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Non-pharmacologic approaches to target inflammation in Myeloproliferative Neoplasms

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

by

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2021

CHAPTER 1

INTRODUCTION, CONCLUSIONS & CHAPTERS 2, 3, 5 & 6

In preparation for submission. Methods and figures in chapter 5

CHAPTER 4

DEDICATION

To

my son.

I love you more than words can describe.

You are my greatest blessing.

"Do the things that interest you and do them with all your heart.
Don't be concerned about whether people are watching you or criticizing you.
The chances are that they aren't paying any attention to you.
It's your attention to yourself that is so stultifying.
But you have to disregard yourself as completely as possible.
If you fail the first time then you'll just have to try harder the second time.
After all, there's no real reason why you should fail. Just stop thinking about yourself."

Eleanor Roosevelt

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VITA

EDUCATION

2016-2021 Ph.D. in Biomedical Sciences

University of California, Irvine, School of Medicine

2016-2020 M.S. in Biomedical Sciences

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2007-2012 Bachelor's Degree in Medicine

Autonomous University of Baja California (UABC)

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2018-2021 Graduate Student Researcher (Ph.D.)

Dr. Angela Fleischman's laboratory, University of California Irvine

Project: NUTRIENT Trial (NUTRitional Intervention among MyEloproliferative Neoplasms): feasibility phase.

2016-2018 Graduate Student Researcher

Dr. Eric Pearlman's laboratory, University of California Irvine

Project: Investigating the role of Osteopontin (OPN) protein in mouse fungal keratitis

2014-2015 UABC's Diagnostic Center

Assisted as MD, treating acute and chronic illness, providing preventive care and health education to patients.

2014-2015 UABC's Microbiology and Parasitology Laboratory

Lab assistant, preparing culture mediums for bacteria and fungi, as well as basic staining procedures, sterilized laboratory materials in autoclave,

developed microscopy abilities and collaborated in an antibiotic resistance research protocol.

2013-2014 Mexicali's General Hospital (Internship)

Served as MD, rotating in several areas, including: surgery, medical emergencies, internal medicine, gynecology and obstetrics, pediatrics, traumatology and orthopedics, and epidemiology.

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Participated in the research unit for cardiovascular and metabolic diseases in an observational study that correlated the presence of metabolic syndrome in DM individuals under no glycemic control, establishing that insulin resistance is a determining factor in both pathologies.

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2016-2021 UC MEXUS-CONACYT Doctoral Fellowship for Mexican Students

PUBLICATIONS

Andrew Oliver*, Kenza El Alaoui*, Carolyn Haunschild, Julio Avelar-Barragan, **Laura F. Mendez Luque**, Katrine Whiteson, Angela G. Fleischman. Fecal microbial community composition in myeloproliferative neoplasm patients is associated with an inflammatory state. Submitted 2021

Brianna M. Craver, Gajalakshmi Ramanathan, Summer Hoang, Xinyue Chang, **Laura F. Mendez Luque**, Stefan Brooks, Hew Yeng Lai, Angela G. Fleischman. N-acetylcysteine inhibits thrombosis in a murine model of myeloproliferative neoplasms. Blood Adv. 2020;4(2):312–321

Laura F. Mendez Luque, Amanda L. Blackmon, Gajalakshmi Ramanathan, Angela G. Fleischman. Key Role of Inflammation in Myeloproliferative Neoplasms: Instigator of Disease Initiation, Progression. and Symptoms. Current Hematologic Malignancy Reports. 2019

ABSTRACT OF THE DISSERTATION

Non-pharmacologic approaches to target inflammation in Myeloproliferative Neoplasms

by

Laura Fernanda Mendez Luque

Doctor of Philosophy in Biomedical Sciences

University of California, Irvine, 2021

Associate Professor Angela G. Fleischman, Chair

Myeloproliferative neoplasms (MPNs) are hematologic malignancies that result from a somatic mutation, most commonly in the Janus kinase ($JAK2^{V617F}$) causing the constitutive activation of the blood cell production pathway, JAK-STAT which leads to an overproduction of mature myeloid cells. Inflammation is a key feature in the development of these blood cancers and drives clinical manifestations. This thesis centers on diet as a non-pharmacologic approach to reduce inflammation. We designed a 15-week clinical trial to test the anti-inflammatory properties of the Mediterranean diet. The trial consists of a two-week "lead-in" period, a ten-week active intervention and a three-week follow up period. Our primary aim is to evaluate the feasibility of a dietary intervention and the adherence to a Mediterranean diet among a MPN cohort. The study's exploratory endpoints include reduction in inflammatory biomarkers, reduction in symptom burden, and change in the gut microbiome.

Our findings demonstrated that a Mediterranean diet is as feasible to follow for MPN patients as the standard US Dietary Guidelines for Americans. We concluded that with

dietician counseling and written education MPN patients can achieve proper adherence to a Mediterranean diet. However, there were no specific symptoms being impacted by the Mediterranean diet more than the other. Also, no significant changes on cytokine levels were detected in either of the diet groups throughout the course of the study.

Interestingly, we observed that MPN subtype is associated with gut microbiome composition, and that patients with myelofibrosis (MF) had a more dissimilar community composition of microbes in the gut that those suffering from polycythemia vera (PV) or essential thrombocythemia (ET).

We also developed mouse models to test the impact of diet on the trajectory of mutant hematopoietic cells. We adapted a mouse bone marrow transplant technique to yield mice with a small percentage of mutant cells in the blood, recapitulating what is seen in humans with clonal hematopoiesis of indeterminate potential (CHIP), a precursor to hematologic malignancy. With our mouse model we did not find that high fat diet (HFD) nor N-acetylcysteine (N-AC) had a significant impact on the trajectory of *Tet2-/-* cells (a common CHIP mutation)but we did see less weight gain in mice on a HFD + N-AC water.

INTRODUCTION

Myeloproliferative neoplasm (MPN) is a hematologic malignancy including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) characterized by the clonal outgrowth of hematopoietic cells with a somatically acquired mutation most commonly in JAK2 (*JAK2*^{V617F}) [1-5]. This mutation endows upon myeloid progenitors cytokine independent growth and consequently leads to excessive production of myeloid lineage cells. MPN patients present a broad-spectrum of symptoms even in early-stage disease and vary among individuals, though some symptoms are more prevalent in ET, PV, or PMF. Regardless of the subtype, the most common complaint is fatigue (80.7%), followed by pruritus (52.2%), night sweats (49.2%), bone pain (43.9%) and fever (13.7%) [6]. Fatigue in MPN not only stems from the disease itself but can also be caused by cytoreductive therapies used in MPN treatment, such as hydroxyurea, anagrelide, and interferon-alpha. Interestingly, specific symptoms and complaints have been correlated with elevated levels of specific inflammatory biomarkers [7].

Recently developed National Comprehensive Cancer Network (NCCN) guidelines for MPN address the importance of symptom burden and recommend intervention to reduce symptom burden regardless of prognosis scoring category. Early stage MPN can spontaneously progress to myelofibrosis, a more aggressive stage of the disease as well acute myeloid leukemia (AML) which has a particularly dismal prognosis when preceded by an MPN. Therapeutic intervention is focused on patients with late stage disease, mostly due to the lack of currently available therapies that halt or slow disease progression or alter the disease course. There remains a critical need for interventions that reduce

symptom burden and ultimately impact disease progression in MPN at early stages of the disease.

Chronic inflammation is a characteristic feature of MPN [8,9]. Inflammation drives many of the debilitating constitutional symptoms associated with MPN, inflammation also plays a role in promoting the survival advantage and proliferation of *JAK2*^{V617F} neoplastic hematopoietic stem cells [10,11]. Our team has shown that inflammation inhibits the growth of *JAK2*^{WT} cells, while allowing for robust growth of *JAK2*^{V617F} mutant cells [12]. Moreover, *JAK2*^{V617F} mutant cells themselves produce pro-inflammatory cytokines (most notably tumor necrosis factor-alpha, TNF) and induce bystander normal cells to produce excessive pro-inflammatory cytokines [13].

Diet relates to the incidence of various types of malignancies, vascular events, and metabolic syndromes [14]. The Mediterranean diet, characterized by increased consumption of extra virgin olive oil (EVOO), nuts, legumes, vegetables, fruits, fish, and whole grain products, has proven to be beneficial in diseases where chronic subclinical inflammation plays a key role [15]. For example, the PREDIMED (Prevención con Dieta Mediterránea) study demonstrated that a Mediterranean diet supplemented with EVOO or nuts reduced the incidence of major cardiovascular events [16]. The Mediterranean diet's anti-inflammatory properties are attributed to its richness in phenolic compounds and main nutrients, such as: fiber, monounsaturated fatty acids, n-3 polyunsaturated fatty acids, vitamins C and E, and carotenoids [17]. Overproduction of pro-inflammatory cytokines like IL-6 and TNF is a characteristic feature of Myeloproliferative Neoplasm (MPN) and correlates with high symptom burden and may also play a role in disease progression.

Nutritional control of inflammation represents a unique low risk therapeutic approach to alleviate the symptom burden of MPN patients and to also possibly blunt disease progression. To date, only one therapy has been identified to alleviate the symptom profile of MPN patients through JAK inhibition [18]. The JAK1/2 inhibitors ruxolitinib and fedratinib reduce inflammatory cytokines and improve symptoms but not without significant side effects including thrombocytopenia, anemia, increased risk of skin cancer, and immunosuppression. Traditional therapies, including non-pharmacologic therapies such as phlebotomy, have been shown to have no impact on or worsen symptom burden [19]. There remains an unmet need for low-risk interventions, such as nutrition, that impact symptom burden.

CHAPTER 1

Key Role of Inflammation in Myeloproliferative Neoplasms: Instigator of Disease Initiation, Progression. and Symptoms

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Abstract

Purpose of review: Chronic inflammation is a characteristic feature of myeloproliferative neoplasm (MPN) and impacts many aspects of the disease including initiation, progression, and symptomatology.

Recent findings: The chronic inflammatory state of MPN results from disruption of immune signaling pathways leading to overproduction of inflammatory cytokines by both the neoplastic clones and bystander immune cells. This chronic inflammation may allow for the neoplastic clone to gain a selective advantage. The symptomatic burden felt by MPN patients may be a result of the chronic inflammation associated with MPN, as several cytokines have been linked with different symptoms. Pharmacologic as well as nonpharmacologic treatments of the inflammatory component of this disease may lead to

decreased symptomatic burden, prevention of disease progression and improvement in overall disease trajectory.

Summary: Inflammation plays a key role in the pathogenesis of MPN and represents an important therapeutic target.

Introduction

Inflammation plays a crucial role in the development and progression of myeloproliferative neoplasms (MPN). Myeloid malignancies are characterized by elevated levels of inflammatory cytokines, which correlate with disease initiation and progression, symptomatic burden, and prognosis. Increased inflammatory cytokines, including tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6), are typically observed in MPN patients [1, 2] as well as MPN mouse models [3]. These findings suggest that inflammatory cytokines are involved in the natural course of the disease and are driving the clinical manifestations.

The clinical burden of MPN develops from a combination of unrestrained production of mature myeloid cells by the neoplastic clone, and a bone marrow microenvironment of inflammatory products from activated leukocytes and platelets, which leads to the accumulation of reactive oxygen species (ROS) in the hematopoietic stem cell (HSC) compartment [4].

Role of Inflammation in MPN Disease Initiation

Inflammation may create an environment which is highly favorable for growth of the *JAK2*^{V617F} neoplastic hematopoietic stem cells. The *JAK2*^{V617F} mutant hematopoietic progenitors are resistant to inflammation, while *JAK2*^{WT} cells are suppressed by inflammation, possibly through the induction of apoptosis, quiescence, or reduced self-renewal/increased differentiation of HSC [5]. Moreover, *JAK2*^{V617F} mutant cells themselves produce inflammatory cytokines (most notably TNF-α) and also induce bystander normal cells to produce inflammatory cytokines [6, 7].

A chronic inflammatory state may increase one's risk of developing MPN. MPN patients are more likely to have a preceding diagnosis of autoimmune disease [8]. Interestingly, the JAK2 46/1 haplotype identified as associated with JAK2-mutated MPN is also associated with Crohn's disease [9]. Lai et al found that MPN patient monocytes produce TNF-α for prolonged periods of time after Toll-like Receptor (TLR) stimulation because they are less able to respond to the anti-inflammatory cytokine IL-10 [7]. This defective negative regulation of TLR signaling was observed in both the mutant and wild-type cells alike from MPN patients, suggesting this is not a cell intrinsic consequence of *JAK2*^{V617F}. Moreover, the unaffected identical twin of an MPN patient was found to have prolonged TNF-α production following stimulation similar to her twin with MPN, suggesting this abnormality may be a genetic feature rather than a consequence of MPN. This work also demonstrates that the chronic inflammatory state in MPN may be due to defects in quelling inflammation after stimulation.

Aging is considered a pro-inflammatory state due to the increased release of inflammatory cytokines and immunosenescence which may likely play a role throughout myeloid malignancies. MPN is generally a disease of the elderly with the average age of onset being 65 years old, further highlighting the association between inflammation and development of MPN.

Source of Inflammation in MPN and Pathway Targets

The mechanisms driving inflammation in MPN are multifactorial and not fully understood (**Figure 1.1**). Production of inflammatory cytokines is not exclusive to the *JAK2*^{V617F} mutant clone, demonstrating that the presence of the mutant clone creates an environment which induces bystander normal cells to produce inflammatory cytokines. Using single-cell profiling, Kleppe et al showed that hematopoietic cells from MPN mouse models as well as primary MPN patient samples aberrantly secrete inflammatory cytokines [6]. In addition, Stat3 was found to be critical for the production of inflammatory cytokines in MPN. Pan hematopoietic deletion of Stat3 reduced inflammatory cytokines and attenuated disease severity, however deleting Stat3 in the MPN cells while preserving Stat3 in non-mutant cells did not reduce cytokine production nor attenuate disease pathology further supporting the key role of bystander cells as producers of inflammatory cytokines in MPN.

The ability of the mutant clone to induce inflammation may create a self-perpetuating environment for its continued selection. An inflammatory environment may be critical to the maintenance and/or expansion of the mutant clone. If so, blocking this

inflammation induced by the mutant clone could be a useful therapeutic approach to potentially blunt the expansion of mutant over wild-type cells. Blockade of mutant clone induced inflammation would also likely have an impact on the negative consequences of the mutant clone such as accelerated atherosclerosis or potentially even thrombosis.

Derangement of JAK/STAT signaling is not the sole contributor to inflammation in MPN. Although ruxolitinib reduces inflammatory cytokines in MPN patients, it may not be enough to fully return cytokines to normal. Fisher et al found a modest difference in pre versus post ruxolitinib plasma levels cytokines such as VEGF, TNF-α, IL-6, IL-10, and IL-16 in MF patients, demonstrating that JAK inhibition may not be sufficient to normalize inflammatory cytokines [10].

Hyperactivation of the NFκB signaling pathway is a key contributor to chronic inflammation in MPN. Using mass cytometry Fisher et al found that primary samples from myelofibrosis (MF) and secondary acute myeloid leukemia samples had constitutive NFκB signaling and many NFκB target genes were found to have increased expression in MF patient $CD34^+$ cells. Moreover, NFκB inhibition suppressed colony formation from MF $CD34^+$ cells implicating NFκB as a therapeutic target in MPN [11].

Yang et al found a significant enrichment of the NFκB signaling pathway in sorted hematopoietic stem cells from $JAK2^{V617F}$ mice as compared to wild-type mice [12]. They found that the NFκB inhibitor dimethylaminoparthenolide (DMAPT) reduced proliferation of Ba/F3-EpoR-JAK2V617F cells, inhibited the growth of human $JAK2^{V617F}$-positive cell lines, and inhibited the hematopoietic progenitor colony outgrowth in $JAK2^{V617F}$ mice BM and MPN patient peripheral blood $CD34^+$ cells. Treatment of combination therapy with

ruxolitinib and DMAPT reduced mutant myeloid precursors in the bone marrow and spleen of JAK2^{V617F} mice and reduced bone marrow fibrosis.

Kleppe et al [13] also identified activation of NFκB in both malignant and non-malignant cells in MPN and found that inhibition of BET bromodomain proteins attenuated NFκB signaling and reduced cytokine production *in vivo*. They also found that combined treatment with ruxolitinib and the BET inhibitor JQ1 reduced inflammatory cytokines, reduced disease burden, and reversed bone marrow fibrosis in mouse MPN models. There is abundant pre-clinical evidence to support evaluation of NFκB inhibition in MPN patients. A clinical trial is currently open at Washington University in St. Louis investigating the combination of the NFKB inhibitor pevonedistat in combination with ruxolitinib in patients with myelofibrosis (NCT03386214).

Figure 1

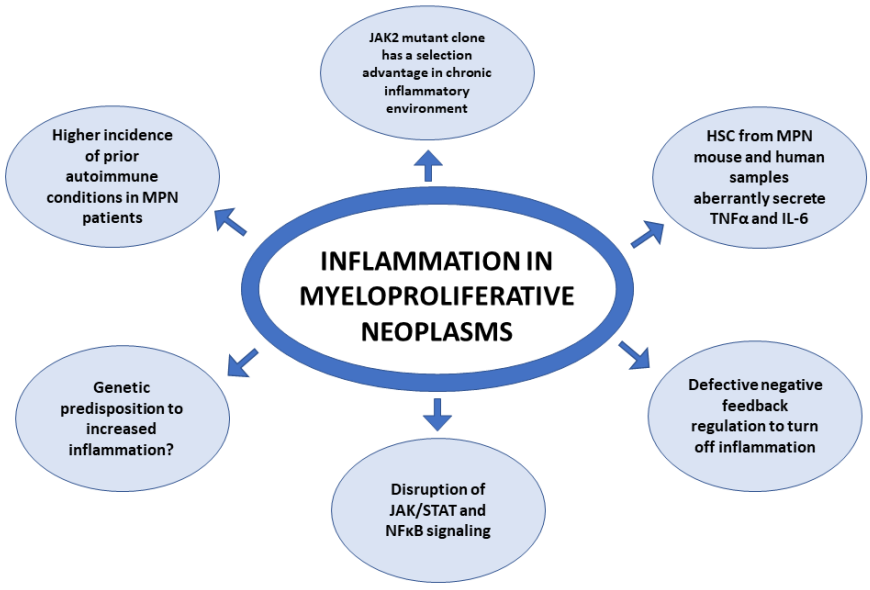

Figure 1.1 Factors influencing inflammation in myeloproliferative neoplasms. Chronic inflammation is a multifactorial process in MPN. It is difficult to assess if it is the result from a preceding pro- inflammatory environment or if the neoplastic clone itself predisposes to its development. Understanding the different drivers of inflammation in MPN may lead to targeted treatments in the future.

Inflammation and Symptoms In MPN

MPN patients can experience a variety of symptoms including fatigue, sleep disturbance, night sweats, weight loss, depression, anxiety, early satiety, pruritus, and bone pain. Fatigue is the predominant symptom, which affects 81-95% of MPN patients, and has been reported as an important distressing factor causing decreased quality of life (QoL) by cancer survivors [14-19]. Fatigue in MPN not only stems from the disease itself which will be discussed below, but can also be caused by cytoreductive therapies used in MPN treatment, such as hydroxyurea, anagrelide, and interferon-alpha. In MPN, a patients QoL can also be affected by disease complications including thrombosis, hemorrhage, hepatosplenomegaly, anemia, cachexia, and weight loss [15].

Several studies have shown a correlation with inflammatory markers and perceived symptoms of disease. Elevated levels of IL-6, a highly expressed cytokine within MPNs, has been linked to cancer-related fatigue, and has shown a correlation with depression (32% in a cohort of 1788 MPN patients) [20-22]. Similarly, fever and night sweats are well known to be influenced by pyrogenic cytokines (IL-1, IL-2, IL-6, TNF-α, and IFN) [23]. Clinically, Bower et al reported a positive correlation among TNF- α levels and post-chemotherapy fatigue in women with breast cancer [24].

Abdominal symptoms are common among MPN patients and can be mostly attributed to splenomegaly, portal hypertension, mechanical obstruction, and splenic infarcts. Splenomegaly has been associated with the expansion of the malignant clone from the bone marrow microenvironment to extramedullary sites, and with specific cytokines including MIG, HGF, and IL-1RA [2].

Thrombosis, one of the principal targets of MPN therapy particularly in essential thrombocythemia (ET) and polycythemia vera (PV) patients, may result in a variety of abdominal complaints. An evaluation of 244 PV and ET patients demonstrated a positive association between the highest CRP protein tertile and the highest rate of major thrombotic events [25]. Likewise, patients with the lowest pentraxin 3 levels were at higher risk for major thrombotic events.

Microvascular events also affect MPN patients, which can result in headaches, concentration problems, lightheadedness, dizziness, vertigo, numbness/tingling, and sexual dysfunction [26]. Inflammation has been identified as a cause for cognitive impairment in both animal and human models. IL-6 deficient animals are protected from lipopolysaccharide (LPS) induced cognitive impairment, suggesting that IL-6 plays a key role in interrupting the process of memory and learning [27]. In patients suffering from hematological disorders, the presence of high levels of IL-6 usually correlates with poor executive function [28].

Weight loss in MPN is complex and multifactorial. Cancer patients often suffer from cancer cachexia, a dysregulation of carbohydrate and fat metabolism in which TNF-α is responsible for the proteolysis of skeletal muscle and the enhancement of genes related to enzymes in the ubiquitin dependent proteolytic pathway [29-32].

Pruritus has also been linked to the inflammatory cascade, which affects over 50% of the MPN population. Pruritis is predominantly observed in PV patients (65%), who have been noted to have increased number of constitutively activated and hypersensitive circulating basophils [33]. Interestingly, an increased number of mast cells was demonstrated in *Jak2*^{V617F} transgenic mice with the PV phenotype, which also may

contribute to pruritis [34]. These mast cells are a source of prostaglandin, leukotriene, histamine, and tryptase, mediators of the inflammatory response involved in pruritus. Recent studies evaluating the effects of infrared thermography have documented mast cell degranulation due to changes in temperature with the release of pyrogenic factors such as interleukins, histamine, and leukotrienes [35, 36], this may provide some explanation for pruritis after hot showers in MPN.

Lifestyle and Environmental Influence MPN Development

Although there is a familial predisposition to acquire MPN most cases are sporadic which suggests that lifestyle choices and environment may play an important role in MPN disease initiation. Cigarette smoking leads to a chronic inflammatory state that is the pre-stimulus for several chronic illnesses including cardiovascular disease, chronic obstructive pulmonary disease (COPD) and cancer. The association of smoking with different hematologic malignancies has been investigated and sufficient evidence indicates a role in the development of acute myeloid leukemia [37, 38]. Evidence for the role of cigarette smoking in MPN development has, until recently, been limited. Two studies of women only cohorts found a correlation between exposure to tobacco smoke and the incidence of myeloproliferative neoplasms. The UK Million Women Study was one of the largest studies to provide sufficient power to assess tobacco smoking as a risk for subtypes of hematological malignancies and they found that current smokers had a higher risk for developing myelodysplastic syndrome (MDS) and MPN compared to never-smokers [39]. The Iowa Women's Health Study found that current cigarette smoking was associated with

an increased risk of all MPNs and for the particular subtypes there was a stronger association for PV than ET [40]. In addition, a case-control study carried out by Sørensen and Hasselbalch studied the relationship between smoking and MPN using chronic lymphoid leukemia (CLL) patients as controls and found that a history of smoking increased the odds of developing MPN compared to CLL [41]. Very recently, a Danish population-based study found smoking to be a significant risk factor for developing MPN when comparing smokers to never-smokers [42]. Also, a meta-analysis that combined several published studies reported an increased odds ratio for MPN when comparing ever-smokers to never-smokers [43]. Interestingly, the most common somatic mutation in MPN, the *JAK2*^{V617F} mutation, is more common in smokers than non-smokers, further supporting the idea that the pro-inflammatory effects of tobacco smoke induce genetic changes in hematopoietic stem cells (HSCs) [44, 45]. The positive association between a history of smoking and the risk of MPN has been postulated to occur via chronic inflammation and oxidative stress leading to genomic instability, derivation of a malignant clone and clonal expansion in HSCs [46].

