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**OMEGA-3 FATTY ACIDS AS THERAPEUTIC OPTIONS FOR THE TREATMENT OF
B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA**

A dissertation submitted to
the Graduate College of
Marshall University

In partial fulfillment of
the requirements for the degree of

Doctor of Philosophy
in
Biomedical Sciences

by
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LIST OF ABBREVIATIONS

AA- arachidonic acid

ADP- adenosine diphosphate

Akt- protein kinase B

ALA- alpha-linolenic acid

ALC- absolute lymphocyte count

AraC- arbinosylcytosine

ATM- ataxia telangiectasia

BAFF- B-cell activating factor

Bcl-2- B-cell CLL/lymphoma 2

Bcl-3- B-cell lymphoma 3

BCR- B-cell receptor

BIRC3- baculoviral IAP repeat-containing protein 3

cAMP- cyclic adenosine monophosphate

CAP- cyclophosphamide, doxorubicin, prednisone

CBC- complete blood cell count

CCND1/3- cyclins

CDK6- cyclin-dependent kinase 6

CFAR- cyclophosphamide, fludarabine, alemtuzumab, rituximab

c-FLIP- FLICE inhibitory protein

CHOP- cyclophosphamide, doxorubicin, vincristine, prednisone

cIAPS- inhibitors of apoptosis

CLL- Chronic Lymphocytic Leukemia

COX- cyclooxygenase

CRTH2- chemoattractant receptor-homologous molecule expressed on TH2 cells

c-Src- proto-oncogene tyrosine-protein kinase

CT- closure time

CYP- cytochrome p450

Del11q22-23- deletion of chromosomal region 11q22-23

Del13q14- deletion of chromosomal region 13q14

Del17p13- deletion of chromosomal region 17p13

DHA- docosahexaenoic acid

DLEU-1/2- deleted in leukemia-1/2

DOX- doxorubicin

DP- D prostanoid receptor

DPA- docosapentaenoic acid

ECOG- Eastern Cooperative Oncology Group

EDTA- Ethylenediaminetetraacetic acid

EET- epoxyeicosatrienoic acid

EETeTrs- epoxyeicosatetraenoic acids

EFAs- essential fatty acids

EGFR- epidermal growth factor receptor

EGR-1- early growth response protein 1

EP- E prostanoid receptor

EPA- eicosapentaenoic acid

EPI- epinephrine

ERK- extracellular signal-regulated kinase

FA- fatty acid

FADD- Fas-associated death domain

FCR- fludarabine, cyclophosphamide, rituximab

FISH- fluorescence in situ hybridization

FLICE- caspase 8/FADD-like IL-1 β converting enzyme

FP- F prostanoid receptor

GEPA- gene expression profile analyses

GLA- gamma-linolenic acid

GM-CSF- granulocyte-macrophage colony-stimulating factor

GPCRs- G-protein-coupled rhodopsin receptors

GSK3 α - glycogen synthase kinase 3

HEPE- hydroxyeicosapentaenoic acid

HETEs- hydroxyeicosatetraenoic acids

HIF-1 α - hypoxia inducible factor-1 alpha

HLA- homogamma linolenic acid

ICD- International Classification of Disease

IgVH- immunoglobulin heavy variable chain

I κ B- intracellular inhibitor of kappa B

IKK- inhibitor of I κ B kinase

IL-1- interleukin-1

IL-1R- IL-1 Receptor

iNOS- inducible nitric oxide synthase

IP- I prostanoid receptor

IRF- IFN regulatory factor

ITP- idiopathic thrombocytopenic purpura

LA- linoleic acid

LC-1- irreversible-IKK inhibitor

LDT- lymphocyte doubling time

LOX- lipoxygenase

LPS- lipopolysaccharide

LTs- leukotrienes

MAPK- MAP kinase

MBL- monoclonal B-cell lymphocytosis

miR- microRNA

MRP1- multidrug-resistance-associated protein 1

MTT- (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

N-3- omega-3

N-6- omega-6

NCI SEER- Surveillance Epidemiology and End Results Study

NFκB- nuclear factor kappa B

NOTCH1- notch homolog 1

NSCLC- non-small cell lung cancer

OA- oleic acid

OFAR- oxaliplatin, fludarabine, alemtuzumab, rituximab

OS- overall survival

PGES-1- prostaglandin E synthase-1

Pgp- p-glycoprotein

PGs- prostaglandins

PI3K- phosphoinositide 3-kinase

IPN- paclitaxel induced peripheral neuropathy

PKA- protein kinase A

PLL- Pro-lymphocytic Leukemia

PPARs- peroxisome proliferator-activated receptors

PTMs- post-translational modifications

PUFA- polyunsaturated fatty acids

RANKL- receptor activator of NF κ B ligand

RHD- Rel homology domain

RNS- reactive nitrogen species

ROS- reactive oxygen species

SA- stearic acid

SF3B1- slicing factor 3B subunit 1

SLL- small lymphocytic lymphoma

SOC- standard of care

SPEP- serum protein electrophoresis

β 2M- β 2- microglobulin

T(14;19)- translocation between chromosome 14 and chromosome 19

TADS- transactivation domains

TBARs- thiobarbituric acid reactive substances

Tcf- T-cell factor

TGF α - transforming growth factor-alpha

TLR4- toll-like receptor 4

TNFR- TNF Receptor

TNF- α - tumor necrosis factor alpha

TP- thromboxane receptor

TP53- tumor suppressor protein p53

TRAP- tartrate-resistance acid phosphatase

TT- time to treatment

TXs- thromboxanes

VEGF- vascular endothelial growth factor

WBC- white blood cells

WHO- World Health Organization

X:Yn-3/6- X denotes carbon number, Y denotes number of unsaturations, n-3- omega-3, n-6-
omega-6

ZAP-70- zeta-associated protein 70

ABSTRACT

B-cell chronic lymphocytic leukemia (CLL) is the most common form of adult leukemia in the western world. CLL is often diagnosed in the asymptomatic (early-stage) stages. However, approximately 50% of these patients will progress to advanced, symptomatic disease and require therapy. Current treatment options are limited due to progressive drug resistance and severe drug-induced toxicities which are often too toxic for the elderly or those with co-morbidities. Therefore, a non-toxic therapeutic intervention that could slow the progression of asymptomatic CLL to symptomatic CLL or enhance the effects of actively used chemo-therapeutic drugs in patients who require therapy would be clinically beneficial.

In our studies, we evaluated the use of omega-3 (n-3) fatty acids as therapeutic options for CLL utilizing both a clinical supplementation model (FWA #00002704) as well as a cell culture model. The primary objective of the initial study was to determine whether consumption of n-3 could suppress activation of nuclear factor kappa B (NFκB) in lymphocytes from patients diagnosed with early stage CLL. The primary objective of the follow-up study was to evaluate whether n-3 eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA) could enhance the chemo-sensitivity of CLL-derived cell lines EHEB and MEC-2 and pro-lymphocytic leukemia-derived cell line JVM-2 to anti-cancer drugs doxorubicin, vincristine or fludarabine *in vitro*.

Our initial study indicated that consumption of an EPA + DHA containing n-3 supplement 1) suppressed activation of NFκB in lymphocytes from patients diagnosed with early stage CLL, 2) increased molar concentrations of n-3 in the plasma, 3) increased the chemo-sensitivity of lymphocytes to doxorubicin in an *in vitro* assay and 4) decreased the expression of 32 genes in lymphocytes. Targeting the NFκB pathway is proposed as a therapy for CLL. Suppression of NFκB activation by n-3 may slow the progression of the disease to symptomatic

disease where therapy is required. However, a definitive clinical trial will be needed to determine if n-3 can slow the progression of CLL.

The aim of the second study was to expanding upon the chemo-sensitization capabilities of n-3. In these trials, cell-cycle analyses, Annexin-V assays, assays for malondialdehyde (a measure of lipid peroxidation) and DCF fluorescence assays (a measure of reactive oxygen species (ROS) generation) were performed to explore potential mechanism(s) through which enhanced chemo-sensitivity was achieved.

Results indicated that: 1) EPA and DHA differentially sensitized B-leukemic cell lines EHEB, JVM-2 and MEC-2 to doxorubicin, vincristine and fludarabine *in vitro*; 2) n-3 alone and with drug treatment increased cell death and induced G2/M arrest in a cell-type specific manner; 3) lipid peroxidation increased in the presence of n-3; 4) there was higher lipid peroxidation in MEC-2 cells in presence of DHA and doxorubicin than with either alone; 5) n-3 increased generation of ROS in MEC-2, and 6) the addition of vitamin-E abrogated the increase in ROS generation and chemo-sensitivity of MEC-2 to doxorubicin by DHA.

N-3 are a promising therapeutic intervention for the treatment of CLL with the capacity to potentially slow the progression of the disease and enhance the chemo-sensitivity of malignant cells to chemo-therapeutic drugs and warrants further investigation. Slowing the progression of the disease would be clinically beneficial. Enhanced chemo-sensitivity would be expected to increase drug efficacy and potential reductions in drug dosage and drug-induced toxicities.

CHAPTER 1: INTRODUCTION

CHRONIC LYMPHOCYTIC LEUKEMIA

EPIDEMIOLOGY, CHARACTERIZATION

B-cell chronic lymphocytic leukemia (CLL) is the most common form of adult leukemia in the western world (Gaidano, Foa, & Dalla-Favera, 2012) and accounts for 30% of all mature B-cell malignancies (Linnet et al., 2007). CLL involves progression toward malignant disease with most cases of CLL being preceded by monoclonal B-cell lymphocytosis (MBL), an indolent B-cell expansion defined by less than 5×10^3 MBL/ μ L in the peripheral blood (Gaidano et al., 2012). The incidence of CLL in the United States is approximately 100,000 individuals with an estimated 15,000 new diagnoses per year (Shanafelt & Call, 2004). CLL is typically diagnosed in the asymptomatic stages incidentally during a complete blood cell count (CBC) for unrelated indications (Shanafelt & Call, 2004). However, approximately 50% of these patients experience rapid progressive disease, require therapy and have a significantly shortened lifespan (Shanafelt & Call, 2004). It is approximated that 4,500 individuals will die of CLL or CLL-related complications in the United States per year (Lanasa, 2010; Shanafelt & Call, 2004). The life expectancy of an individual with CLL is highly variable with a median survival from diagnosis varying between 18 months to >10 years (Eichhorst, Dreyling, Robak, Montserrat, & Hallek, 2011; Gaidano et al., 2012; Shanafelt & Call, 2004), a trait largely attributed to the marked clinical heterogeneity of the disease.

CLL is considered to be a disease of the elderly with a median age range of 60-68 years (Shanafelt & Call, 2004). According to the NCI SEER (Surveillance Epidemiology and End Results) study, which enrolled 20,000 CLL and small lymphocytic lymphoma (SLL) patients in

the United States from 1987 to 2004, the incidence of CLL increased with age and showed a 70-90% higher propensity in males than females when adjusting for age (Linnet et al., 2007).

Compared to Caucasians, the incidence in African-Americans and Asian/Pacific Islanders was found to be 25-28% and 69-80% lower, respectively (Linnet et al., 2007). While the exact origin of CLL remains unknown, immunogenetic studies and gene expression profiling analyses have provided insight into the putative CLL progenitor and suggest that the origin of CLL is derived from antigen-experienced B cells (including both memory B cells and marginal zone B-cells)(Gaidano et al., 2012).

Historically, CLL is viewed as a tumor caused by the accumulation of long-lived but mainly indolent lymphocytes (Gaidano et al., 2012). However, studies have shown that a small fraction of CLL cells have proliferative capacity resulting in approximately 2% new CLLs being produced per day (Messmer et al., 2005). While lymphocytes in the peripheral blood are in a predominantly resting, non-proliferative state, specific structures known as proliferation centers (bone marrow and lymph nodes) replenish the CLL population and aid to the accumulation of CLL cells (Gaidano et al., 2012).

Although designated as a single entity, CLL is characterized by biological, clinical and cytogenetic heterogeneity with a characteristic immunophenotype of IgM^{weak}, CD5+, CD19+, CD20^{weak}, CD22^{weak}, CD79a+, CD23+, CD43+, CD11c^{weak}, CD10^{negative}, and cyclin D1 negative (Linnet et al., 2007).

CLASSIFICATION AND STAGING

Chronic lymphocytic leukemia has been recognized as a distinct clinical entity for nearly 100 years (Linnet et al., 2007). However, disease classification systems did not systematically distinguish 'chronic' from 'acute' forms of leukemia until the late 1960s with the adoption of the

International Classification of Disease (ICD) (Linnet et al., 2007). The absence of standardized disease definitions, staging schemes and markers of behavior hampered clinical trials and epidemiological studies until the mid-1970s (Linnet et al., 2007). It was during this time that proposals for staging systems by Rai *et al.* and Binet *et al.* (1977) were accepted. Clinical trial guidelines from the International Workshop of CLL, Binet *et al.* and Cheson *et al.* were subsequently implemented in the 1980s and 1990s. The staging system and clinical trial guidelines were instrumental to advances in the treatment and understanding of CLL biology (Linnet et al., 2007; Shanafelt & Call, 2004). The improved knowledge of normal B- and T-cell differentiation and genetic changes led to the revised European and American Lymphoma classification in the mid-1990s, followed by the World Health Organization (WHO) classification of all hematopoietic and lymphoproliferative disorders and set the basis for modern classification and staging of CLL (Linnet et al., 2007).

Before the advent of automated instruments for performing blood cell counts, CLL was typically diagnosed when patients exhibited symptoms such as lymphadenopathy, cytopenias, frequent infections, and abnormal morphology of peripheral blood lymphocytes (Shanafelt & Call, 2004). At this point in time, CLL was thought to be an indolent disease of the elderly (Shanafelt & Call, 2004). Clinical management of CLL was predominately mediated by “watchful waiting” and most clinicians often counseled patients that they would likely die of causes unrelated to CLL before they would require therapy (Shanafelt & Call, 2004). The infectious complications associated with CLL, increased risk of autoimmune disorders, and increased risk of secondary malignancies were less defined and clinically underappreciated with treatment being reserved for patients with advanced-stage disease (Shanafelt & Call, 2004).

Nearly all of these paradigms have changed with the advent of automated instruments, immunophenotyping, flow cytometry, and cytogenetic evaluations (Shanafelt & Call, 2004).

Currently, CLL should be considered anytime a patient presents an absolute lymphocyte count (ALC) $> 5 \times 10^9/L$ without clear etiology over a duration of at least 3 months (Eichhorst et al., 2011; Shanafelt & Call, 2004). Furthermore, according to the WHO classification, SLL and CLL are considered the same entity. SLL requires the presence of lymphadenopathy and/or splenomegaly, with the number of B-lymphocytes in the peripheral blood not exceeding $5 \times 10^9/L$ and must be excluded during the diagnosis of CLL (Eichhorst et al., 2011). Similarly, reactive causes of lymphocytosis and leukemic phase of other lymphoproliferative disorders, particularly mantle cell lymphoma, must be excluded before diagnosis of CLL (Eichhorst et al., 2011; Shanafelt & Call, 2004). Diagnostic criteria for CLL are described in Table 1.1.

Upon diagnosis of CLL, patients staging is determined according to the clinical “staging” criteria outline by Rai *et al.* (Rai & Jain, 2011) which separates patients into different prognostic “risk” groups based on the presence of lymphadenopathy, splenomegaly, hepatomegaly, and cytopenias (Shanafelt & Call, 2004). The three stage system categorizes patients as having low risk (original Rai stage 0), intermediate risk (Rai stage I-II), or high risk (Rai stage III-IV) disease (Shanafelt & Call, 2004). Staging criteria are described in Table 1.2. Additional cytogenetic and phenotypic tests are performed to assist with predicting diagnosis and guide treatment (Shanafelt & Call, 2004) (outlined in next section).

| Table 1. 1. Diagnostic Criteria for CLL | |
|---|--|
| Diagnostic Criteria | Typical Method of Testing |
| 1. ALC, >5.0 x 10 ⁹ /L | Automated blood counter |
| 2. Clonal B-cell proliferation | Exclusively κ or γ light chain use on flow cytometry Immunophenotype consistent with CLL: CD5+, CD19+, CD20 ^{dim} , dim surface immunoglobulin |
| 3. Rule out leukemic phase of other lymphoproliferative disorders (especially mantle cell lymphoma) | Mantle cell lymphoma: Flow cytometry*: CD23 ^{dim or absent} , CD5+, bright CD20+ FISH**: t(11:14) Cyclin D1 positive Nodal marginal zone lymphoma: Flow cytometry*: CD5-, bright CD20+ Hairy cell lymphoma: Flow cytometry*: CD5-, bright CD11c/CD22+, CD19+, bright CD20+, CD103+ TRAP positive Lymphoplasmacytic lymphoma: Flow cytometry*: CD5+/-, CD19+, CD20+ SPEP-associated monoclonal protein (>2.5g/dL) Follicular lymphoma (leukemic phase): Flow Cytometry*: CD5-, CD19+, CD20+, CD10+/- FISH**: t(14:18) |
| <i>Material obtained from (Shanafelt & Call, 2004)</i> | |

Table 1.1 Diagnostic Criteria for Chronic Lymphocytic Leukemia. Abbrev: ALC- absolute lymphocyte count; CLL- chronic lymphocytic leukemia; FISH- fluorescence in situ hybridization; SPEP- serum protein electrophoresis; TRAP- tartrate-resistance acid phosphatase; *Flow cytometric results must be correlated with careful pathologic assessment of morphologic features; **FISH- translocation of indicated chromosome. Material was obtained from (Shanafelt & Call, 2004).

| Table 1. 2. Rai Staging Criteria | | | | | | |
|--|---------------------------------------|-----------------------|------------------------------|---------------------------------|---|-----------------------------------|
| Physical Examination | | | | | | |
| Rai Stage | ALC >5.0 x 10⁹/L | Enlarged nodes | Enlarged liver/spleen | Hemoglobin <10.5 g/dL | Platelets <100 x 10⁹/L | Median Survival (months) * |
| 0 | + | - | - | - | - | 150 |
| I | + | + | - | - | - | 101 |
| II | + | +/- | + | - | - | 71 |
| III | + | +/- | +/- | + | - | 19 |
| IV | + | +/- | +/- | +/- | + | 19 |
| <i>Material obtained from (Shanafelt & Call, 2004)</i> | | | | | | |

Table 1.2. Rai Staging Criteria. Abbrev: ALC- absolute lymphocyte count; +: present, -: absent; +/-: may be present or absent; *: according to original series by Rai *et al.* Material was obtained from (Shanafelt & Call, 2004).

SYMPTOMS, RISK ASSESSMENT, PROGNOSTICS

Clinical manifestations associated with CLL often include fever, night sweats, weight loss defined as >10% of body weight, fatigue, frequent infections, splenomegaly, hepatomegaly, lymphadenopathy, autoimmune complications and cytopenias (Shanafelt & Call, 2004).

Given the heterogeneity of CLL, various prognostic indicators have been established to aid in predicting prognosis and guiding treatment. The staging criteria outlined by Rai *et al.* (Rai & Jain, 2011) provided a useful tool for grouping patients into low-, intermediate- or high-risk groups and has been shown to correlate with prognosis of disease (Table 1.2). The lymphocyte doubling time (LDT) also provided insight into the kinetics of CLL. The LDT is a clinical measurement that addresses the kinetics of cell growth by calculating the number of months the ALC takes to double. Since patients with advanced disease (Rai stages III and IV) typically

require therapy, the prognostic utility of LDT is most important for patients with early stage disease (Rai stages 0, I and II) (Shanafelt & Call, 2004). Studies have found that patients with CLL exhibiting a LDT of less than 12 months had shortened survival time as compared to those patients with a LDT greater than 12 months (Montserrat, Sanchez-Bisono, Vinolas, & Rozman, 1986). While historically clinical stage and LDT have been the most accurate and widely used prognostic tools for counseling patients with CLL, both are imprecise. Clinical staging can underappreciate the prognosis of patients due to marked heterogeneity in the clinical progression of disease in patients at similar age (Shanafelt & Call, 2004). Similarly, the LDT can be confounded by factors that cause fluctuations in the ALC (Shanafelt & Call, 2004). As such, other diagnostic tools such as flow cytometry, gene expression profiling, chromosomal analysis and fluorescence *in situ* hybridization (FISH) have been established to aid in risk assessment and prognosis of CLL.

