be diminished in patients receiving daily aspirin doses greater than 100-150 mg daily. Management: Avoid daily aspirin doses greater than 100 mg in patients receiving ticagrelor. Canadian recommendations are to avoid daily aspirin doses greater than 150 mg. Daily low-dose aspirin (U.S.: 75-100 mg; Canada: 75-150 mg) is recommended. *Risk D: Consider therapy modification*

Tiludronate: Aspirin may decrease the serum concentration of Tiludronate. *Risk C: Monitor therapy*

Tipranavir: May enhance the antiplatelet effect of Agents with Antiplatelet Properties. *Risk C: Monitor therapy*

Tositumomab and Iodine I 131 Tositumomab: Agents with Antiplatelet Properties may enhance the adverse/toxic effect of Tositumomab and Iodine I 131 Tositumomab. Specifically, the risk of bleeding-related adverse events may be increased. *Risk C: Monitor therapy*

Treprostinil: May enhance the adverse/toxic effect of Salicylates. Bleeding may occur. *Risk C: Monitor therapy*

Urokinase: Agents with Antiplatelet Properties may enhance the anticoagulant effect of Urokinase. *Risk X: Avoid combination*

Valproic Acid and Derivatives: Salicylates may increase the serum concentration of Valproic Acid and Derivatives. *Risk C: Monitor therapy*

Varicella Virus-Containing Vaccines: Salicylates may enhance the adverse/toxic effect of Varicella Virus-Containing Vaccines. Reye's Syndrome may develop. *Risk D: Consider therapy modification*

Vitamin E: May enhance the antiplatelet effect of Agents with Antiplatelet Properties. *Risk C: Monitor therapy*

Vitamin K Antagonists (eg, warfarin): Salicylates may enhance the anticoagulant effect of Vitamin K Antagonists. *Risk D: Consider therapy modification*