In addition to the risk for developing MPN due to smoking behavior, the effects of past or current tobacco use on MPN symptom burden is largely unknown. An internet-based survey developed by a team of MPN investigators for MPN patients has found significant differences in symptom burden between ever-smokers and never-smokers. Of the 435 patient participants, 58% reported no history of tobacco use while 42% of the population consisted of former or current users of some form of tobacco. In terms of severity of symptom burden, current and former smokers were more likely to experience significantly higher levels of fatigue, inactivity, concentration difficulties and decreased

quality of life compared to never smokers (personal communication, R. Scherber). While smoking behavior is gaining attention in MPN, it is also important to evaluate the molecular effects of smoking on HSCs in the MPN setting.

The human intestinal gut microbiome is increasingly appreciated for its role in metabolism and interaction with host immune cell populations. The host microbiome can also influence MPN phenotype since dysbiosis is now well-recognized to play a role in autoimmune diseases such as inflammatory bowel disease (IBD) including Crohn's disease and ulcerative colitis (UC) [47, 48], and graft-versus-host disease (GVHD) [49]. There also exists described associations between microbiota and hematopoiesis such as size of the bone marrow myeloid pool [50] steady-state hematopoiesis [51]. Now, it has been shown that microbiota induced differentiation of Th17 cells drives the appearance of multiple myeloma in transgenic mice and the presence of IL-17 in the bone marrow also predicted disease progression in multiple myeloma patients [52]. Interestingly, we have preliminary data indicating that the Prevotellaceae family of bacterium, as also reported by Calcinotto et al [52], is increased in MPN patients compared to normal controls and found that the cytokines TNF-alpha and IL-17a explained the most variance in microbiome data (Fleischman lab unpublished data). Further studies with larger cohorts are required to address the causal role of microbiota in driving inflammation in MPN. Considering that the enteric microbiome plays an integral role in host immunity, there are various efforts targeting the intestinal microbial communities such as fecal microbial transplantation (FMT) [53], antibiotics and anti-fungals, and dietary interventions [54] to manage or prevent chronic inflammatory conditions.

Clonal hematopoiesis is the expansion of peripheral blood cells derived from a single HSC and has been recently reported to occur during aging [55, 56]. Age-related clonal hematopoiesis has been associated with adverse clinical outcomes relating to hematologic cancer, coronary heart disease, ischemic stroke and overall mortality [55]. Clonal hematopoiesis of indeterminate potential (CHIP) refers to mutations occurring in a candidate driver genes for hematological malignancy such as DNMT3A or TET2 at a variant allele frequency greater than 2% [57]. CHIP is rare under the age of 40 but the frequency increases with age with 1 in 10 (10%) people over the age of 70 exhibiting detectable mutant peripheral blood cells and represents precursor cells for neoplasia [55]. Clonal hematopoiesis (CH) is very common in the elderly and is significantly associated with smoking behavior and was also found to associate with the number of mutations detected in CH [58]. Tobacco use was also associated with a higher likelihood of clonal hematopoiesis in patients with non-hematologic cancers [59].

Ionizing radiation is a human carcinogen with an established casual role in leukemias, particularly acute myeloid leukemia in Japanese A-bomb survivors [60]. Although no direct evidence exists for increased MPN incidence following radiation exposure, it is interesting to note that MPN patients in Ukraine, exposed to the radiation from the Chernobyl nuclear accident, exhibit a different genetic profile when compared to unexposed patients. Radiation exposed patients had a lower rate of $JAK2^{V617F}$ mutation, higher rate of the type I CALR mutation and increased number of triple negative cases in exposed subjects when compared to unexposed MPN patients [61]. These findings indicate that MPNs acquired via exposure to ionizing radiation display distinct genomic characteristics that require further investigation.

A cluster of PV exists in Pennsylvania, which raises the question of local environmental exposure, where elevated levels of radon gas was found in indoor air and radium in the soil. In 2009 The Agency for Toxic Substances and Disease Registry (ATSDR) collected peripheral blood samples from 1170 full time residents of Luzerne, Schuylkill, or Carbon County who had lived in the tri-county area for 1-year or longer and tested them for *JAK2*^{V617F} using a PCR-based method with a detection limit of 0.05% allele burden [62]. They found 19 (1.6%) residents who tested positive for *JAK2*^{V617F}, 14 of whom did not have a diagnosis or symptoms of MPN. In the majority of JAK2 positive cases without an MPN diagnosis the JAK2 allele burden was 1.2% or less. It is difficult to compare the prevalence of JAK2 positivity in this area of Pennsylvania with published reports of JAK2 screening in other populations due to differences in analytical methodologies and participant selection.

Nonpharmacological Approaches to Reduce Inflammation in MPN

Our current pharmacologic treatment of MPN often inadequately control symptom burden and have significant side effect profiles. Traditional therapies like therapeutic phlebotomies in patients with PV [63] have been shown to be ineffective and in some cases worsen symptom burden. The JAK1/2 inhibitor ruxolitinib reduces inflammatory cytokines and improves symptoms, but not without significant side effects such as thrombocytopenia, anemia, increased risk of skin cancer, and immunosuppression [64]. There is a crucial need to explore low-risk therapies to diminish inflammation in MPN, particularly in patients with early stage disease, as this may be an ideal way to reduce inflammation and empower MPN patients to change their disease trajectory (**Figure 1.2**). Besides the minority of

patients who are on interferon-alpha, little attention is placed on preventing disease progression, instead we wait until the patient progresses to myelofibrosis and then attempt to intervene. This "watch and wait" approach can make both MPN patients and physicians alike feel powerless.

Figure 2

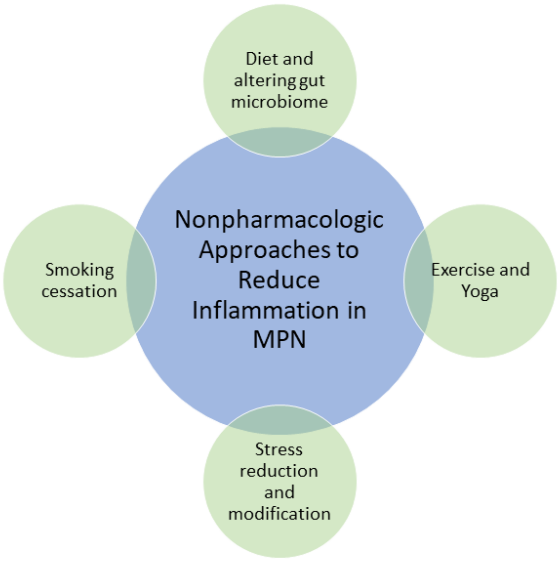

Figure. 1.2 Nonpharmacologic approaches to reduce inflammation in MPN. Maintaining a healthy lifestyle may help prevent a chronic inflammatory environment in patients with strong family history of MPN and/or patients on early stages of the disease. These nonpharmacologic approaches may be able to reduce overall inflammation with the goal of reversing some of these factors or mitigating their effects

The use of exercise to combat fatigue in malignancy is an emerging field. Recently, a study found that MPN patients who were physically active reported less fatigue than those who were not [19]. Yoga has been used effectively in various conditions related to stress and inflammation, including cancer (breast, lung, pancreatic and most recently in MPN) since it has improved the QoL by relieving stress, anxiety, depression, fatigue, and

emotional and social function [65-68]. A randomized trial investigating yoga in a breast cancer cohort demonstrated a reduction in inflammatory cytokines [69].

Yoga can produce stimulating effects on physical and mental energy, and thereby could alleviate levels of fatigue in MPN patients [68, 70]. A feasibility study consisting of 38 analyzable participants [71] followed by a qualitative study with a sample size of 39 participants [72] were conducted to explore the benefits of yoga in MPN. In both studies, MPN patients were required to complete an hour of weekly yoga for 12 weeks. Preliminary data demonstrated that online yoga was a more feasible option compared with in-person yoga because many patients travel significant distances to receive specialty MPN care. Seventy-one percent (20 of 28) of patients traveled out of town and 36% (10 of 28) traveled out of state for their MPN care, suggesting that many MPN patients may not have easy access to a facility to actively engage with for in-person interventions. Furthermore, participating in online yoga may help MPN patients overcome other limitations of in-person activities, such as fatigue, pain, transportation, and scheduling difficulties [72, 73].

At the end of both trials the research team conducted a 15- to 20-minute phone interview with participants consisting of 10 questions pertaining to patients' thoughts, feelings, and perceptions of their experiences practicing online yoga. Almost all patients reported a positive impact on their physical health as a result of yoga practice. The most common mentioned benefits were increases in physical activity, reductions in fatigue, and improved sleep. Some participants reported reduced pain, easier breathing, improved circulation, improved eating habits, improvement of their MPN symptoms, or just feeling better or more health conscious in general. It was noted, however, that the primary limitations in these studies were an overrepresentation of women (n = 34/39) and that

several participants (n = 25/39) had prior yoga experience. This prevents the ability to generalize the findings of the present trials to all MPN patients, the majority of whom likely have no experience with yoga. Larger studies investigating the impact of yoga on MPN patient QoL, symptom burden, inflammatory cytokines are forthcoming.

Diet is another nonpharmacological approach to reduce inflammation. The Mediterranean diet which is rich in fruits, vegetables, legumes, whole grains, fish, nuts, and low-fat dairy products has proven to effectively reduce CRP ($p = 0.015$) and IL-6 levels ($p = 0.025$) [74]. In addition, implementation of the Mediterranean diet has been associated with decreased incidence of various cancers such as breast, lung and colon [75-77], suggesting that it may have some preventative effects. Low-inflammation diets have been found to induce changes in thrombotic markers with decreases in homocysteine levels ($p =0.031$), white blood cell counts ($p = 0.001$), and fibrinogen levels ($p =0.025$) [78].

The PREDIMED (Prevención con Dieta Mediterránea) trial was a Mediterranean diet nutritional intervention among individuals with high cardiovascular risk, but who had not yet developed a cardiovascular disease [79]. Participants were randomized into two different Mediterranean diet groups and one control group. One of the Mediterranean diet arm was given provisions of extra-virgin olive oil and the other one was given provisions of mixed nuts. Participants in both Mediterranean arms had a significantly lower incidence of major cardiovascular events (hazard ratio 0.70 for group assigned to Mediterranean diet with extra virgin olive oil and 0.72 to the group assigned to Mediterranean diet with nuts).

Diet is an attractive tool to empower MPN patients to reduce inflammation, manage symptoms, and prevent disease progression. In February 2017, an online survey hosted by the Mayo Clinic Survey Research Center was advertised on multiple internet websites and

communities that focused on MPN [80, 81]. The purpose of this questionnaire was to collect data on demographics, MPN characteristics, nutritional habits, supplement use, and symptom burden using the MPN-10. 1,329 MPN patients from all over the world (37 countries) responded to the online survey, out of which 24% were diagnosed with MF, 37% with PV, and 38% with ET. Some of the respondents (34%) reported that they were already using diet as a measure to help control their symptoms. A wide variety of sources such as books (28.2%), websites (27.1%), health care providers such as physicians, naturopaths (28.2%), online forums (23.2%), friends (12.2%), nutritionists (9.5%), phone or tablet applications (9.1%), or videos (4.2%) were used for nutritional education. About 96% of MPN patients responded that they would be willing to restrict their diet by eating only certain foods if it helped control symptom burden and the great majority, 98% would do so if it could help stabilize or improve the course of their disease. A correlation was made between the intake of at least once per week of fast food (P=0.0007), fried foods (p=0.0198), pre-made snacks (P=0.03), soda (P<0.0001), refined sugar (P=0.01), and tacos (P=0.03) with worsened symptom score compared with no intake at all. Otherwise, patients who ingested at least once per week alcohol (p<0.0001) and rice (p=0.0452) noticed significant improvement in their symptom burden. With these last two being basic components of the Mediterranean diet, preliminary data suggests that diet may play a role in MPN symptoms and disease course.

This data provides rationale for looking at nutritional control of inflammation as a new alternative therapy to the limited interventions that have currently shown to alleviate the symptom burden of MPN patients. A feasibility trial investigating a Mediterranean Diet intervention in MPN is currently open and enrolling at University of California, Irvine. This

study will determine whether MPN patients are able to adhere to a Mediterranean Diet if given in-person dietician counseling and weekly written curriculum. The exploratory endpoints of this study are improvement of MPN symptom burden and reduction of plasma inflammatory cytokines.

Conclusion

There is an emerging body of evidence to support the importance of inflammation in disease pathogenesis of MPN. A combination of over-production of inflammatory cytokines and the inability to regulate and reduce these cytokines can lead to a state of chronic inflammation which self-perpetuates the neoplastic clone. Interventions that can reduce inflammation overall or target specific cytokines may play a role in the prevention of and future treatments for MPN. Lifestyle modification, including diet and exercise has been associated with qualitative improvement of MPN disease burden. Future research may aim to develop measurable inflammatory markers of MPN that can help define the disease and may correlate to prognosis, phenotype, progression and remission of disease. These markers may be used in conjunction with molecular studies, cell counts, age and co-morbidities to risk stratify patients' disease and therefore direct treatment.

References

Papers of particular interest, published recently, have been highlighted as:
•• Of major importance

1. Hoermann G, Greiner G, Valent P. Cytokine regulation of microenvironmental cells in myeloproliferative neoplasms. Mediat Inflamm. 2015;2015:869242.
2. Tefferi A, Vaidya R, Caramazza D, Finke C, Lasho T, Pardanani A. Circulating interleukin (IL)-8, IL-2R, IL-12, and IL-15 levels are independently prognostic in primary myelofibrosis: a comprehensive cytokine profiling study. J Clin Oncol Off J Am Soc Clin Oncol. 2011;29(10):1356–63.
3. Tyner JW, Bumm TG, Deininger J, Wood L, Aichberger KJ, Loriaux MM, et al. CYT387, a novel JAK2 inhibitor, induces hematologic responses and normalizes inflammatory cytokines in murine myeloproliferative neoplasms. Blood. 2010;115(25):5232–40.
4. Hasselbalch HC. Chronic inflammation as a promotor of mutagenesis in essential thrombocythemia, polycythemia vera and myelofibrosis. A human inflammation model for cancer development? Leuk Res. 2013;37(2):214–20. https://doi.org/10.1016/j.leukres. 2012.10.020.
5. •• Fleischman AG, Aichberger KJ, Luty SB, Bumm TG, Petersen CL, Doratotaj S, et al. TNFalpha facilitates clonal expansion of JAK2V617F positive cells in myeloproliferative neoplasms. Blood. 2011;118(24):6392–8 **This study demonstrates that JAK2 mutant hematopoietic progenitors are resistant to inflammation and that this may allow for the emergence of JAK2 mutant cells in the context of chronic inflammation.**
6. •• Kleppe M, Kwak M, Koppikar P, Riester M, Keller M, Bastian L, et al. JAK-STAT pathway activation in malignant and nonmalignant cells contributes to MPN pathogenesis and therapeutic response. Cancer Discov. 2015;5(3):316–31 **This study reveals that both mutant and non-mutant cells in MPN contribute to the inflammatory state in MPN.**
7. •• Lai HY, Brooks SA, Craver BM, Morse SJ, Nguyen TK, Haghighi N, et al. Defective negative regulation of Toll-like receptor signaling leads to excessive TNF-alpha in myeloproliferative neoplasm. Blood Adv. 2019;3(2):122–31 **This study shows that MPN monocytes have difficulty turning off inflammatory signals and this contributes to inflammation in MPN.**
8. Kristinsson SY, Landgren O, Samuelsson J, Bjorkholm M, Goldin LR. Autoimmunity and the risk of myeloproliferative neoplasms. Haematologica. 2010;95(7):1216–20.
9. Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat Genet. 2008;40(8):955–62.
10. •• Fisher DAC, Miner CA, Engle EK, Hu H, Collins TB, Zhou A, et al. Cytokine production in myelofibrosis exhibits differential responsiveness to JAKSTAT, MAP kinase, and NFκB signaling. Leukemia. 2019. https://doi.org/10.1038/s41375-019-0379-y. **This study demonstrates that JAK inhibition is not sufficient to return cytokines to normal in MPN.**

11. Fisher DAC, Malkova O, Engle EK, Miner CA, Fulbright MC, Behbehani GK, et al. Mass cytometry analysis reveals hyperactive NF kappa B signaling in myelofibrosis and secondary acute myeloid leukemia. Leukemia. 2017;31(9):1962–74.
12. Yang Y, Nath D, Dutta A, Crooks PA, Mohi G. The NF-KB inhibitor DMAPT in combination with ruxolitinib displays efficacy in Jak2V617F knock-in mouse model of myeloproliferative neoplasms. American Society of Hematology Annual Meeting; December 1, 2018; San Diego; 2018.
13. •• Kleppe M, Koche R, Zou L, van Galen P, Hill CE, Dong L, et al. Dual targeting of oncogenic activation and inflammatory signaling increases therapeutic efficacy in myeloproliferative neoplasms. Cancer Cell. 2018;33(4):785–7 **This paper highlights the importance of targeting inflammation in MPN.**
14. Johansson P, Mesa R, Scherber R, Abelsson J, Samuelsson J, Birgegard G, et al. Association between quality of life and clinical parameters in patients with myeloproliferative neoplasms. Leuk Lymphoma. 2012;53(3):441–4.
15. Mesa RA, Niblack J, Wadleigh M, Verstovsek S, Camoriano J, Barnes S, et al. The burden of fatigue and quality of life in myelo- proliferative disorders (MPDs): an international internet-based survey of 1179 MPD patients. Cancer. 2007;109(1):68–76.
16. Mitra D, Kaye JA, Piecoro LT, Brown J, Reith K, Mughal TI, et al. Symptom burden and splenomegaly in patients with myelofibrosis in the United States: a retrospective medical record review. Cancer Med. 2013;2(6):889–98.
17. Abelsson J, Andreasson B, Samuelsson J, Hultcrantz M, Ejerblad E, Johansson B, et al. Patients with polycythemia vera have worst impairment of quality of life among patients with newly diagnosed myeloproliferative neoplasms. Leuk Lymphoma. 2013;54(10): 2226–30.
18. •• Scherber R, Dueck AC, Johansson P, Barbui T, Barosi G, Vannucchi AM, et al. The Myeloproliferative Neoplasm Symptom Assessment Form (MPN-SAF): international prospective validation and reliability trial in 402 patients. Blood. 2011;118(2):401–8 **This paper describes the validation of the MPN-SAF symptom assessment form.**
19. Scherber RM, Kosiorek HE, Senyak Z, Dueck AC, Clark MM, Boxer MA, et al. Comprehensively understanding fatigue in patients with myeloproliferative neoplasms. Cancer. 2016;122(3): 477–85.
20. Bower JE, Ganz PA, Desmond KA, Bernaards C, Rowland JH, Meyerowitz BE, et al. Fatigue in long-term breast carcinoma survivors: a longitudinal investigation. Cancer. 2006;106(4):751–8.
21. Musselman DL, Miller AH, Porter MR, Manatunga A, Gao F, Penna S, et al. Higher than normal plasma interleukin-6 concentrations in cancer patients with depression: preliminary findings. Am J Psychiatry. 2001;158(8):1252–7.
22. Scherber RM, Geyer HL, Mesa RA. Quality of life in MPN comes of age as a therapeutic target. Curr Hematol Malig Rep. 2014;9(4): 324–30.
23. Kurzrock R. The role of cytokines in cancer-related fatigue. Cancer. 2001;92(6 Suppl):1684–8.
24. Bower JE, Ganz PA, Irwin MR, Arevalo JM, Cole SW. Fatigue and gene expression in human leukocytes: increased NF-kappaB and decreased glucocorticoid signaling in breast cancer survivors with persistent fatigue. Brain Behav Immun. 2011;25(1):147–50.

25. Barbui T. How to manage thrombosis in myeloproliferative neo- plasms. Curr Opin Oncol. 2011;23(6):654–8.
26. •• Geyer HL, Dueck AC, Scherber RM, Mesa RA. Impact of inflammation on myeloproliferative neoplasm symptom development. Mediat Inflamm. 2015;2015:284706 **This paper correlates specific cytokines with specific symptoms in MPN.**
27. Sparkman NL, Buchanan JB, Heyen JR, Chen J, Beverly JL, Johnson RW. Interleukin-6 facilitates lipopolysaccharide-induced disruption in working memory and expression of other proinflammatory cytokines in hippocampal neuronal cell layers. J Neurosci. 2006;26(42):10709–16.
28. Meyers CA, Albitar M, Estey E. Cognitive impairment, fatigue, and cytokine levels in patients with acute myelogenous leukemia or myelodysplastic syndrome. Cancer. 2005;104(4):788–93.
29. Tisdale MJ. Cancer cachexia: metabolic alterations and clinical manifestations. Nutrition. 1997;13(1):1–7.
30. Bossola M, Muscaritoli M, Costelli P, Bellantone R, Pacelli F, Busquets S, et al. Increased muscle ubiquitin mRNA levels in gas- tric cancer patients. Am J Physiol Regul Integr Comp Physiol. 2001;280(5):R1518–23.
31. Ramos EJ, Suzuki S, Marks D, Inui A, Asakawa A, Meguid MM. Cancer anorexia-cachexia syndrome: cytokines and neuropeptides. Curr Opin Clin Nutr Metab Care. 2004;7(4):427–34.
32. Argiles JM, Busquets S, Lopez-Soriano FJ. The pivotal role of cytokines in muscle wasting during cancer. Int J Biochem Cell Biol. 2005;37(10):2036–46.
33. Pieri L, Bogani C, Guglielmelli P, Zingariello M, Rana RA, Bartalucci N, et al. The JAK2V617 mutation induces constitutive activation and agonist hypersensitivity in basophils from patients with polycythemia vera. Haematologica. 2009;94(11):1537–45.
34. Jin X, Zhao W, Kirabo A, Park SO, Ho WT, Sayeski PP, et al. Elevated levels of mast cells are involved in pruritus associated with polycythemia vera in JAK2V617F transgenic mice. J Immunol. 2014;193(2):477–84.
35. Jackson N, Burt D, Crocker J, Boughton B. Skin mast cells in polycythaemia vera: relationship to the pathogenesis and treatment of pruritus. Br J Dermatol. 1987;116(1):21–9.
36. Wang J, Ishii T, Zhang W, Sozer S, Dai Y, Mascarenhas J, et al. Involvement of mast cells by the malignant process in patients with Philadelphia chromosome negative myeloproliferative neoplasms. Leukemia. 2009;23(9):1577–86.
37. Boyle P. Cancer, cigarette smoking and premature death in Europe: a review including the Recommendations of European Cancer Experts Consensus Meeting, Helsinki, October 1996. Lung Cancer. 1997;17(1):1–60.
38. Fircanis S, Merriam P, Khan N, Castillo JJ. The relation between cigarette smoking and risk of acute myeloid leukemia: an updated meta-analysis of epidemiological studies. Am J Hematol. 2014;89(8):E125–32.
39. Kroll ME, Murphy F, Pirie K, Reeves GK, Green J, Beral V. Alcohol drinking, tobacco smoking and subtypes of haematological malignancy in the UK Million Women Study. Br J Cancer. 2012;107(5):879–87.