Extensive molecular investigations of the B-Cell Receptor (BCR) indicated that 60-65% of CLL harbor somatic hypermutations in the immunoglobulin heavy variable chain (IgVH) genes. In 1999, Damle *et al.* (Damle et al., 1999) and Hamblin *et al.* (Hamblin, Davis, Gardiner, Oscier, & Stevenson, 1999) reported the prognostic significance of somatic mutations in the IgVH genes in CLL. When patients with early stage CLL were stratified as IgVH-mutated or IgVH-nonmutated, significant differences in survival times were observed (Damle et al., 1999; Hamblin et al., 1999). Median survival time for early-stage patients with nonmutated IgVH genes was less than 95 months (8 years) whereas early-stage patients with a mutated IgVH had a median survival time greater than 293 months (24 years) (Damle et al., 1999; Hamblin et al., 1999). Whereas tests for IgVH mutational status are not typically performed in the United States

(Shanafelt & Call, 2004), its utilization as a prognostic marker is beneficial for patients with CLL.

Phenotypic evaluations of CLL cells have indicated the presence of surface protein marker CD38 in subsets of CLL. Numerous reports have substantiated the prognostic significance of CD38 expression (Del Poeta et al., 2001; Lekovic et al., 2011; Shanafelt & Call, 2004). Del Poeta *et al.* reported an 8-year survival of 92% for patients who were CD38 negative but only 50% for patients who were CD38 positive (Del Poeta et al., 2001). However, the consistency of CD38 expression on CLL cells has been controversial because many groups reported changes in the CD38 status in as many as 10-25% of patients with CLL (Shanafelt & Call, 2004). Despite the controversy of CD38 expression, it appears to be correlated with poor prognosis (Lekovic et al., 2011).

Gene expression profiles have also aided to the identification of prognostic markers for CLL. Gene expression profile analyses (GEPAs) have identified a restricted set of genes that helped discriminate between mutated- and unmutated-IgVH clones in patients with CLL (Shanafelt & Call, 2004). Of particular interest was the tyrosine kinase gene, zeta-associated protein 70 (ZAP-70). Under normal conditions, ZAP-70 is involved with T-cell signaling; however, it has been shown to be differentially expressed by patients with unmutated IgVH CLL clones (Shanafelt & Call, 2004). Subsequent studies have shown that ZAP-70 positive CLL cells correctly predict the mutational status (unmutated) in approximately 78-90% of patients (Crespo et al., 2003; Wiestner et al., 2003). More importantly, ZAP-70 expression was also a prognostic marker for time to treatment (TT) and overall survival (OS) (Crespo et al., 2003). Crespo *et al.* reported that patients who had at least 20% ZAP-70-positive CLL cells had more rapid

progression and poorer survival than those with less than 20% ZAP-70-positive CLL cells (Crespo et al., 2003).

Similarly, GEPA have identified other markers thought to aid in prognosis including notch homolog 1 (NOTCH1), splicing factor 3B subunit 1 (SF3B1) and baculoviral IAP repeat-containing protein 3 (BIRC3) (Gaidano et al., 2012). Descriptions of these genes and their influence on prognosis are provided in Table 1.3.

| Table 1. 3. GEPA Identified Prognostic Markers for Chronic Lymphocytic Leukemia | | | |
|--|---|------------------|--|
| Gene | Description | Frequency | Prognostic Indication |
| NOTCH1 (Notch Homolog 1) | Encodes a ligand-activated transcription factor that regulates several downstream pathways involved in cell growth. Mutations in NOTCH1 impair NOTCH1 degradation leading to increased intensity and duration of NOTCH1 activity. | 10% | Poor Prognosis |
| SF3B1 (Splicing Factor 3B Subunit 1) | Critical component of spliceosome. Thought to control cell cycle progression and apoptosis. | 5-10% | Associated with shortened survival time. |
| BIRC3 (Baculoviral IAP Repeat-containing Protein 3) | Negative regulator of MAP3K14. Mutations in BIRC3 disrupts proteosomal degradation of MAP3K14 and leads to constitutive activation of non-cannonical NFκB. | 4% | Poor Prognosis |
| <i>Material obtained from (Gaidano et al., 2012)</i> | | | |

Chromosomal analysis and the development of FISH aided the detection of chromosomal abnormalities and provided numerous prognostic markers for CLL (Shanafelt & Call, 2004). Studies have demonstrated that over 80% of all CLL patients have chromosomal abnormalities (Rodriguez-Vicente, Diaz, & Hernandez-Rivas, 2013). Descriptions of the most common chromosomal abnormalities and their influence on prognosis are illustrated in Table 1.4.

In summary, CLL, although designated as a single entity, is a highly variable disease. While clinical staging remains the foundation for determining the prognosis of patients, it fails to recognize a substantial subset of patients who are likely to experience rapid progression to advanced stage disease. The use of LDT and identification of various prognostic markers outlined above have aided the identification of patients with early-stage CLL who are at risk of early progression. All of these tools are vital for guiding clinical treatment of CLL and identifying at risk patients who may benefit from shorter follow-up intervals.

| Table 1. 4. Prognosis by FISH Results for Patients with CLL | | | |
|--|---|------------------|--------------------------------|
| Chromosomal Abnormality | Description | Frequency | Median Survival (years) |
| None | - | - | 9.0 |
| Del13q14 | Contains the DLEU-1/2 genes which encode for miR-15a and miR-16a. MiR-15a/16a regulates a variety of genes including anti-apoptotic Bcl-2 gene, cyclins CCND1/3 and CDK6. | 50-60% | 11.0 |
| Trisomy 12 positive | Thought to alter dosage of one or more proto-oncogenes. | 15% | 9.0 |
| Del11q22-23 | Affects the ATM gene. Deficiency in ATM causes genomic instability. | unknown | 6.6 |
| Del17p13 | Disrupts the TP53 tumor suppressor gene. | 5-10% | 2.5 |

Table 1.4. Prognosis by FISH Results for Patients with CLL. Del13q14: deletion of chromosomal region 13q14; Del11q22-23: deletion of chromosomal region 11q22-23; Del17p13: deletion of chromosomal region 17p13; DLEU-1/2: deleted in leukemia-1/2; miR: microRNA; Bcl-2: B-cell lymphoma 2; CCND1/3: cyclins; CDK6: cyclin-dependent kinase 6; ATM: ataxia telangiectasia; TP53: tumor suppressor protein p53. Data obtained from (Gaidano *et al.*, 2012).

TREATMENT

Currently, no curative therapy exists for CLL with the exception of allogeneic transplantation (Dreger, 2009). Historically, the goal of treatment was to alleviate symptoms and prolong survival (Shanafelt & Call, 2004). Additionally, chemotherapy for all (non-selected)

patients has been found to be ineffective for patients with early-stage disease. Treatment of early-stage CLL has been associated with severe toxicity and no increase in survival (Shanafelt & Call, 2004). As such, treatment for early-stage CLL is not recommended outside of clinical trials (Shanafelt & Call, 2004). Watchful waiting with active support care measures remains the standard of care for early-stage (asymptomatic) patients (Shanafelt & Call, 2004).

However, approximately 50% of patients with early-stage CLL will progress to aggressive symptomatic disease and require therapy (Shanafelt & Call, 2004). Criteria for treatment, according to the National Cancer Institute, are outlined in Table 1.5. Treatment options are largely dependent on the performance status of the patient (ECOG-Eastern Cooperative Oncology Group), prior treatment, duration of remission, co-morbidities, and adverse effects associated with prior treatment (Shanafelt & Call, 2004). Current therapies for CLL include alkylating agents, purine analogues, monoclonal antibodies, combinatorial treatments, and allogeneic transplantation (Shanafelt & Call, 2004). Descriptions of the various treatment options and their mechanism(s) of action are in Table 1.6.

Although numerous treatment options exist for the treatment of CLL, most of them are undermined due to progressive drug resistance and the severe toxicities associated with treatments which are often too toxic for the elderly and those with co-morbidities. Similarly, certain prognostic indicators, such as del17p13, have been shown to contribute to chemorefractoriness further limiting the application of certain drugs (Gaidano et al., 2012). Given the age group of individuals diagnosed with CLL, a non-toxic therapeutic intervention that could 1) have cytotoxic effects against malignant cells, 2) increase the sensitivity of CLL cells to chemotherapy or 3) reduce anti-cancer drug-induced toxicities would be highly desirable and clinically beneficial. Increased chemo-sensitivity would be expected to increase drug efficacy,

and potentially reduce drug dosage resulting in reduced drug-induced toxicities. Similarly, a reduction in drug-induced toxicities would allow for potential increases in the number of chemotherapy cycles an individual can receive, thereby, increasing the likelihood of achieving a response.

| Table 1. 5. Clinical Treatment of CLL Criteria | |
|--|---|
| Indication for Treatment | Exception |
| Worsening Anemia (hemoglobin, <10.5g/dL), Thrombocytopenia (platelets, <100 x 10 ⁹ /L) due to marrow failure | Rule out autoimmune hemolytic anemia, pure red blood cell aplasia, or ITP as etiology of cytopenias |
| >50% increase in ALC in <2 months or anticipated doubling lymphocyte doubling time of <6 months | Exclude acute infection or transient reactive etiology as cause of increase in ALC |
| Progressive or massive splenomegaly (>6 cm below costal margin) | Consider Richter transformation if rapid enlargement |
| Progressive or massive lymphadenopathy (>10 cm in longest dimension) | Consider Richter transformation if rapid enlargement |
| Constitutional Symptoms: Weight loss >10% body weight in < 6 months Fever >38°C for > 2 weeks without infection Night sweats without evidence of infection Extreme Fatigue (unable to work or perform usual activities) Autoimmune anemia or ITP poorly responsive to other therapies | Exclude infection as the etiology of constitutional symptoms |
| <i>Material obtained from (Shanafelt & Call, 2004)</i> | |

Table 1.5. Clinical Treatment of CLL Criteria. Abbrev. ALC: absolute lymphocyte count; ITP: Idiopathic thrombocytopenic purpura. Material was obtained from (Shanafelt & Call, 2004).

| Table 1. 6. Treatment Regimes for Chronic Lymphocytic Leukemia | | |
|---|--|---|
| Type | Therapies | Mechanism of Action |
| Allogeneic Transplantation | Transplantation | The anti-leukemic principle of allogeneic transplantation is thought to be through the immune-mediated anti-host activities conferred with the graft (graft-versus-leukemia effect (GVL)). Only curative therapy. Limited in use due to compatibility issues and chronic graft-versus-host complications. |
| Alkylating Agents | Chlorambucil Cyclophosphamide Bendamustine (Bifunctional) | Irreversible DNA cross-linking ultimately leading to cell death. |
| Purine Nucleoside Analogs | Fludarabine* Cladrine Pentostatin Bendamustine (Bifunctional) | Purine analog, inhibits DNA synthesis by inhibiting DNA polymerase, DNA primase, DNA ligase and accessory protein that synthesizes an RNA primer ultimately leading to cell death. |
| Immunotherapies | Rituximab (Anti-CD20) Alemtuzumab (Anti-CD52) | Exert cytotoxic effects by inducing complement-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity. |
| Combinatorial Therapies | CHOP, CAP, FCR**, OFAR CFAR | Multi-factorial. |

Table 1.6. Treatment Regimes for Chronic Lymphocytic Leukemia. CHOP: cyclophosphamide, doxorubicin, vincristine, prednisone; CAP: cyclophosphamide, doxorubicin, prednisone; FCR: fludarabine, cyclophosphamide, rituximab; OFAR: oxaliplatin, fludarabine, alemtuzumab, rituximab; CFAR: cyclophosphamide, fludarabine, alemtuzumab, rituximab. *: Standard of Care as Monotherapy; **: Standard of Care

NUCLEAR FACTOR KAPPA B BACKGROUND

Nuclear factor kappa B (NF κ B) is a multi-protein complex transcriptional regulator that is made up of different protein dimers (Karin et al., 2002). The NF κ B family of transcription factors is composed of homo- and hetero-dimers derived from five distinct subunits: Rel A (p65), c-Rel, RelB, p50 (NF κ B1) and p52 (NF κ B2) (Lawrence, 2009; F. Li & Sethi, 2010). All family members share a highly conserved Rel homology domain and interactions with inhibitors of kappa B (I κ Bs), the intracellular inhibitors of NF κ B (F. Li & Sethi, 2010). NF κ B proteins bind κ B sites as either as homo- or hetero-dimers, and can exert both positive and negative effects on target gene transcription (Hayden & Ghosh, 2012). NF κ B proteins are characterized by the presence of an N-terminal Rel homology domain (RHD) which comes into contact with DNA and supports dimerization of NF κ B subunits (Hayden & Ghosh, 2012). Only Rel A, c-Rel, and RelB possess C-terminal transactivation domains (TADs) that confer the ability to initiate transcription (Hayden & Ghosh, 2012). Despite p50 and p52 lacking TADs, they can positively regulate transcription through heterodimerization with TAD-containing NF κ B subunits or through interaction with non-Rel proteins that have transactivating capability (Hayden & Ghosh, 2012). Inversely, p50 and p52 homodimers can negatively regulate transcription by competing with TAD-containing dimers for binding κ B sites. Similarly, p50 and p52 dimers may also constitutively occupy some κ B sites thereby enforcing an activation threshold for certain NF κ B target genes (Hayden & Ghosh, 2012).

A hallmark of the NF κ B pathway is its regulation by I κ B proteins. I κ B proteins I κ B α , I κ B β , I κ B ϵ , I κ B γ , Bcl-3 (B-cell lymphoma 3) and I κ Bns and the precursor proteins p100 (NF κ B2) and p105 (NF κ B1) are defined by the presence of multiple ankyrin repeats (Hayden &

Ghosh, 2012). Activation of NF κ B is achieved through phosphorylation of I κ Bs on conserved serine residues inducing polyubiquitination of the nuclear export signal, which, under normal conditions keeps a steady-state cytoplasmic localization of NF κ B dimers, thus preventing DNA binding (Hayden & Ghosh, 2012). Proteosomal degradation of I κ B from the NF κ B complex allows NF κ B dimers to accumulate in the nucleus and bind DNA κ B sites (Hayden & Ghosh, 2012). The degenerative nature of the κ B site sequence, ability of individual NF κ B subunits to form homo- or hetero-dimers, heterotypic interactions with other transcription factors, and the regulation of transcriptional activity by post-translational modifications (PTMs) targeting NF κ B subunits allow for both positive and negative regulation of transcription of a wide variety of target genes (Hayden & Ghosh, 2012). The termination of transcription response depends on both resynthesis of typical I κ B proteins and removal of active NF κ B dimers from the DNA (Hayden & Ghosh, 2012).

Collectively, the regulation and activity of NF κ B are intricate in nature and can be influenced by a variety of factors. The following sections are to explore the signaling pathways which lead to NF κ B activation, its physiological role and its implications in cancer.

ACTIVATION OF NF κ B

NF κ B is activated by many divergent stimuli, including pro-inflammatory cytokines (e.g. tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1)), T- and B-cell mitogens, bacteria, lipopolysaccharide (LPS), viruses, viral proteins, double-stranded RNA, physical and chemical stresses and cellular stresses such as ionizing radiation and chemo-therapeutic agents (F. Li & Sethi, 2010). Activation of NF κ B can be broadly classified into two types of pathways: canonical and non-canonical (Hayden & Ghosh, 2012; F. Li & Sethi, 2010).

The canonical pathway is triggered by microbial products and pro-inflammatory cytokines such as TNF- α and IL-1 (Hayden & Ghosh, 2012; F. Li & Sethi, 2010). Upon recognition of ligand, cytokine receptors such as TNF receptor (TNFR), IL-1 receptor (IL-1R) or toll-like receptor 4 (TLR4) trigger signaling cascades that culminate in activation of inhibitor of κ B kinase- β (IKK β). IKK β exists in a complex with IKK α and the regulatory protein NEMO (IKK γ) (Hayden & Ghosh, 2012; Karin et al., 2002). Activation of IKK β phosphorylates κ B proteins such as I κ B α resulting in I κ B α 's polyubiquitination and proteosomal degradation, thereby allowing the NF κ B complex to translocate into the nucleus and bind κ B sites (Figure 1.1) (Hayden & Ghosh, 2012; Karin et al., 2002). It should also be noted that IKK α and IKK β can mediate cross-talk with additional signaling pathways, including p53, MAP kinase (MAPK) and IFN regulatory factor (IRF) pathways, and directly regulate aspects of transcriptional responses (Hayden & Ghosh, 2012).

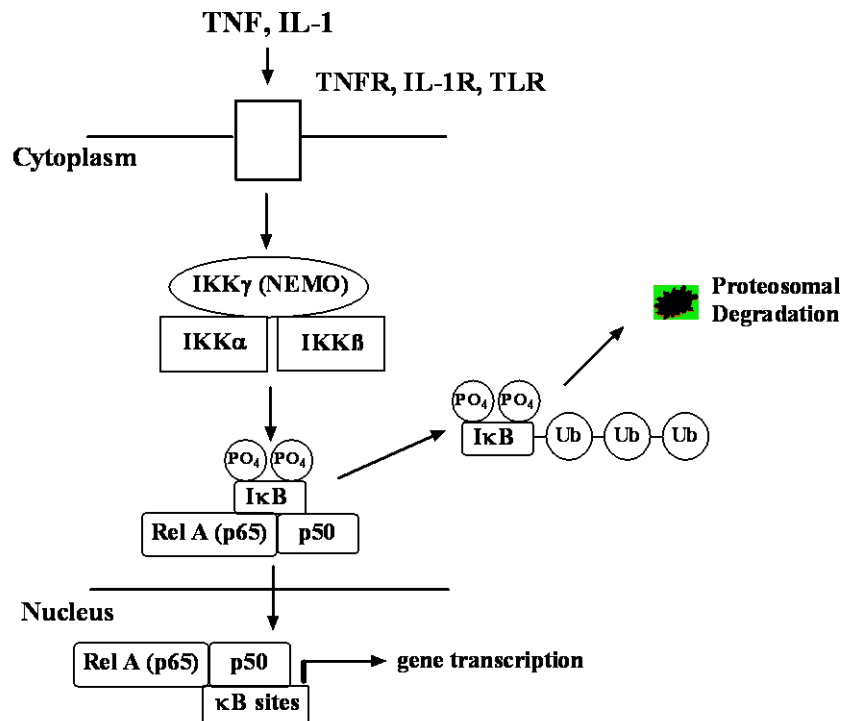


Figure 1. 1. Canonical NFκB Pathway.

Upstream ligand activated cytokine receptors, such as TNFR and IL-1R, activate the IKKβ subunit of the NEMO/IKKα/IKKβ complex. Activated IKKβ phosphorylates the IκB proteins (bound to the NFκB heterodimers). Phosphorylation of IκB leads to polyubiquitination of IκB and ubiquitin-dependent degradation of IκB by the 26s proteasome, resulting in the nuclear translocation of RelA/p50 (or c-Rel/p50) heterodimers and subsequent binding to κB sites and transcriptional activation of target genes. Figure is redrawn from (Karin, Cao, Greten, & Li, 2002; Lawrence, 2009).

The non-canonical, or alternative, pathway is triggered by specific members of the TNF cytokine family, such as CD40 ligand, receptor activator of NF κ B ligand (RANKL) and B-cell activating factor (BAFF) (Hayden & Ghosh, 2012; Sun, 2011). In contrast to the canonical pathway, the non-canonical pathway depends on IKK α and is independent of NEMO (Hayden & Ghosh, 2012; Lawrence, 2009). These cytokines (CD40L/BAFF/RANKL) activate NF κ B-inducing kinase (NIK) which, in turn, activates IKK α (Lawrence, 2009). Activation of IKK α leads to phosphorylation of p100 and the generation of p52/RelB complexes and regulates genes involved in lympho-organogenesis and B-cell activation (Figure 1.2) (Hagemann, Biswas, Lawrence, Sica, & Lewis, 2009; Hayden & Ghosh, 2012).

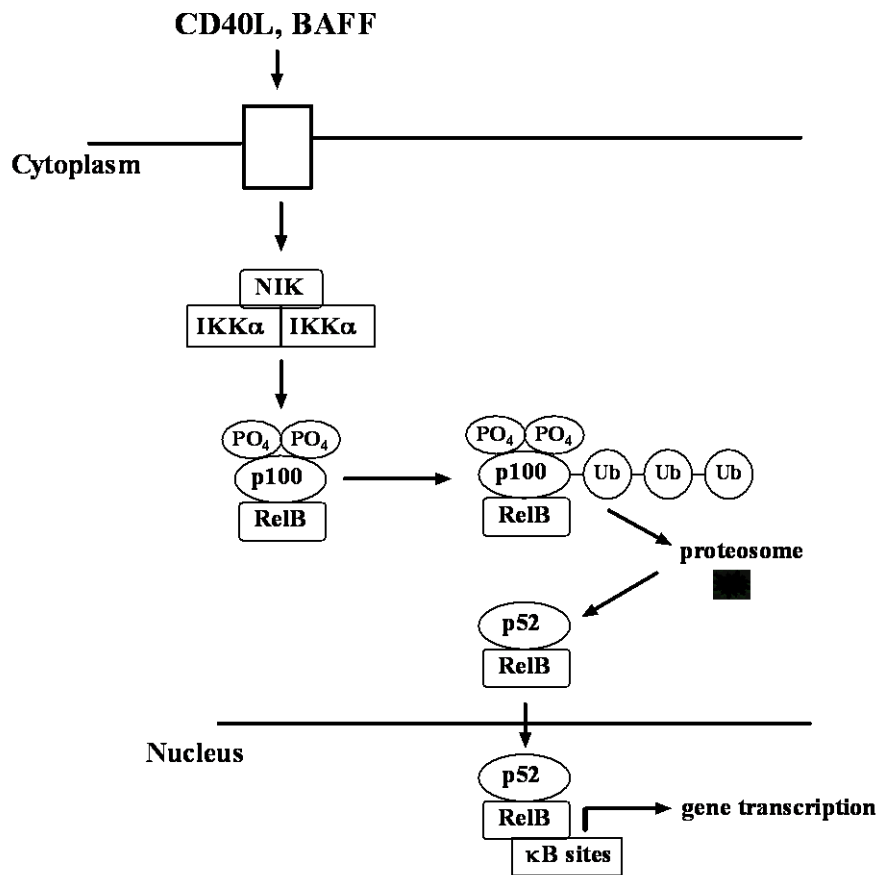


Figure 1. 2. Non-canonical NFκB Pathway.

Upstream activation by TNF cytokine family members, such as CD40L and BAFF, activates the kinase, NIK. NIK in turn activates IKKα which phosphorylates the p100 subunit of the p100/RelB complexes. Phosphorylation of p100 leads to its polyubiquitination and triggering the ubiquitin-dependent degradation of the carboxyl terminal half of p100, releasing its amino acid half, the p52 polypeptide, which together with its heterodimer partner, RelB, translocates into the nucleus to activate transcription. Figure is redrawn from (Karin et al., 2002; Lawrence, 2009).

Although various stimuli can activate NF κ B, most are mediated through either the canonical or non-canonical (alternative) pathways to regulate a wide variety of genes. Although each NF κ B dimer is likely to have distinct regulatory functions, many of the target genes are conserved to several, if not all NF κ B proteins (Karin et al., 2002). These genes fall into four major categories: immunoregulatory and inflammatory genes, anti-apoptotic genes, genes that positively regulate cell proliferation, and genes that encode for negative regulators of NF κ B (Karin et al., 2002). Collectively, the transcriptional programs regulated by NF κ B are essential for the development and maintenance of immune systems, skeletal systems, and epithelium (Hayden & Ghosh, 2012). In these settings, the NF κ B pathways contributes to control of cell survival, differentiation and proliferation (Hayden & Ghosh, 2012). While NF κ B aids in various processes necessary for normal functioning cells, the same processes can be performed in a variety of diseases in which aberrant activation of NF κ B has been associated, including cancer (Hayden & Ghosh, 2012). In this context, the initiation of tumorigenesis requires six essential alterations to normal cell physiology: self-sufficiency in growth signals, insensitivity to contact inhibition, evasion of apoptosis, immortalization, sustained angiogenesis and tissue invasion and metastasis (Karin et al., 2002). NF κ B is known to induce several of these cellular alterations (Karin et al., 2002). Thus, the likelihood that NF κ B is involved in cancer is high, a notion which will be explored in the following section.

NF κ B AND CANCER

Increased constitutively active NF κ B has been observed in various solid tumors as well as hematological malignancies (Braun et al., 2006; Darnell, 2002; Ditsworth & Zong, 2004). Several mechanisms have indicated how NF κ B transcription factors bypass their normal modes

of regulation. For example, the avian REV-T oncovirus produces a constitutively active v-Rel oncoprotein which can induce constitutive NF κ B activation and causes rapidly progressing lymphomas and leukemias (Gilmore, 1999). Chromosomal abnormalities can also disrupt genes which encode NF κ B and I κ B proteins, thereby uncoupling NF κ B factors from their regulators and causing constitutive NF κ B activation. Finally, autocrine and paracrine production of pro-inflammatory cytokines, oncogenic activation of upstream signaling molecules can persistently stimulate IKK activity leading to constitutive NF κ B activation (Karin et al., 2002). Together, constitutively active NF κ B activation can promote various aspects of tumorigenesis including promoting cancer-cell proliferation, inhibition of apoptosis and increasing tumor's angiogenic and metastatic potential (Karin et al., 2002).

NF κ B can contribute to cell-proliferation by activating genes such as IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF) and CD40L which encode for various growth factors that stimulate the proliferation of lymphoid and myeloid cells. The continuous production of these cytokines can induce a chronic stimulation of cell proliferation through an autocrine or paracrine fashion. Additionally, NF κ B has also been shown to stimulate production of cyclin D1, thereby promoting cell-cycle progression (Guttridge, Albanese, Reuther, Pestell, & Baldwin, 1999; Hinz et al., 1999).

NF κ B can also inhibit apoptosis by promoting transcription of anti-apoptotic genes (Karin et al., 2002). These factors include inhibitors of apoptosis (cIAPs), caspase-8/FADD (FAS-associated death domain)-like IL-1 β converting enzyme (FLICE) inhibitory protein (c-FLIP) and members of the Bcl-2 family (Karin & Lin, 2002). Furthermore, NF κ B can also attenuate the apoptotic response of genotoxic anti-cancer therapies and ionizing radiation thereby

contributing to chemo-resistance (F. Li & Sethi, 2010; C. Y. Wang, Cusack, Liu, & Baldwin, 1999; C. Y. Wang, Mayo, & Baldwin, 1996).

In addition to the ability of NF κ B to promote cell proliferation and inhibit apoptosis, NF κ B can also increase angiogenesis and metastatic potential. This is achieved by NF κ B-targeted transcription of various chemokines, such as IL-8, which increase the migratory and invasive capacity (Guo et al., 2011; Kim, Choi, Park, & Kim, 2012) and by transcription of the stimulator of angiogenesis- vascular endothelial growth factor (VEGF) (Huang, Robinson, Deguzman, Bucana, & Fidler, 2000; Tanaka et al., 2012). NF κ B has also been shown to induce transcription of several metalloproteinases- proteolytic enzymes that promote tumor invasion of surrounding tissues (J. Li et al., 2012; Qin et al., 2012).

Consistent with the notions that NF κ B can promote oncogenesis, various studies have demonstrated that NF κ B activation is positively associated with disease progression. Studies by Tang et al. found that high levels of immunohistochemical expression of NF κ B p65 were detected in lung cancers (X. Tang et al., 2006). Interestingly, the NF κ B p65 expression in lung adenocarcinomas was significantly higher in advanced TNM (tumor, lymph nodes, metastasis) stages (III-IV) than in earlier stages (I-II), suggesting that NF κ B is associated with disease progression (X. Tang et al., 2006). These findings are further supported by Zhang et al. who found that expression of NF κ B/Rel A was higher in non-small cell lung cancer (NSCLC) tissue as compared to normal adjacent tissue and normal lung tissue (Zhang et al., 2007). Additionally, there was a positive correlation between NF κ B activation (nuclear translocation of Rel A) and tumor clinicopathological features such as tumor grade and TNM staging, supporting the findings by Tang et al (Zhang et al., 2007). Zhang et al. also demonstrated that nuclear Rel A

binding and cytoplasmic phosphorylated-inhibitor of kappa-B-alpha (pI κ B- α) were positively associated with poor prognosis (Zhang et al., 2007).

Although the involvement of NF κ B activation in various malignancies has been evaluated, the activation of NF κ B in CLL remains of primary interest in this dissertation. Targeting NF κ B activation has also come into light as a promising candidate for intervention for the treatment of CLL. (F. Li & Sethi, 2010). Increased constitutive activation of NF κ B has been observed in primary CLL cells (Hewamana, Alghazal, et al., 2008; Hewamana, Lin, et al., 2008; Hewamana et al., 2009). It has also been found that increased binding of Rel A, a subunit of NF κ B which contains the transactivation domain, was positively correlated with white blood cell count, a measure of tumor burden, and lymphocyte doubling time, a measure of tumor kinetics (Hewamana, Alghazal, et al., 2008). Furthermore, it has been shown that Rel A DNA binding was strongly associated with Binet staging, predictive of time to initial treatment and subsequent treatment, and predictive of overall survival (Hewamana et al., 2009). Rel A DNA binding has also been shown to be negatively correlated with induction of spontaneous apoptosis *in vitro* suggesting that NF κ B is an important mediator of survival (Hewamana, Alghazal, et al., 2008). This is supported by the fact that inhibition of NF κ B activation, through the use of an irreversible-IKK inhibitor (LC-1), induced dose-responsive increases in apoptosis (Hewamana, Lin, et al., 2008). The addition of LC-1 was also able to overcome the cytoprotective effects of CD40L and IL-4, upstream agonists of NF κ B activation, providing further support of the importance of NF κ B in CLL (Hewamana, Lin, et al., 2008). Additionally, studies by Pepper et al. indicated that the cytotoxic effects of aspirin and its analogues were mediated by both COX-2 inhibition as well as inhibition of Rel A DNA (Pepper et al., 2011). It is also important to note that in the studies by Hewamana et al., inhibition of NF κ B activation by LC-1 exhibited strong

synergy with fludarabine, a commonly used first-line treatment in CLL (Riches, Ramsay, & Gribben, 2011; Silber et al., 1994), in both good prognostic CLL patients who were fludarabine-sensitive and poor prognostic CLL patients who displayed chemorefractoriness toward fludarabine (Hewamana, Lin, et al., 2008). Given the ability of NF κ B to promote survival of CLL cells *in vitro* and aid in various biological processes such as proliferation, migration, inhibition of apoptosis and chemo-resistance, therapeutic interventions which target NF κ B would be of considerable interest and clinically relevant.

OMEGA-3 FATTY ACIDS

BACKGROUND

Omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids (PUFAs) are essential fatty acids (EFAs). These fats are referred to as essential because humans, like all mammals, are unable to synthesize them and thereby must obtain them from the diet. The n-6 EFA is represented by linoleic acid (LA; 18:2n-6) and n-3 EFA is represented by alpha-linolenic acid (ALA; 18:3n-3) (Figure 1.1). LA is primarily found in seeds of most plants, whereas, ALA is primarily found in green leafy vegetables, flax seed, and walnuts (Simopoulos, 2008). The distinction of these two essential fatty acids (FAs) is based on the location of the first double bond from the methyl (omega) end of the FA molecule located between carbon 6-7 (n-6) or 3-4 (n-3) (Figure 1.1) (Simopoulos, 1991, 2000, 2002, 2008).

Both EFAs are capable of being metabolized in humans to their longer chain 20-carbon and 22-carbon derivatives. LA is metabolized to 20-carbon arachidonic acid (AA; 20:3n-6) and ALA is metabolized to the 20-carbon eicosapentaenoic acid (EPA; 20:5n-3) and 22-carbon docosahexaenoic acid (DHA; 22:6n-3). This is achieved by increasing the carbon chain length and degree of unsaturation through addition of extra carbons to the carboxyl end of the fatty acid chain (Figure 1.1) and then desaturating a bond. While AA can be metabolized from its precursor, LA, it can also be obtained from meats of animals that consume LA and then store AA in their meat. Similarly, EPA and DHA can be obtained from fish or fish oils (Simopoulos, 1991, 2000, 2002, 2008).

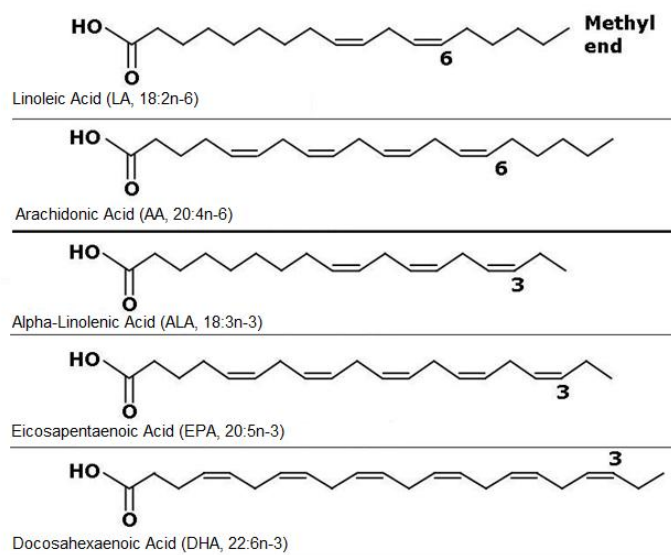


Figure 1. 3. Chemical Structures of Omega-3 and Omega-6 Fatty Acids.

Picture modified from the European Food Information Council. Abbrev. X:Y- X denotes number of carbon atoms; Y denotes number of unsaturations; n-3: omega-3; n-6: omega-6. Picture modified from the European Food Information Council.

EFA's are important components of cellular membranes and are selectively found amongst lipid classes. ALA and LA are mostly found in triglycerides, cholesterol esters with a relatively small abundance found in phospholipids. AA and EPA are found in triglycerides, cholesterol esters and phospholipids. Unlike the other n-3 FAs, DHA is predominantly found in phospholipids (Simopoulos, 2008). Furthermore, n-6 and n-3 FA compete with each other for incorporation into the cell and are substrates for the same metabolizing enzymes (reviewed in the following section). As such, consumption of n-3 such as EPA or DHA can partially displace n-6, especially AA, in the membranes of cells (Simopoulos, 1991, 2000, 2002, 2008).

Currently, the western diet is heavily favored toward the n-6 FA with little to no n-3 FA intake. The average ratio of n-6 to n-3 in the western diet is 15-20 to 1 (Simopoulos, 2008) whereas the ideal ratio of n-6 to n-3 is 4-1:1 (Patterson, Wall, Fitzgerald, Ross, & Stanton, 2012). Furthermore, mammals lack the ability to convert n-6 to n-3 as they lack the necessary converting enzyme, omega-3 desaturase (Simopoulos, 2008). This is an important consideration;

both n-6 and n-3 are integral parts of cellular membranes and are structurally similar. Yet these two classes of EFA are not interconvertible, and are metabolically and functionally distinct, often having important opposing physiological functions (Simopoulos, 2008). As such, this imbalance toward n-6 can lead to increased risk of various diseases. In support of this notion, various studies have illustrated that a high intake of n-6 and a high n-6/n-3 ratio can promote pathogenesis of many diseases including cardiovascular, autoimmune, cancer, and inflammatory disease (Simopoulos, 2008). Inversely, consumption of n-3 has been shown to have beneficial health effects and often has suppressive effects on pathogenesis (Simopoulos, 2008).

Beneficial health effects of n-3, particularly EPA and DHA, were described first in Greenland Eskimos who consumed high quantities of fish and displayed lower rates of coronary heart disease, asthma, type 1 diabetes mellitus, and multiple sclerosis (Simopoulos, 2008) than populations that consumed less fish. Since then, beneficial effects of n-3 have been extended to include cancer, inflammatory bowel disease, rheumatoid arthritis and psoriasis (Simopoulos, 2008).

The application of n-3 as potential therapeutic options for CLL is of great interest and the principle focus of this thesis. The metabolism of PUFAs and their physiological role in humans as well as their implications on CLL will be discussed in the following sections.

METABOLISM

As introduced above, both LA and ALA are able to be metabolized to their longer 20-carbon and 22-carbon derivatives. This is achieved through a series of elongation and desaturation reactions mediated by specific elongase and desaturase enzymes illustrated in Figure 1.2 with delta-6 desaturase being the rate limiting enzyme (Patterson et al., 2012; Simopoulos, 2008). Since both n-3 and n-6 FA utilize the same enzymes their conversion to their

longer derivatives can be influenced if there is a high intake of one type of EFA. For instance, a high intake of ALA can interfere with the elongation and desaturation of LA. This concept has been demonstrated in human studies which illustrated that a constant consumption of ALA with a decreasing consumption of LA led to increases in EPA in the plasma phospholipids (Liou, King, Zibrik, & Innis, 2007). Additionally, the elongation and desaturation of n-3 and n-6 FA can also be hindered by the present of trans-fats, which inhibit the delta-6 desaturase (Patterson et al., 2012; Simopoulos, 2008). As such, the propensity of PUFAs to undergo elongations and desaturations is largely dependent on the type of fat intake of the individual.

The elongation and desaturation of LA and ALA is an important process as the bioactivity of these EFAs is thought to be predominately mediated through their longer chain metabolites. Of the various n-6 and n-3 derivatives, AA and EPA are especially important as these FAs are precursors for eicosanoid production (Haeggstrom, Rinaldo-Matthis, Wheelock, & Wetterholm, 2010; Serhan & Petasis, 2011). Eicosanoids are biologically active, 20-carbon lipids which are important mediators of acute inflammation, and have been implicated in several specific diseases such as thrombosis, cancer, atherosclerosis, asthma and rhinitis (Haeggstrom et al., 2010; Patterson et al., 2012). These AA-derived and EPA-derived eicosanoids are produced by three major pathways: the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) pathways (Haeggstrom et al., 2010; Luo & Wang, 2011; Patterson et al., 2012). Collectively, eicosanoids consist of prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), hydroxyeicosatetraenoic acids (HETEs), epoxyeicosatrienoic acids (EET), epoxyeicosatetraenoic acids (EETeTrs) and hydroxyeicosapentaenoic acids (HEPE) (Arnold, Konkel, Fischer, & Schunck, 2010; Luo & Wang, 2011; Patterson et al., 2012). The synthesis of the various eicosanoids is illustrated in Figure 1.3.

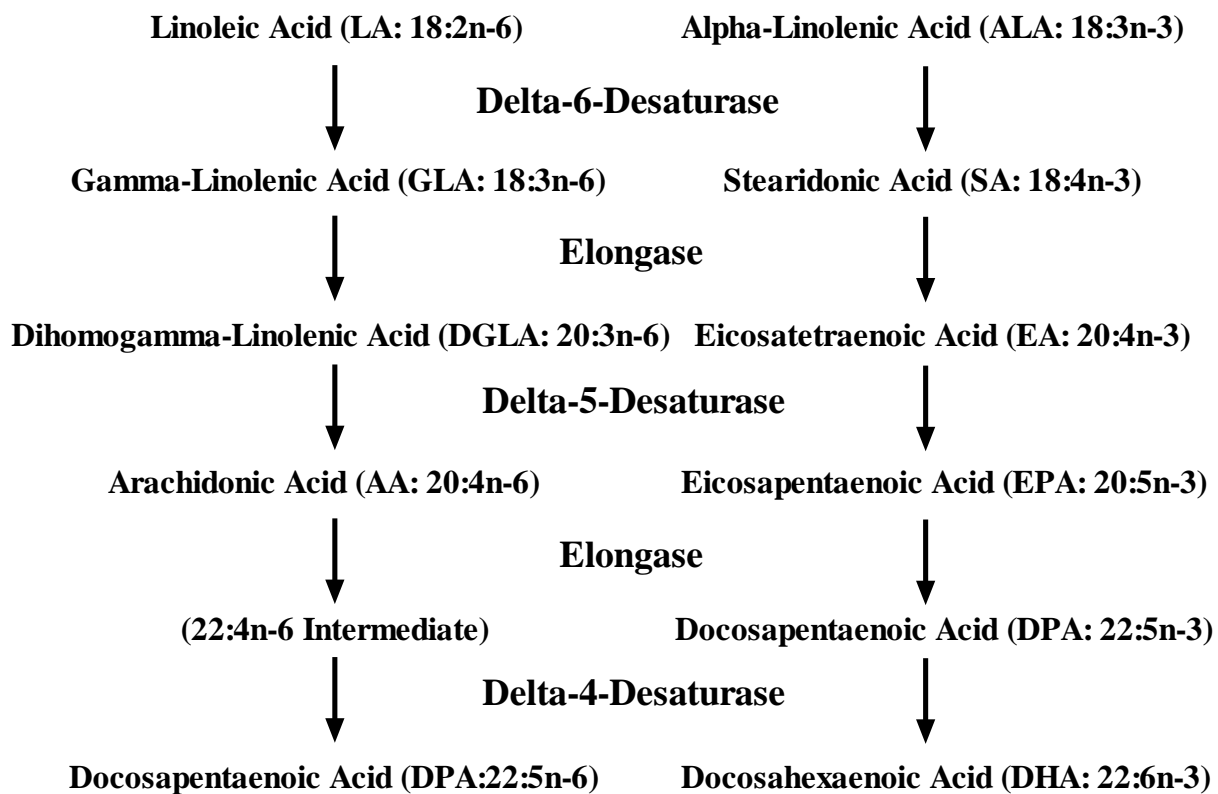


Figure 1. 4. Metabolism of Linoleic Acid and Alpha-Linolenic Acid to their longer-chain derivatives.