40. Leal AD, Thompson CA, Wang AH, Vierkant RA, Habermann TM, Ross JA, et al. Anthropometric, medical history and lifestyle risk factors for myeloproliferative neoplasms in the Iowa Women's Health Study cohort. Int J Cancer. 2014;134(7):1741–50.
41. Lindholm Sorensen A, Hasselbalch HC. Smoking and Philadelphia-negative chronic myeloproliferative neoplasms. Eur J Haematol. 2016;97(1):63–9.
42. Pedersen KM, Bak M, Sorensen AL, Zwisler AD, Ellervik C, Larsen MK, et al. Smoking is associated with increased risk of myeloproliferative neoplasms: a general population-based cohort study. Cancer Med. 2018;7(11):5796–802.
43. Jayasuriya NA EC, Hasselbalch HC, Sørensen A. Cigarette smoking, complete blood count, and myeloproliferative neoplasms – a meta-analysis. American Society of Hematology Annual Meeting. 2017;Abstract 3199.
44. Nielsen C, Birgens HS, Nordestgaard BG, Bojesen SE. Diagnostic value of JAK2 V617F somatic mutation for myeloproliferative cancer in 49 488 individuals from the general population. Br J Haematol. 2013;160(1):70–9.
45. Weinberg I, Borohovitz A, Krichevsky S, Perlman R, Ben-Yehuda A, Ben-Yehuda D. Janus kinase V617F mutation in cigarette smokers. Am J Hematol. 2012;87(1):5–8.
46. Hasselbalch HC. Smoking as a contributing factor for development of polycythemia vera and related neoplasms. Leuk Res. 2015;39: 1137–45.
47. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ, et al. Faecalibacterium prausnitzii is an anti- inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A. 2008;105(43):16731–6.
48. Sokol H, Leducq V, Aschard H, Pham HP, Jegou S, Landman C, et al. Fungal microbiota dysbiosis in IBD. Gut. 2017;66(6):1039–48.
49. Staffas A, Burgos da Silva M, van den Brink MR. The intestinal microbiota in allogeneic hematopoietic cell transplant and graft- versus-host disease. Blood. 2017;129(8):927–33.
50. Balmer ML, Schurch CM, Saito Y, Geuking MB, Li H, Cuenca M, et al. Microbiota-derived compounds drive steady-state granulopoiesis via MyD88/TICAM signaling. J Immunol. 2014;193(10):5273–83.
51. •• Josefsdottir KS, Baldridge MT, Kadmon CS, King KY. Antibiotics impair murine hematopoiesis by depleting the intestinal microbiota. Blood. 2017;129(6):729–39 **This paper demonstrates the negative effects of chronic inflammation on hematopoietic stem cells.**
52. Calcinotto A, Brevi A, Chesi M, Ferrarese R, Garcia Perez L, Grioni M, et al. Microbiota-driven interleukin-17-producing cells and eosinophils synergize to accelerate multiple myeloma progression. Nat Commun. 2018;9(1):4832.
53. Keshteli AH, Millan B, Madsen KL. Pretreatment with antibiotics may enhance the efficacy of fecal microbiota transplantation in ulcerative colitis: a meta-analysis. Mucosal Immunol. 2017;10(2):565–6.
54. Cotillard A, Kennedy SP, Kong LC, Prifti E, Pons N, Le Chatelier E, et al. Dietary intervention impact on gut microbial gene richness. Nature. 2013;500(7464):585–8.
55. •• Jaiswal S, Fontanillas P, Flannick J, Manning A, Grauman PV, Mar BG, et al. Age-related clonal hematopoiesis associated with adverse outcomes. N Engl J Med.

2014;371(26):2488–98 **This paper describes clonal hematopoiesis of indeterminate potential and its association with adverse clinical outcomes.**
56. •• Xie M, Lu C, Wang J, McLellan MD. Age-related mutations associated with clonal hematopoietic expansion and malignancies. Nat Med. 2014;20(12):1472–8 **This paper demonstrates that clonal hematopoiesis is common among people with cancers.**
57. Steensma DP. Clinical consequences of clonal hematopoiesis of indeterminate potential. Blood Adv. 2018;2(22):3404–10.
58. Zink F, Stacey SN, Norddahl GL, Frigge ML, Magnusson OT, Jonsdottir I, et al. Clonal hematopoiesis, with and without candidate driver mutations, is common in the elderly. Blood. 2017;130(6): 742–52.
59. •• Coombs CC, Zehir A, Devlin SM, Kishtagari A, Syed A, Jonsson P, et al. Therapy-related clonal hematopoiesis in patients with non- hematologic cancers is common and associated with adverse clinical outcomes. Cell Stem Cell. 2017;21(3):374–82.e4 **This paper demonstrates clonal hematopoiesis is common in people with cancer.**
60. Preston DL, Kusumi S, Tomonaga M, Izumi S, Ron E, Kuramoto A, et al. Cancer incidence in atomic bomb survivors. Part III. Leukemia, lymphoma and multiple myeloma, 1950-1987. Radiat Res. 1994;137(2 Suppl):S68–97.
61. Poluben L, Puligandla M, Neuberg D, Bryke CR, Hsu Y, Shumeiko O, et al. Characteristics of myeloproliferative neoplasms in patients exposed to ionizing radiation following the Chernobyl nuclear accident. Am J Hematol. 2019;94(1):62–73.
62. Community health screening for JAK2(V617F) mutation Luzerne, Schuylkill and Carbon Counties, Pennsylvania In: Services USDoHaH, editor. Atlanta; 2011.
63. Barbui T, Barosi G, Birgegard G, Cervantes F, Finazzi G, Griesshammer M, et al. Philadelphia-negative classical myeloproliferative neoplasms: critical concepts and management recommendations from European LeukemiaNet. J Clin Oncol Off J Am Soc Clin Oncol. 2011;29(6):761–70.
64. Verstovsek S, Mesa RA, Gotlib J, Levy RS, Gupta V, DiPersio JF, et al. A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. N Engl J Med. 2012;366(9):799–807.
65. Buffart LM, van Uffelen JG, Riphagen II, Brug J, van Mechelen W, Brown WJ, et al. Physical and psychosocial benefits of yoga in cancer patients and survivors, a systematic review and meta- analysis of randomized controlled trials. BMC Cancer. 2012;12: 559.
66. Cote A, Daneault S. Effect of yoga on patients with cancer: our current understanding. Can Fam Physician. 2012;58(9):e475–9.
67. Harder H, Parlour L, Jenkins V. Randomised controlled trials of yoga interventions for women with breast cancer: a systematic literature review. Support Care Cancer. 2012;20(12):3055–64.
68. Sadja J, Mills PJ. Effects of yoga interventions on fatigue in cancer patients and survivors: a systematic review of randomized con- trolled trials. Explore. 2013;9(4):232–43.
69. Bower JE, Greendale G, Crosswell AD, Garet D, Sternlieb B, Ganz PA, et al. Yoga reduces inflammatory signaling in fatigued breast cancer survivors: a randomized controlled trial. Psychoneuroendocrinology. 2014;43:20–9.

70. Carson JW, Carson KM, Porter LS, Keefe FJ, Seewaldt VL. Yoga of awareness program for menopausal symptoms in breast cancer survivors: results from a randomized trial. Support Care Cancer. 2009;17(10):1301–9.
71. Huberty J, Eckert R, Gowin K, Mitchell J, Dueck AC, Ginos BF, et al. Feasibility study of online yoga for symptom management in patients with myeloproliferative neoplasms. Haematologica. 2017;102(10):e384–e8.
72. Huberty J, Eckert R, Larkey L, Gowin K, Mitchell J, Mesa R. Perceptions of myeloproliferative neoplasm patients participating in an online yoga intervention: a qualitative study. Integr Cancer Ther. 2018;17(4):1150–62.
73. van Waart H, van Harten WH, Buffart LM, Sonke GS, Stuiver MM, Aaronson NK. Why do patients choose (not) to participate in an exercise trial during adjuvant chemotherapy for breast cancer? Psycho-oncology. 2016;25(8):964–70.
74. Smidowicz A, Regula J. Effect of nutritional status and dietary patterns on human serum C-reactive protein and interleukin-6 concentrations. Adv Nutr. 2015;6(6):738–47.
75. Turati F, Carioli G, Bravi F, Ferraroni M, Serraino D, Montella M, et al. Mediterranean Diet and Breast Cancer Risk. Nutrients. 2018;10(3):326. https://doi.org/10.3390/nu10030326.
76. Castello A, Amiano P, Fernandez de Larrea N, Martin V, Alonso MH, Castano-Vinyals G, et al. Low adherence to the western and high adherence to the Mediterranean dietary patterns could prevent colorectal cancer. Eur J Nutr. 2018. https://doi.org/10.1007/s00394- 018-1674-5.
77. Schulpen M, van den Brandt PA. Adherence to the Mediterranean diet and risk of lung cancer in the Netherlands Cohort Study. Br J Nutr. 2018;119(6):674–84.
78. Chrysohoou C, Panagiotakos DB, Pitsavos C, Das UN, Stefanadis C. Adherence to the Mediterranean diet attenuates inflammation and coagulation process in healthy adults: the ATTICA Study. J Am Coll Cardiol. 2004;44(1):152–8.
79. Estruch R, Ros E, Salas-Salvado J, Covas MI, Corella D, Aros F, et al. Primary prevention of cardiovascular disease with a Mediterranean diet supplemented with extra-virgin olive oil or nuts. N Engl J Med. 2018;378(25):e34.
80. Scherber RM, Langlais BT, Geyer HL, Dueck AC, Kosiorek HE, Johnston C, et al editors. Nutrition and supplement use characteristics in the myeloproliferative neoplasms: results from the Nutrient Survey. American Society of Hematology Annual Meeting; Atlanta; 2017.
81. Scherber R GH, Dueck A, Johnston C, Langlais B, Padrnos L, Palmer J, Fleischman A, Mesa R. Nutritional needs and preferences of myeloproliferative neoplasm patients: phase 1A of the Nutrient Study. 22nd Congress of the European Hematology Association Madrid; 2017. p. P377.

CHAPTER 2

The NUTRIENT Trial: study design and primary objectives

Previous work in our lab has aimed to understand and address the inflammatory environment involving MPN. In doing so we have identified that the need to manage cytokine levels and alleviate symptom burden among MPN populations remains. Recently, studies have begun to investigate interventions with low or no risk such as lifestyle changes in the context of MPN. Scientific evidence supports that inflammation can be modulated through diet. Given the positive outcome that the Mediterranean dietary pattern has had in other inflammatory diseases, we hypothesized that MPN patients suffering from chronic inflammation may benefit from the Mediterranean diet's anti-inflammatory properties.

This study proposes a 15-week non-pharmacologic intervention that seeks to improve the quality of life among MPN patients, and to our knowledge it is the first nutritional intervention to be tried of its kind among a cohort of MPN patients. This chapter will give a detailed explanation of our study's design and setup including participant's accrual and characteristics, as well as data collection and management. Our main interest was to determine if patients were willing to restrict their diet if it helped improve symptom burden, and if following a diet with written curriculum and verbal consult was feasible for them.

Study design and participants

The NUTRIENT study was a single center interventional proof-of concept study of a dietary intervention among MPN patients performed at University of California, Irvine from October 2018 through December 2019. The primary objective of the NUTRIENT study was to assess whether MPN patients can adopt a Mediterranean eating pattern if given dietician counseling and educational materials. Thirty-one MPN patients were randomly assigned to receive either dietician counseling and written educational materials on the Mediterranean Diet (MED) or the 2015-2020 United States Dietary Guidelines for Americans (USDA), 28 participants completed the study. Participants were told they would be randomized to one of two diets that are conventionally regarded to be healthful but were not informed of the specific diets being studied nor which group they were randomized to. The US Dietary Guidelines for Americans was chosen as an intervention that would provide the participants with equal counseling attention but did not encourage a Mediterranean diet eating pattern.

We recruited individuals who were 18 years of age or older and who had been previously diagnosed with a Philadelphia chromosome negative MPN including essential thrombocythemia (ET), polycythemia vera (PV), or myelofibrosis (MF, includes primary myelofibrosis as well as post-ET or post-PV myelofibrosis). Because the intervention required active participation in counseling from a registered dietician, written educational materials delivered via email, and online surveys participants were required to fluently speak, understand, and read English and have access to email and the internet. Although this trial explored diet as a non-pharmacologic therapy, because diet was not intended to

replace a patient's ongoing treatment any type of MPN directed therapy was allowed. A complete list of inclusion and exclusion criteria are provided in **Table 2.1**.

Inclusion Criteria: • Age ≥ 18 with a previous diagnosis of a Philadelphia chromosome negative MPN including ET, PV, or MF • Any type of previous therapy is allowed • ECOG performance status ≤2 • Life expectancy of greater than 20 weeks • Has an email address and can access the internet • Able to read and understand English
Exclusion Criteria: • Pregnant women or planning to become pregnant over the course of the study • Weight loss of more than 10 pounds or 10% of the total body weight over the last 6 months • History of allergic reactions attributed to nuts or olive oil

Table 2.1 Inclusion and exclusion criteria

We screened 47 potential participants. Five did not meet the inclusion criteria, 11 participants were consented and met inclusion criteria, however they did not complete the observation period surveys and so did not progress to the intervention phase and were not randomized. Of the 31 participants who were randomized, two withdrew due to family illness and one was lost to follow-up. Demographics of the 28 patients who completed the study are shown in **Table 2.2**.

	USDA (n=13)	Mediterranean Diet (n=15)
Female n (%)	10 (76%)	10 (67%)
Age mean (range)	58 (21-77)	57 (25-71)
Disease n (%) **PV** **ET** **MF**	 6 (46%) 3 (23%) 4 (31%)	 8 (53%) 3 (20%) 4 (27%)
Mutation n (%) **JAK2** **CALR** **MPL**	 12 (92%) 0 1 (8%)	 13 (87%) 2 (13%) 0
Treatment n (%) **Hydroxyurea** **Ruxolitinib** **Interferon** **Other**	 4 (31%) 1 (8%) 2 (15%) 6 (46%)	 5 (34%) 2 (13%) 3 (20%) 5 (33%)
MPN-SAF mean (range)	15 (0-67)	11 (0-39)
Mediterranean Diet Adherence Score prior to starting intervention mean (range)	5 (1.5-9)	7 (3.5-10)

Table 2.2 Demographics of trial participants

Endpoints

We had a combined primary endpoint of both feasibility of and adherence to a Mediterranean diet assessed via online surveys. Feasibility was assessed via a single-item question on each of the online surveys administered while participants were actively receiving intervention of "how easy do you feel this diet is to follow?" on a 0 to 10 numerical score (0 very easy to 10 very difficult) with a score of <5/10 being regarded as reasonably easy to follow. Adherence was assessed using the 14-point Mediterranean Diet Adherence Screener (MEDAS) [20]. We defined good adherence to a Mediterranean style eating pattern as a score of ≥ 8 on the MEDAS. As a second mode of assessment dietary adherence completed an online 24hour diet recall using the National Cancer Institute Automated Self-Administered 24-hour (ASA24®) dietary assessment tool at weeks 1, 2, 3,

6, 9, 12, and 15. The Mediterranean-Style Dietary Pattern Score (MSDPS) was calculated for each entry using the algorithm developed by Rumawas et al [21], with a score of >25 (≥forth quintile) defined as adequate adherence.

Exploratory endpoints include reduction in inflammatory biomarkers, reduction in symptom burden, changes in hematologic parameters, lipids, and change in the gut microbiome.

Study Schedule

The total duration of the study was 15 weeks (**Figure 2.1**). During weeks 1-2 participants were monitored without intervention during which we obtained two baseline measures of unannounced dietary intake and symptom burden and one set of biological samples (blood and stool at any day during week 1). Participants received a total of 10 weeks active dietary intervention (week 3-12) starting with an in-person meeting with a dietician at week 3 followed by the distribution of dietary resources, during which 4 unannounced surveys, 4 dietary recalls, and 2 biological samples were collected (weeks 3, 6). Throughout this time they also received provisions of extra virgin olive oil (Mediterranean diet group) or gift cards (USDA group). During weeks 13-15 participants no longer received weekly educational materials. At any time during week 15, participants completed one survey, one unannounced 24 hour dietary recall, and contributed one set of biological samples (blood, stool).

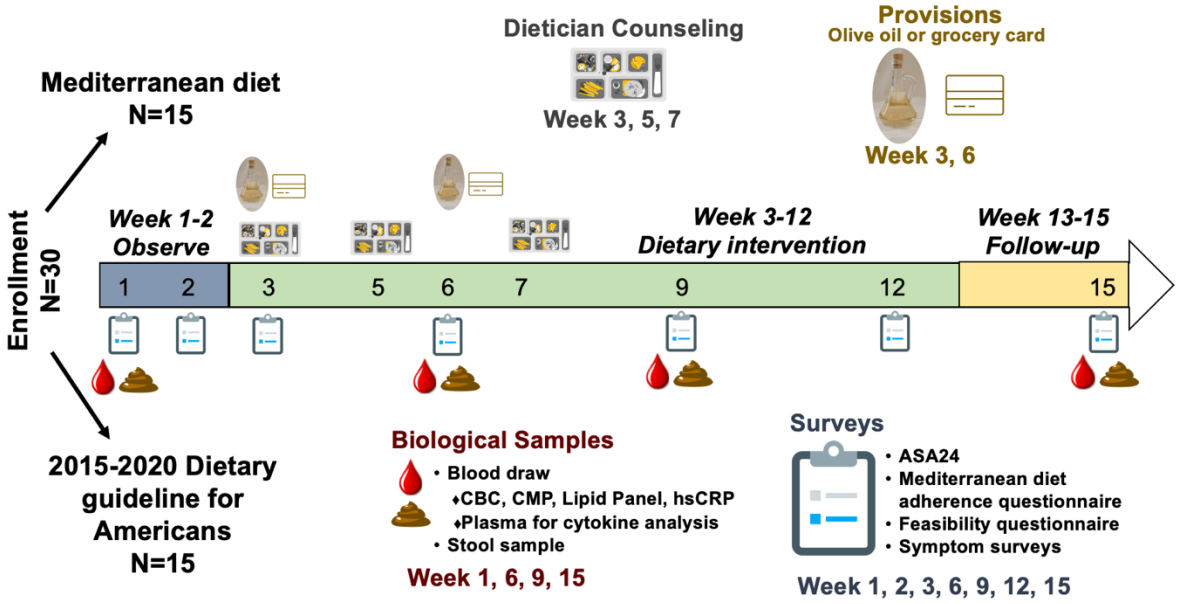

Figure 2.1 NUTRIENT study design

Intervention

Study subjects were randomized by simple randomization (1:1) before the start of the intervention phase to receive either a 10-week long Mediterranean diet or control diet (US Dietary Guidelines for Americans) intervention with data collection extending two weeks before and three weeks after diet intervention. All participants met once at the start of the intervention period (week 3) with a registered dietician for one-on-one counseling to educate the participant on the central components of the Mediterranean diet or the US Dietary guidelines, and to tailor the diet to meet each participant's medical needs and/or cultural preferences. Participants had two brief follow up dietary counseling visits during week 5 and 7. Participants were emailed 10 weekly installments of educational materials

on their respective diet in a colorful pdf format during week 3-13. All participants in the Mediterranean diet arm were given 750ml of extra virgin olive oil (EVOO) at week 3 and 6, and all participants following the standard US Guidelines diet were given a $10 grocery gift card at week 3 and 6. During this time period participants completed four online surveys (week 3, 6, 9, 12) and donated two sets of blood, stool, and urine samples (weeks 6, 9).

Data Collection

Patient Reported Outcomes:

Besides two initial assessments, six unannounced surveys and 24-hour food recalls were collected throughout the duration of the 15-week study. Conformity with the Mediterranean dietary pattern was assessed by the 14-item Mediterranean diet adherence score (MEDAS). Symptom burden was assessed using the MPN symptom assessment form (MPN-SAF TSS or MPN-10), a standardized tool used in clinical trials.

Laboratory Studies:

Four biological sample data points were collected during the 15-week study which included collection of blood, stool, and urine. A certified phlebotomist drew a total amount of 50cc of blood from participating subjects at week 1, 6, 9, and 15. A portion of this blood was sent to pathology for measurement of complete blood count (CBC), comprehensive metabolic panel, lipid panel, and hs-CRP. 3-4 ml of blood were centrifuged to obtain plasma and stored at -80°C for cytokine measurements to be performed. The remainder of blood was flash frozen into 1ml aliquots. Participants were given a urine specimen cup to provide

a urine sample of at least 50ml during weeks 1, 6, 9, and 15 to measure urine total polyphenol (TPE), a biomarker of adherence to the Mediterranean diet. The samples were aliquoted into 500ul tubes (in triplicate) and flash frozen. They are currently stored at -80°C for measurement of TPE in future studies. Stool sample collection is discussed in the microbiome section. These specimens were used to measure changes in the gut microbiome with the diets.

Anthropometric measures:

Weights were measured at every visit (weeks 1, 6, 9, 15) although the purpose of this study was not to lose weight. Recording an accurate body weight is fundamental for nutrition screening. In the context of myeloproliferative neoplasms this is critical because undesired weight loss can be a sign of disease progression, especially in patients with myelofibrosis [22].

Data management

Participants were blinded to which diets were being tested and the diet that they were randomized to. Consent and HIPAA forms were signed on the day of enrollment and all the paperwork was kept in a separate file folder for each individual. For privacy, their personal identifications were replaced for an FEA number which stands for feasibility. We shared their data such as laboratory studies (blood counts, chemistries, lipids) with them upon request throughout the course of the study. The surveys were administered via Qualtrics and the 24-hour food recalls were entered using the Automated Self-

Administered 24-hour (ASA24®) Dietary Assessment Tool from the NCI's website. Access links were automatically sent on due dates from our We Are MPN research email. All data was entered into an Excel sheet for ease of analysis.

Compliance Behavior with Online Surveys

Participants completed the week 1 survey and 24 hour diet recall (ASA24®) at time of enrollment. Subsequently participants received an email alerting them to complete a Qualtrics survey and a 24 hour diet recall on a random day during weeks 2, 3, 6, 9, 12, and 15. If surveys had not been completed, email reminders were sent every two days, if surveys had not been completed after two emails a follow up phone call was done. With the use of reminders 100% of surveys and 24 hour diet recalls were completed. We investigated whether demographics such as age or gender correlated with the need for reminders to complete the surveys. We reasoned that older participants would be less technology savvy and may not check email regularly, resulting in reduced compliance with survey requests. However, older participants did not require more reminders than younger participants (**Figure 2.2A**), demonstrating that older individuals are just as capable of participating in online studies as younger individuals. In addition, we found similar needs for reminders based on gender (**Figure 2.2B**) and dietary intervention group (**Figure 2.2C**).

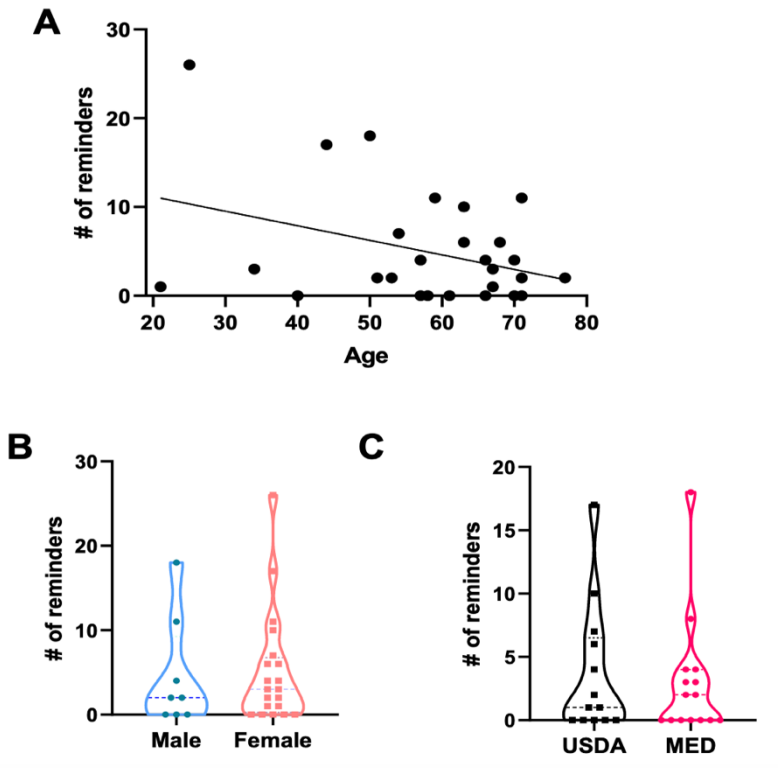

Figure 2.2 Compliance behavior as a function of age and gender. (A) Total number of reminders required as a function of participant age. (B) Total number of reminders required based on gender. (C) Total number of reminders required by treatment group.

Feasibility of a Mediterranean Diet in MPN Patients

The primary objective of this study was to assess whether a Mediterranean diet intervention is feasible in the MPN patient population. To assess ease of the diet intervention among MPN patients during the 10 week active intervention phase surveys also included a single feasibility question, asking "how easy is it for you to follow this diet, with 1 being very easy to follow and 10 being very difficult to follow". We defined feasibility as reporting a score of <5 on all surveys. Seventy percent of the patients on the USDA diet achieved this feasibility benchmark, and 79% of patients in the Mediterranean arm achieved this feasibility benchmark (**Figure 2.3A**). This demonstrates that a

Mediterranean diet is at least as feasible to follow for MPN patients as the standard US Dietary Guidelines for Adults.