Abbrev. X:Y- X denotes number of carbon atoms; Y denotes number of unsaturations; n-3: omega 3; n-6: omega 6.

Whereas PUFA are able to undergo enzymatic metabolism, they are also able to undergo non-enzymatic degradation. Non-enzymatic degradation of PUFA is mediated by various reactive oxygen and nitrogen species (ROS/RNS) which oxidize lipids, collectively referred to as lipid peroxidation, and yield a diverse array of products (Niki, 2009). The degree of lipid peroxidation is dependent on the number of unsaturations possessed by the FA molecule. As such, the higher the degree of unsaturation the FA molecule possesses the greater the amount of lipid peroxidation that can ensue (Niki, 2009).

Lipid peroxidation can be mediated by free radical-mediated oxidation or free radical-independent non-enzymatic oxidation (Niki, 2009). Free radical-mediated lipid peroxidation is achieved by a chain mechanism in which one initiating free radical can oxidize many molecules of lipids (Niki, 2009). The type of free radical which oxidizes the lipid molecules is dependent on the condition of the cell (Niki, 2009). Free radical-independent non-enzymatic oxidation is mediated by singlet oxygen and ozone (Niki, 2009). Singlet oxygen oxidizes unsaturated lipids mainly by the ene-reaction to give hydroperoxide with concomitant double-bond migration, with minor side reactions such as 1,4 addition to give 1,4-endoperoxide and 1,2 addition to give dioxetane, which readily decomposes to yield carbonyl compounds (Niki, 2009).

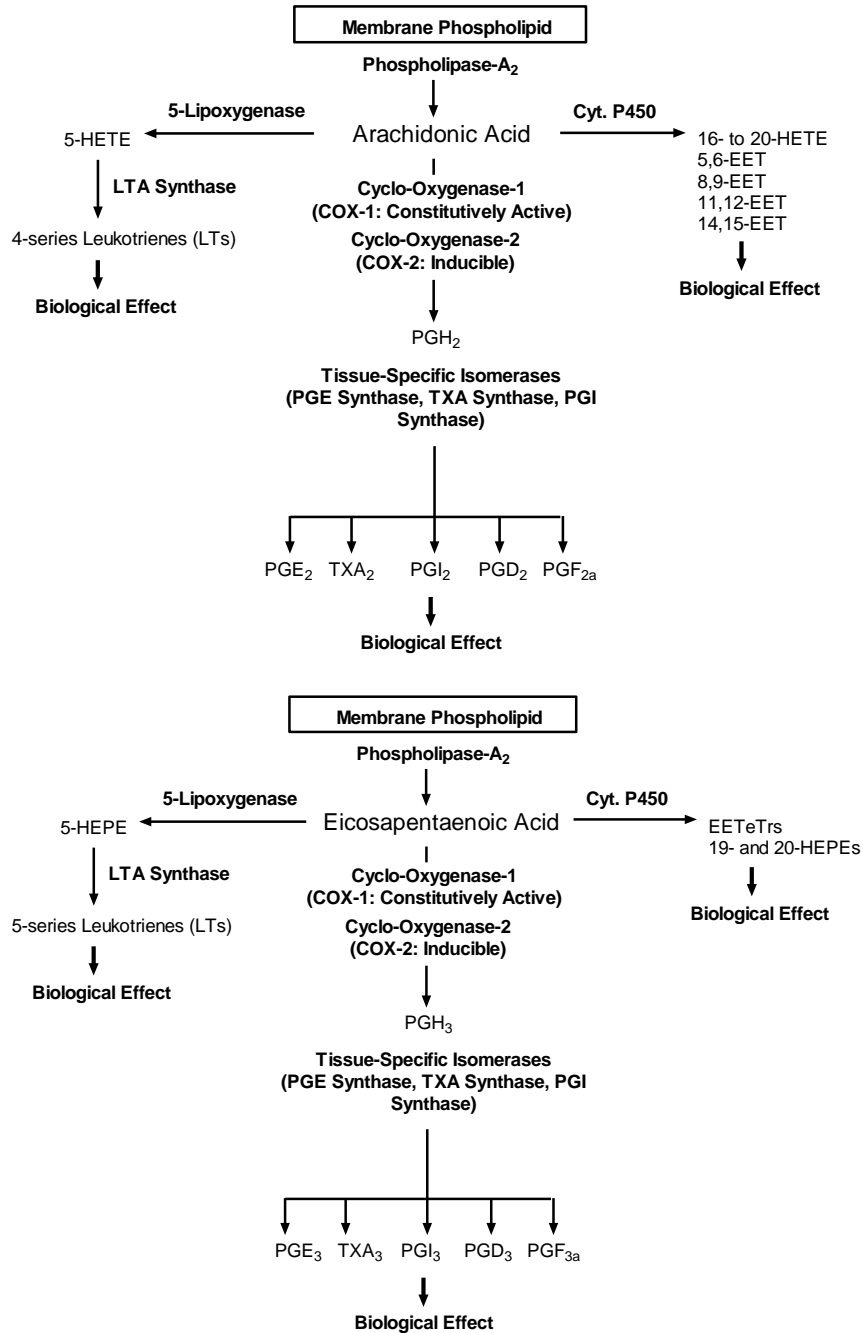


Figure 1. 5. Schematic of Eicosanoid Production.

Within a cell, AA and EPA is released from the membrane phospholipids by the enzyme phospholipase-A2 (PLA₂) where after they will be metabolized by the cyclo-oxygenase enzymes (COX-1, a constitutive enzyme, or COX-2, an inducible enzyme), 5-lipoxygenase (LOX) or cytochrome p450 (CYP). AA COX-derived eicosanoid PGH₂ is subsequently metabolized by a series of specific prostaglandin synthase enzymes leading to the production of the 2-series prostaglandins: PGE₂, PGI₂, PGD₂, and PGF_{2α} and thromboxanes A₂ and B₂. AA LOX-derived eicosanoid 5-hydroperoxyeicosatetraenoic acid (5-HPETE) is subsequently metabolized by Leukotriene Synthase to produce the 4-series of leukotrienes: LTA₄, LTB₄, LTC₄, LTD₄ and LTE₄. Metabolism of AA by CYP produces the 16- to 20-HETEs and 5,6-, 8,9-, 11,12- and 14,15-epoxyeicosatrienoic acids (EETs). In contrast, EPA can also act as a substrate for COX, LOX and CYP enzymes and give rise to an entirely different set of eicosanoids. These are the 3-series prostaglandins and thromboxanes and the 5-series leukotrienes and epoxyeicosatetraenoic acids (EETeTrs) and 19- and 20-hydroxyeicosapentaenoic acids (HEPE).

Collectively, PUFA are substrates for a variety of enzymatic and non-enzymatic reactions. The type of PUFA, degree of unsaturation and intake all influence the rate and type of by-products that are produced aiding to the complexity of PUFA metabolism and the versatility of their actions.

MECHANISMS OF ACTION

The mechanisms through which PUFA exert their actions are diverse and multifaceted, a trait aided by the diversity of their by-products. However, one of the most prominent roles of PUFA is their involvement with inflammation.

Inflammation is an important physiological host defense mechanism that protects the host from infection and other insults. Inflammation initiates pathogen killing and tissue repair processes as well as restoration of homeostasis at an infected or damaged site (Niki, 2009). Under normal conditions, inflammation is tightly regulated to prevent excessive damage to the host. Regulation is often mediated through negative feedback mechanisms such as anti-inflammatory cytokines, inhibition of pro-inflammatory cascades, shedding of receptors for inflammatory mediators and through activation of regulatory cells (Niki, 2009). As such, controlled, regulated inflammatory responses are essential to remain healthy and maintain body homeostasis. However, a loss of tolerance and/or regulation of inflammatory processes can lead to excessive, irreparable damage to the host tissue leading to various pathologies, including cancer (Niki, 2009).

Eicosanoids are key mediators and regulators of inflammation (Calder, 2009; Haeggstrom et al., 2010; Serhan & Petasis, 2011). As previously mentioned, the eicosanoids produced by AA are comprised of the 2-series PGs and TXs and the 4-series LTs. PGE₂ and the 4-series LTs are particularly important as these eicosanoids modulate both the intensity and

duration of the inflammatory response (Calder, 2009). However, eicosanoid production is not solely derived from AA, but can also be derived from EPA. The eicosanoids produced from EPA, which includes the 3-series PGs/TXs and 5-series LTs, are functionally different from their AA-derived counterparts and often times have antagonistic actions on inflammation (Table 1.7) (Calder, 2009).

Prostaglandins and thromboxanes, collectively referred to as prostanoids, exert their biological effects by binding to cell-surface prostanoid receptors belonging to the family of G-protein-coupled rhodopsin receptors (GPCRs) or nuclear receptors such as the peroxisome proliferator-activated receptors (PPARs) (Cathcart, Lysaght, & Pidgeon, 2011; Ricciotti & FitzGerald, 2011). The prostanoid receptor family is composed of 8 members: E prostanoid receptor (EP 1-4); D prostanoid receptor (DP1); F prostanoid receptor (FP); I prostanoid receptor (IP); and thromboxane receptor (TP) (Ricciotti & FitzGerald, 2011). These prostanoid receptors are coupled to a range of intracellular signaling pathways summarized in Table 1.8.

Of particular interest are the EP receptors and PGE₂ as PGE₂ is one of the most abundant eicosanoids produced in the body (Ricciotti & FitzGerald, 2011). EP2 and EP4 activate adenylyl cyclase to generate cyclic adenosine monophosphate (cAMP) leading to activation of protein kinase A (PKA) (Alfranca et al., 2006; Ricciotti & FitzGerald, 2011). PKA can, in turn, inhibit glycogen synthase kinase 3 alpha (GSK3 α), thereby promoting transcription of T-cell factor (Tcf)-dependent genes such as COX-2. Additionally, EP4 specifically activates the phosphoinositide 3-kinase (PI3K)/ protein kinase B (Akt) pathway which, in some cell types, augments extracellular signal-regulated kinase (ERK) activity and mediates expression of early growth response protein 1 (EGR-1) and subsequent transcription of genes such as prostaglandin synthase-1 (mPGES-1) (Alfranca et al., 2006). PGE₂ has also been shown to activate nuclear

factor kappa B (NF κ B), a key mediator of the inflammatory processes(Ditsworth & Zong, 2004), and hypoxia inducible factor-1 alpha (HIF-1 α) (Alfranca et al., 2006). Additionally, PGE₂ has been shown to transactivate the epidermal growth factor receptor (EGFR) via activation of proto-oncogene tyrosine-protein kinase Src (c-Src), which activates EGFR directly or indirectly by inducing metalloproteinase activity that releases membrane bound transforming growth factor-alpha (TGF α) (Buchanan, Wang, Bargiacchi, & DuBois, 2003; Pai et al., 2002). Collectively, the bioactivity of PGE₂ is diverse and cell-type specific. However, PGE₂ is typically associated with promotion of increased states of inflammation and has been implicated in various tumorigenic processes (Reader, Holt, & Fulton, 2011).

Table 1. 7. Pro-inflammatory Effects of N-6 Fatty Acid-derived Eicosanoids and Anti-inflammatory Effects of N-3 Fatty Acid-derived Eicosanoids

| Pro-inflammatory effects of the n-6 fatty-acid-derived eicosanoids | | | |
|--|--|--|---|
| | Arachidonic Acid (n-6) Derived Eicosanoids | Physiological Effect | Organ or Cells |
| Prostaglandins | PGD ₂ | Bronchoconstriction Pro-inflammatory Pro-arrhythmic | Bronchi Activation of Eosinophils Vessels |
| | PGE ₂ | Induces Fever Causes Pain Increases Production of IL-6 | Nociceptor Sensory Neurons |
| | PGF ₂ | Bronchoconstriction Pro-arrhythmic | Bronchi Vessels |
| | PGI ₂ | Causes Pain | Nociceptor Sensory Neurons |
| Thromboxanes | TXA ₂ /TXB ₂ | Pro-aggregation Vasoconstriction Bronchoconstriction | Platelets Vessels Bronchi |
| Leukotrienes | LTA ₄ /LTB ₄ /LTC ₄ /LTD ₄ /LTE ₄ | Pro-inflammatory Chemotaxis Release of ROS | Leukocytes Leukocytes Granulocytes |
| Anti-inflammatory effects of the n-3 fatty-acid-derived eicosanoids | | | |
| | Eicosapentaenoic Acid and Docosahexaenoic Acid (n-3) Derived Eicosanoids | Physiological Effect | Organ or Cells |
| Prostaglandins | PGD ₃ /PGE ₃ /PGF ₃ /PGI ₃ | Anti-arrhythmic | Vessels |
| Thromboxanes | TXA ₃ /TXB ₃ | Anti-aggregation | Platelets |
| Leukotrienes | LTA ₅ /LTB ₅ /LTC ₅ /LTD ₅ /LTE ₅ | Anti-inflammatory | Leukocytes |
| Resolvins | RVE ₁ | Anti-aggregation Anti-inflammatory | Platelets Dendritic Cells |
| | RVD | Anti-inflammatory | Dendritic Cells |
| Neuroprotectin | NPD ₁ | Anti-inflammatory Anti-apoptotic Decreases Oxidative Stress | Retina (photoreceptor cells) and Brain |
| <i>Material obtained from (Calder, 2009).</i> | | | |

| Table 1. 8. Signal Transduction of AA-derived Prostanoids | | | |
|--|-----------------------------------|---|-----------------------------|
| Class | Subtype | G-Protein Coupled | Second Messenger(s) |
| PGE₂ | EP1 | G _q | ↑ IP ₃ |
| | EP2 | G _s | ↑ cAMP |
| | EP3 | G _i , G ₁₂ , G _{Rho} | ↓ cAMP, ↑ Ca ²⁺ |
| | EP4 | G _s | ↑ cAMP |
| PGD₂ | DP | G _s | ↑ cAMP |
| | CRTH2 | G _i | ↓ cAMP, ↑ Ca ²⁺ |
| PGF_{2α} | FP _A , FP _B | G _q , G _{Rho} | ↑ IP ₃ |
| PGI₂ | IP-IP | G _s , G _q , G _i | ↑ IP ₃ , ↑↓ cAMP |
| | IP-TP _α | G _s | ↑ cAMP |
| TxA₂ | TP _α , TP _β | G _q , G _s (α), G _i (β), G _h , | ↑ IP ₃ , ↑↓ cAMP |
| | | G _{12/13} | ↑ Ca ²⁺ |
| <i>Material obtained from (Alfranca et al., 2006).</i> | | | |

Table 1.8. Signal Transduction of AA-derived Prostanoids. Abbrrev.: PGE₂: prostaglandin E₂; EP: prostaglandin E₂ receptor; IP₃: inositol triphosphate; cAMP: cyclic adenosine monophosphate; PGD₂: prostaglandin D₂; DP: prostaglandin D₂ receptor; CRTH2: chemoattractant receptor-homologous molecule expressed on Th2 cells; PGF_{2α}: prostaglandin F_{2α}; FP: prostaglandin F receptor; PGI₂: prostacyclin 2; IP: prostacyclin receptor; TP: thromboxane receptor; TxA₂: thromboxane A₂. Data obtained from (Alfranca, Iniguez, Fresno, & Redondo, 2006).

Whereas eicosanoids are primary mediators of inflammation, products of lipid peroxidation can also contribute to an inflammatory state. While the mechanisms, dynamics and products of lipid peroxidation are still under investigation, it has been shown that lipid peroxidation induces: 1) disturbances of fine structures, 2) alterations in integrity, fluidity and permeability of cell membranes leading to functional loss, 3) modifies low-density lipoproteins to pro-atherogenic and pro-inflammatory forms, and 4) can generate potentially toxic products (Greenberg et al., 2008; Niki, 2009). Despite the potential mutagenic and genotoxic effects that lipid peroxides can elicit, it has also become evident that lipid peroxidation products exert various biological functions *in vivo* such as regulation of gene expression, signaling messengers, activators of receptors and nuclear transcription factors, and inducers of adaptive responses (Niki, 2009). Recent studies have provided evidence that many products of lipid peroxidation

exert opposite dual effects, depending on conditions, such as cytotoxic or cytoprotective effects, pro- and anti-atherogenic effects, pro- and anti-apoptotic effects and pro- and anti-inflammatory effects (Parthasarathy, Santanam, Ramachandran, & Meilhac, 2000; Zmijewski et al., 2005). Thus, the contribution of lipid peroxides to a pro-inflammatory state is complex and context dependent.

The mechanisms of action exerted by n-6 and n-3 are diverse and multi-faceted. Typically, n-6 FAs are considered to be pro-inflammatory; whereas n-3 FAs are considered to be anti-inflammatory. States of chronic inflammation are an underlying cause for various pathologies including cancer (Balkwill & Coussens, 2004; Clevers, 2004). A diet rich in n-6 will shift the balance toward a pro-inflammatory and pro-thrombotic state and can, thereby, promote tumor promotion and progression. Inversely, the addition of n-3 can antagonize the pro-inflammatory effects of n-6 thereby providing a potential therapy for the treatment of cancer, the focal point of this thesis.

OMEGA-3 FATTY ACIDS AND CANCER

Recently, numerous studies have supported the notion that AA and its metabolites have a significant role in tumor promotion (Hyde & Missailidis, 2009). Although the role of AA and its metabolites on tumor promotion is intricate and multi-faceted, much attention has been placed on COX-2 and PGE₂. It is clear that overexpression of COX-2 is implicated in various forms of human cancers including lung (Krysan et al., 2004), breast (Barnes, Haywood, Flint, Knox, & Bundred, 2006), colorectal (Chan, Ogino, & Fuchs, 2007), prostate (Lee, Pan, Cheng, Chi, & Liu, 2001), head and neck (D. W. Tang et al., 2003), and chronic lymphocytic leukemia (Ryan et al., 2006; Secchiero et al., 2005). In conjunction, AA-COX derived eicosanoid, PGE₂, is the most abundantly formed prostaglandin (Ricciotti & FitzGerald, 2011) and has been implicated in

various aspects of tumorigenesis via induction of cell proliferation, anti-apoptosis, angiogenesis, invasion, and metastasis (Greenhough et al., 2009). In addition, PGE₂ has also been linked to upregulation of B-cell CLL/lymphoma 2 (Bcl-2), an anti-apoptotic protein, and induction of NFκB (D. Wang & Dubois, 2010).