Adherence to the Mediterranean diet was assessed at weeks 1, 2, 3, 6, 9, 12, and 15. Using a 14-point Mediterranean Diet Adherence Score (MEDAS). Because in some cases week 3 data was collected before the dietician counseling sessions and in other cases after the dietician counseling session this week's data was not included in the analysis. For future studies we will ensure all participants undergo dietician counseling prior to any data collection during the defined intervention period. A MEDAS score of ≥8 was defined as having good adherence to a Mediterranean style diet. Our pre-defined goal was to have at least 80% of the participants in the Mediterranean group maintaining a MEDAS score of ≥8 over the entire active intervention period (**Figure 2.3B**). At weeks 6 and 9 >80% of participants on the Mediterranean arm achieved a score of ≥8. At week 12, 78% achieved a score of ≥8 which was slightly below our predefined adherence goal. In contrast, at no time point did at least 80% of the USDA group achieve a MEDAS score of ≥8. This demonstrates that with dietician counseling and education MPN patients can achieve adequate adherence to a Mediterranean diet eating pattern. However, the lull in adherence at week 12 suggests that ongoing active dietician counseling is helpful to maintain adequate adherence to a Mediterranean diet eating pattern. In this current study dietician counseling sessions occurred during weeks 3, 5, and 7 and participants received written curriculum weekly during weeks 3-13. For future studies dietician counseling session will occur throughout the active intervention period.

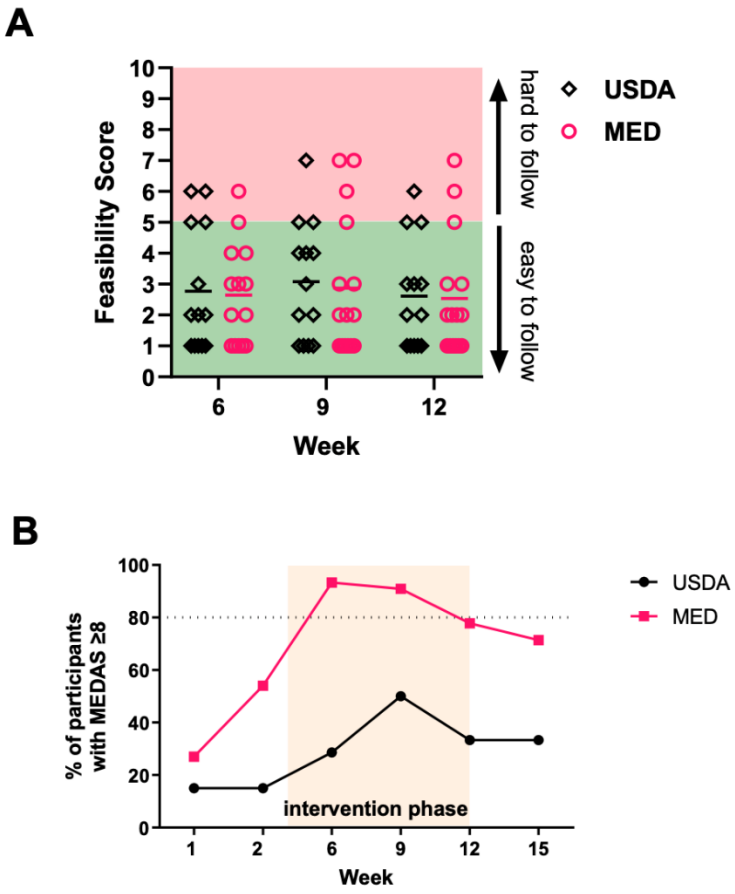

Figure 2.3 Feasibility and adherence score. (A) Participants who reached the feasibility benchmark during the diet intervention phase. (B) Percentage of participants with MEDAS ≥8 over the course of the 15-week trial.

Conclusions

In summary, we observed that older individuals are just as capable of participating in online studies as younger individuals since they did not need more reminders when compared to the younger population. This is important to know as the majority of patients suffering from MPN are over 65 years old. We also found that there was really no difference in the number of reminders needed between males and females as well as when comparing both of the dietary interventions.

The primary objective of this study was to assess the feasibility of a Mediterranean dietary intervention among MPN patients. We demonstrated that for the patients in our cohort a Mediterranean diet is at least as feasible to follow as is a standard USDA diet. We achieved our predefined goal for Mediterranean diet adherence score as greater than 80% of the participants in the Mediterranean diet group maintained a MEDAS ≥8 for the majority of the active intervention period. We can conclude that with dietician counseling and written education MPN patients can achieve adequate adherence to a Mediterranean diet eating pattern. However, ongoing active dietitian counseling might be needed towards the end of the intervention to maintain adequate adherence to the diet that they were assigned to.

CHAPTER 3

The NUTRIENT Trial: biological correlates and exploratory endpoints

Abnormally high levels of inflammatory cytokines contribute to the MPN disease phenotype characterized by thrombosis, bone marrow fibrosis, splenomegaly, and constitutional symptom burden which cause a negative impact in the quality of life of patients. Our diet intervention examined the preliminary effects of a well-established anti-inflammatory diet on cytokine expression, symptom burden management and disease related markers. This non-traditional approach to a chronic hematologic malignancy represents an innovative and integrative method to target inflammation. All biological correlates and exploratory endpoints will be addressed in this chapter.

Because of the overproduction of myeloid lineage cells in MPN it was reasonable to examine blood tests, though no clinically relevant impact was expected from the diet. Complete blood counts (CBC) are performed routinely to monitor signs of disease progression and disease management. Preventing blood clots and bleeding is particularly important in treating MPN. These disease related markers show alterations according to the MPN subset and will be discussed in detail further along. This chapter also presents analyses of the nutritional information gathered from the self-reported food recalls which include intake of total calories and fats, macro and micronutrients, and fiber. We only focused on those relevant to the Mediterranean dietary pattern.

Changes in eating patterns over the course of the study

The 24-hour diet recalls using the ASA24® platform gave us detailed information about their dietary intake over the preceding 24 hours. The total calories consumed did not change during the active intervention period in either group (**Figure 3.1A**) nor was there an impact on the participant's weights (**Figure 3.1B**). To mention that this study was not geared towards weight loss, but rather focused on maintaining a healthy lifestyle. Participants following the Mediterranean diet can consume a different amount of energy (i.e. calories) and still be following the diet. The total number of calories eaten will depend on whether they're trying to lose, gain, or maintain weight. Following a Mediterranean diet can significantly improve metabolic health even in the absence of weight loss. For future studies measuring the body mass index (BMI) rather than simply weight may be a more accurate indicator of health.

Comparison of intake of macronutrients on diet arms

According to the Mediterranean dietary pattern, the daily intake of macronutrients aims to be: 50% of total calories from carbohydrates (emphasizing whole grains, fruits and vegetables), 35% of total calories from fat (with primary source as extra virgin olive oil), and 15% of total calories from proteins. We also displayed visually the intake of macronutrients on both diet arms during the intervention period (**Figures 3.1C and 3.1D**).

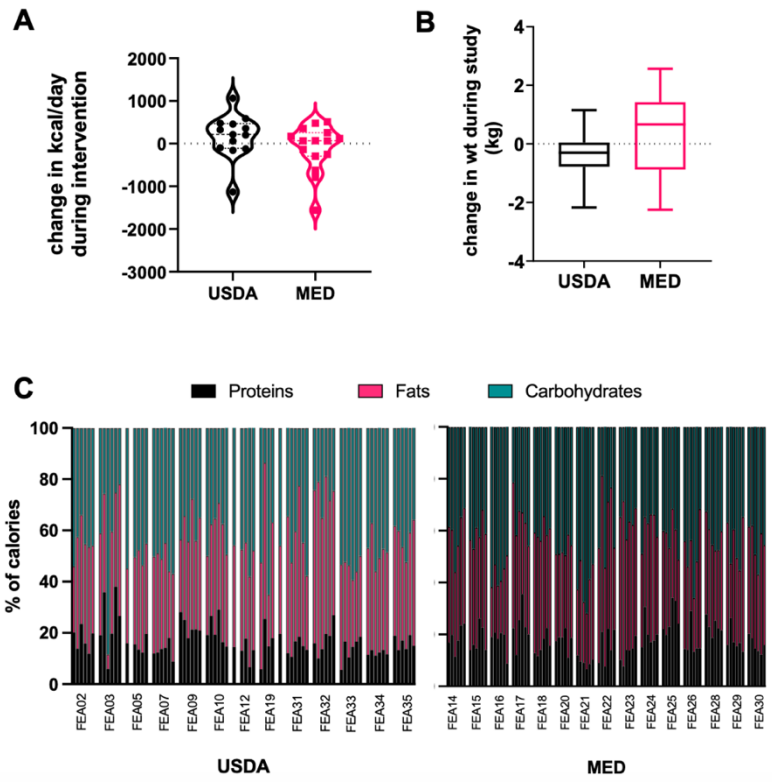

Figure 3.1 Changes in kilocalories, weights, and macronutrients. (A) Changes in the intake of kilocalories (kcal) per day by both diet groups during the diet intervention. Baseline calories was the average of the total calorie intake on the two ASA24s during the lead-in period, intervention calories was the average calories consumed on the ASA24s during weeks 6, 9, and 15. (B) Changes in weight for each of the arms throughout the study. We compared the average weight during weeks 6, 9, 15 versus the baseline weight. (C) Visual representation of the percentage of calories coming from the macronutrients consumed by the participants in both of the diet groups during the intervention period.

Fats

Fat intake under the Mediterranean diet is moderately high (35% total calories). It consists predominately of monounsaturated fats (e.g. olive oil) as opposed to polyunsaturated (e.g. canola oil) and saturated (e.g. animal) fats. Less than 10% of total calorie intake should be from saturated fats. We expected the Mediterranean diet group to show a decrease in consumption of saturated fats and an increase in monounsaturated fats. The increase in monounsaturated fats would be consistent with an increased intake of

extra virgin olive oil, vegetables, nuts, seeds, and fish as part of this dietary pattern. We did not find any significant change in the percentage coming from the different types of fats when comparing the USDA versus MED group, however perhaps this is due to participants not being specifically screened for normative dietary pattern (**Figure 3.2**).

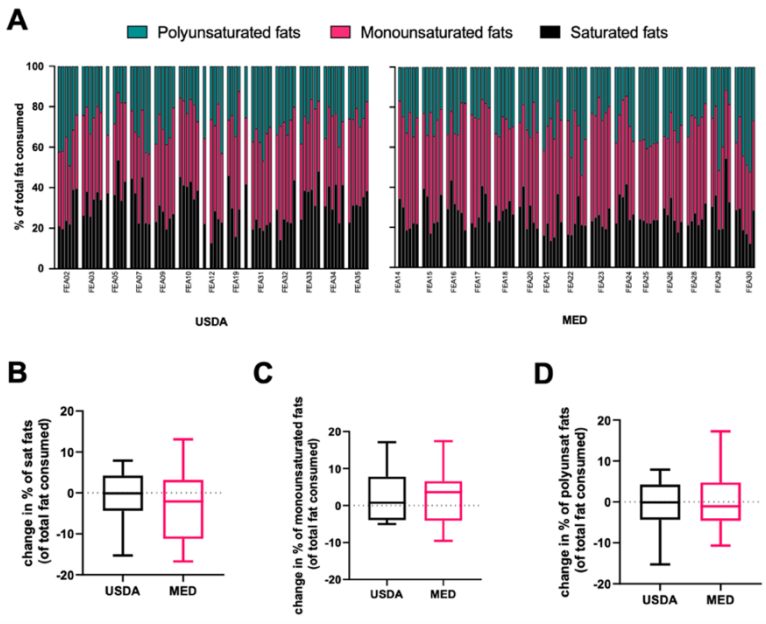

Figure 3.2 Consumption of fats during the intervention period. (A) Percentage of each type of fat consumed by both study groups during the diet intervention. Percent change in intake of (B) saturated fats, (C) monounsaturated fats, (D) and polyunsaturated fats throughout the intervention period for each diet arm. We calculated the average intake on the 2 ASA24s during the lead-in period as our baseline and the average intake of the ASA24s during weeks 6 and 9 as the intervention period.

Dietary Fiber

A distinctive characteristic of the Mediterranean dietary pattern is the abundance of foods rich in fiber content, such as fruit, vegetables, legumes, cereals, and nuts. According to the American Heart Association (AHA), the daily value for fiber is 25 grams per day on a 2,000-calorie diet for adults. This number may also depend on age or sex. For women

under 50 an intake of 25 grams of fiber per day is recommended, as are 21 grams of fiber per day for women over 50 years old. Men under 50 should consume 38 grams of dietary fiber per day, and men over 50 about 30 grams per day. During the active intervention we expected the participants in the Mediterranean diet group to reach their daily fiber intake target or to go over it. However, about half of the participants in both groups (53 and 54%) managed to reach their respective targets (**Figure 3.3A**). Only 25% of the males reached their daily fiber intake as compared to 65% of the females in both diet arms. This result suggests that females are more likely to reach their fiber per day target than males. To further explore the consumption of dietary fiber we looked at the percent change of fiber intake while on the intervention period. Similarly, about a quarter of the participants (23 and 27%) had a 50% increase in their fiber intake during the active intervention (**Figure 3.3B**).

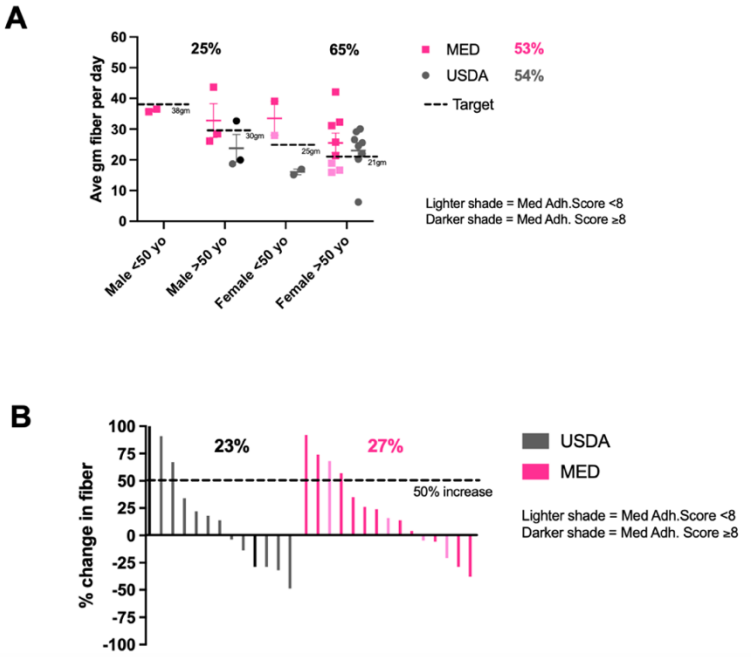

Figure 3.3 Daily intake target and percent change in fiber. (A) Average grams of fiber consumed per group during the diet intervention according to daily targets recommended for age and sex. (B) Percent change in the intake of fiber comparing both diet groups during the diet intervention period.

Vitamin and antioxidant intake

From the information reported by the 24 hour food recalls we were able to look at vitamins and minerals that are abundant in a Mediterranean style diet. The main vitamins acquired from a Mediterranean dietary pattern are vitamin C and E, as well as B-carotene as precursor of vitamin A. We were interested in knowing whether they were differences between the study groups in reaching their daily intake targets during the intervention period.

There are different daily intake targets for vitamins A and C according to sex: 900 mcg RAE for males and 700 mcg RAE for females. When comparing the intake of these vitamins during the intervention we found that a higher percentage of women were able to reach and go over their target than men. While looking at the two diet groups we didn't see a drastic difference (**Figures 3.4A and 3.4B**). About half of the participants in the Mediterranean diet started the trial having achieved their intake target of vitamin E and kept it stable during the intervention period. Most of the patients who were part of the USDA group were under their vitamin E target and did not manage to increase their levels while following the dietary pattern (**Figure 3.4C**).

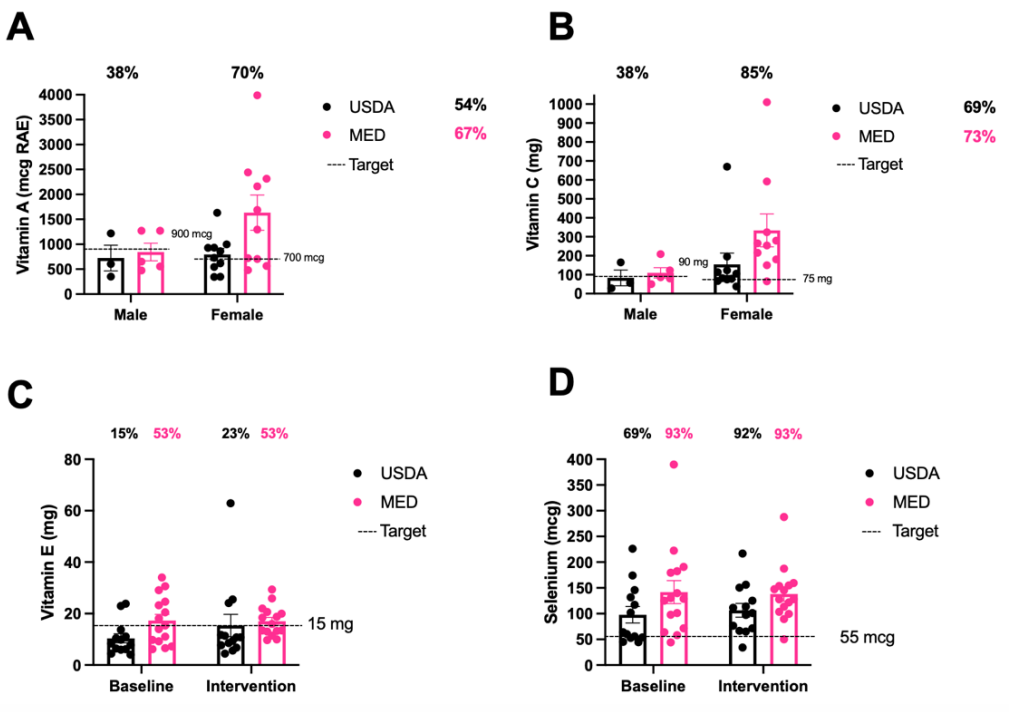

Figure 3.4 Targets for vitamin and antioxidant intake. Average intake of (A) vitamin A and (B) vitamin C during the intervention period separated by sex and diet groups. Average intake of (C) vitamin E and (D) selenium comparing baseline to intervention between both diet arms.

The Mediterranean diet provides high amounts of antioxidants. We focused on selenium because of its protective effect in cancer. Almost all of the participants in the Mediterranean group had already met their selenium daily intake target at baseline and maintained it throughout the diet intervention. Starting with 69% the USDA group obtained a higher selenium intake per day during the active intervention (92%) which is comparable to that of the Mediterranean group (93%) (**Figure 3.4D**).

Impact of diet on symptom burden

Symptom burden was assessed using the MPN-SAF TSS/ MPN-10, which grades the 10 most clinically relevant symptoms of MPN patients. The MPN-10 measures the worst level of the symptom (e.g., fatigue) on a 0 (no fatigue) to 10 (worst imaginable) in the past week. This tool has been validated to measure symptom burden in the MPN population. In addition extended questions were asked regarding gastrointestinal symptoms.

The waterfall plots shown on **Figure 3.5A** represent the percent change in MPN symptom burden for both diet arms throughout the course of the intervention period (weeks 6-12) and at the end of the study (week 15). We felt it important to quantify symptom burden at week 15 to determine if perhaps the diet intervention had a lasting impact that went beyond the active intervention period. A slightly larger number of participants following the Mediterranean diet had a 50% reduction in symptom burden when compared to the USDA group. Although, a smaller percentage of the patients following the USDA diet reached the symptom reduction benchmark we can identify a downward trend that extends until the end of the trial, suggesting that adopting a healthy eating pattern improves symptom burden regardless of a specific diet. **Figure 3.5B** shows the raw change in score for every individual symptom evaluated by the MPN-SAF TSS. In our surveys we also included questions about gastrointestinal symptoms. We can conclude that no particular symptom is being affected more than the other by either of the diets. The Mediterranean diet has no greater impact than the USDA diet over any specific symptom.

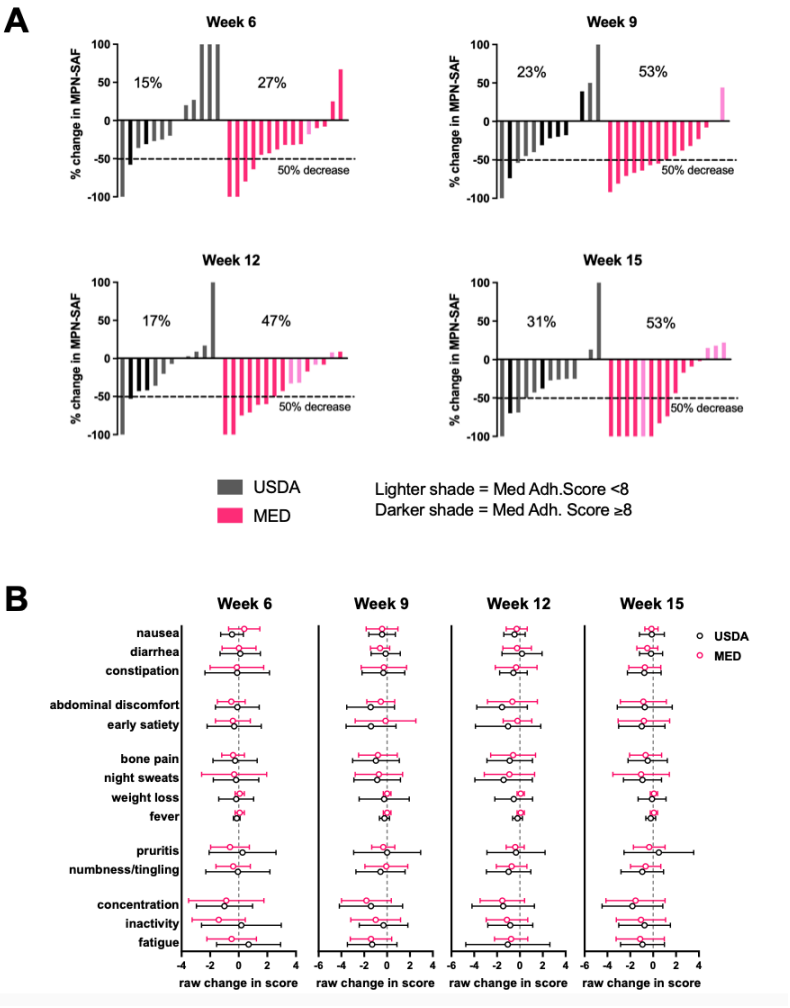

Figure 3.5 Effect of diet intervention on symptom burden. (A) Percent change in MPN symptom burden score for both diet arms throughout the intervention period (weeks 6-12) and at the end of the study (week 15). (B) Raw change in MPN symptom burden score for every individual symptom evaluated by the MPN-SAF.

Impact of diet on inflammatory biomarkers

Many factors can be associated with elevated C-reactive protein (CRP) levels in plasma. Therefore, it is considered a nonspecific marker of inflammation and infection [23]. CRP is a pentameric acute-phase protein produced by the liver. It rises following the secretion of interleukin 6 (IL-6) by macrophages [24] and adipocytes [25].

The high-sensitivity C-reactive protein (hs-CRP) is a blood test that can detect even lower levels of chronic inflammation in the body. Average levels range from 0.1 to 0.3 mg/dL. High-sensitivity C-reactive protein was measured at each visit (weeks 1,6,9, and 15). Although most participants fall in a normal range, among those who are elevated in the Mediterranean diet group there seems to be a downward trend towards the end of the trial (**Figure 3.6**). This observation suggests that perhaps a longer intervention is needed to see a possible effect of the Mediterranean diet in decreasing levels of hs-CRP. Also, this data may help inform future studies, those with high hs-CRP may be ideal candidates for interventions.

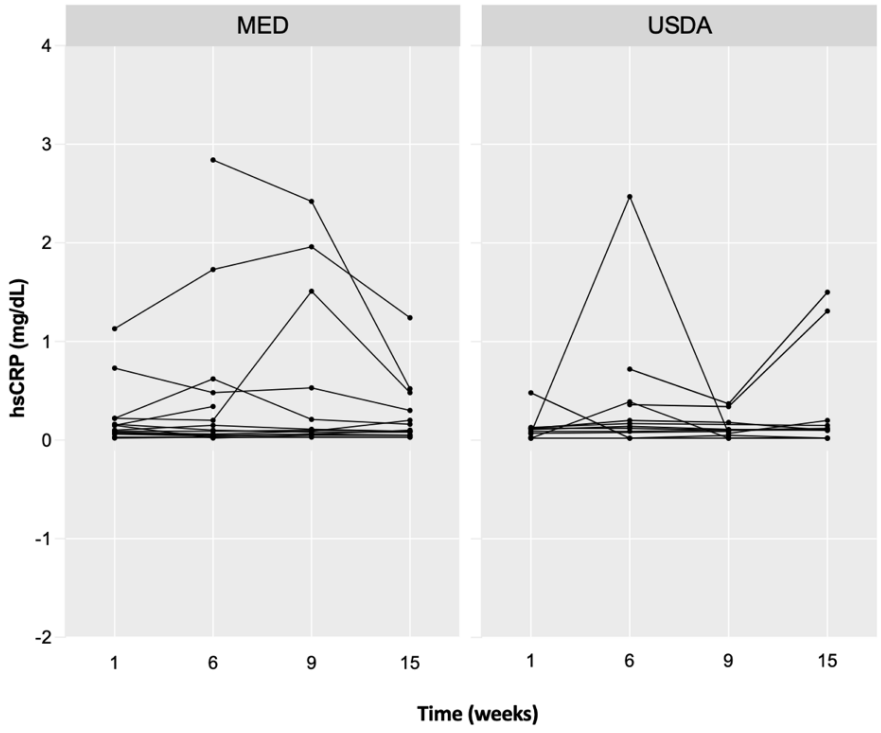

Figure 3.6 Longitudinal measurements of high-sensitivity CRP throughout the course of the study. Average levels range from 0.1 to 0.3 mg/dL.

In addition, plasma was stored to perform a multiplexed cytokine assay to quantify and investigate the potential impact of a Mediterranean diet on the circulating levels of inflammatory cytokines in MPN patients. Pro-inflammatory cytokines, particularly tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6) are overexpressed in patients with myeloid malignancies, and they are linked to disease initiation, symptom burden, disease progression and a worsened prognosis. This suggests that these cytokines play a role in the fundamental aspects of the development and/or manifestations of hematologic malignancies. Likewise, the anti-inflammatory cytokines, IL-4, IL-10 and IL-13 are commonly elevated in patients suffering from myeloid malignancies, conceivably as an attempt to dampen the chronic inflammatory state [26].

The panel consisted of 10 cytokines (IL-12p70, IL-1B, IL-4, IL-5, IFNγ, IL-6, IL-8, IL-22, TNFα, and IL-10) out of which only 50% of them came into the detectable range. The longitudinal measurements for these 5 cytokines (TNFα, IL-6, IL-8, IL-10, IL-22) are shown in **Figure 3.7**. No significant changes on cytokine expression levels were detected in either of the diet groups throughout the course of the trial. Potentially longer time periods or other more sensitive methods for detecting subtle changes in inflammatory markers are needed.

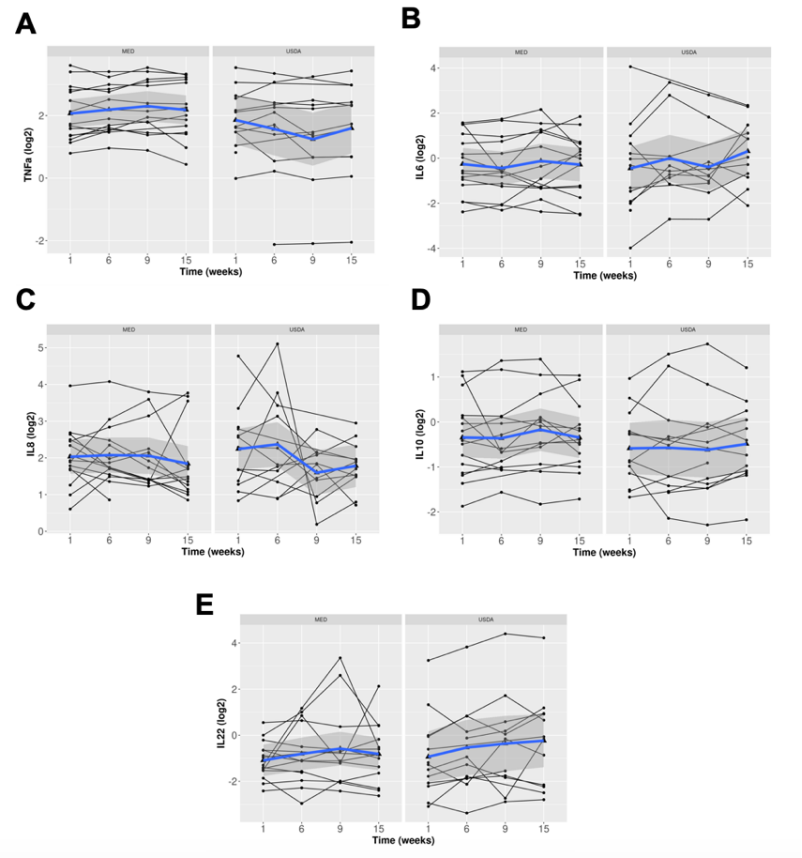

Figure 3.7 Expression levels of different cytokines by time – (A) TNF-α, (B) IL-6, (C) IL-8, (D) IL-10 and (E) IL-22.

Impact of diet on blood counts

Patients suffering from MPN have abnormal blood counts. These alterations depend on the subset of the disease. Generally, those with essential thrombocythemia will have elevated platelet counts, those with polycythemia vera will have excessive red blood cells, and those with primary myelofibrosis may have immature white cells in the peripheral blood and often have low red blood cells and platelets.

The normal range of red blood cells per microliter is usually 4.7 million to 6.1 million for men and 4.2 million to 5.4 million for women. The normal range of white blood

cells (WBC) per microliter is usually 5,000 to 10,000 for men and 4,500 to 11,000 for women. Normal hematocrit (HTC) levels are 42 percent to 52 percent of the total blood count for men and 37 percent to 47 percent for women. Platelet counts (PLT), regardless of age or gender, are considered normal at 150,000 to 400,000 per microliter.

Complete blood counts (CBC) are of particular interest in these blood cancers because they allow for proper monitoring of disease progression and dictate the treatment to follow. Thrombosis and bleeding are major complications in essential thrombocythemia. The first-line treatment for ET as well as for PV involves antiplatelet therapy with low-dose aspirin. Occasionally, aspirin will cause excessive bleeding as a side effect, reason why its use needs to be carefully monitored. For polycythemia vera the treatment goal is to keep the blood's hematocrit level below 45 percent. Phlebotomy (therapeutic blood drawing) is commonly used to reduce the number of red blood cells. However, if given too frequently, it can result in anemia from removal of the red cells. This is monitored by periodic blood testing.

Changes over time were reported on blood counts. **Figure 3.8A** shows that the white blood cells count for almost all of the participants in both diet groups fell in normal range. No trend could be identified out of the few that were above 10, 500 cells per microliter. Throughout the entire trial, the majority of the participants in both the Mediterranean diet and USDA groups were able to maintain their hematocrit below 45 percent (**Figure 3.8B**). Most of the participants in both study arms had elevated platelet counts, above 400,000 per microliter. Interestingly, each participant's platelet levels were remained relatively stable over the course of the study (**Figure 3.8C**). In summary, we did not detect an obvious impact of either of the diets on MPN patients' blood counts.

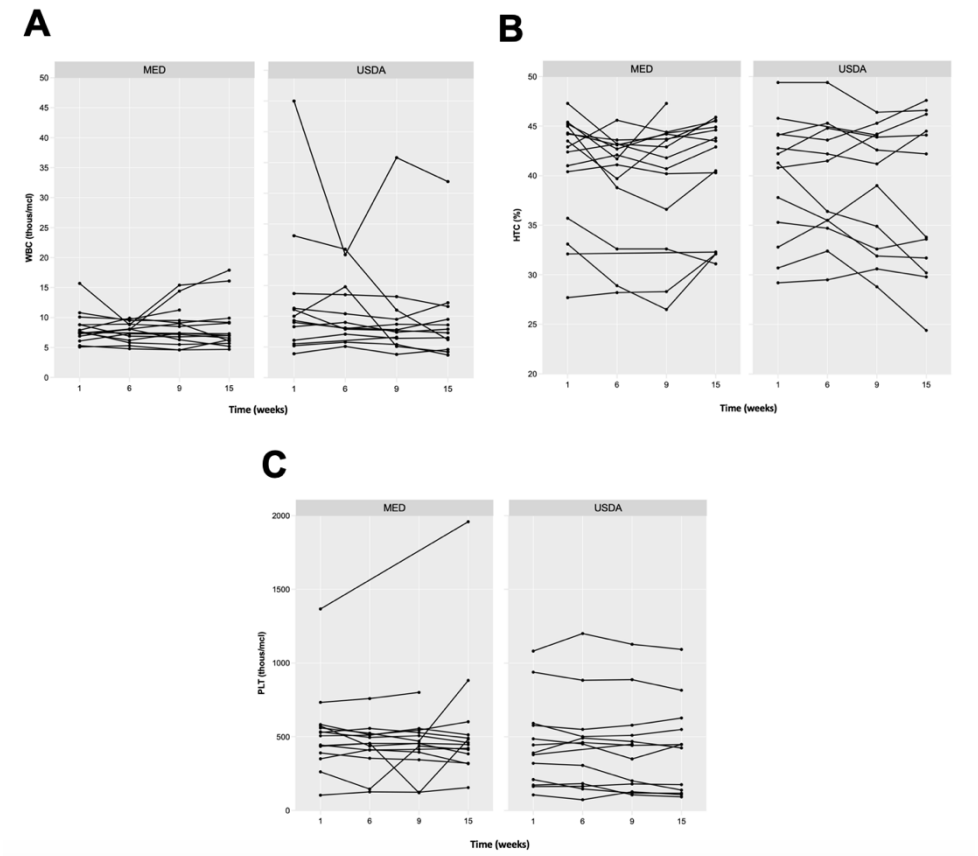

Figure 3.8 Longitudinal measurements of complete blood counts comparing both diet groups. (A) White blood cells (B) Hematocrit (C) Platelets.

Conclusions

From the data presented in this chapter we can conclude that the impact of the Mediterranean diet is not necessarily on consumption of total calories, weight changes, or macro and micronutrients intake. We observed that the diet intervention didn't change the percentage of calories coming from proteins, fats, and carbohydrates, nor was there a significant change in the distribution of the different types of fats. Therefore, we identified the need to give specific guidance on healthy and unhealthy fats to help participants in the Mediterranean diet increase their consumption of monounsaturated fats as we expected

they would do. Subsequent Mediterranean diet interventions should focus on increasing the intake of fiber. To achieve this we should also offer detailed guidance on daily intake targets according to age and sex groups. We noticed that females are most likely to reach their intake targets not only for fiber, but for also for other vitamins and antioxidants that we looked at in this study. Therefore, we may need to personalize guidance based on gender.

About half of the participants in the Mediterranean diet group had at least a 50% reduction in symptom burden towards the end of the study. A smaller percentage of participants following the USDA diet also showed a reduction in symptoms. This observation suggests that adopting a healthy eating pattern improves symptom burden regardless of the type of diet. In the Mediterranean diet group, among the participants with high levels of hs-CRP a downward trend was observed at the end of the trial. Meaning that perhaps a longer intervention is needed to see a possible effect of the Mediterranean diet in decreasing levels of hs-CRP. Because we did not detect significant changes on cytokine levels in either of the diet groups throughout the course of the study, we propose that longer time periods or other more sensitive methods for detecting subtle changes in inflammatory markers are needed. Lastly, there was no impact of any of the diets on blood counts and we weren't really expecting for diet alone to modify this parameter.

CHAPTER 4

Fecal microbial community composition in myeloproliferative neoplasm patients is associated with an inflammatory state

Short title: Gut microbiome in myeloproliferative neoplasm

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Data Sharing: protocol methods will be available on github

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Contributions

AO, JA-B, and CH analyzed data and wrote the paper, KEA collected data, analyzed results, and wrote the paper, LML collected data, KW analyzed data and wrote the paper, AGF conceived the study, analyzed results, and wrote the paper.

Conflict of Interest

No authors declare any conflict of interest

MAIN TEXT

The human microbiome may influence inflammatory and malignant diseases, with widespread implications for diagnostics, prevention, and therapy. Moreover, there is an expanding appreciation for associations between the gut microbiome and hematopoiesis. Studies involving the microbiome in hematologic malignancies have primarily focused on acute myeloid leukemia and hematopoietic stem cell transplantation, specifically evaluating the impact of the microbiome on infection [1], hematopoietic reconstitution and graft versus host disease (GVHD) [2]. To date, no studies have investigated the gut microbiome of myeloproliferative neoplasm (MPN) patients.

MPN is a hematologic malignancy with a hallmark feature of chronic inflammation. The inflammation in MPN is multifactorial; the neoplastic clone itself induces inflammation, however chronic inflammation may precede the development of MPN and play a critical role in disease initiation. Disease manifestations are variable among MPN patients, even those with identical MPN driver mutations. This suggests that other forces modulating inflammation play an instructive role in MPN disease manifestation. We conducted a pilot study to test the hypothesis that the microbiome of MPN patients is distinct from healthy controls, and that changes in gut microbiome composition may be associated with MPN development and clinical manifestation.

A total of 25 patients with MPN and 25 non-MPN controls participated in this initial pilot study, exclusion criteria included gastrointestinal disease (e.g. inflammatory bowel disease, malabsorption, malignancy), and history of pelvic irradiation. All participants completed a survey which included questions about demographics, lifestyle, and other

clinical covariates of interest (**Table 4.1**). The MPN cohort additionally completed questions on disease characteristics, treatment regimens and symptom burden using the Myeloproliferative Neoplasm Symptom Assessment Form (MPN-SAF) (**Table S4.1**). The questionnaire was completed at time of enrollment in the study.

	MPN group (N=25)	Non-MPN controls (N=25)
Age - median (range)	66 (26-91 years)	52.5 (26-78 years)
Sex - N (%)		
Male	13 (52%)	9 (36%)
Female	12 (48%)	16 (64%)
Race		
Caucasian	24 (96%)	19 (76%)
Asian	1 (4%)	6 (24%)
African American	0	0
Native American	0	0
Ethnicity		
Hispanic	1 (4%)	1 (4%)
Non-Hispanic	24 (96%)	24 (4%)
Antibiotics exposure (> 2 weeks)	6 (24%)	1 (4%)
Auto-immune disease	6 (24%)	1 (4%)
Family history		
Hematologic malignancy	7 (28%)	3 (16%)
MPN	2 (8%)	3 (16%)
Auto-immune disease	6 (24%)	3 (16%)
Solid cancer	8 (32%)	7 (28%)
Born by Cesarean section	23 (92%)	19 (76%)
Any Supplements	22 (88%)	15(65%)
Probiotics	3 (12%)	1 (4%)
Antioxidants	2 (8%)	4 (18%)
Specific diet	9 (36%)	6 (24%)
Breast milk fed at birth	9 (36%)	14 (56%)
Formula fed	5 (20%)	4 (18%)
Both	2 (9%)	4 (18%)
Unknown	8 (32%)	2 (9%)

Table 4.1 Characteristics of Subjects

Participants collected three fecal samples over the course of one week. We performed 16S rRNA gene sequencing. To investigate whether having MPN was associated with changes in alpha diversity within the gut microbiome, we analyzed the number of distinct species (richness) and the distribution (evenness) of those species. Both richness and evenness did not significantly differ between MPN patients and healthy controls (**Figure 4.1A**). The most abundant bacterial taxa found in this cohort came from the taxonomic families *Ruminococcaceae* (mean 32.1%), *Lachnospiraceae* (26.7%), and *Bacterioidaceae* (21.7%) (**Figure 4.1B**).

We next asked whether there were specific taxa that differed between patients with MPN and healthy controls. A random forest was capable of distinguishing between patients with MPN and healthy controls using microbiome composition alone (**Figure 4.1C**). While several taxa informed the random forest model (**Figure 4.1D**), we found an operational taxonomic unit (OTU) from the genus *Phascolarctobacterium* was critical in differentiating patients with MPN from healthy controls. Furthermore, gut microbiomes from healthy controls have significantly higher raw abundances of sequence reads mapping to *Phascolarctobacterium* (**Figure 4.1E**). Using linear discriminant analysis to confirm the random forest results showing differential abundance of *Phascolarctobacterium* between patients with MPN and healthy controls also revealed significantly lower relative abundance of *Phascolarctobacterium* in patients with MPN (**Figure S4.1**). Increased *Phascolarctobacterium* is associated with benefits that include protection from *Clostridium difficile* infection [3] and lower levels of C-reactive protein (CRP) [4]. Further, decreased abundance of *Phascolarctobacterium* is observed in autoimmune diseases such as primary sclerosing cholangitis and ulcerative colitis [5] and may be associated with decreases in the

short chain fatty acid propionate in the gut, which in turn can influence inflammation [6]. *Phascolarctobacterium* may protect from inflammation, thus lower *Phascolarctobacterium* in MPN patients corroborates a chronic inflammatory state in this disease.

Changes in taxonomic composition may indicate differences in the functional potential of microbial communities. We inferred gene composition from taxonomic composition and found the microbiomes of MPN patients were enriched for genes involved in D-Glucuronate metabolism (**Figure 4.1F**). Changes in abundances of β-D-glucuronidases are associated with colon cancer and other inflammatory diseases [7].

The taxonomic composition of gut microbiomes within this cohort were largely personalized (PERMANOVA, $R^2 = 0.65$, $p = 0.001$), reflecting the individualistic nature of the microbiome. An MPN diagnosis explained 1.7% (PERMANOVA, $p = 0.001$) of the between-cohort variance in the microbiome (**Figure 4.1G**), suggesting subtle but significant differences between the microbiomes of patients with MPN compared to healthy controls. To contextualize this variation, consider the extreme intervention of ileocecal resection in Crohn's disease, which explained 5% of microbiome variance [8]. Other factors are known to shape microbiome composition, such as diet and cohabitation. In the present cohort, approximately half of the study participants were cohabitants, including nine MPN patient-normal pairs and three normal-normal pairs, comprising 12 different households. Of the MPN patients and healthy subjects cohabitating, living together explained 50% of the variance in the microbiome (**Figure 4.1G**), consistent with reports [9] showing that cohabiting people usually have the same diet, hygiene, and lifestyle, all of which strongly affect microbiome composition.

Since MPN can be stratified into subtypes based on phenotype, we sought to determine if there were specific microbial signatures between the subtypes measured in our cohort. Unsupervised ordination analysis of MPN subtypes showed that microbiomes of myelofibrosis (MF) patients had more similar community composition than those from Polycythemia Vera (PV) or Essential Thrombocythemia (ET) (**Figure 4.1H**). However, when the analysis was performed using a supervised random forest (RF) approach, distinct differences between early (PV/ET) and late stage (MF) MPN were observed (**Figure 4.1I**). Moreover, clustering from PV and ET patients was dense whereas that from MF was more dispersed. Dysbiotic individuals with a larger spread in microbial community composition than healthy individuals has been called the 'Anna Karenina principle' for animal microbiomes [10]—paralleling Leo Tolstoy's dictum that "all happy families look alike; each unhappy family is unhappy in its own way". These data suggest that early and late stage subtypes of MPN might be differentiated by the composition of gut microbes.

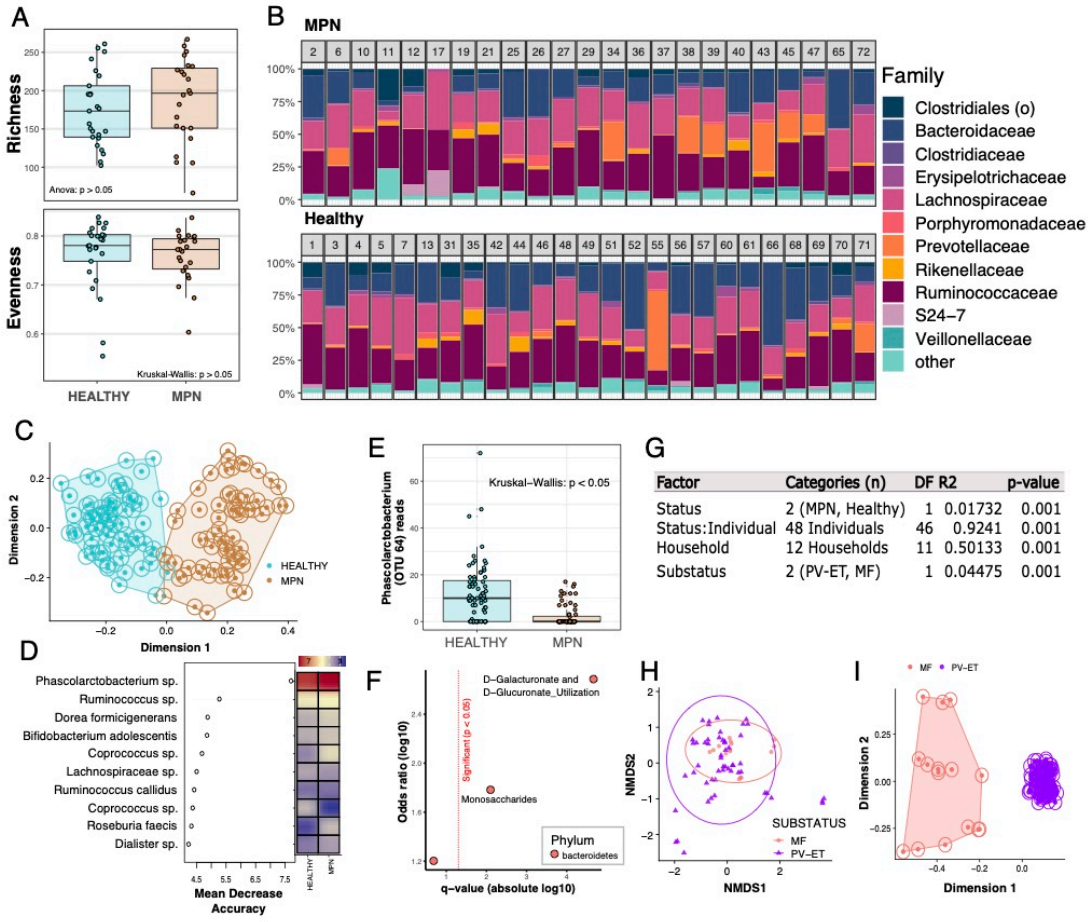


Figure 4.1 Characterization of the gut microbiome in patients with myeloproliferative neoplasms. (A) Alpha diversity was averaged within individual, showing no significant differences between health status for richness (number of distinct operational taxonomic units (OTU)) and evenness (distribution of those species). (B) Gut microbial families in MPN (top) and healthy (bottom) subjects averaged within individual (numbers on top of bars). (C) Permutated random forest plot of all samples from MPN and healthy individuals, identifying (D) taxa that were indicative of health status. (E) Normalized number of reads mapping to *Phascolarctobacterium* sp. from all samples of MPN patients and healthy individuals. (F) Using Phylogenize to identify functional potential of the communities enriched among MPN patients. (G) PERMANOVA results showing the significance and variance in microbiome composition explained by each tested factor. (H) Unsupervised ordination of the microbiomes from patients with PV and ET versus MF, and (I) a random forest proximity plot distinguishing MPN sub status based on the gut microbial community.

We sought to correlate plasma cytokines with microbial composition in the MPN cohort. We measured twelve cytokines in a subset of 20 individuals which included 15 MPN patients and 5 healthy controls (**Figure 4.2A**). We found increased plasma concentrations

of TNFα and IP10 in MPN patients (**Figure 4.2C)**, consistent with other studies [11]. Notably, although a random forest could clearly distinguish MPN and healthy using the microbiome (**Figure 4.1E**), the classification suffered considerably when using only cytokine abundances (**Figure 4.2B**). The largest contributing factor to the cytokine RF model was TNFα (**Figure 4.2C**), which plays a critical role in MPN pathogenesis by creating an environment that is conducive to the growth of the neoplastic clone [11]. Integrating the microbiome and cytokine data revealed associations between cytokine-taxa pairs (**Figure 4.2D**). We found an association between TNFα and the genus *Veillonella* (**Figure 4.2D**). *Veillonella* stimulates TNFα production of peripheral blood mononuclear cells in a dose dependent fashion [12]. Species of *Veillonella* have also been implicated in Crohn's disease [13], highlighting a potential role in inflammation. Using undirected Spearman correlations as an alternative way to investigate TNFα and the microbiome, we found the genus *Parabacteroides* may also be associated with TNFα production in MPN; however, this trend was not significant after correcting for multiple comparisons ($r = 0.65$, $p = 0.016$, $q = 0.77$, **Supplemental Figure 4.2**). Interestingly, one study found a species of *Parabacteroides* to be enriched in patients with colorectal carcinoma [14]. Further, *Parabacteroides* abundance was negatively correlated with intake of fruits and vegetables. Conceivably, dietary nutrients such as vitamins and fiber may be an important covariate in the management of inflammatory diseases such as MPN. For example, low fiber diets are associated with colonic inflammation, and can be lessened by switching to a high fiber diet coincident with changes in colonic microbial metabolism [15]. Future studies should examine how dietary interventions in MPN patients could be helpful to reduce inflammation in part via modulation of the microbiome.