Nuclear factor kappa B belongs to a family of transcription factors that play a pivotal role in inflammatory and stress responses as well as in tumor cell resistance to apoptosis (Balkwill & Coussens, 2004; Darnell, 2002). NFκB controls various cytokines (e.g. IL-1, IL-2, IL-6, IL-12, TNF-α), chemokines (e.g. IL-8, MIP-1α, MCP1), adhesion molecules (e.g. ICAM, VCAM and E-selectin) and inducible effector enzymes such as inducible nitric oxide synthase (iNOS) and COX-2 (Schmitz & Ecker, 2008). Several studies have demonstrated that NFκB can promote oncogenesis by virtue of its ability to regulate the expression of a wide array of genes that modulate apoptosis, proliferation, inflammation, tumor metastasis, and angiogenesis (Clevers, 2004). Increased constitutively active NFκB has been observed in various solid tumors as well as hematological malignancies (Braun et al., 2006; Darnell, 2002; Ditsworth & Zong, 2004). Activation of NFκB has been linked to aggressive tumor growth and resistance to both chemotherapy and radiotherapy (F. Li & Sethi, 2010). NFκB regulates COX-2 (Schmitz & Ecker, 2008). COX-2 increases production of PGE₂ (Calder, 2009). PGE₂ in turn can stimulate various pathways associated with tumorigenesis including activation of NFκB (Ditsworth & Zong, 2004; D. Wang & Dubois, 2010). Thus, the relationship between NFκB, COX-2 and PGE₂ is inherently intertwined and potentially self-propagating.

N-3 and n-6 compete with each other for incorporation and metabolism. The effects of n-3 derived metabolites are antagonistic of n-6 derived metabolites. Given the involvement of AA

and its metabolites in tumor promotion, the use of n-3, particularly EPA and DHA, as therapeutic options for various malignancies presents a promising avenue of research.

Consistent with this thought, n-3 fatty acids have consistently been shown to slow the growth and increase the chemo-sensitivity of various malignancies *in vitro* and in animal models (Das & Madhavi, 2011; Das, Madhavi, Sravan Kumar, Padma, & Sangeetha, 1998; Dekoj et al., 2007; Hardman, Avula, Fernandes, & Cameron, 2001). However, the capacity of n-3 to translate into clinical benefit is of primary interest. Clinical studies utilizing n-3 in breast and lung cancer have demonstrated promising results. Studies by Bougnoux et al. indicated that response to therapy was positively correlated with n-3 levels in breast adipose tissue in patients with localized breast carcinoma given a therapy of mitoxantrone, vindesine, cyclophosphamide, and 5-fluorouracil (Bougnoux et al., 1999). Notably, increased DHA especially stood out as a positive predictor of chemo-sensitivity (Bougnoux et al., 1999). In line with these observations, studies by Bougnoux et al. also illustrated that supplementation with 1.8 g/daily of DHA improved the efficacy of anthracycline-based therapy against the progression of visceral metastases (Bougnoux et al., 2009). In this study, significant increases in time to progression and median overall survival were achieved in patients with the highest plasma DHA levels as compared to the lowest plasma DHA levels (8.7 months versus 3.5 months and 34 months versus 22 months; respectively) (Bougnoux et al., 2009). Importantly, it was also found that supplementation with DHA did not induce any additional adverse effects. It should be noted that this was a small study (n= 25) and the relationship between DHA content and metastatic cancer outcome subsequent to chemotherapy does not ensure a causality (Bougnoux et al., 2009). Similarly, studies by Murphy et al. indicated that supplementation with fish oil increased the first-line chemotherapy efficacy in patients with advanced non-small cell lung cancer (NSCLC)

(Murphy et al., 2011b). In this study, patients with clinical diagnosis of stage IIIB or IV NSCLC who were chemotherapy-naïve were either supplemented with fish oil (2.5g of EPA + DHA/day; n=15) or no fish oil (standard of care group (SOC); n=31) in conjunction with first-line platinum-based doublet chemotherapy of palliative intent consisting of either carboplatin with vinorelbine or carboplatin with gemcitabine (Murphy et al., 2011b). Primary endpoints of this study were response rates, defined as complete response plus partial response, and clinical benefit, defined as the sum of complete response, partial response and stable disease divided by the number of patients (Murphy et al., 2011b). Collectively, results from Murphy et al. illustrated that patients in the fish oil group had increased response rates and greater clinical benefit compared to the SOC group (60.0% versus 25.8%, p-value: 0.008 and 80.0% versus 41.9%, p-value: 0.02, respectively) (Murphy et al., 2011b). It should also be noted that patients in the fish oil group had greater 1-year survival rates as compared to the SOC group (60.0% versus 38.7%), although this was not statistically significant (Murphy et al., 2011b). Furthermore, patients in the fish oil group did not display any additional toxicities associated with treatment as compared to the SOC group. Inversely, as compared to the SOC group, patients in the fish oil received a significantly higher number of chemotherapy cycles (number of chemotherapy cycles received \pm SD: 3.9 ± 0.9 versus 3.0 ± 1.4 , p-value: 0.02) and longer, almost significant, time on chemotherapy (time on chemotherapy (days) \pm SD: 78.9 ± 23.5 versus 60.3 ± 31.1 , p-value: 0.05) (Murphy et al., 2011b).

N-3 may also be able to reduce the side effects of treatments. In support of this notion, Ghoreishi et al. illustrated that a daily dose of 630 mg n-3 partially prevented paclitaxel induced peripheral neuropathy (PIP), a dose-limiting side effect of paclitaxel therapy (Ghoreishi et al., 2012). Additionally, Heller et al. indicated that the addition of 0.2g/kg/daily fish oil increased

liver and pancreas function while reducing weight loss in patients recovering from major elective abdominal surgery (Heller et al., 2004). Studies by van der Meij et al. indicated that patients with stage III NSCLC who consumed oral n-3 supplements (2.02g EPA + 0.92g DHA) during multimodality treatment had significantly greater quality of life parameters, physical and cognitive function, global health status and social function, and higher physical activity as compared to the placebo group (van der Meij et al., 2012). Similarly, Murphy et al. also found that patients with NSCLC who consumed fish oil (2.2g of EPA/day) retained weight and either gained or maintained muscle mass as compared to the non-intervention group during first-line therapy (Murphy et al., 2011a).

Collectively, these findings highlight the application of n-3 at both the basic research level as well as in a clinical setting. However, the primary purpose of this research was to evaluate the potential of n-3 as therapeutic interventions for the treatment of CLL. Although limited, *in vitro* and *in vivo* studies on B-leukemic cells have provided insight into the application of n-3 as potential therapeutic options for the treatment of B-cell malignancies (such as CLL). Studies by Cha et al. indicated that supplementation of mice bearing L1210 B-leukemia with a DHA containing diet, as compared to a chow diet, in combination with arabinosylcytosine (AraC) chemotherapy displayed improved survival (Cha, Meckling, & Stewart, 2002). In this study, compared to the chow diet, the 1.5% DHA-containing diet (average intake of 1.8 g of DHA/kg/day) prolonged the life span (33 ± 3.4 days, $p < 0.01$) of L1210-bearing mice following AraC treatment with no incidence of death due to drug toxicity (Cha et al., 2002). However, it should be noted that a high intake of DHA (4.5 g of DHA/kg/day) in combination with AraC did not shown benefit. Inversely, the high DHA-containing diet was associated with a shorter survival time (26.5 ± 2.0 days), increased circulating tumor cell burden, and lowered red blood

cell concentrations (Cha et al., 2002). Studies by Guffy et al. illustrated that *in vitro* treatment of L1210 lymphoblastic leukemia cells with DHA increased the sensitivity of cells to the anti-cancer drug Adriamycin in a dose-responsive manner as compared to cells treated with oleic acid (Guffy, North, & Burns, 1984). Additionally, increased DHA content indicate dose-responsive decreases in viability at a fixed concentration of Adriamycin (Guffy et al., 1984). Petersen et al. indicated that treatment of L1210 cells with DHA increased the sensitivity of cells to the thioether lipid BM 41.400 as compared to cells treated with oleic acid (Petersen, Kelley, Modest, & Burns, 1992). Whereas the use of n-3 has actively been explored in various malignancies, including B-cell malignancies, it has not been demonstrated whether or not n-3 can provide benefit to patients diagnosed with CLL in clinical settings.

It should be reminded that CLL is the most common form of adult leukemia in the western world (Riches et al., 2011). CLL is primarily a disease of the elderly with two thirds of patients being 65 years of age at the time of diagnosis (Riches et al., 2011). CLL is often initially diagnosed in the asymptomatic stages where watchful waiting is the primary course of treatment (Riches et al., 2011). However, approximately 50% of these patients will progress to advanced, symptomatic stages (Shanafelt & Call, 2004). Progression to symptomatic stages is associated with shortened survival and need for therapy (Shanafelt & Call, 2004). Despite the success of current treatment options, such as FCR (fludarabine, cyclophosphamide and rituximab), many patients develop drug resistance and disease relapse (Riches et al., 2011; Shanafelt & Call, 2004; Silber et al., 1994). Additionally, clinical treatment of CLL in patients who require aggressive treatment is limited due to significant side-effect profiles which are often too toxic for the elderly or those with co-morbidities (Riches et al., 2011). As such, clinical treatment of CLL is often hindered by progressive drug resistance and drug-induced toxicities (Riches et al., 2011).

Therefore, a non-toxic therapeutic intervention that could slow the progression of asymptomatic CLL to symptomatic CLL or enhance the effects of actively used chemotherapeutic drugs in patients who require therapy would be clinically beneficial. Thus, the application of n-3 as a novel therapeutic option for CLL is of considerable interest. In the following chapters, the application of n-3 fatty acids, particularly EPA and DHA, as therapeutic options for CLL will be explored through both *in vitro* studies as well as clinical studies with the aim of providing scientific insight into the potential use of n-3 as therapeutic options for the treatment of CLL.

REFERENCES

- Alfranca, A., Iniguez, M. A., Fresno, M., & Redondo, J. M. (2006). Prostanoid signal transduction and gene expression in the endothelium: role in cardiovascular diseases. *Cardiovasc Res*, *70*(3), 446-456. doi: 10.1016/j.cardiores.2005.12.020
- Arnold, C., Konkel, A., Fischer, R., & Schunck, W. H. (2010). Cytochrome P450-dependent metabolism of omega-6 and omega-3 long-chain polyunsaturated fatty acids. *Pharmacol Rep*, *62*(3), 536-547.
- Balkwill, F., & Coussens, L. M. (2004). Cancer: an inflammatory link. *Nature*, *431*(7007), 405-406. doi: 10.1038/431405a
- Barnes, N., Haywood, P., Flint, P., Knox, W. F., & Bundred, N. J. (2006). Survivin expression in situ and invasive breast cancer relates to COX-2 expression and DCIS recurrence. *Br J Cancer*, *94*(2), 253-258. doi: 10.1038/sj.bjc.6602932
- Bougnoux, P., Germain, E., Chajes, V., Hubert, B., Lhuillery, C., Le Floch, O., . . . Calais, G. (1999). Cytotoxic drugs efficacy correlates with adipose tissue docosahexaenoic acid level in locally advanced breast carcinoma. *Br J Cancer*, *79*(11-12), 1765-1769. doi: 10.1038/sj.bjc.6690281
- Bougnoux, P., Hajjaji, N., Ferrasson, M. N., Giraudeau, B., Couet, C., & Le Floch, O. (2009). Improving outcome of chemotherapy of metastatic breast cancer by docosahexaenoic acid: a phase II trial. *Br J Cancer*, *101*(12), 1978-1985. doi: 10.1038/sj.bjc.6605441
- Braun, T., Carvalho, G., Fabre, C., Grosjean, J., Fenaux, P., & Kroemer, G. (2006). Targeting NF-kappaB in hematologic malignancies. *Cell Death Differ*, *13*(5), 748-758. doi: 10.1038/sj.cdd.4401874

- Buchanan, F. G., Wang, D., Bargiacchi, F., & DuBois, R. N. (2003). Prostaglandin E2 regulates cell migration via the intracellular activation of the epidermal growth factor receptor. *J Biol Chem*, 278(37), 35451-35457. doi: 10.1074/jbc.M302474200
- Calder, P. C. (2009). Polyunsaturated fatty acids and inflammatory processes: New twists in an old tale. *Biochimie*, 91(6), 791-795. doi: 10.1016/j.biochi.2009.01.008
- Cathcart, M. C., Lysaght, J., & Pidgeon, G. P. (2011). Eicosanoid signalling pathways in the development and progression of colorectal cancer: novel approaches for prevention/intervention. *Cancer Metastasis Rev*, 30(3-4), 363-385. doi: 10.1007/s10555-011-9324-x
- Cha, M. C., Meckling, K. A., & Stewart, C. (2002). Dietary docosahexaenoic acid levels influence the outcome of arabinosylcytosine chemotherapy in L1210 leukemic mice. *Nutr Cancer*, 44(2), 176-181. doi: 10.1207/s15327914nc4402_09
- Chan, A. T., Ogino, S., & Fuchs, C. S. (2007). Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. *N Engl J Med*, 356(21), 2131-2142. doi: 10.1056/NEJMoa067208
- Clevers, H. (2004). At the crossroads of inflammation and cancer. *Cell*, 118(6), 671-674. doi: 10.1016/j.cell.2004.09.005
- Crespo, M., Bosch, F., Villamor, N., Bellosillo, B., Colomer, D., Rozman, M., . . . Montserrat, E. (2003). ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. *N Engl J Med*, 348(18), 1764-1775. doi: 10.1056/NEJMoa023143

- Damle, R. N., Wasil, T., Fais, F., Ghiotto, F., Valetto, A., Allen, S. L., . . . Chiorazzi, N. (1999). Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood*, *94*(6), 1840-1847.
- Darnell, J. E., Jr. (2002). Transcription factors as targets for cancer therapy. *Nat Rev Cancer*, *2*(10), 740-749. doi: 10.1038/nrc906
- Das, U. N., & Madhavi, N. (2011). Effect of polyunsaturated fatty acids on drug-sensitive and resistant tumor cells in vitro. *Lipids Health Dis*, *10*, 159. doi: 10.1186/1476-511X-10-159
1476-511X-10-159 [pii]
- Das, U. N., Madhavi, N., Sravan Kumar, G., Padma, M., & Sangeetha, P. (1998). Can tumour cell drug resistance be reversed by essential fatty acids and their metabolites? *Prostaglandins Leukot Essent Fatty Acids*, *58*(1), 39-54.
- Dekoj, T., Lee, S., Desai, S., Trevino, J., Babcock, T. A., Helton, W. S., & Espat, N. J. (2007). G2/M cell-cycle arrest and apoptosis by n-3 fatty acids in a pancreatic cancer model. *J Surg Res*, *139*(1), 106-112. doi: S0022-4804(06)00559-2 [pii]
10.1016/j.jss.2006.10.024
- Del Poeta, G., Maurillo, L., Venditti, A., Buccisano, F., Epiceno, A. M., Capelli, G., . . . Amadori, S. (2001). Clinical significance of CD38 expression in chronic lymphocytic leukemia. *Blood*, *98*(9), 2633-2639.
- Ditsworth, D., & Zong, W. X. (2004). NF-kappaB: key mediator of inflammation-associated cancer. *Cancer Biol Ther*, *3*(12), 1214-1216.
- Dreger, P. (2009). Allotransplantation for chronic lymphocytic leukemia. *Hematology Am Soc Hematol Educ Program*, 602-609. doi: 10.1182/asheducation-2009.1.602

- Eichhorst, B., Dreyling, M., Robak, T., Montserrat, E., & Hallek, M. (2011). Chronic lymphocytic leukemia: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*, *22 Suppl 6*, vi50-54. doi: 10.1093/annonc/mdr377
- Gaidano, G., Foa, R., & Dalla-Favera, R. (2012). Molecular pathogenesis of chronic lymphocytic leukemia. *J Clin Invest*, *122*(10), 3432-3438. doi: 10.1172/JCI64101
64101 [pii]
- Ghoreishi, Z., Esfahani, A., Djazayeri, A., Djalali, M., Golestan, B., Ayromlou, H., . . . Darabi, M. (2012). Omega-3 fatty acids are protective against paclitaxel-induced peripheral neuropathy: a randomized double-blind placebo controlled trial. *BMC Cancer*, *12*, 355. doi: 10.1186/1471-2407-12-355
- Gilmore, T. D. (1999). Multiple mutations contribute to the oncogenicity of the retroviral oncoprotein v-Rel. *Oncogene*, *18*(49), 6925-6937. doi: 10.1038/sj.onc.1203222
- Greenberg, M. E., Li, X. M., Gugiu, B. G., Gu, X., Qin, J., Salomon, R. G., & Hazen, S. L. (2008). The lipid whisker model of the structure of oxidized cell membranes. *J Biol Chem*, *283*(4), 2385-2396. doi: 10.1074/jbc.M707348200
- Greenhough, A., Smartt, H. J., Moore, A. E., Roberts, H. R., Williams, A. C., Paraskeva, C., & Kaidi, A. (2009). The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcinogenesis*, *30*(3), 377-386. doi: 10.1093/carcin/bgp014
- Guffy, M. M., North, J. A., & Burns, C. P. (1984). Effect of cellular fatty acid alteration on adriamycin sensitivity in cultured L1210 murine leukemia cells. *Cancer Res*, *44*(5), 1863-1866.

- Guo, D. L., Zhou, H., Wu, Y., Zhou, F., Zhang, X. M., Xu, G. Y., & Wen, H. P. (2011). [Role of NF-kappaB in factor VIIa-induced proliferation and migration of colon cancer cell line SW620 cells]. *Zhonghua Zhong Liu Za Zhi*, *33*(9), 649-653.
- Guttridge, D. C., Albanese, C., Reuther, J. Y., Pestell, R. G., & Baldwin, A. S., Jr. (1999). NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. *Mol Cell Biol*, *19*(8), 5785-5799.
- Haeggstrom, J. Z., Rinaldo-Matthis, A., Wheelock, C. E., & Wetterholm, A. (2010). Advances in eicosanoid research, novel therapeutic implications. *Biochem Biophys Res Commun*, *396*(1), 135-139. doi: 10.1016/j.bbrc.2010.03.140
- Hagemann, T., Biswas, S. K., Lawrence, T., Sica, A., & Lewis, C. E. (2009). Regulation of macrophage function in tumors: the multifaceted role of NF-kappaB. *Blood*, *113*(14), 3139-3146. doi: 10.1182/blood-2008-12-172825
- Hamblin, T. J., Davis, Z., Gardiner, A., Oscier, D. G., & Stevenson, F. K. (1999). Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood*, *94*(6), 1848-1854.
- Hardman, W. E., Avula, C. P., Fernandes, G., & Cameron, I. L. (2001). Three percent dietary fish oil concentrate increased efficacy of doxorubicin against MDA-MB 231 breast cancer xenografts. *Clin Cancer Res*, *7*(7), 2041-2049.
- Hayden, M. S., & Ghosh, S. (2012). NF-kappaB, the first quarter-century: remarkable progress and outstanding questions. *Genes Dev*, *26*(3), 203-234. doi: 10.1101/gad.183434.111
- Heller, A. R., Rossel, T., Gottschlich, B., Tiebel, O., Menschikowski, M., Litz, R. J., . . . Koch, T. (2004). Omega-3 fatty acids improve liver and pancreas function in postoperative cancer patients. *Int J Cancer*, *111*(4), 611-616. doi: 10.1002/ijc.20291

- Hewamana, S., Alghazal, S., Lin, T. T., Clement, M., Jenkins, C., Guzman, M. L., . . . Pepper, C. (2008). The NF-kappaB subunit Rel A is associated with in vitro survival and clinical disease progression in chronic lymphocytic leukemia and represents a promising therapeutic target. *Blood*, *111*(9), 4681-4689. doi: 10.1182/blood-2007-11-125278
- Hewamana, S., Lin, T. T., Jenkins, C., Burnett, A. K., Jordan, C. T., Fegan, C., . . . Pepper, C. (2008). The novel nuclear factor-kappaB inhibitor LC-1 is equipotent in poor prognostic subsets of chronic lymphocytic leukemia and shows strong synergy with fludarabine. *Clin Cancer Res*, *14*(24), 8102-8111. doi: 10.1158/1078-0432.ccr-08-1673
- Hewamana, S., Lin, T. T., Rowntree, C., Karunanithi, K., Pratt, G., Hills, R., . . . Pepper, C. (2009). Rel a is an independent biomarker of clinical outcome in chronic lymphocytic leukemia. *J Clin Oncol*, *27*(5), 763-769. doi: 10.1200/jco.2008.19.1114
- Hinz, M., Krappmann, D., Eichten, A., Heder, A., Scheidereit, C., & Strauss, M. (1999). NF-kappaB function in growth control: regulation of cyclin D1 expression and G0/G1-to-S-phase transition. *Mol Cell Biol*, *19*(4), 2690-2698.
- Huang, S., Robinson, J. B., Deguzman, A., Bucana, C. D., & Fidler, I. J. (2000). Blockade of nuclear factor-kappaB signaling inhibits angiogenesis and tumorigenicity of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin 8. *Cancer Res*, *60*(19), 5334-5339.
- Hyde, C. A., & Missailidis, S. (2009). Inhibition of arachidonic acid metabolism and its implication on cell proliferation and tumour-angiogenesis. *Int Immunopharmacol*, *9*(6), 701-715. doi: 10.1016/j.intimp.2009.02.003
- Karin, M., Cao, Y., Greten, F. R., & Li, Z. W. (2002). NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer*, *2*(4), 301-310. doi: 10.1038/nrc780

- Karin, M., & Lin, A. (2002). NF-kappaB at the crossroads of life and death. *Nat Immunol*, 3(3), 221-227. doi: 10.1038/ni0302-221
- Kim, H., Choi, J. A., Park, G. S., & Kim, J. H. (2012). BLT2 up-regulates interleukin-8 production and promotes the invasiveness of breast cancer cells. *PLoS One*, 7(11), e49186. doi: 10.1371/journal.pone.0049186
- Krysan, K., Dohadwala, M., Luo, J., Lin, Y., Zhu, L., Heuze-Vourc'h, N., . . . Dubinett, S. (2004). Cyclooxygenase-2-dependent expression of survivin in non-small cell lung cancer. *Chest*, 125(5 Suppl), 140S.
- Lanasa, M. C. (2010). Novel insights into the biology of CLL. *Hematology Am Soc Hematol Educ Program*, 2010, 70-76. doi: 10.1182/asheducation-2010.1.70
- Lawrence, T. (2009). The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol*, 1(6), a001651. doi: 10.1101/cshperspect.a001651
- Lee, L. M., Pan, C. C., Cheng, C. J., Chi, C. W., & Liu, T. Y. (2001). Expression of cyclooxygenase-2 in prostate adenocarcinoma and benign prostatic hyperplasia. *Anticancer Res*, 21(2B), 1291-1294.
- Lekovic, D., Mihaljevic, B., Kraguljac-Kurtovic, N., Peruncic-Jovanovic, M., Bogdanovic, A., Colovic, M., & Gotic, M. (2011). [Prognostic significance of new biological markers in chronic lymphocytic leukaemia]. *Srp Arh Celok Lek*, 139(11-12), 753-758.
- Li, F., & Sethi, G. (2010). Targeting transcription factor NF-kappaB to overcome chemoresistance and radioresistance in cancer therapy. *Biochim Biophys Acta*, 1805(2), 167-180. doi: 10.1016/j.bbcan.2010.01.002
- Li, J., Lau, G., Chen, L., Yuan, Y. F., Huang, J., Luk, J. M., . . . Guan, X. Y. (2012). Interleukin 23 promotes hepatocellular carcinoma metastasis via NF-kappa B induced matrix

- metalloproteinase 9 expression. *PLoS One*, 7(9), e46264. doi:
10.1371/journal.pone.0046264
- Linnet, M. S., Schubauer-Berigan, M. K., Weisenburger, D. D., Richardson, D. B., Landgren, O., Blair, A., . . . Dores, G. M. (2007). Chronic lymphocytic leukaemia: an overview of aetiology in light of recent developments in classification and pathogenesis. *Br J Haematol*, 139(5), 672-686. doi: 10.1111/j.1365-2141.2007.06847.x
- Liou, Y. A., King, D. J., Zibrik, D., & Innis, S. M. (2007). Decreasing linoleic acid with constant alpha-linolenic acid in dietary fats increases (n-3) eicosapentaenoic acid in plasma phospholipids in healthy men. *J Nutr*, 137(4), 945-952.
- Luo, P., & Wang, M. H. (2011). Eicosanoids, beta-cell function, and diabetes. *Prostaglandins Other Lipid Mediat*, 95(1-4), 1-10. doi: 10.1016/j.prostaglandins.2011.06.001
- Messmer, B. T., Messmer, D., Allen, S. L., Kolitz, J. E., Kudalkar, P., Cesar, D., . . . Chiorazzi, N. (2005). In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. *J Clin Invest*, 115(3), 755-764. doi: 10.1172/jci23409
- Montserrat, E., Sanchez-Bisono, J., Vinolas, N., & Rozman, C. (1986). Lymphocyte doubling time in chronic lymphocytic leukaemia: analysis of its prognostic significance. *Br J Haematol*, 62(3), 567-575.
- Murphy, R. A., Mourtzakis, M., Chu, Q. S., Baracos, V. E., Reiman, T., & Mazurak, V. C. (2011a). Nutritional intervention with fish oil provides a benefit over standard of care for weight and skeletal muscle mass in patients with nonsmall cell lung cancer receiving chemotherapy. *Cancer*, 117(8), 1775-1782. doi: 10.1002/cncr.25709
- Murphy, R. A., Mourtzakis, M., Chu, Q. S., Baracos, V. E., Reiman, T., & Mazurak, V. C. (2011b). Supplementation with fish oil increases first-line chemotherapy efficacy in

- patients with advanced nonsmall cell lung cancer. *Cancer*, 117(16), 3774-3780. doi: 10.1002/cncr.25933
- Niki, E. (2009). Lipid peroxidation: physiological levels and dual biological effects. *Free Radic Biol Med*, 47(5), 469-484. doi: 10.1016/j.freeradbiomed.2009.05.032
- Pai, R., Soreghan, B., Szabo, I. L., Pavelka, M., Baatar, D., & Tarnawski, A. S. (2002). Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. *Nat Med*, 8(3), 289-293. doi: 10.1038/nm0302-289
- Parthasarathy, S., Santanam, N., Ramachandran, S., & Meilhac, O. (2000). Potential role of oxidized lipids and lipoproteins in antioxidant defense. *Free Radic Res*, 33(3), 197-215.
- Patterson, E., Wall, R., Fitzgerald, G. F., Ross, R. P., & Stanton, C. (2012). Health implications of high dietary omega-6 polyunsaturated Fatty acids. *J Nutr Metab*, 2012, 539426. doi: 10.1155/2012/539426
- Pepper, C., Mahdi, J. G., Buggins, A. G., Hewamana, S., Walsby, E., Mahdi, E., . . . Fegan, C. (2011). Two novel aspirin analogues show selective cytotoxicity in primary chronic lymphocytic leukaemia cells that is associated with dual inhibition of Rel A and COX-2. *Cell Prolif*, 44(4), 380-390. doi: 10.1111/j.1365-2184.2011.00760.x
- Petersen, E. S., Kelley, E. E., Modest, E. J., & Burns, C. P. (1992). Membrane lipid modification and sensitivity of leukemic cells to the thioether lipid analogue BM 41.440. *Cancer Res*, 52(22), 6263-6269.
- Qin, J., Wang, Y., Bai, Y., Yang, K., Mao, Q., Lin, Y., . . . Xie, L. (2012). Epigallocatechin-3-gallate inhibits bladder cancer cell invasion via suppression of NF-kappaB mediated

- matrix metalloproteinase-9 expression. *Mol Med Rep*, 6(5), 1040-1044. doi: 10.3892/mmr.2012.1054
- Rai, K. R., & Jain, P. (2011). Advances in the clinical staging of chronic lymphocytic leukemia. *Clin Chem*, 57(12), 1771-1772. doi: 10.1373/clinchem.2010.159004
- Reader, J., Holt, D., & Fulton, A. (2011). Prostaglandin E2 EP receptors as therapeutic targets in breast cancer. *Cancer Metastasis Rev*, 30(3-4), 449-463. doi: 10.1007/s10555-011-9303-2
- Ricciotti, E., & FitzGerald, G. A. (2011). Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol*, 31(5), 986-1000. doi: 10.1161/atvbaha.110.207449
- Riches, J. C., Ramsay, A. G., & Gribben, J. G. (2011). Chronic lymphocytic leukemia: an update on biology and treatment. *Curr Oncol Rep*, 13(5), 379-385. doi: 10.1007/s11912-011-0188-6
- Rodriguez-Vicente, A. E., Diaz, M. G., & Hernandez-Rivas, J. M. (2013). Chronic lymphocytic leukemia: a clinical and molecular heterogenous disease. *Cancer Genet*, 206(3), 49-62. doi: 10.1016/j.cancergen.2013.01.003
- Ryan, E. P., Pollock, S. J., Kaur, K., Felgar, R. E., Bernstein, S. H., Chiorazzi, N., & Phipps, R. P. (2006). Constitutive and activation-inducible cyclooxygenase-2 expression enhances survival of chronic lymphocytic leukemia B cells. *Clin Immunol*, 120(1), 76-90. doi: 10.1016/j.clim.2005.12.012
- Schmitz, G., & Ecker, J. (2008). The opposing effects of n-3 and n-6 fatty acids. *Prog Lipid Res*, 47(2), 147-155. doi: 10.1016/j.plipres.2007.12.004
- Secchiero, P., Barbarotto, E., Gonelli, A., Tiribelli, M., Zerbinati, C., Celeghini, C., . . . Zauli, G. (2005). Potential pathogenetic implications of cyclooxygenase-2 overexpression in B

- chronic lymphoid leukemia cells. *Am J Pathol*, 167(6), 1599-1607. doi: 10.1016/s0002-9440(10)61244-8
- Serhan, C. N., & Petasis, N. A. (2011). Resolvins and protectins in inflammation resolution. *Chem Rev*, 111(10), 5922-5943. doi: 10.1021/cr100396c
- Shanafelt, T. D., & Call, T. G. (2004). Current approach to diagnosis and management of chronic lymphocytic leukemia. *Mayo Clin Proc*, 79(3), 388-398. doi: 10.4065/79.3.388
- Silber, R., Degar, B., Costin, D., Newcomb, E. W., Mani, M., Rosenberg, C. R., . . . Potmesil, M. (1994). Chemosensitivity of lymphocytes from patients with B-cell chronic lymphocytic leukemia to chlorambucil, fludarabine, and camptothecin analogs. *Blood*, 84(10), 3440-3446.
- Simopoulos, A. P. (1991). Omega-3 fatty acids in health and disease and in growth and development. *Am J Clin Nutr*, 54(3), 438-463.
- Simopoulos, A. P. (2000). Human requirement for N-3 polyunsaturated fatty acids. *Poult Sci*, 79(7), 961-970.
- Simopoulos, A. P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother*, 56(8), 365-379.
- Simopoulos, A. P. (2008). The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp Biol Med (Maywood)*, 233(6), 674-688. doi: 10.3181/0711-mr-311
- Sun, S. C. (2011). Non-canonical NF-kappaB signaling pathway. *Cell Res*, 21(1), 71-85. doi: 10.1038/cr.2010.177
- Tanaka, T., Nakayama, H., Yoshitake, Y., Irie, A., Nagata, M., Kawahara, K., . . . Shinohara, M. (2012). Selective inhibition of nuclear factor-kappaB by nuclear factor-kappaB essential

- modulator-binding domain peptide suppresses the metastasis of highly metastatic oral squamous cell carcinoma. *Cancer Sci*, 103(3), 455-463. doi: 10.1111/j.1349-7006.2011.02174.x
- Tang, D. W., Lin, S. C., Chang, K. W., Chi, C. W., Chang, C. S., & Liu, T. Y. (2003). Elevated expression of cyclooxygenase (COX)-2 in oral squamous cell carcinoma--evidence for COX-2 induction by areca quid ingredients in oral keratinocytes. *J Oral Pathol Med*, 32(9), 522-529.
- Tang, X., Liu, D., Shishodia, S., Ozburn, N., Behrens, C., Lee, J. J., . . . Wistuba, II. (2006). Nuclear factor-kappaB (NF-kappaB) is frequently expressed in lung cancer and preneoplastic lesions. *Cancer*, 107(11), 2637-2646. doi: 10.1002/cncr.22315
- van der Meij, B. S., Langius, J. A., Spreeuwenberg, M. D., Slootmaker, S. M., Paul, M. A., Smit, E. F., & van Leeuwen, P. A. (2012). Oral nutritional supplements containing n-3 polyunsaturated fatty acids affect quality of life and functional status in lung cancer patients during multimodality treatment: an RCT. *Eur J Clin Nutr*, 66(3), 399-404. doi: 10.1038/ejcn.2011.214
- Wang, C. Y., Cusack, J. C., Jr., Liu, R., & Baldwin, A. S., Jr. (1999). Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-kappaB. *Nat Med*, 5(4), 412-417. doi: 10.1038/7410
- Wang, C. Y., Mayo, M. W., & Baldwin, A. S., Jr. (1996). TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. *Science*, 274(5288), 784-787.
- Wang, D., & Dubois, R. N. (2010). Eicosanoids and cancer. *Nat Rev Cancer*, 10(3), 181-193. doi: 10.1038/nrc2809

Wiestner, A., Rosenwald, A., Barry, T. S., Wright, G., Davis, R. E., Henrickson, S. E., . . .

Staudt, L. M. (2003). ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. *Blood*, *101*(12), 4944-4951. doi: 10.1182/blood-2002-10-3306

Zhang, D., Jin, X., Wang, F., Wang, S., Deng, C., Gao, Z., & Guo, C. (2007). Combined prognostic value of both RelA and IkappaB-alpha expression in human non-small cell lung cancer. *Ann Surg Oncol*, *14*(12), 3581-3592. doi: 10.1245/s10434-007-9560-z

Zmijewski, J. W., Landar, A., Watanabe, N., Dickinson, D. A., Noguchi, N., & Darley-Usmar, V. M. (2005). Cell signalling by oxidized lipids and the role of reactive oxygen species in the endothelium. *Biochem Soc Trans*, *33*(Pt 6), 1385-1389. doi: 10.1042/bst20051385

CHAPTER 2: INHIBITION OF NUCLEAR FACTOR KAPPA B ACTIVATION IN EARLY-STAGE CHRONIC LYMPHOCYtic LEUKEMIA BY OMEGA-3 FATTY ACIDS

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ABSTRACT

Targeting the nuclear factor kappa B (NFκB) pathway is proposed as a therapy for chronic lymphocytic leukemia (CLL). We hypothesized that an omega-3 fatty acids (n-3) supplement would suppress NFκB activation in lymphocytes of Rai Stage 0-1 CLL patients. The initial dose of 2.4 g n-3/day was gradually increased to 7.2 g n-3/day. After n-3 consumption: (1) plasma n-3 increased; (2) NFκB activation was suppressed in lymphocytes; (3) *in vitro* sensitivity of lymphocytes to doxorubicin was increased; and (4) expression of 32 genes in lymphocytes was significantly decreased.

INTRODUCTION

Chronic lymphocytic leukemia is a B-cell leukemia. During the early stages (Rai Stage 0-1) of CLL patients, no therapy is required (Robak, 2007). However, about 50% of these patients will progress to more advanced stages (Shanafelt & Call, 2004). Progression to later stages (Rai Stage 2-4) disease is associated with shortened survival and the need for chemotherapy (Shanafelt & Call, 2004). Therefore, early identification of progression potential and a therapeutic intervention that could prevent or delay disease progression without undesirable side effects would be clinically beneficial.

Nuclear factor kappa B (NF κ B) belongs to a family of transcription factors that plays a key role in inflammatory and stress responses as well as in tumor cell resistance to apoptosis (Balkwill & Coussens, 2004; Darnell, 2002). Several studies suggest that NF κ B family members are involved in cancer development (Clevers, 2004; Munzert et al., 2002). NF κ B can promote oncogenesis by virtue of its capacity to regulate the expression of a large number of genes that modulate apoptosis, proliferation, inflammation, tumor metastasis and angiogenesis (Clevers, 2004). Increased, constitutive activity of NF κ B is observed in various solid tumors and in hematological malignancies (Cilloni, Martinelli, Messa, Baccarani, & Saglio, 2007; Darnell, 2002; Ditsworth & Zong, 2004).

There is increasing evidence that NF κ B may play a key role in controlling apoptosis and disease progression in hematologic malignancies (Cilloni et al., 2007). Previous studies have demonstrated that CLL cells have elevated NF κ B DNA-binding activity compared to normal B cells (Braun et al., 2006; Endo et al., 2007) and that inhibition of NF κ B activation *in vitro* resulted in apoptosis of the malignant cells (Braun et al., 2006; Endo et al., 2007). NF κ B is

currently considered an important target for the development of novel drugs for the treatment of hematological malignancies, especially CLL, and of solid tumors (Braun et al., 2006; Cilloni et al., 2007; Cilloni et al., 2006; Darnell, 2002; Hewamana et al., 2008; Hewamana et al., 2009).

Omega-3 and omega-6 polyunsaturated fatty acids are essential fatty acids which must be obtained from diet. The ratio of omega-3 to omega-6 in the average western diet is heavily weighted in favor of omega-6 (Simopoulos, 2000, 2002). Long chain omega-3 fatty acids (eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)) are primarily found in fish oils (Simopoulos, 1991). However, the 18 carbon n-3 FA, alpha linolenic acid (ALA), is also found in plant derived foods, especially canola or flax oil and walnuts. Omega-3 fatty acids have consistently been shown *in vitro* and *in vivo* to slow growth of cancer of various types and to increase the efficacy of chemotherapy (Bougnoux et al., 1999; Hardman, 2002). The omega-6 fatty acid, linoleic acid (LA), is found in corn and soybean oils. The elongated, desaturated product of LA, arachidonic acid (AA), is found in the meat of animals that consumed corn or soybeans and can be synthesized by humans. Growing evidence supports the notion that AA and its metabolites have a significant role in tumor promotion (Hyde & Missailidis, 2009).

Our preliminary work and that of others (Narayanan, Narayanan, & Reddy, 2005; Ross, Maingay, Fearon, Sangster, & Powell, 2003) indicates that supplementation with omega-3 fatty acids (n-3) inhibits NFκB activity of many cancer types *in vitro* and in mouse models. We did not know if this inhibitory effect would translate to humans. The primary objective of this study was to determine if consumption of an n-3 supplement would suppress the activation of NFκB in the lymphocytes of patients with early stage (Rai Stage 0-1) CLL. We hypothesized that dietary omega-3 supplementation would decrease the activation of NFκB in malignant lymphocytes from patients with CLL in early stages. In this pilot study, we assayed NFκB activity in

lymphocytes from patients with early stage (Rai Stage 0-1) CLL before and after up to 12 months of oral omega-3 supplementation. Secondary objectives were to evaluate the effects of n-3 supplementation on 1) fatty acid composition of lymphocytes, 2) concentration of fatty acids in the plasma before and after consumption of n-3, 3) the *in vitro* sensitivity of the patients' lymphocytes to doxorubicin and 4) changes in lymphocyte gene expression.

METHODS

IRB and informed consent

The Marshall University Institutional Review Board reviewed and approved the protocol for this study under a Federal Wide Assurance (FWA #00002704) with the Office of Human Research Protections. The trial was listed at www.clinicaltrials.gov/, number NCT00899353. The purpose of the study and possible risks and benefits were explained prior to obtaining signed informed consent from all research participants. Whenever possible, specimens were obtained at the same time as blood was being obtained for routine clinical tests.

Subject Population

Subjects participating in this pilot study had been diagnosed with chronic lymphocytic leukemia in the early stages (Rai stage 0-1) at the time of entry according to standard clinical practice, and did not require therapy. General eligibilities were that participants had to be older than 18 years of age with no maximum age limit, free of co-morbid conditions with a life expectancy of more than 12 months, patients who have taken omega-3 or other fish oil containing nutritional supplements prior to study accrual, discontinued their use for minimum of 2 months before enrollment, and patients should have an ECOG (Eastern Cooperative Oncology Group) performance score of 0, 1 or 2.