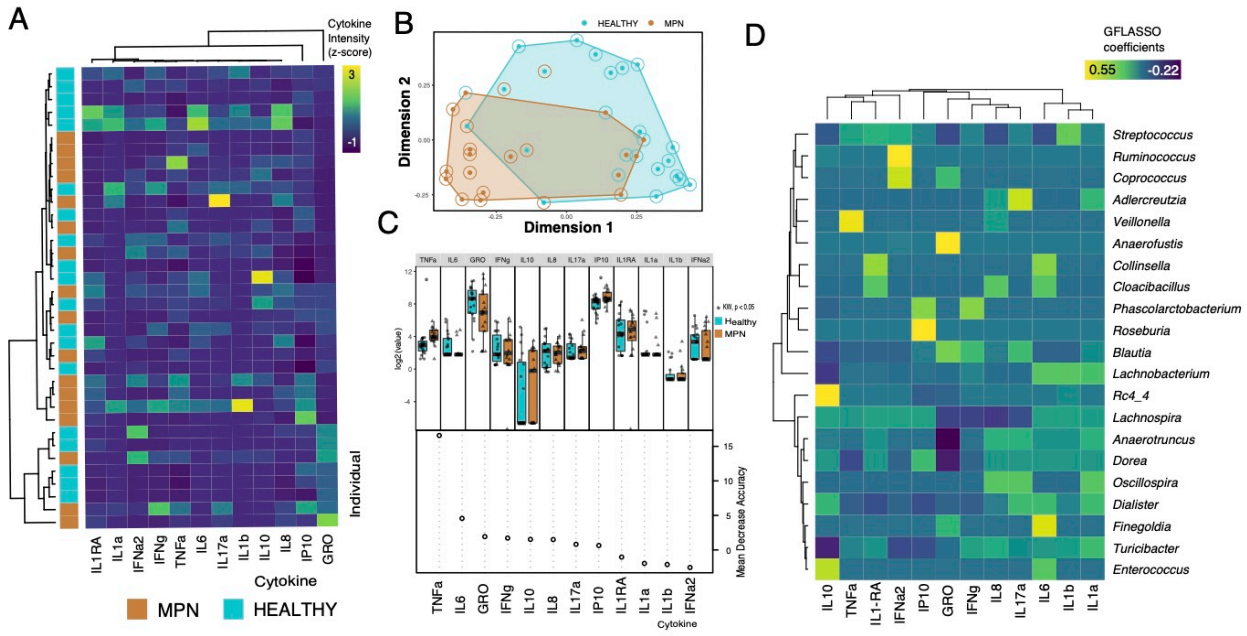


Figure 4.2 Cytokines and the microbiome in MPN. (A) Heat map of plasma cytokine concentrations in a subset of MPN patients and additional healthy controls with cytokines scaled using z-scores. (B) Random forest plot utilizing cytokine profile to distinguish MPN from healthy, the dots represent the actual health status, and the circles around the dots represent the RF classification, and (C) the cytokines relied on most heavily to make the classification of MPN versus normal, particularly TNFα. (D) Grid-fused LASSO regression to select microbes that best predicted cytokine abundances in MPN patients identified several OTUs that may have correlative relationships with various cytokines.

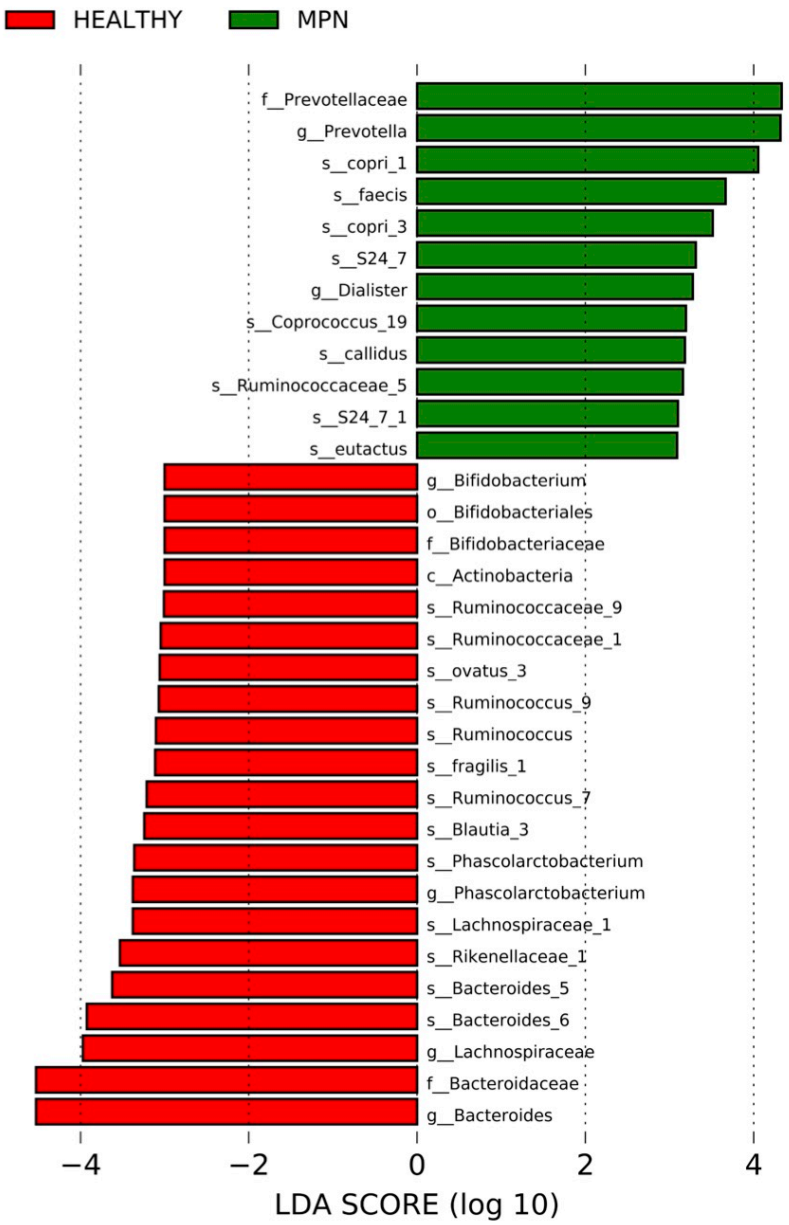
It is difficult to distinguish whether the microbiome affects MPN initiation and symptoms or whether the inflammatory response to the microbiome is exaggerated in MPN as it is widely influenced by multiple factors. A potential caveat of this study is the heterogeneity of treatments for the individuals with MPN; indeed we suspect different medications will have variable effects on the microbiome. However, due to the pilot nature of this initial investigation we are unable to confidently determine these differences and surmise this is an important avenue for future research. Furthermore, therapeutic

manipulation of the microbiome using diet, probiotics, or potentially fecal transfer with the intent of reducing inflammation in MPN remains unexplored. Despite the limitations of this initial pilot study, however this study is an important step in the path to better understand the role of the microbiome in MPN.

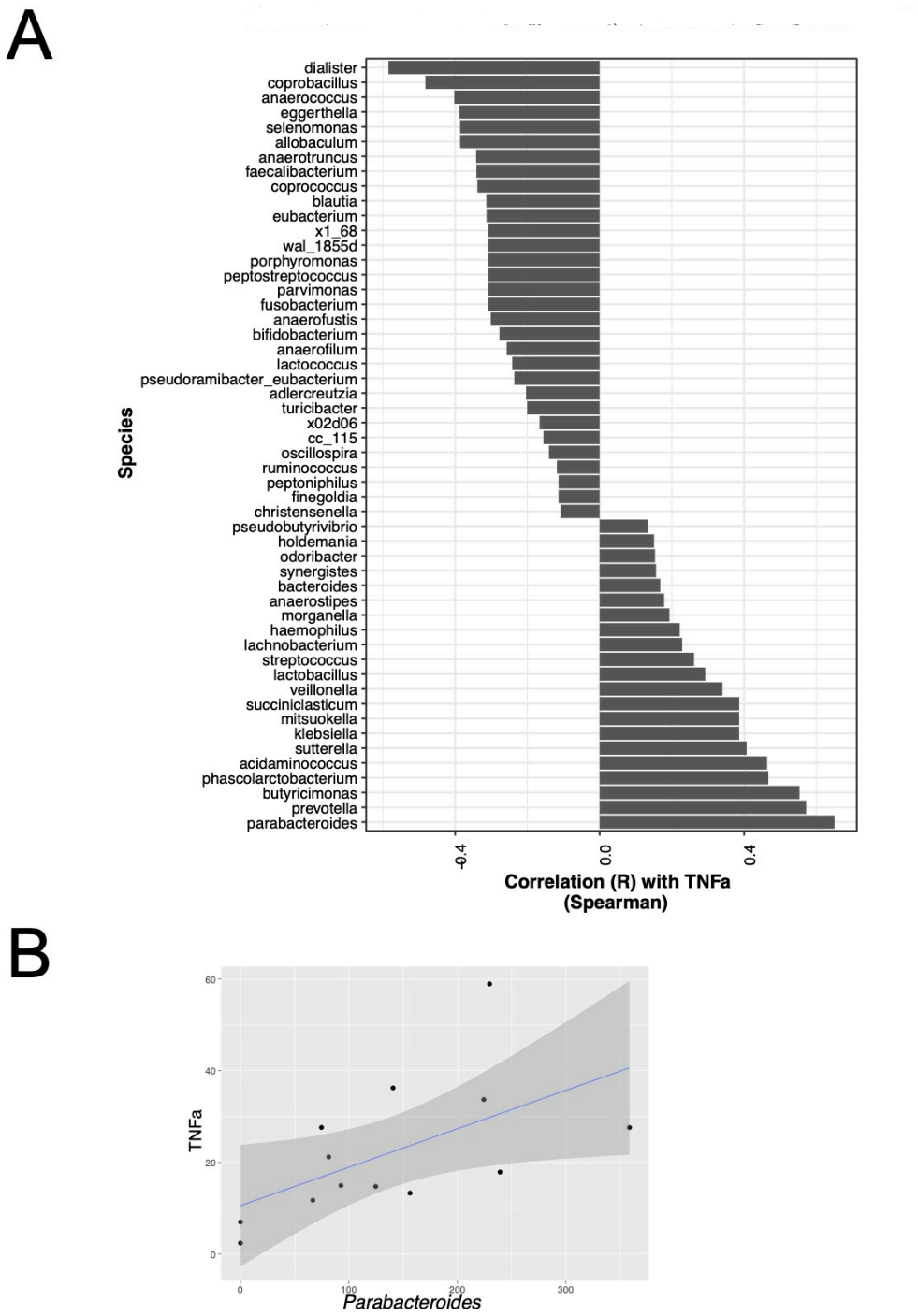
Supplemental Table and Figures

Characteristic of the disease	MPN Patients (n=25)
Type of disease PV ET MF	 11(44%) 8 (32%) 6 (24%)
Mutation type JAK2V617F CALR	 23 (92%) 2 (8%)
Thrombosis DVT Stroke STEMI or NSTEMI	5 (20%) 1 (4%) 1 (4%) 3 (12%)
Medication Aspirin Ruxolitinib Interferon alpha Anagrelide Hydroxyurea	 22 (88%) 6 (24%) 2 (8%) 1 (4%) 7 (28%)
Symptom burden (MPN-SAF score) High (>20 total score) Low	 7 (28 %) 18 (72%)

Supplemental Table 4.1 Disease characteristics of the MPN cohort



Supplemental Figure 4.1 Lefse (linear discriminant analysis) between healthy and MPN individuals for all microbiome samples.



Supplemental Figure 4.2 (A) Spearman correlations between TNFα and microbial genera using all samples from patients with MPN. (B) Scatterplot of between TNFα and Parabacteroides.

REFERENCES

1. Galloway-Peña JR, Shi Y, Peterson CB, et al. Gut microbiome signatures are predictive of infectious risk following induction therapy for acute myeloid leukemia. Clin Infect Dis. 2019;
2. Staffas A, Burgos da Silva M, Slingerland AE, et al. Nutritional Support from the Intestinal Microbiota Improves Hematopoietic Reconstitution after Bone Marrow Transplantation in Mice. Cell Host & Microbe. 2018;23(4):447-457.e444.
3. Nagao-Kitamoto H, Leslie JL, Kitamoto S, et al. Interleukin-22-mediated host glycosylation prevents Clostridioides difficile infection by modulating the metabolic activity of the gut microbiota. Nature medicine. 2020;
4. Citronberg JS, Curtis KR, White E, et al. Association of gut microbial communities with plasma lipopolysaccharide-binding protein (LBP) in premenopausal women. The ISME Journal. 2018;12(7):1631-1641.
5. Bajer L, Kverka M, Kostovcik M, et al. Distinct gut microbiota profiles in patients with primary sclerosing cholangitis and ulcerative colitis. World journal of gastroenterology. 2017;23(25):4548-4558.
6. Trompette A, Gollwitzer ES, Yadava K, et al. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nature medicine. 2014;20(2):159-166.
7. Dashnyam P, Mudududdla R, Hsieh T-J, et al. β-Glucuronidases of opportunistic bacteria are the major contributors to xenobiotic-induced toxicity in the gut. Scientific reports. 2018;8(1):16372.
8. Vich Vila A, Imhann F, Collij V, et al. Gut microbiota composition and functional changes in inflammatory bowel disease and irritable bowel syndrome. Science translational medicine. 2018;10(472):
9. Dill-McFarland KA, Tang Z-Z, Kemis JH, et al. Close social relationships correlate with human gut microbiota composition. Scientific reports. 2019;9(1):703.
10. Zaneveld JR, McMinds R, Vega Thurber R. Stress and stability: applying the Anna Karenina principle to animal microbiomes. Nat Microbiol. 2017;2(17121.
11. Fleischman AG, Aichberger KJ, Luty SB, et al. TNFalpha facilitates clonal expansion of JAK2V617F positive cells in myeloproliferative neoplasms. Blood. 2011;118(24):6392-6398.
12. Matera G, Muto V, Vinci M, et al. Receptor recognition of and immune intracellular pathways for Veillonella parvula lipopolysaccharide. Clin Vaccine Immunol. 2009;16(12):1804-1809.
13. Ananthakrishnan AN, Luo C, Yajnik V, et al. Gut Microbiome Function Predicts Response to Anti-integrin Biologic Therapy in Inflammatory Bowel Diseases. Cell Host Microbe. 2017;21(5):603-610.e603.
14. Feng Q, Liang S, Jia H, et al. Gut microbiome development along the colorectal adenoma-carcinoma sequence. Nature communications. 2015;6(6528.
15. O'Keefe SJD, Li JV, Lahti L, et al. Fat, fibre and cancer risk in African Americans and rural Africans. Nature communications. 2015;6(1):6342.

CHAPTER 5

Exploring the influence of diet on the MPN gut microbiome

It has been recognized that diet has a direct impact in shaping the gut microbiome. The ingestion of high- fiber diets, animal fats, and plant-based diets are associated with distinct patterns of gut microbiota composition [27]. These microorganisms are important for the development of the host's immune system, as mice lacking gut microbes have altered immune responses [28]. Microbiota dysregulation (or dysbiosis) is seen in different inflammatory conditions [29] including inflammatory bowel disease, rheumatoid arthritis, and systemic lupus erythematosus indicating that manipulating the microbiota may be an innovative therapeutic strategy for inflammatory diseases. The focus of this chapter is to determine how the Mediterranean diet influences the microbiome of MPN patients. Stool samples were used to measure changes in the gut microbiome with the diets. We believe that therapeutically manipulating the intestinal microbiota through an anti-inflammatory diet, the Mediterranean diet, might increase the overall bacterial diversity and alter the composition in the MPN gut microbiome.

Methods

Collection of fecal samples:

Study participants were asked to provide a fecal samples from 4 different timepoints (weeks 1, 6, 9 and 15) stored in Zymo DNA/RNA shield preservation buffer (Cat. #R1101). Samples were frozen at -80°C once returned.

Extraction of DNA from fecal samples:

Fecal samples stored in DNA/RNA shield were thawed on ice, vortexed to homogenize, then 1000 uL of fecal slurry was extracted using ZymoBiomics DNA Miniprep Kit (Cat. #D4300) according to the manufacturer's protocol. Bead lysis during the extraction was performed at 6.5 m/s for 5 minutes total (MPBio FastPrep-24).

Shotgun library preparation and sequencing:

Libraries for shotgun sequencing were prepared using the Illumina DNA prep kit (Cat. # 20018705), using an adapted low-volume protocol [30]. Briefly, a maximum of 5 uL or 50 ng (whichever was reached first) of DNA from each sample was tagmented for 15 min at 55°C. Next, 1.25 uL of 1 uM forward and 1.25 uL of 1 uM reverse barcodes were added to each sample and annealed via PCR using KAPA HiFi DNA Polymerase (Cat. # NC0465187). Samples were then pooled and cleaned of smaller fragments using the included sample purification beads according to the published protocol. The pooled sample libraries were quantified using Quanti-iT PicoGreen dsDNA (Cat. #P7589). Multiple positive (ZymoBIOMICS Microbial Community DNA Standard, Cat. #D6305) and negative sequencing controls were included to identify potential contaminants and sequencing bias. Libraries were shipped overnight on dry ice to Novogene Corporation Inc. (Sacramento, CA) for sequencing using Illumina's Hiseq 4000. An average of 2,819,107 +/- 670,543 paired-end reads per sample, 150 bases in length, were obtained.

OTU table generation and analysis:

Raw data was first cleaned to remove sequencing adapters and artifacts using BBMap [31]. BBMap was also used to demultiplex and remove barcodes from samples. Sequences were quality filtered so that the minimum quality score did not fall below 25 using PRINSEQ++ [32]. Visualization of sequence quality was done using FastQC [33]. Removal of human-derived reads was performed in Bowtie2 by aligning samples to reference human genome hg38 [34]. Taxonomy of the resulting sequences was characterized using MetaPhlAn3 to produce an OTU table [35]. Filtering of microbial contaminants was performed in R v3.6.3 using the negative sequencing controls. Additionally, microbes which appeared in the positive control, but who were not part of the original composition, were also removed from the OTU table. Richness and evenness metrics were calculated using the Vegan v2.5-6 package in R [36]. Significance testing of richness and evenness metrics was done using a linear-mixed effect model in the nlme v3.1-148 package in R [37]. The adonis function in Vegan was used to generate a Bray-Curtis dissimilarity matrix and to perform PERMANOVA significance testing. Non-metric multidimensional scaling of microbiome data was performed using the metaMDS function in Vegan. Plots were generated using ggplot2 v3.3.0 [38]. The code used for this analysis is available at https://github.com/Javelarb/MPN_diet_intervention.

Results

MPN subtype is associated with gut microbiome composition: alpha diversity

Since MPN can be stratified into subtypes based on phenotype, we sought to investigate if either ET, MF or PV were associated with changes in alpha diversity within the gut microbiome. We analyzed the number of distinct species (richness) and the distribution (evenness) of those species. The richness in our cohort significantly decreased when comparing PV with MF, as well as when comparing ET with MF, meaning that people with MF have a reduced total number of unique species (**Figure 5.1A**). The evenness was also decreased in participants with myelofibrosis (**Figure 5.1B**). This subtype of MPN tends to be dominated by one particular microbe instead of having a more even distribution.

MPN subtype is associated with gut microbiome composition: beta diversity

We sought to determine if there were specific microbial signatures between the subtypes measured in our cohort. Analysis of MPN subtypes showed that microbiomes of myelofibrosis (MF) patients tend to be more disparate from each other than those from polycythemia vera (PV) or essential thrombocythemia (ET). Although, the microbes of patients with PV and ET are also separating away from each other (**Figure 5.1C**).

Fecal microbial community composition across MPN subtypes

The most abundant taxonomic families among our cohort are visually represented in Figure 5.1D. We observed that each person's microbial community composition appears

dissimilar from each other, but at the same time they look more like their own self during the course of the diet study.

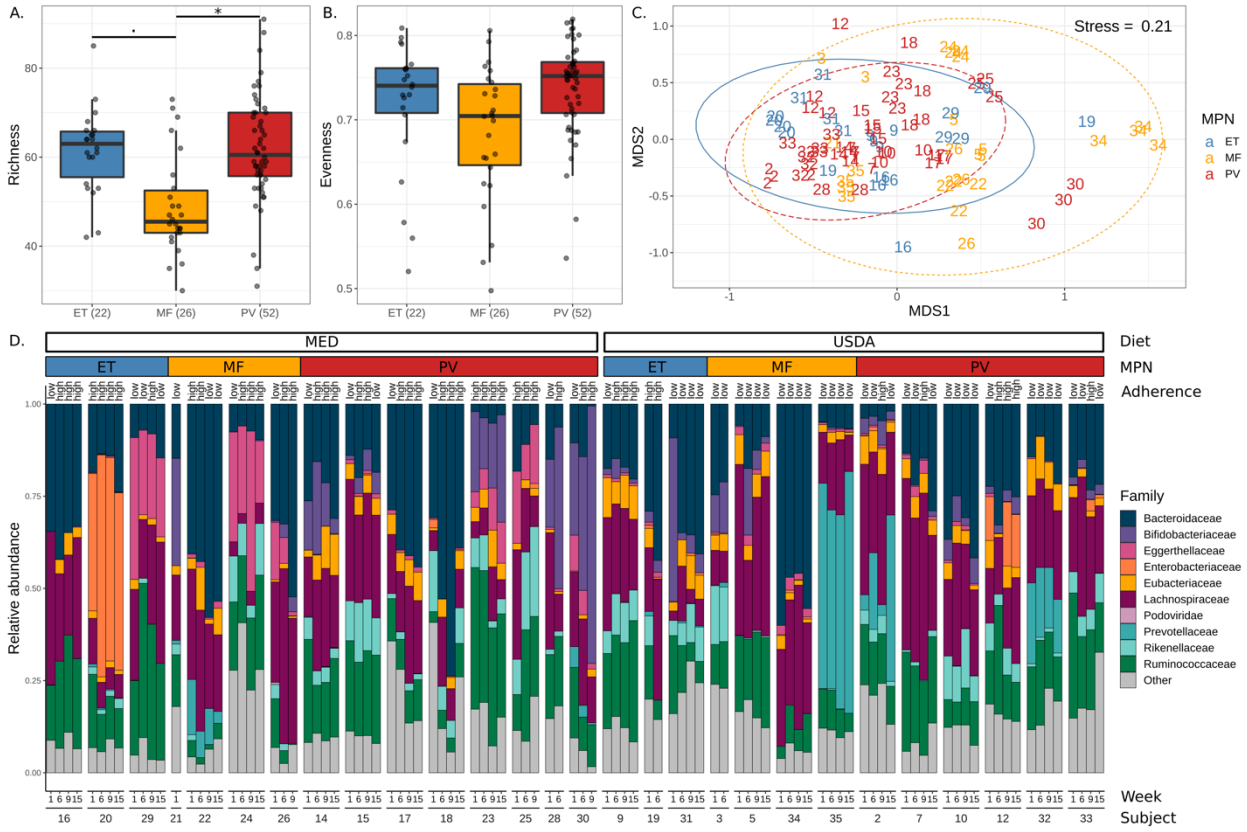


Figure 5.1 MPN subtype is associated with gut microbiome composition. (A) The number of unique microbial species (richness) and (B) the evenness of the gut microbiome across MPN subtypes. Each point is one sample, with up to four samples per individual. The number of samples per MPN subtype is shown in parenthesis. Significant differences are labeled as; `.` = (p = 0.05), `*` = (p < 0.05), and `**` = (p < 0.005). (C) Non-metric multidimensional scaling of Bray-Curtis dissimilarities across MPN subtypes to represent microbial composition. Points closer together are more similar in microbial composition when compared to points further apart. Points are labeled by the subject of origin, with up to four samples per subject. (D) The relative abundance of the top ten microbial families across MPN subtypes and diet types. Samples are grouped by subject over time. The 'high' and 'low' adherence labels indicate whether an individual had a Mediterranean adherence score of > 8 or < 8, respectively, for each time point.