After obtaining informed consent, two 7 ml tubes of Ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood were obtained (time 0) as a baseline and at each subsequent monthly visit. One month after the initial specimen, each patient started n-3 consumption with a dose of 3 capsules (2.4g of n-3) per day (1 capsule/meal). Dosage was increased to 6 capsules (4.8g of n-3) and then to 9 capsules (7.2g of n-3) per day (up to 3/meal) at monthly intervals. A schematic of dosing scheme is provided in Appendix, Supplementary Figure 2.1). If a patient did

not want to consume the increased dosages, he/she was allowed to continue in the study at a lower dose. Results of fatty acid composition in lymphocytes and plasma, NFκB activity, doxorubicin sensitivity, and mRNA expression of the lymphocytes up to consumption of 7.2 g per day for three months are reported. No placebo was used, rather after-consumption data were compared to baseline data.

Normal participants

Five normal healthy volunteers were recruited. These volunteers did not receive omega-3 supplementation and served to establish normal values for NFκB activation in lymphocytes.

Description of omega 3 supplement

The supplement was a commercially available (Res-Q 1250, N3 Oceanic, Palm, PA) fish oil concentrate prepared by molecular distillation. It was free from pollutants, PCB's, pesticides and mercury. Each capsule contained about 400mg of EPA, 300mg of DHA and 100-150 mg of other omega 3 fatty acids. Thus, consumption of 3, 6, or 9 capsules/day yielded 2.4 g, 4.8 g, or 7.2 g/day of n-3, respectively. These amounts are all below the maximum tolerated dose of 13.1 g/day EPA + DHA reported by Burns et al. (Burns et al., 1999). Potential dose limiting events were diarrhea, stomach upset or dislike of taking the capsules.

Platelet function

Tests of platelet function were performed in the clinical laboratory at Cabell Huntington Hospital. For these tests, anticoagulated fresh, whole blood is rapidly passed through a small hole in a membrane coated with either collagen and adenosine diphosphate (ADP) (Collagen/ADP) or collagen and epinephrine (Collagen/EPI). Platelets adhere to and gradually block the small hole in the center of each membrane. The time, in seconds, for blood to close the hole is the Closure Time (CT).

Molecular Testing

Tests for cytogenetic alterations were performed using standard tests (culture and banding) at Cabell Huntington Hospital. Interphase fluorescence in situ hybridization (FISH) was performed using a multiprobe panel (Vysis, Abbot Molecular, Abbott Park, IL, USA.) targeting five prognosis related CLL anomalies according to routine techniques at the Laboratory Corporation of America.

Lymphocyte Isolation

EDTA anticoagulated blood specimens (7 ml x 2) were placed on ice until retrieved by research laboratory personnel (within 2 hours of collection). The sample was centrifuged at 400 x g for 10 minutes and about 2 ml of plasma was removed, divided to 0.5 ml aliquots and stored at -80°C for future assay. The buffy coat, some plasma and adjacent red blood cells were removed. A Ficoll-Paque (GE Healthcare, Piscataway, NJ) gradient was used to isolate lymphocytes, according to manufacturer's protocol. Following Ficoll-Paque separation, greater than 95% of cells were lymphocytes (Silber et al., 1994). Since there was not 100% lymphocyte isolation, samples were referred to as WBCs. WBCs were washed twice with a glucose containing balanced salt solution, counted and divided into appropriate aliquots for NFκB activity assay, doxorubicin sensitivity, mRNA assay and for gas chromatography analysis.

White and red blood cell fatty acid composition

The fatty acid compositions of WBCs or RBCs were assessed using gas chromatography of samples collected prior to initiation of the omega 3 supplement (baseline, time 0) and after consumption of each dose of the supplement for 1 month. The assay was performed as previously published (Witte, Salazar, Ballester, & Hardman, 2010).

Plasma Fatty Acid Concentrations

The concentrations of 10 analyzed fatty acids in plasma were assessed using gas chromatography, according to our routine techniques, prior to initiation of the omega-3 supplement (baseline, time 0) and after consumption of each dose of the supplement for 1 month. Two hundred forty microliters to 400 μ L of plasma was added to distilled water with 0.1% BHT for a final volume of 700 μ L to prevent fatty acid oxidation. One hundred microliters of 1mM nervonic acid was added to each sample. Nervonic acid served as the internal standard as previously published (Shantha & Ackman, 1990). Lipids were extracted with chloroform/methanol, then methylated. Methylated lipids were separated and identified using gas chromatography as previously published (Witte et al., 2010). Fatty acid methyl ester standards (Nu-Chek-Prep, Elysian, MN) were used for peak identification. Respective fatty acids were corrected for FID response and carbon response as previously described by others (Huang Yieru, 1990; William, 1989) and initial plasma volume used (see Equation 1). We were especially interested in total omega 6 fatty acids, LA, gamma linolenic acid (GLA), 20:3n-6, and AA, and total omega-3 fatty acids, ALA, EPA, docosapentaenoic acid (DPA) and DHA.

Equation 1: [Fatty Acid]

$$[\text{Fatty Acid}] = \frac{\text{AUC of fatty acid} \times [\text{Nervonic acid}]}{\text{AUC of Nervonic Acid}} \times \text{FID Response Factor} \times \text{Carbon Response Factor} \times \frac{\text{Final Volume of Fatty Acid}}{\text{Initial Volume of Fatty Acid}}$$

Protein extraction

Protein extraction from lymphocytes was performed using Thermo Scientific Mammalian Protein Extraction Reagent (M-PER) according to manufacturer's protocol. Total protein concentration was determined using Pierce Coomassie (Bradford) Protein Assay Kit protocol.

A standard curve was used to determine the protein concentration of each unknown sample.

NFκB Activity Assay

NFκB activities in patient specimens, positive and negative controls were evaluated using a Thermo Scientific Transcription Factor kit for NFκB p50, according to manufacturer's protocol. The kit contains streptavidin-coated 96-well plates and an NFκB biotinylated-consensus sequence. Only the active form of NFκB will bind to the DNA consensus sequence. Protein extracts containing 1-15μg of protein/well were added in triplicates to appropriate microplate wells. Luminescence resulting from a reaction with bound NFκB was detected using a Berthold Centro LB960 Luminometer and analyzed with MikroWin 2000 ver. 1.08. NFκB activity was normalized by luminescence units/μg of protein per well.

Doxorubicin sensitivity assay

About 5×10^5 lymphocytes were diluted into 500 μl of RPMI/15% fetal bovine serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin media. Cells were aliquoted in triplicates in microtubes and treated with 0, 0.5, 1, 1.5, 2, or 2.5 μM doxorubicin (DOX) for 20 hours at 37°C. Lymphocytes were centrifuged at 600 x g for 10 minutes then DOX/media was removed. A standard colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine remaining live cells compared to the untreated sample. MTT is a yellow tetrazolium dye that is reduced to a purple formazan dye in living cells by reductase enzymes. The relative number of viable cells can be assessed by spectrophotometric measurement of formazan production relative to control specimens.

mRNA extraction, microarray and statistical analyses

Expression profiling was performed on mRNA from 5 patients characterized with higher initial NFκB activity and 4 patients with lower initial NFκB activity. Remaining patients were not assayed due to limited samples or to insufficient n-3 consumption time points.

About 1 million WBCs from each patient were homogenized in Tri-Reagent LS (Sigma) following the manufacturer's protocol to isolate mRNA. RNA concentration and purity was determined using a Nanodrop ND-1000 spectrophotometer. Total RNA quality was determined using an Agilent BioAnalyzer (Agilent, Santa Clara, CA). All samples had RNA Integrity Numbers higher than 7.

RNA expression profiles were obtained using a universal reference microarray design. Total RNA, 250 ng, was labeled using QuickAmp labeling kit (Agilent) and Cy3-CTP and Cy5-CTP from Perkin Elmer (Waltham, MA) according to previously described methods (Syed & Threadgill, 2006). The RNA samples isolated from patient's peripheral blood were labeled using Cy3-CTP and the universal human reference RNA was labeled with Cy5-CTP. Following purification of labeled cRNA, 825 ng of Cy3- and Cy5-labeled samples were combined and hybridized for 17 hours at 65°C onto Whole Human Genome 4x44k microarrays (Agilent). Arrays were scanned and lowest-normalized data extracted to tab-delimited text files using Agilent Feature Extraction software (version 10.5.1.1).

The log ratio of expression of the sample to that of universal reference RNA was computed, with values removed for control spots, spots identified as not well above background on both channels, and spots identified as non-uniform or saturated on either channel. Values from all arrays were compiled into a single table. Spots for which more than 50% of the samples had missing values were removed from further analysis. The Agilent array platform contains a

number of probes that are spotted multiple times on the array; for these probes the average across replicate spots was computed. Missing values were then imputed using the K nearest neighbor algorithm as implemented by the R/Bioconductor (www.r-project.org, www.bioconductor.org) `impute.knn` function in the "impute" package. A two-class Significance Analysis of Microarrays (Tusher, Tibshirani, & Chu, 2001), using the SAM function in the R/Bioconductor package "siggenes", was performed between the baseline mRNA expressions and the mRNA expressions after consumption of highest amount of omega-3 on: 1) samples with higher initial NF κ B activity (patients 1 to 5, n= 5 patients) or 2) with lower initial NF κ B activity (patients 6 to 9, n= 4 patients). A threshold delta value was chosen using the `findDelta` function from the same package to give a false discovery rate of 10%. The resulting list of probes was filtered for a minimum fold change of 1.5. For any genes represented by multiple probes in this list of significantly differentially expressed genes, the maximum fold change among those probes was reported. These analyses follow the method detailed in Tusher VG, et al. (Tusher et al., 2001). Microarray data are available in MIAME-complaint form at the NCBI GEO repository, accession number GSE29678.

Statistical Analysis, other data

SPSS (IBM SPSS Statistics) Version 14.0 was used for statistical analysis of other numeric data by Multiple Comparison using Games-Howell Post-Hoc Test. Correlation coefficients were calculated from least squares linear regression plots. Prism[®] software (Graphpad, Inc., La Jolla, CA) was used for preparation of graphs.

RESULTS

Subject characteristics

Fifteen CLL and 5 normal participants were recruited for this pilot study. All available data from these patients is reported. Individual characteristics are indicated in Table 2.1.

Graphic display of NFκB data seemed to indicate 2 populations of patients. Thus, patient characteristics were analyzed on the basis of subjects which fell below the median NFκB activation ($< 21.4 \times 10^5$ NFκB luminescence units/μg of protein) or above the median NFκB activation ($> 21.4 \times 10^5$ NFκB luminescence units/μg of protein). Subject characteristics, with subjects divided into higher and lower initial NFκB activation groups are shown in Table 2.2. There were no significant differences in mean age or gender of the groups. Data indicated that the 'lower initial activation' patients had significantly lower absolute lymphocyte counts (Mean \pm SEM: $11.9 \pm 3.2 \times 10^3$ lymphocytes/μl vs $28.8 \pm 4.0 \times 10^3$ lymphocytes/μl; p: 0.009) and tended to be at lower Rai stage (stage 0, 84% vs 58%, not statistically different by Fisher exact test) than patients exhibiting 'higher initial activation' of NFκB at baseline.

Table 2. 1. Individual Patient Characteristics

| Patient | Age/Gender | RAI Stage (0-1) | Time From Diagnosis to N-3 Initiation (Months) | Duration of N-3 Consumption (Months) | Baseline ALC ($10^3/\mu\text{L}$) | ALC post n-3 consumption ($10^3/\mu\text{L}$) | Fold Change in ALC | T(14;19) | Del.13q | Trisomy 12 | Del. 17p | CD38 | β2M (mg/L) |
|---------|------------|-----------------|--|--------------------------------------|-------------------------------------|---|--------------------|----------|---------|------------|----------|---------|-------------------------|
| 1 | 63/M | 0 | 16 | 12 | 25.4 | 41.3 | 1.62 | - | - | + | - | - | 1.7 |
| 2 | 70/F | 0 | 8 | 12 | 30.5 | 52.1 | 1.71 | + | - | - | - | - | 1.4 |
| 3 | 64/F | 0 | 39 | 12 | 15.2 | 11.9 | 0.78 | - | + | - | - | Unknown | 1.6 |
| 4 | 59/F | 1 | 133 ^a | 12 | 26.3 | 25.2 | 0.96 | - | + | - | - | - | 2.1 |
| 5 | 58/M | 1 | 2 | 5 | 20.7 | 27.3 | 1.32 | - | Unknown | - | - | + | 1.3 |
| 6 | 77/M | 0 | 157 | 12 | 35.5 | 38.2 | 1.08 | - | + | - | - | - | unknown |
| 7 | 68/M | 1 | 2 | 12 | 5.7 | 5.7 | 1.00 | - | + | - | - | - | 1.4 |
| 8 | 65/F | 0 | 5 | 12 | 20.3 | 18.9 | 0.92 | - | + | - | - | - | 3.5 |
| 9 | 72/M | 0 | 5 | 12 | 5.7 | 8.5 | 1.49 | - | - | + | - | - | 1.8 |
| 10 | 63/F | 0 | 28 | 12 | 7.5 | 7.9 | 1.05 | - | - | - | Unknown | - | 1.9 |
| 11 | 72/F | 0 | 35 | 12 | 8.5 | 9.9 | 1.16 | - | - | - | - | - | 1.8 |
| 12 | 62/F | Unknown | 79 | 8 | Unknown | 2.5 | N/A | - | Unknown | - | - | unknown | Unknown |
| 13 | 63/M | 1 | 61 | 8 | 48.1 | 49.7 | 1.03 | - | - | - | - | - | 1.5 |
| 14 | 82/F | 0 | 11 | 5 | 7.9 | 9.6 | 1.22 | - | - | - | - | - | 2.4 |
| 15 | 73/M | 0 | 14 | 1 | 23.6 | Unknown | N/A | - | - | + | - | + | 3.8 |

Table 2.1. Individual Patient Characteristics. Individual patient's age, gender, staging, time from diagnosis to n-3 initiation, duration of n-3 consumption, absolute lymphocyte counts (ALC) before and after n-3 consumption, fold change in ALC as compared to baseline, cytogenetics and phenotype is shown. Prognostics were done on samples at diagnosis of CLL. Tests for IGVH status were not performed at this institution. Prognosis - translocation between chromosome 14 and chromosome 19 [t(14;19)]; and deletion of long arm of chromosome 13 (Del.13q), are associated with good prognosis; trisomy 12 is associated with intermediate prognosis; deletion of short arm of chromosome 17 (Del.17p), CD38 positive, and high levels of $\beta\text{2-microglobulin}$ (β2M mg/L) > 2 are associated with poor prognosis; ^a patient referral, exact time of diagnosis unknown.

| Table 2. 2. Patient Lymphocyte Count, Cytogenetics, Phenotype and NFκB Activity | | | | |
|--|--|--|--|----------------|
| | Total Population | Baseline NFκB Activity > Median | Baseline NFκB Activity < Median | P-value |
| Patients (n) | 15 | 7 | 6 | |
| Age (Mean ± SEM) | 66; 63 - 82 (median; range) | 64.9 ± 2.5 | 69.2 ± 1.6 | 0.192 |
| Rai Stage (Stage 0/1) | 11/4 | 4/3 | 5/1 | |
| Absolute Lymphocyte Count (10³/μl) (Mean ± SEM) | 20.07; 5.7 – 48.1 (median; range) | 28.8 ± 4.0 | 11.9 ± 3.2 | 0.009 |
| # with T (14;19) | 1 | 1 | 0 | |
| # with Del. 13q | 5 | 3 | 2 | |
| # with Trisomy 12 | 3 | 1 | 2 | |
| # CD38 +/- | 2/10 | 1/5 | 1/5 | |
| β2M mg/L (Mean ± SEM) | 1.8; 1.3 - 3.8 (median; range) | 1.60 ± 0.12 | 2.37 ± 0.41 | 0.134 |
| NFκB Activity (Mean ± SEM) | 21.4 x 10 ⁵ ; 0.945 x 10 ⁵ – 231 x 10 ⁵ (median; range) | 127. x 10 ⁵ ± 27.7 x 10 ⁵ | 7.75 x 10 ⁵ ± 2.67 x 10 ⁵ | 0.005 |

Table 2.2. Patient Characteristics and NFκB Activity. Median and Range of patient’s age, staging, median and range of absolute lymphocyte counts, cytogenetics, phenotype and median and range of NFκB activity for all patients are shown. Mean ± SEM patient age, staging, mean ± SEM absolute lymphocyte count, cytogenetics, phenotype and mean ± SEM NFκB activity on the basis of whether patients fell above or below the median initial NFκB activity are shown. NFκB values were done on entry into the study and after n-3 consumption. NFκB activity is presented in NFκB luminescence units/μg protein. Subjects were divided into two groups based on whether initial NFκB activation fell below or above the median (21.4 x 10⁵ NFκB luminescence units/ μg protein) NFκB activation in lymphocytes from all but 2 patients. Three (3) patients were excluded as baseline NFκB Activity was not determined.

Omega-3 fatty acid supplementation did not have detrimental effects on platelet function

Consumption of omega 3 fatty acids is known to slightly slow blood clotting. To assess the impact of omega 3 fatty acids on platelet function, standard clinical tests, collagen/ADP and collagen/EPI (epinephrine), were performed at baseline and after 6 months of omega-3 consumption (dosage up to 7.2 g of n-3/day).

There was no difference between either collagen/ADP or collagen/EPI times at baseline and after omega-3 consumption. Omega-3 consumption slightly increased collagen/EPI time but this increase was neither statistically nor clinically significant and remained within normal parameters (Appendix, Supplementary Figure 2.1).

Omega-3 consumption had minimal side effects

Known side effects associated with omega-3 consumption are diarrhea, nausea, fishy odors, bloating and slowed blood coagulation. Side effects experienced by participants were most commonly associated with mild diarrhea (grade 1 and 2, n=4 and 1, respectively) or nausea (n=2). As a result of associated side effects, patients 3, 4, 8, 10 and 14 reduced intakes to 4.8, 2.4, 4.8, 2.4, and 4.8 g of n-3/day, respectively.

Omega-3 fatty acid fraction in RBCs and WBCs increased upon fish oil consumption

The content of fatty acids in tissue membranes is generally indicative of consumption of those fats and can be used as a measure of compliance with the supplement. There was a statistically significant reduction of AA and there were significant increases in EPA, DPA, and DHA in red blood cells following omega-3 consumption (data not shown, previously published

(Witte et al., 2010)). Differences in the levels of omega-6 and omega-3 fatty acids in white blood cells showed a trend similar to that of red cells but were not mathematically significant.

Consumption of n-3 increased n-3 fatty acid content in plasma

Mean \pm SEM baseline (prior to n-3 supplementation) plasma concentrations of 10 fatty acids analyzed from 6 patients with early-stage CLL are shown in Table 2.3. Mean baseline [μ M] of analyzed fatty acids were in agreement with previously published results (Harper, Edwards, DeFilippis, & Jacobson, 2006). Only 6 patients were analyzed as these were the only patients who consumed all three proposed doses of n-3 (2.4g, 4.8g and 7.2 g of n-3/day).

Mean \pm SEM fatty acid plasma concentrations of 10 fatty acids analyzed from 6 subjects at baseline (0 g supplement), 2.4, 4.8, and 7.2 g of n-3 consumption per day and discontinued n-3 consumption (n=2) are shown in Figure 2.1 and Table 2.3. Graphs are provided for visual comparison (Fig. 2.1) and Table 2.3 provides means \pm SEM molar concentrations for use in future comparisons.

Figure 2.1A shows the mean [μ M] \pm SEM of stearic acid (SA) and oleic acid (OA) with increasing n-3 consumption and discontinued n-3 consumption. Plasma OA was significantly decreased following consumption of 2.4 g of n-3 per day (p-value: 0.033 (Table 2.3)). No differences were observed in SA during the course of n-3 consumption.

Figure 2.1B shows the mean [μ M] \pm SEM of n-6 fatty acids: LA, GLA, homogamma linolenic acid (HLA) and AA following increasing doses of n-3 or discontinued consumption of n-3. Compared to baseline, there were significant decreases in LA at 2.4 g, and GLA at 4.8 and 7.2 g of n-3 per day (p-value: 0.007, 0.037, and 0.03, respectively (Table 2.3)). Dose-responsive reductions in AA were observed at 4.8 g and 7.2 g of n-3 per day (p-value: 0.045 and 0.005, respectively) compared to baseline (Table 2.3).