MPN subtype associated with depletion of specific microbe

Next we explored if there were differentially abundant microbes between the MPN subtypes and we found significantly (FDR < 0.05) lower relative abundance of *Faecalibacterium prausnitzii* in individuals with MF when compared to those with PV (**Figure 5.2**). These differences were present at the beginning of the trial and cannot be attributed to the diet intervention. Still, this is an interesting finding that suggests that the gut microbiome between MPN subtypes is different.

F. prausnitzii is one of the most important butyrate (short chain fatty acid) producers found in the intestine [39, 40]. This anti-inflammatory compound has a crucial role in maintaining intestinal barrier integrity and host wellbeing. Butyrate is the main source of energy for the colonocytes and it has protective properties against colorectal cancer (CRC) and inflammatory bowel diseases [41, 42]. In addition, butyrate can have beneficial effects on glucose and energy homeostasis by activating intestinal gluconeogenesis [43].

Transplantation of *F. prausnitzii* has been shown to be an effective therapeutic approach for diabetes and its complications [44]. On the other hand, low abundance of this microbe has been associated with increased inflammatory processes and atherosclerosis. *F. prausnitzii* may have a protective effect from inflammation, thus lower *F. prausnitzii* in MF patients corroborates a chronic inflammatory state in this particular subtype of MPN.

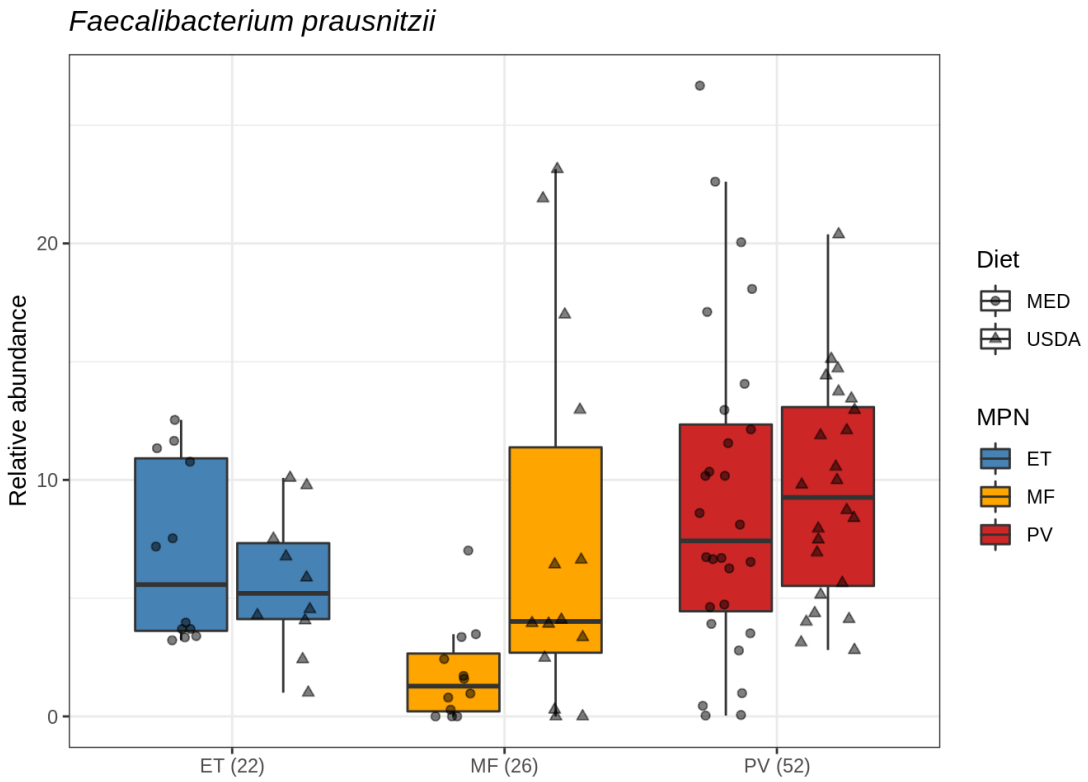


Figure 5.2 F. prausnitzii is differentially abundant between MPN subtypes. *F. prausnitzii* was significantly depleted in myelofibrosis when compared to polycythemia vera.

Conclusions

Our findings suggest that early and late stage subtypes of MPN might be differentiated by the composition of intestinal microbes. Participants from our trial with myelofibrosis had reduced microbial diversity as well as a significant depletion of *F. prausnitzii* in their gut. It is interesting that in two independent studies we found that the MF population was different from ET and PV patients. A possible explanation for this observation could be that patients bearing this disease have the most inflammation in their guts of the two MPN subtypes. This further highlights that myelofibrosis is a different and more serious disease than PV or ET.

CHAPTER 6

Creation of a mouse model that represents CHIP to test the impact of a high fat diet on expansion of CHIP mutant cells

Abstract

Clonal hematopoiesis of indeterminate potential (CHIP) is the presence of cells with mutations in the blood that are also seen in hematologic malignancies, but in a hematologically normal person. CHIP is common in the aging population, with up to 20% of people over the age of 60 having CHIP. CHIP may progress to an overt hematologic malignancy and also has non-cancer consequences such as increased inflammation leading to accelerated atherosclerosis. Lifestyle choices such as smoking behavior is associated with an increased risk of CHIP. We hypothesize that diet may also impact the emergence of CHIP and its progression to hematologic malignancy. Specifically, high inflammatory diets may increase one's risk of CHIP, and low inflammatory diets such as a Mediterranean diet may reduce one's risk of CHIP. Genetic mouse models exist for common CHIP mutations, such as *Tet2*, *Dnmt3a*, *Asxl1*, and *Jak2*. However, these genetic models do not accurately recapitulate human CHIP where only a small percentage of cells is mutant in the context of wild-type cells. Here, we develop a more physiologically relevant CHIP model using transplantation into minimally irradiated donors and test the impact of a high fat diet, a common high inflammatory diet, in these mice.

Introduction

Clonal hematopoiesis of Indeterminant Potential (CHIP) is a newly described phenomenon whereby cells with mutations associated with hematologic malignancies are present in people without apparent hematologic abnormalities [45-49]. CHIP is likely a requisite precursor to hematologic malignancy but only a fraction of people with CHIP will go on to develop a hematologic malignancy. The incidence of CHIP increases with age, it is present in 10-15% of people over 65 [45]. In CHIP, pre-existing mutant clones expand only when they gain a selective advantage in the context of an aged HSC pool. CHIP is more common in current and former smokers [45, 50] implicating a role for lifestyle choices in CHIP development.

Diet is emerging as a potential determinate of CHIP. An ongoing cohort study using longitudinal data from over 44,000 participants in the UK Biobank examined whole-exome sequencing data and survey-based information on health-associated behaviors of people in the United Kingdom. Results from this study suggested that unhealthy diet quality may be associated with a higher prevalence of CHIP and higher rates of adverse cardiovascular events and death independent of CHIP status [51]. Another study, the Women's Health Initiative (WHI), analyzed deep-coverage whole genome sequencing of 8,709 postmenopausal women who were free of cancer or cardiovascular disease. Across individual lifestyle factors, a normal body mass index was associated with a lower prevalence of CHIP [52]. These findings support the idea that lifestyle choices such as diet impact the selective outgrowth of CHIP mutant hematopoietic cells.

The most common CHIP mutations in descending order are in the genes DNMT3A, TET2, ASXL1, TP53, and JAK2, and mouse models are available for all of them. A fraction of mice bearing mutations in DNMT3A and TET2 develop spontaneous hematologic malignancies [53, 54], and TET2 and DNMT3A cell outcompete wild-type hematopoietic cells in competitive repopulation assays. Experimentally induced inflammatory stressors such as exogenous TNFα enhances the selective advantage of CHIP mutant cells [55]. Chronic inflammation induces the production of reactive oxygen species (ROS) leading to DNA damage and HSC exhaustion [10]. We hypothesize that inflammation induced ROS may be responsible for the selection of CHIP mutant cells by reducing the fitness of their wild-type counterparts.

Tet2 knockout mouse models have been generated to study the function of TET2 *in vivo.* As early as 2-4 months of age, *Tet2*−/− mice can display a disordered hematopoiesis and resemble characteristics of CMML, indicated by monocytosis/neutrophilia, hepatosplenomegaly, and increased cellularity in the bone marrow (BM) [53]. Competitive transplant assays have shown that hematopoietic stem cells (HSC) harboring *Tet2* mutations have a growth advantage when compared to normal HSC [56]. Zhe Li et al. demonstrated that transplantation of TET2-/- BM cells led to increased white blood cell (WBC) counts, monocytosis, and splenomegaly in WT recipient mice [53]. *Tet2* mutations on their own are not enough to cause AML in mice, implying that perhaps other mutations and/or environmental stressors are necessary for their selective advantage leading to the development of leukemias [57].

An inflammatory environment originated by external factors, infections, autoimmunity and aging may cause mutations and genomic instability in somatic cells.

Inflammation can also modulate the tumor microenvironment through angiogenesis and the expression of chemokines and cytokines [57]. Cai et al. studied if TET2-/-hematopoietic stem and progenitor cells (HSPCs) produce inflammatory cytokines and how they respond to an inflammatory challenge. Their findings show that serum IL-6 levels and the expression of IL-6 are significantly upregulated in TET2-/- HSPCs with or without LPS stimulation, suggesting that TET2-/-HSPCs are powered with a selection advantage under conditions of inflammation-induced stress. *In vitro* studies from Abegunde et al. show that TET2-deficient murine and human macrophages are hyperinflammatory. This phenotype arises on chronic TNFα exposure and is associated with myeloid biasing and resistance to apoptosis [55]. Their evidence suggests that *Tet2* mutations promote clonal dominance with aging by conferring TNFα resistance to hematopoietic progenitors while also propagating such an inflammatory environment [58].

The main objective of this chapter is to assess the impact of an inflammatory high-fat diet (HFD) on the clonal expansion of malignant myeloid cells, specifically TET2 knockout ($TET2^{-/-}$) cells. A study looking at the effects of diet-induced obesity in mice concluded that HSCs were negatively altered as it promoted the expansion of pro-inflammatory myeloid cell production from the bone marrow [59]. We hypothesize that an unhealthy high fat diet will create an inflammatory environment that will allow for the clonal expansion of malignant cells. Moreover, we reasoned that intervention with an antioxidant such as N-acetylcysteine will suppress oxidative stress and inflammation, thus restraining the outgrowth of mutant cells.

Materials and Methods

Mice

All animal procedures were performed under approval of the Institutional Animal Care and Use Committee at the University of California, Irvine. The TET2 knockout mutant mice were obtained from The Jackson Laboratory (TET2 , JAX stock #023359). Mouse genotyping was performed with PCR reaction per the protocols provided from The Jackson laboratory. Complete blood counts were measured using the ABCVet Hemalyzer (scil, Viernheim, Germany) using blood diluted 1:1 with 100mM EDTA. Spleen and liver weights were recorded upon sacrifice or death.

Diets

Diets were purchased from Research Diet Inc. We used the rodent diet D12450B, which is a type of control diet with 10 kcal% from fat (low fat). Fat is distributed the following way: from soybean oil, USP (25.00 g), lard (20.00 g). The rodent diet D12451 causes metabolic disease and has 45 kcal% from fat (high fat). Fat is distributed the following way: from lard (177.50 g), soybean oil, USP (25.00 g).

N-AC water

For N-AC treatment, mice were given drinking water with 2gm/L N-AC (Bulk Supplements, Henderson, NV) beginning at 16 weeks post-transplant and this was replenished weekly.

Flow Cytometry

Flow cytometry for chimerism of mature cells in peripheral blood was performed using CD45.1 and CD45.2 antibodies. Data was acquired on the BD Accuri C6 (BD Biosciences, San Jose CA).

Competitive transplants

Whole bone marrow was harvested from both femurs and tibias of donor mice (CD45.1/CD45.1/2). RBCs were lysed with Ammonium-Chloride-Potassium (ACK) buffer, washed, then cells were stained for 30 minutes on ice. B6.SJL-Ptprca Pepcb/Boy (BoyJ, CD45.1) mice were purchased from The Jackson Laboratory. Recipient mice (CD45.2) were minimally irradiated with 50cGy the day of transplantation. To develop chimeric mice, donor whole bone marrow cells from WT C57B6 or TET2 knockout mice (CD45.1/CD45.1/2) were mixed in a ratio of 50:50 ($5x10^6$ each) with competitor cells (CD45.1/CD45.1/2) and injected intravenously into irradiated recipients. Peripheral blood was collected at monthly intervals to assess the donor cell engraftment.

Statistical Analysis

All statistical analyses were performed using Graphpad Prism version 9.0 (Graphpad, La Jolla, CA, USA).

Results

Development of CHIP model with a low percentage of CHIP mutant cells

Genetic mouse models are readily available for the most common CHIP mutations. In these models all hematopoietic cells bear the CHIP mutations. However, in human CHIP only a small percentage of cells bear the CHIP mutation. Because our central purpose is to test how modulation of inflammation through diet may impact the competition between CHIP mutant and WT hematopoietic cells we needed to develop a system in which only a small percentage of cells bear the CHIP mutation. Therefore, we established transplantation models to recapitulate human CHIP bearing *TET2* and *DNMT3A* mutations.

We chose to use the recently established *Dnmt3a*^{R878H} mouse line (B6(Cg)-Dnmt3atm1Trow/J, JAX stock# 032289) rather than *Dnmt3a*^{KO} mice because the *Dnmt3a*^{R878H} line recapitulates the common *DNMT3A*^{R822H} hotspot mutation seen in human CHIP. We performed competitive repopulation assays both with TET2-/-(**Figure 6.1A**) and *Dnmt3a*^{R878H} (**Figure 6.1B**) donors at a 1:1 ratio of mutant:WT whole bone marrow. We conditioned recipient mice with either 800cGy (lethal dose for C57B/6 mice) which clears the bone marrow for full engraftment of the donor cells or 50cGy which does not obliterate the recipient's bone marrow but allows for a small amount of engraftment from donor cells. The modest engraftment of mutant cells into recipients conditioned with 50cGy irradiation achieved our goal of a small percentage of mutant cells in the blood.

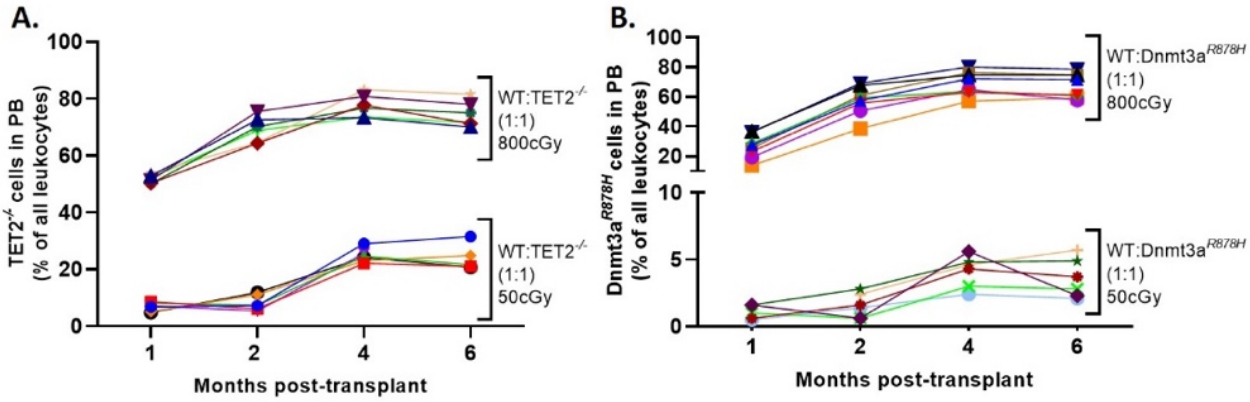


Figure 6.1 Engraftment of CHIP mutant cells can be modulated by altering the intensity of recipient irradiation. TET$^{-/-}$ (A) and Dnmt3a^{R878H} (B) whole bone marrow cells were injected at a 1:1 ratio along with WT bone marrow cells into mice conditioned with either 800cGy or 50cGy X-ray irradiation. The percentage of mutant cells was followed in the blood over 6 months.

Testing the impact of a high fat diet (HFD) and the anti-oxidant N-Acetylcysteine (N-AC) on the selection of TET2-/- mutant cells

We transplanted TET2-/- whole bone marrow into 50cGy to determine how diet may impact the selection of mutant cells. Moreover, we also wanted to investigate how the antioxidant N-AC would impact the trajectory of TET2-/- mutant cells. The C57B6 mouse model is ideal for competitive repopulation assays. The standard C57B6 strain has the CD45.2 haplotype. PepBoy mice have the CD45.1 haplotype but otherwise are congenic with C57B6 mice. CD45.2 C57B6 and CD45.1 PepBoy mice can be bred to create CD45.1/2 heterozygotes. Therefore, this system allows one to easily distinguish three different sources of cells in a competitive repopulation assay. We backcrossed our TET2-/- into CD45.2, CD45.1, and CD45.1/2 backgrounds to give us more flexibility for transplant planning.

We transplanted TET2-/- mice from either a CD45.1 or CD45.1/2 background along with wild-type cells from a CD45.1/2 or CD45.1 background, respectively into CD45.2 mice which were conditioned with 50cGy X ray irradiation (**Figure 6.2**).

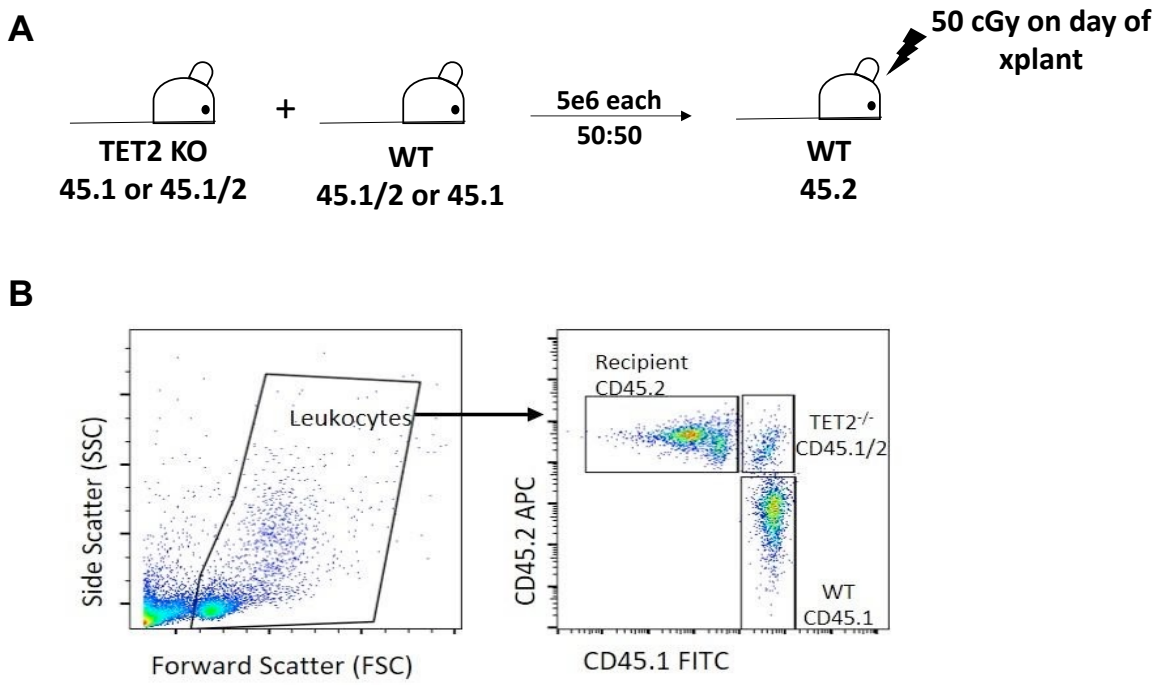


Figure 6.2 Experimental Design for the TET2-/- transplantation experiments. (A) Transplantation schematic, (B) Representative flow cytometry plot in the case where TET2-/- is in a CD45.1/2 background.

Mice rested for 16 weeks post-transplant before starting the HFD and N-AC treatment. N-acetylcysteine (N-AC) is an antioxidant agent that comes from the amino acid L-cysteine. It has been approved by the FDA for the use in acetaminophen (Tylenol) overdose. Investigations from our lab demonstrated that in a murine model of MPN, N-AC reduced thrombus formation *in vivo* and improved the animal's survival [60]. Other studies have shown that N-AC has a thrombolytic effect on arterial thrombi [61], as well as proven

to reduce the formation of platelet-leukocyte aggregates (PLAs) in a model of experimental diabetes [62]. In accordance with its anti-inflammatory properties, oral N-AC treatment in hemodialysis patients during a 3 month period significantly decreased serum levels of hs-CRP (22.4 vs. 5.2), IL-6 (8.1 vs. 3.6), parathyroid hormone (iPTH) (257.2 vs. 158.8), ferritin (632.0 vs. 515.1) and erythrocyte sedimentation rate (ESR) (54.2 vs. 38.3). The N-AC was supplemented in the drinking water at a concentration of 2gm/L. Fresh N-AC water was replaced once weekly. Below is a scheme of or experimental setup (**Figure 6.3**).

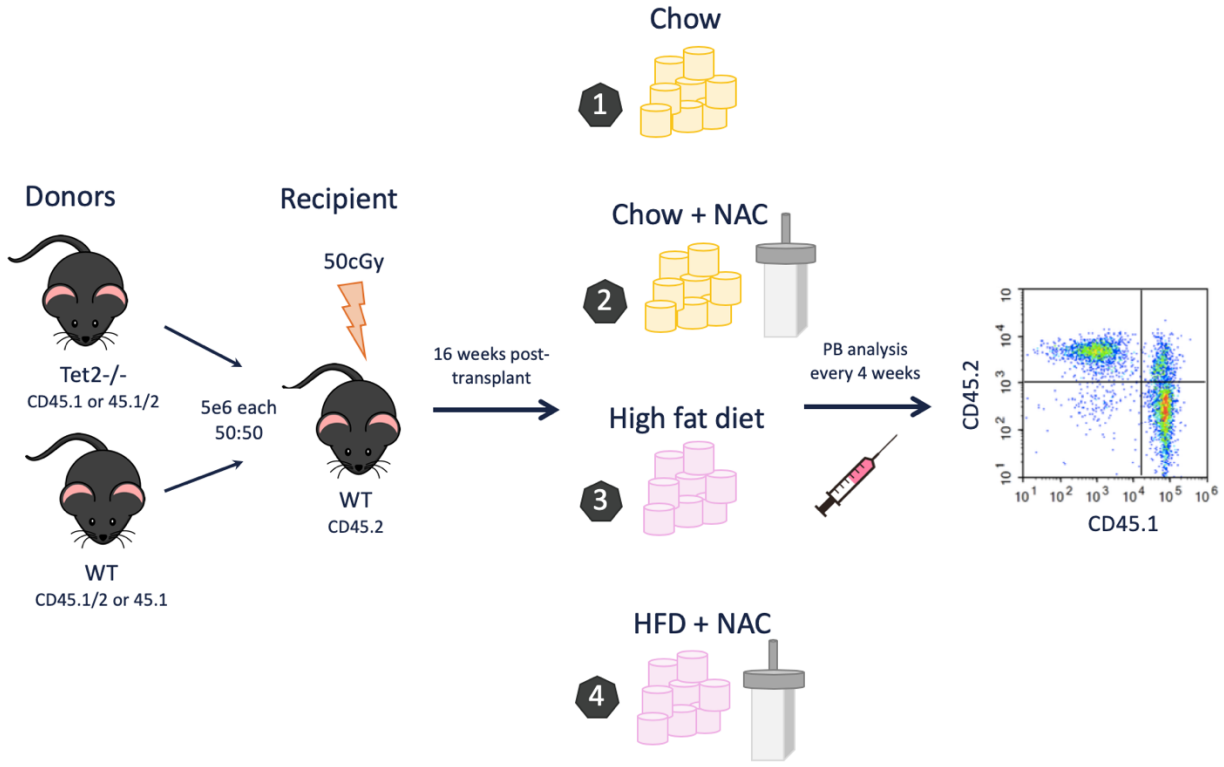


Figure 6.3 In vivo competitive bone marrow transplant testing HFD and supplementation with N-AC water.

Trajectory of TET2-/- mutant cells over time

We collected peripheral blood monthly and measured the percentage of TET2-/- cells by flow cytometry (**Figure 6.4**). We noted that the selective advantage of TET2-/-

differed in the CD45.1 (**Figure 6.4A**) versus the CD45.1/2 (**Figure 6.4B**) background. Exposure groups were designated randomly, however, as shown in Figure 6.4, there was inherent differences in the percentage of TET2-/- cells between groups. Therefore, we calculated the fold change in TET2-/- from the beginning to the end of the exposure period for each mouse (**Figure 6.5**).

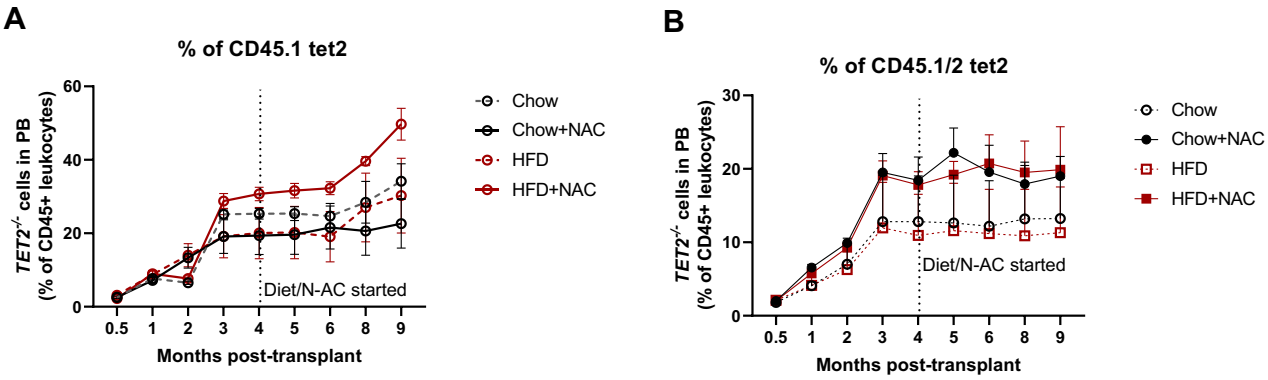


Figure 6.4 Trajectory of TET2-/- cells in the peripheral blood. The percentage of TET2-/- cells from a CD45.1 (A) and CD45.1/2 (B) background were followed monthly in the peripheral blood of transplanted mice.

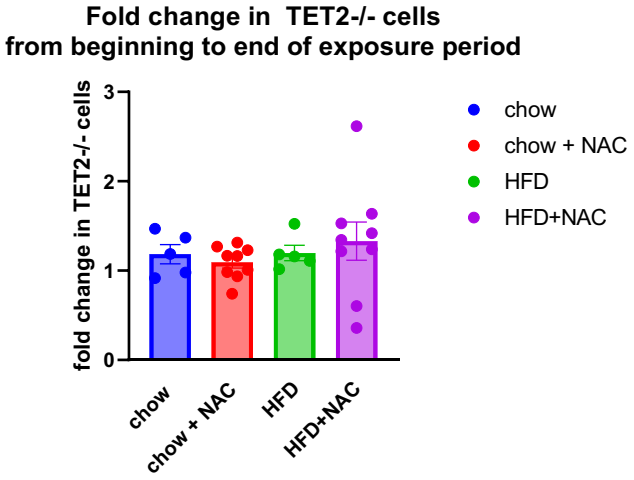


Figure 6.5 Fold change in the percentage of TET2-/- cells from month 4 (beginning of the exposure period) to month 9 (end of the exposure period). No significant difference were found between any of the groups.

When comparing the fold change in TET2-/- cells from the beginning to the end of the exposure period we did not detect any differences between groups.

Because HFD induces weight gain, and increased adipose tissue is pro-inflammatory , we also measured weights in the cohort. Interestingly, at the 6-12 week time points mice on HFD + N-AC gained less weight than mice on HFD + regular water (**Figure 6.6**).

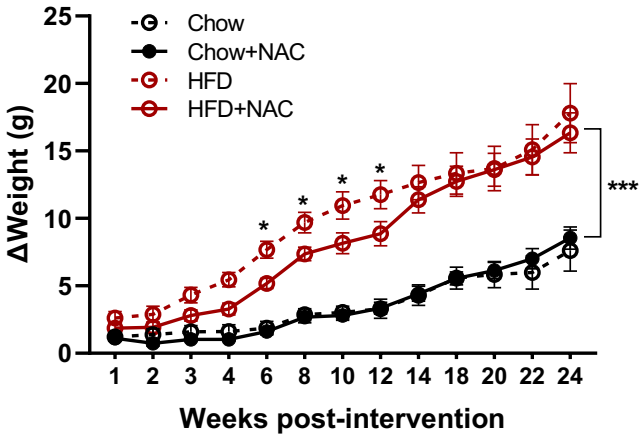


Figure 6.6 N-AC diminishes HFD induced weight gain. Mice were weighed approximately weekly starting at the time HFD and N-AC were introduced.

Discussion

Here, we adapted available genetic mouse models bearing mutations commonly seen in human CHIP to be a more physiologic representation of CHIP. Our goal was to create a transplant model with a fraction of mutant cells, this will allow us to test whether specific experimental interventions augment or decrease the selective advantage of CHIP mutant cells. As expected, TET2-/- cells have a stronger selective advantage than *Dnmt3a* mutant cells (**Figure 1**). For future experiments we can further modulate the number of

mutant cells injected as well as titrate the irradiation dose to alter the desired percentage of mutant cells in the blood.

We did not find that HFD nor N-AC had a significant impact on the trajectory of TET2-/- cells. We are also moving on now to test the impact of HFD on other CHIP mutants such as DNMT3A and JAK2. We used a moderate high fat diet (45% kcal from fat), it is possible that a higher fat diet is required to induce sufficient inflammation to increase the selection of CHIP mutant cells. Other possibilities are that a longer exposure is required to observe the impact of diet on selection of CHIP mutant cells.

Although we did not find that N-AC impacted the trajectory of TET2-/- mutant cells, we did observe less weight gain in mice on a HFD + N-AC. Whether this reduction in weight gain is due to less consumption of food when on N-AC water, increased physical activity, or some other mechanism remains to be seen but will be followed up on.

SUMMARY AND CONCLUSIONS

The NUTRIENT trial is a feasibility study to evaluate if MPN patients are able to follow a healthy diet when given written curriculum and verbal information, and to explore whether a diet rich in anti-inflammatory properties (Mediterranean diet), can improve MPN symptoms. The overreaching goal of this project is to target inflammation in MPN using non-pharmacologic approaches, primarily in a clinical trial setting seeking to reduce inflammation, modulate related symptom burden, and possibly blunt disease progression.

Overall, being this the first dietary intervention among the MPN population, our main purpose was to collect data to design subsequent studies which will test the impact of diet on a larger group of MPN patients. This study was not powered to detect changes in the symptom burden or inflammatory cytokine levels, however these measurements will help guide our power calculation for subsequent studies.

We concluded that older MPN patients are as capable to complete the online assignments as the younger ones. Sending email reminders was effective for most participants to engage, although some needed more than one reminder to answer the surveys. The number of reminders did not differ much between males and females nor was it higher for one diet group compared with the other, meaning that participants regardless of their gender showed similar enthusiasm for both of the diets offered.

Geography was a limitation for this study. Participants who were not from southern California were not as compliant towards the end of the study, failing to complete the assignments on time, not mailing back their fecal samples and cancelling appointments.

Surprisingly, patients who came across the NUTRIENT study through our website, were more responsive and engaged than those recruited by the research team in the field.

Another limitation to our pilot study was the number of enrolled patients who had a moderate to low symptom burden. It was hard to evaluate the anti-inflammatory effect of the diet on symptom burden when patients weren't highly symptomatic to begin with. For future studies we propose to screen potential trial candidates with high symptom burden in order to appreciate the change in MPN symptom score during the dietary intervention.

Yet a third limitation was including participants who at baseline were highly adherent to the Mediterranean diet in conformity with a MEDAS ≥8. We observed that at week 2 (before the intervention phase), almost 60% of the patients in the Mediterranean group were already adherent as compared to less than 20% of the participants in the USDA group. This establishes that there's a need to have the starting point better defined to avoid preexisting adherence to the Mediterranean diet in either group.

We also saw that there was a slight decrease in the percentage of patients who were adherent to de Mediterranean diet towards the end of the intervention phase, suggesting that incorporation of some sort of extra stimulus is needed, such as ongoing active dietitian counseling to maintain the interest.

During the intervention phase we noticed an increase in the amount of USDA group participants who were adherent to the Mediterranean diet. This could be because many of the recommendations overlap (whole grain, fruit, vegetables, nuts/seeds) in both food pyramids, or we also hypothesized that this could be due to a "game effect" in which patients might have gotten clues of the type of food they should be eating from the MEDAS questionnaire and they tried to raise their score thinking they would do better. Considering

this we should rephrase our patient reported outcome survey design as to not reveal the components of the Mediterranean diet that will gain "points" on the survey. Another approach would be to estimate the Healthy Eating Index (HEI) score calculated from the ASA24® food recalls and compare that score over time in the USDA group.

Removal of week 3 from all data sets was necessary since not all of the patients had started the diet intervention as originally planned due to scheduling conflicts with the dietitian. Therefore, some patients had their first dietician visit at the week 3 time point and others had not. Patients reported back to us once they had attended their consult. A more strict scheduling criteria with the dietician is needed for subsequent studies. A follow-up phone call on weeks 5 and 7 was part of this study. However, this wasn't possible for all study subjects. Enforcing the need for the follow up visits and administering them via zoom rather than telephone may increase the compliance with follow up visits for future studies.

Participants donated urine samples during 4 timepoints. We originally had planned to use them as an objective measure of adherence to the Mediterranean diet by quantifying the total polyphenol excretion (TPE). This is done by mass spectrometry and was beyond the scope of our study. Nevertheless, these samples were stored for future use.

Though the Mediterranean diet did not have a greater effect than the USDA diet on specific MPN symptoms as we had predicted, overall the dietary intervention led to a reduced symptom burden in both groups. This is still a positive result as MPN patients would benefit from using diet as a medical nutritional therapy.

Also, no obvious impact of the diet intervention was detected in relation to the change in blood counts, calorie intake and weight. Although weights were measured at

every visit, this nutritional intervention was not geared towards weight loss, but rather mere healthy eating and maintaining the weight. The body mass index (BMI) is a more informative measure in nutrition studies. Height should also be measured in consequent studies to calculate the BMI.

We looked at vitamins and antioxidants that are abundant in a Mediterranean style dietary pattern. We were interested in knowing whether they were different and we identified that all the groups increased their intake targets regardless of the specific diet. This result supports that dietary counseling, regardless of the specific diet counseled, may improve vitamin intake and lead to a general improvement in one's diet.

We detected no significant changes in hs-CRP and the 5 cytokines quantified by our panel. We will need a much larger cohort of patients to detect a difference in these inflammatory biomarkers, and a longer intervention period may be necessary. For future studies, perhaps a more sensitive ways to assess inflammatory markers would be through metabolomics.

In our studies to identify the influence of the Mediterranean diet on the MPN gut microbiome we interestingly found *Faecalibacterium prausnitzii*, an anti-inflammatory microbe to be significantly decreased in myelofibrosis patients at the start of the nutrition trial when compared to those with essential thrombocythemia. This fiber degrading microbe is involved in butyrate, a short chain fatty acid production in the gut. We concluded that in our cohort, more inflammation translated into reduced diversity, but could diversity be an established read out for inflammation in MPN patients? Could the decrease in *F. prausnitzii* be predictive of ET and PV people who would eventually transition to MF?

From this trial we also learned that characterizing the participant's microbiome at the beginning of the study is important. We could potentially use the microbiome as a screening tool by identifying a signature with specific taxa.

Finally, we need to address that there are caveat regional differences. It is difficult to recapitulate Mediterranean diet in the Unites States because the method used to process grains is very different from the Mediterranean countries. Overall, the outcome of this diet study is to inform the design of following studies.

REFERENCES

1. Baxter EJ *et al.* Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet* 365, 1054–1061 (2005).
2. James C *et al.* A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature* 434, 1144–1148 (2005).
3. Kralovics R *et al.* A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N. Engl. J. Med.* 352, 1779–1790 (2005).
4. Levine RL *et al.* Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell* 7, 387–397 (2005).
5. Zhao R *et al.* Identification of an acquired JAK2 mutation in polycythemia vera. *J. Biol. Chem.* 280, 22788–22792 (2005).
6. Emanuel RM *et al.* Myeloproliferative neoplasm (MPN) symptom assessment form total symptom score: prospective international assessment of an abbreviated symptom burden scoring system among patients with MPNs. *J. Clin. Oncol.* 30, 4098–4103 (2012).
7. Geyer HL, Dueck AC, Scherber RM, Mesa RA. Impact of inflammation on myeloproliferative neoplasm symptom development. *Mediators Inflamm.* 2015, 284706 (2015).
8. Hermouet S, Bigot-Corbel E, Gardie B. Pathogenesis of myeloproliferative neoplasms: role and mechanisms of chronic inflammation. *Mediators Inflamm.* 2015, 145293 (2015).
9. Hasselbalch HC, Bjørn ME. Mpns as inflammatory diseases: the evidence, consequences, and perspectives. *Mediators Inflamm.* 2015, 102476 (2015).
10. Fleischman AG. Inflammation as a driver of clonal evolution in myeloproliferative neoplasm. *Mediators Inflamm.* 2015, 606819 (2015).
11. Jutzi JS, Pahl HL. The hen or the egg: inflammatory aspects of murine MPN models. *Mediators Inflamm.* 2015, 101987 (2015).
12. Fleischman AG *et al.* TNFalpha facilitates clonal expansion of JAK2V617F positive cells in myeloproliferative neoplasms. *Blood* 118, 6392–8 (2011).
13. Kleppe M *et al.* JAK-STAT pathway activation in malignant and nonmalignant cells contributes to MPN pathogenesis and therapeutic response. *Cancer Discov.* 5, 316–331 (2015).
14. Giugliano D, Ceriello A, Esposito K. The effects of diet on inflammation: emphasis on the metabolic syndrome. *Journal of the American College…* (2006).
15. Calder PC *et al.* Dietary factors and low-grade inflammation in relation to overweight and obesity. *Br. J. Nutr.* 106 Suppl 3, S5–78 (2011).

16. Estruch R *et al.* Primary prevention of cardiovascular disease with a Mediterranean diet. *N. Engl. J. Med.* 368, 1279–1290 (2013).
17. Estruch R. Anti-inflammatory effects of the Mediterranean diet: the experience of the PREDIMED study. *Proc Nutr Soc* 69, 333–340 (2010).
18. Verstovsek S *et al.* A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. *N. Engl. J. Med.* 366, 799–807 (2012).
19. Geyer H *et al.* Symptomatic Profiles of Patients With Polycythemia Vera: Implications of Inadequately Controlled Disease. *J. Clin. Oncol.* 34, 151–159 (2015).
20. Martinez-Gonzalez MA *et al.* A 14-item Mediterranean diet assessment tool and obesity indexes among high-risk subjects: the PREDIMED trial. PLoS One. 7(8): p. e43134 (2012).
21. Rumawas ME, Dwyer JT, Mckeown NM, Meigs JB, Rogers G, Jaques PF. The Development of the Mediterranean-Style Dietary Pattern Score and Its Application to the American Diet in the Framingham Offspring Cohort. *J Nutr.* 139(6): 1150–1156 (2009).
22. Mesa RA, Niblack J, Wadleigh M, Verstovsek S, Camoriano J, Barnes S, Tan AD, Atherton PJ, Sloan JA, Tefferi A. The burden of fatigue and quality of life in myeloproliferative disorders (MPDs): An international internet-based survey of 1179 MPD patients. Cancer. 109, 68–76 (2007).
23. Hong Seog Seo, MD. The Role and Clinical Significance of High-Sensitivity C-Reactive Protein in Cardiovascular Disease. Korean Circulation Journal (2012).
24. Hirschfield GM, Pepys MB. C-reactive protein and cardiovascular disease: new insights from an old molecule. QJM. 96:793-807 (2003).
25. Lau DC, Dhillon B, Yan H, Szmitko PE, Verma S. Adipokines: molecular links between obesity and atherosclerosis. Am J Physiol Heart Circ Physiol. 288:H2031-41 (2005).
26. Craver BM, El Alaoui K, Scherber RM, Fleischman AG. The Critical Role of Inflammation in the Pathogenesis and Progression of Myeloid Malignancies. *Cancers. 10*, 104; doi:10.3390 (2018).
27. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 505, 559–563 (2014).
28. Round J, Mazmanian S. The gut microbiome shapes intestinal immune responses during health and disease. Nat Rev Immunol. 9(5): 313–323. doi:10.1038/nri2515 (2009).

29. Forbes J, Domselaar GV, Bernstein CN. The Gut Microbiota in Immune-Mediated Inflammatory Diseases. Front. Microbiol.; doi: 10.3389/fmicb.2016.01081 (2016).

30. Adams E, Wandro S, Avelar-Barragan J, Oliver A, Whiteson K. Low Volume Methodology for Nextera DNA Flex Library Prep Kit (96 Samples). Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, CA, United States. dx.doi.org/10.17504/protocols.io.be6rjhd6 (2020).

31. Bushnell B. *BBMap: A Fast, Accurate, Splice-Aware Aligner*. United States: N. p., Web (2014).

32. Cantu V, Sadural J & Edwards R. PRINSEQ++, a multi-threaded tool for fast and efficient quality control and preprocessing of sequencing datasets. 10.7287/peerj.preprints.27553v1 (2019).

33. Andrews S. FastQC: a quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (2010).

34. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nature Methods*. 9(4):357-9. doi: 10.1038/nmeth.1923 (2012)

35. Francesco Beghini. MetaPhlAn 3 (3.0.1). Zenodo. https://doi.org/10.5281/zenodo.3955744 (2020).

36. Oksanen, Jari & Blanchet, F. Guillaume & Kindt, Roeland & Legendre, P. & Minchin, Peter & O'Hara, Bob & Simpson, Gavin & Solymos, Peter & Stevens, Hank & Wagner, Helene. (2015). Package 'vegan' - Community Ecology Package. R News. 8. 48-50.

37. Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team. *nlme: Linear and Nonlinear Mixed Effects Models*. R package version 3.1-153, https://CRAN.R-project.org/package=nlme (2021)

38. Wickham H. *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. ISBN 978-3-319-24277-4, https://ggplot2.tidyverse.org (2016).

39. Barcenilla *et al.* Phylogenetic relationships of butyrate-producing bacteria from the human gut. *Appl Environ Microbiol.* 66: 1654–1661 (2000).

40. Duncan *et al.* Growth requirements and fermentation products of *Fusobacterium prausnitzii*, and a proposal to reclassify it as *Faecalibacterium prausnitzii* gen. nov., comb. nov. *Int J Syst Evol Microbiol.* 52: 2141–2146 (2002).

41. Christl *et al.* Antagonistic effects of sulfide and butyrate on proliferation of colonic mucosa. *Dig Dis Sci.* 41: 2477–2481 (1996).

42. Archer *et al.* Butyrate inhibits colon carcinoma cell growth through two distinct pathways. *Surgery* 124: 248–253 (1998).

43. De Vadder F, Kovatcheva-Datchary P, Goncalves D, Vinera J, Zitoun C, Duchampt A, Bäckhed F, Mithieux G. Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. *Cell* 156, 84–96 (2014).

44. Vrieze A, Van Nood E, Holleman F, Salojärvi J, Kootte RS, Bartelsman JF, Dallinga-Thie GM, Ackermans MT, Serlie MJ, Oozeer R. *et al.* Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology* 143, 913–916.e7 (2012).
45. Jaiswal S, Fontanillas P, Flannick J, Manning A, Grauman PV, Mar BG, Lindsley RC, Mermel CH, Burtt N, Chavez A *et al*. Age-related clonal hematopoiesis associated with adverse outcomes. *The New England journal of medicine* 371(26):2488-2498 (2014).
46. McKerrell T, Park N, Moreno T, Grove CS, Ponstingl H, Stephens J, Crawley C, Craig J, Scott MA, Hodkinson C *et al*. Leukemia-associated somatic mutations drive distinct patterns of age-related clonal hemopoiesis. *Cell reports* 10(8):1239-1245 (2015).
47. Busque L, Patel JP, Figueroa ME, Vasanthakumar A, Provost S, Hamilou Z, Mollica L, Li J, Viale A, Heguy A *et al*. Recurrent somatic TET2 mutations in normal elderly individuals with clonal hematopoiesis. *Nature genetics* 44(11):1179-1181 (2012).
48. Genovese G, Kahler AK, Handsaker RE, Lindberg J, Rose SA, Bakhoum SF, Chambert K, Mick E, Neale BM, Fromer M *et al*. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *The New England journal of medicine* 371(26):2477-2487 (2014).
49. Zink F, Stacey SN, Norddahl GL, Frigge ML, Magnusson OT, Jonsdottir I, Thorgeirsson TE, Sigurdsson A, Gudjonsson SA, Gudmundsson J *et al*. Clonal hematopoiesis, with and without candidate driver mutations, is common in the elderly. *Blood* 130(6):742-752 (2017).
50. Coombs CC, Zehir A, Devlin SM, Kishtagari A, Syed A, Jonsson P, Hyman DM, Solit DB, Robson ME, Baselga J *et al*. Therapy-Related Clonal Hematopoiesis in Patients with Non-hematologic Cancers Is Common and Associated with Adverse Clinical Outcomes. *Cell stem cell* 21(3):374-382.e374 (2017).
51. Bhattacharya R, Zekavat SM, Uddin MM, Pirruccello J, Niroula A, Gibson C, Griffin GK, Libby P, Ebert BL, Bick A *et al*. Association of Diet Quality With Prevalence of Clonal Hematopoiesis and Adverse Cardiovascular Events. *JAMA Cardiol.* (2021).
52. Haring B, Reiner AP, Liu J, Tobias DK, Whitsel E, Berger JS, Desai P, Wassertheil-Smoller S, LaMonte MJ, Hayden KM *et al*. Healthy Lifestyle and Clonal Hematopoiesis of Indeterminate Potential: Results From the Women's Health Initiative. *J Am Heart Assoc.* 10(5):e018789 (2021).

53. Li Z, Cai X, Cai CL, Wang J, Zhang W, Petersen BE, Yang FC, Xu M: Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies. *Blood* 118(17):4509-4518 (2011).
54. Jeong M, Park HJ, Celik H, Ostrander EL, Reyes JM, Guzman A, Rodriguez B, Lei Y, Lee Y, Ding L *et al*. Loss of Dnmt3a Immortalizes Hematopoietic Stem Cells In Vivo. *Cell reports* 23(1):1-10 (2018).
55. Abegunde SO, Buckstein R, Wells RA, Rauh MJ: An inflammatory environment containing TNFα favors Tet2-mutant clonal hematopoiesis. *Experimental hematology* 59:60-65 (2018).
56. Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Masse A, *et al.* Mutation in TET2 in myeloid cancers. *N Engl J Med.* 360(22):2289–301 (2009).
57. Cai *et al.* Inhibition of Inflammatory Signaling in Tet2 Mutant Preleukemic Cells Mitigates Stress-Induced Abnormalities and Clonal Hematopoiesis. *Cell Stem Cell* 23, 833–849 (2018)
58. Cull AH, Snetsinger B, Buckstein R, Wells RA, Rauh MJ. Tet2 restrains inflammatory gene expression in macrophages. *Exp Hematol*. 55:56–70, e13 (2017).
59. Singer K, Del Proposto J, Morris DL, Zamarron B, Mergian T, Maley N, Cho KW, Geletka L, Subbaiah P, Muir L *et al.* Diet-induced obesity promotes myelopoiesis in hematopoietic stem cells. *Molecular metabolism* 3, 664-675 (2014).
60. Craver BM, Ramanathan G, Hoang S, Chang X, Mendez Luque LF, Brooks S, Lai HY, Fleischman AG. N-acetylcysteine inhibits thrombosis in a murine model of myeloproliferative neoplasm. *Blood advances* 4, 312-321 (2020).
61. Martinez de Lizarrondo S *et al.* Potent Thrombolytic Effect of N-Acetylcysteine on Arterial Thrombi. *Circulation* 136, 646-660 (2017).
62. Wang B, Yee Aw T, Stokes KY. N-acetylcysteine attenuates systemic platelet activation and cerebral vessel thrombosis in diabetes. *Redox Biol.* 14, 218-228 (2018).