Figure 2.1C shows the mean [μM] \pm SEM of n-3 fatty acids: ALA, EPA, DPA and DHA following increasing doses of n-3 consumption and discontinued consumption of n-3. DPA was significantly increased at 7.2 g of n-3 per day (p-value: 0.042) compared to baseline (Table 2.3). There were dose-responsive increases in EPA and DHA at 2.4 g, 4.8 g and 7.2 g of n-3 per day (p-value: all <0.001) compared to baseline (Table 2.3). Interestingly, significantly higher plasma levels of DHA remained 3 months after discontinuation of n-3 consumption (p-value: <0.001) compared to baseline (Table 2.3).

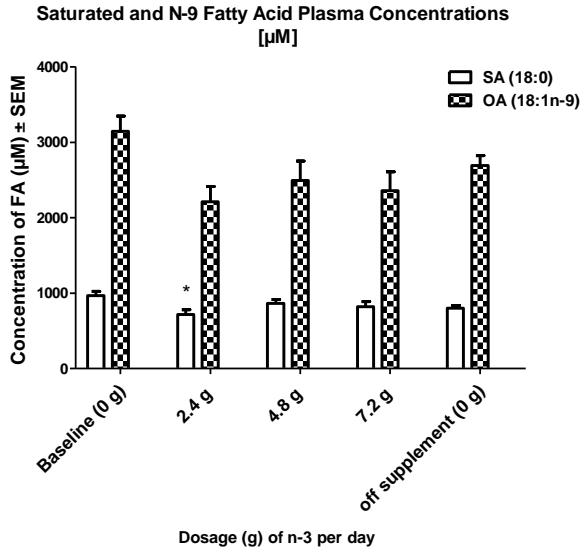
Table 2. 3. Plasma Concentrations of 10 Fatty Acids Analyzed from Subjects Before and Following N-3 Consumption.

| Fatty acid | Baseline 0 g of n-3 per day [μM] ± SEM | Following 2.4 g of n-3 per day [μM] ± SEM | P-values | Following 4.8 g of n-3 per day [μM] ± SEM] | P-values | 7.2 g of n-3 per day [μM] ± SEM | P-values | 3 Months Off supplement 0g of n-3 per day [μM] ± SEM | P- values |
|---------------------------|---|--|----------|---|----------|---------------------------------------|----------|--|--------------|
| SA (18:0) ^b | 967.6 ± 55.10 | 717.0 ± 62.14 | 0.051 | 862.3 ± 55.35 | 0.666 | 819.6 ± 68.23 | 0.462 | 798.4 ± 35.85 | 0.138 |
| OA (18:1n-9) | 3142.2 ± 203.36 | 2209.0 ± 205.72 | *0.033 | 2491.4 ± 257.87 | 0.310 | 2358.8 ± 249.98 | 0.144 | 2690.4 ± 130.98 | 0.383 |
| LA (18:2n-6) | 3411.7 ± 212.53 | 2359.3 ± 146.28 | *0.007 | 2840.8 ± 163.76 | 0.252 | 2726.6 ± 177.10 | 0.137 | 3305.1 ± 329.59 | 0.998 |
| GLA (18:3n-6) | 64.4 ± 8.09 | 40.7 ± 4.39 | 0.129 | 34.9 ± 3.67 | *0.037 | 32.8 ± 4.88 | *0.030 | 45.0 ± 4.72 | 0.292 |
| ALA (18:3n-3) | 104.0 ± 22.51 | 52.4 ± 8.86 | 0.270 | 67.9 ± 11.44 | 0.620 | 76.8 ± 8.70 | 0.791 | 75.4 ± 2.72 | 0.719 |
| HLA (18:3n-6) | 139.2 ± 13.13 | 114.2 ± 13.77 | 0.685 | 110.0 ± 9.63 | 0.408 | 95.7 ± 11.43 | 0.131 | 154.8 ± 14.77 | 0.927 |
| AA (20:4n-6) | 825.4 ± 53.24 | 649.4 ± 58.01 | 0.212 | 629.9 ± 32.37 | *0.045 | 536.8 ± 47.71 | *0.005 | 711.0 ± 56.19 | 0.600 |
| EPA (20:5n-3) | 79.1 ± 7.17 | 154.0 ± 11.04 | *<0.001 | 302.5 ± 14.95 | *<0.001 | 352.0 ± 15.98 | *<0.001 | 91.4 ± 12.55 | 0.904 |
| DPA (22:5n-3) | 65.7 ± 7.17 | 69.0 ± 5.58 | 0.996 | 90.5 ± 5.72 | 0.093 | 93.3 ± 5.17 | *0.042 | 71.4 ± 4.02 | 0.954 |
| DHA (22:6n-3) | 69.4 ± 9.41 | 138.3 ± 8.08 | *<0.001 | 205.2 ± 13.34 | *<0.001 | 202.2 ± 14.43 | *<0.001 | 149.2 ± 6.54 | *<0.001 |

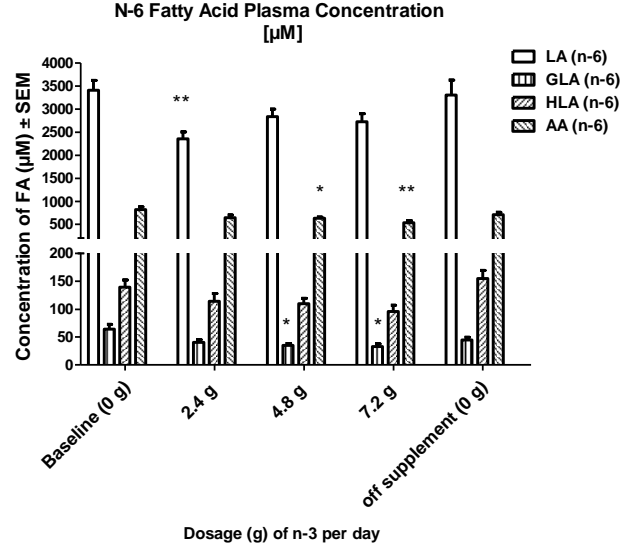
Table 2.3. Plasma Concentrations of 10 Fatty Acids Analyzed from Subjects Before and Following N-3 Consumption.

Mean fatty acid plasma concentrations [μM] ± SEM over the course of n-3 supplementation (n=6) and discontinued n-3 consumption (n=2) from subjects are shown. Omega-3 supplementation was compared against baseline. Statistical significance was determined by Games-Howell Multiple Comparison Test. Abbreviations: SA: Stearic Acid; OA: Oleic Acid; LA: Linoleic Acid; GLA: Gamma-Linolenic Acid; HLA: Homogamma-Linolenic Acid; AA: Arachidonic Acid; ALA: Alpha-Linolenic Acid; EPA: Eicosapentaenoic Acid; DPA: Docosapentaenoic Acid; DHA: Docosahexaenoic Acid. n-9: omega-9 fatty acid; n-6: omega-6 fatty acid; n-3: omega-3 fatty acid. * indicates significance, α = 0.05. ^b A:B; A denotes carbon number, B denotes number of unsaturations.

A



B



C

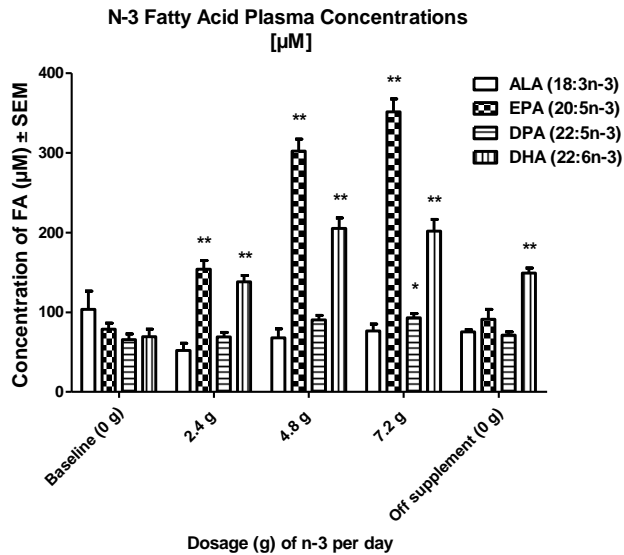


Figure 2.1. Human Plasma Concentrations of 10 Fatty Acids Analyzed Before and Following N-3 Consumption.

Mean fatty acid plasma concentrations [μM] \pm SEM from subjects over the course of n-3 supplementation (n=6) and after discontinued n-3 consumption (n=2) are shown. Figure A illustrates mean [μM] \pm SEM concentrations of SA and OA with increasing n-3 doses and discontinued n-3 consumption. Figure B illustrates mean [μM] \pm SEM concentrations of omega 6 fatty acids LA, GLA, HLA and AA with increasing n-3 doses and discontinued n-3 consumption. Figure C illustrates mean [μM] \pm SEM concentrations of n-3 fatty acids ALA, EPA, DPA and DHA with increasing n-3 doses and discontinued n-3 consumption. Statistical significance was determined by Games-Howell Multiple Comparison Test. Abbreviations: SA: Stearic Acid; OA: Oleic Acid; LA: Linoleic Acid; GLA: Gamma-Linolenic Acid; HLA: Homogamma-Linolenic Acid; AA: Arachidonic Acid; ALA: Alpha-Linolenic Acid; EPA: Eicosapentaenoic Acid; DPA: Docosapentaenoic Acid; DHA: Docosahexaenoic Acid. (A:B:n-9): A denotes carbon number, B denotes number of unsaturations; n-9: omega-9 fatty acid; n-6: omega-6 fatty acid; n-3: omega-3 fatty acid. $\alpha = 0.05$, * < 0.05, ** < 0.01

Omega-3 consumption reduced NFκB activation

Figure 2.2 shows the NFκB activity \pm SD of 15 early (Rai Stage 0-1) stage CLL patients during omega-3 intervention and the mean of 5 healthy controls. In the 7 patients with higher (greater than the median) baseline NFκB activity (patients 1-6, 13), NFκB activity was decreased by the n-3 in a dose responsive manner. Furthermore, 7.2 g of n-3/day returned NFκB activity to levels comparable to that observed in control patients (mean \pm SD: 87,138 \pm 79,040 NFκB luminescence units/ μ g protein). Lower (less than the median) initial activation of NFκB activity was observed in 6 patients (patients 7-10, 11, 15) at baseline and 7.2 g of n-3/day decreased NFκB activity to near normal levels. Patients 12 and 14 could not be categorized as either higher initial NFκB activity or lower initial NFκB activity as baseline NFκB activity was not determined. NFκB activity increased in patients 1, 2, 6 and 8 by three months after they discontinued consumption of omega-3. It is interesting that 2.4 or 4.8 g of n-3 per day, taken for long terms (patient 3, 4 and 10) decreased to or maintained NFκB activity at a near normal level.

Figure 2.3A illustrates NFκB activity (mean \pm SEM) during n-3 consumption for all 15 patients. The greatest apparent decrease in NFκB activity was following 7.2g of n-3 per day compared to baseline NFκB activity but across all patients this decrease was not quite significant (p : 0.079).

Figure 2.3B illustrates mean NFκB activity \pm SEM during the course of n-3 consumption for patients (# 1-6, 13, n=7) with higher ($>$ median) initial NFκB activity. Compared to baseline, there were statistically significant decreases in NFκB activity following consumption of 7.2g of n-3/day (p : 0.027) and at 3 months after n-3 was discontinued (p : 0.040).

Figure 2.3C illustrates mean NFκB activity \pm SEM during the course of n-3 for patients (# 7-10, 11, 15, n=5) with lower initial NFκB activity. There was no significant change in NFκB activity during consumption of n-3. This might be expected since these patients did not initially have NFκB activity significantly different from normal levels.

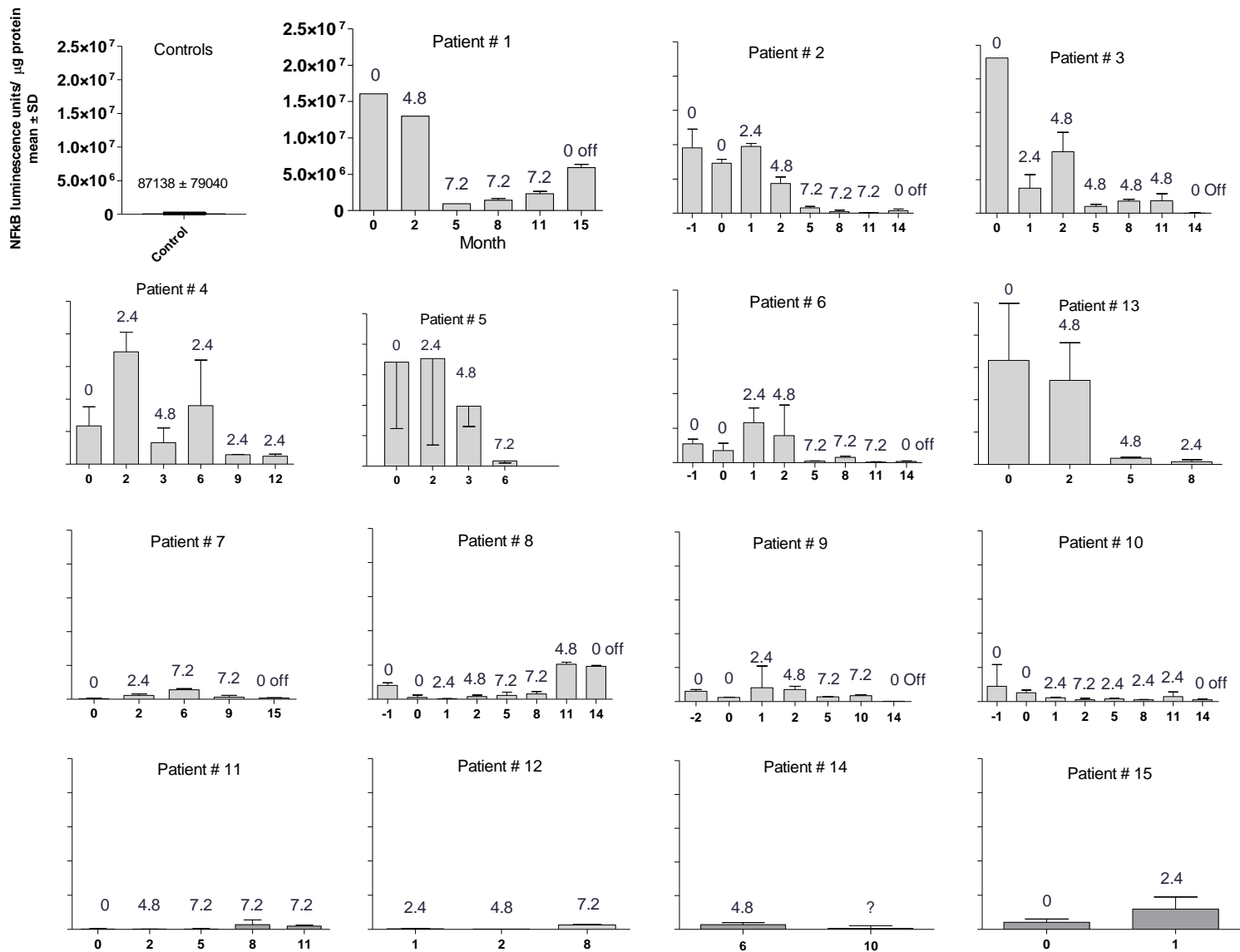


Figure 2.2. Effects of Omega-3 Consumption on NFκB Activity.

The NFκB activities of 15 patients diagnosed with CLL (Rai stage 0-1) before and after consumption of omega-3 and of 5 normal control patients (mean + SD) are shown. NFκB activity was normalized to NFκB luminescence units/μg protein. All Y-axis are the same as for the patient # 1. The X-axis shows the time each sample was collected beginning with month 0. The number above each column indicates the amount of n-3 (grams) being consumed at each time point. All assays were performed in triplicate. Error bars that are not visible are so small they are concealed by the top of the column.

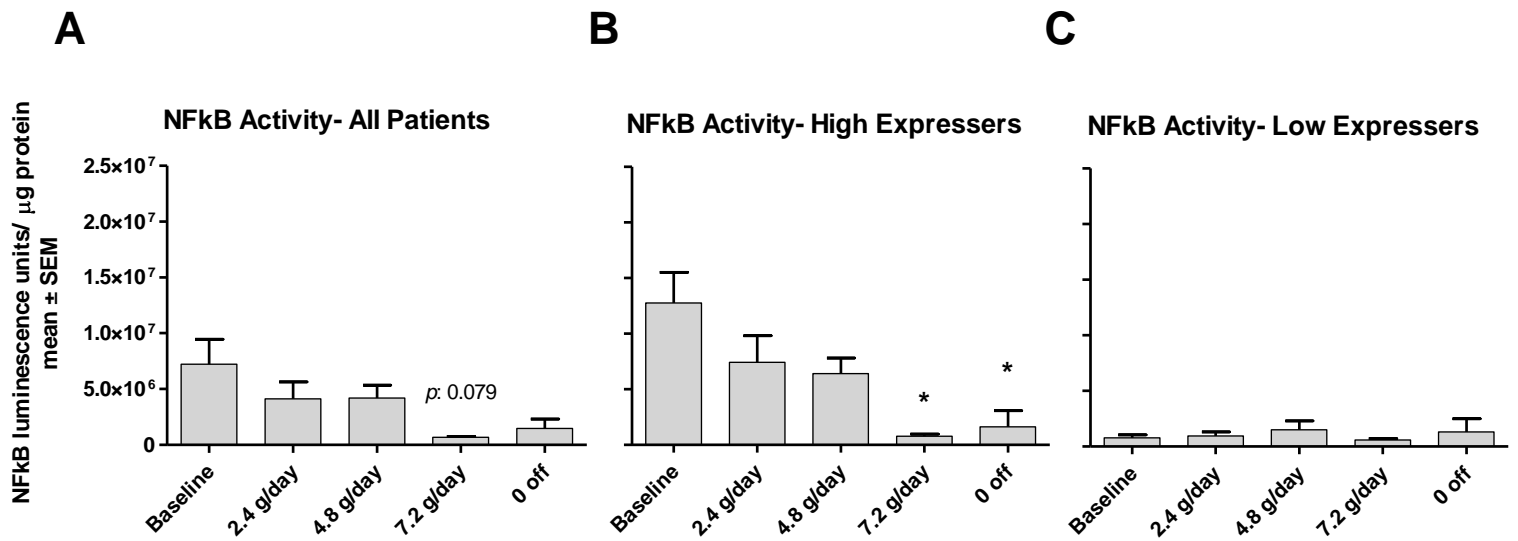


Figure 2.3. NFκB Activity.

NFκB Activity across time is shown. Games-Howell test was performed to determine statistical significance. (A) Mean ± SEM NFκB activity for all patients following n-3 consumption is shown. Across all patients, the decrease in NFκB activity following consumption of 7.2 g of n-3/day compared to baseline was not quite significant (p : 0.079). (B) Mean ± SEM NFκB activity for patients whose baseline NFκB activity was above the median ($>2.14 \times 10^6$) and called “Higher activation” is shown. NFκB activity was significantly less than baseline after 7.2 g of n-3/day and after 3 months off supplement (p : 0.027 and 0.040; respectively). (C) Mean ± SEM NFκB activity for patients whose baseline NFκB activity was below the median ($<2.14 \times 10^6$) and called “Lower activation” is shown. NFκB activity was not significantly different from the normal subjects and no significant changes were seen over the course of n-3 supplementation. All Y-axis are the same as for figure 2.3A. The X-axis shows the n-3 consumption across time.

Omega-3 consumption increases *in vitro* chemo-sensitivity of lymphocytes to doxorubicin

We evaluated the effects of omega-3 consumption on the chemo-sensitivity of malignant lymphocytes to doxorubicin, a therapeutic agent that has been used in treating CLL (Keating, 2010). Figure 2.4 shows the *in vitro* sensitivity to doxorubicin for 12 patients over the course of omega-3 supplementation. Due to inadequate sample required for chemo-sensitivity assays and limited number of data points, data from patients 11, 12 and 13 are not shown. *In vitro* results demonstrate that consumption of omega-3 fatty acids increased the sensitivity (i.e. increased cell death at lower doses of doxorubicin) of malignant lymphocytes to doxorubicin in most patients. Discontinuing omega-3 consumption was associated with decreased sensitivity to doxorubicin, as observed in patients 4, 6, 7 and 8.

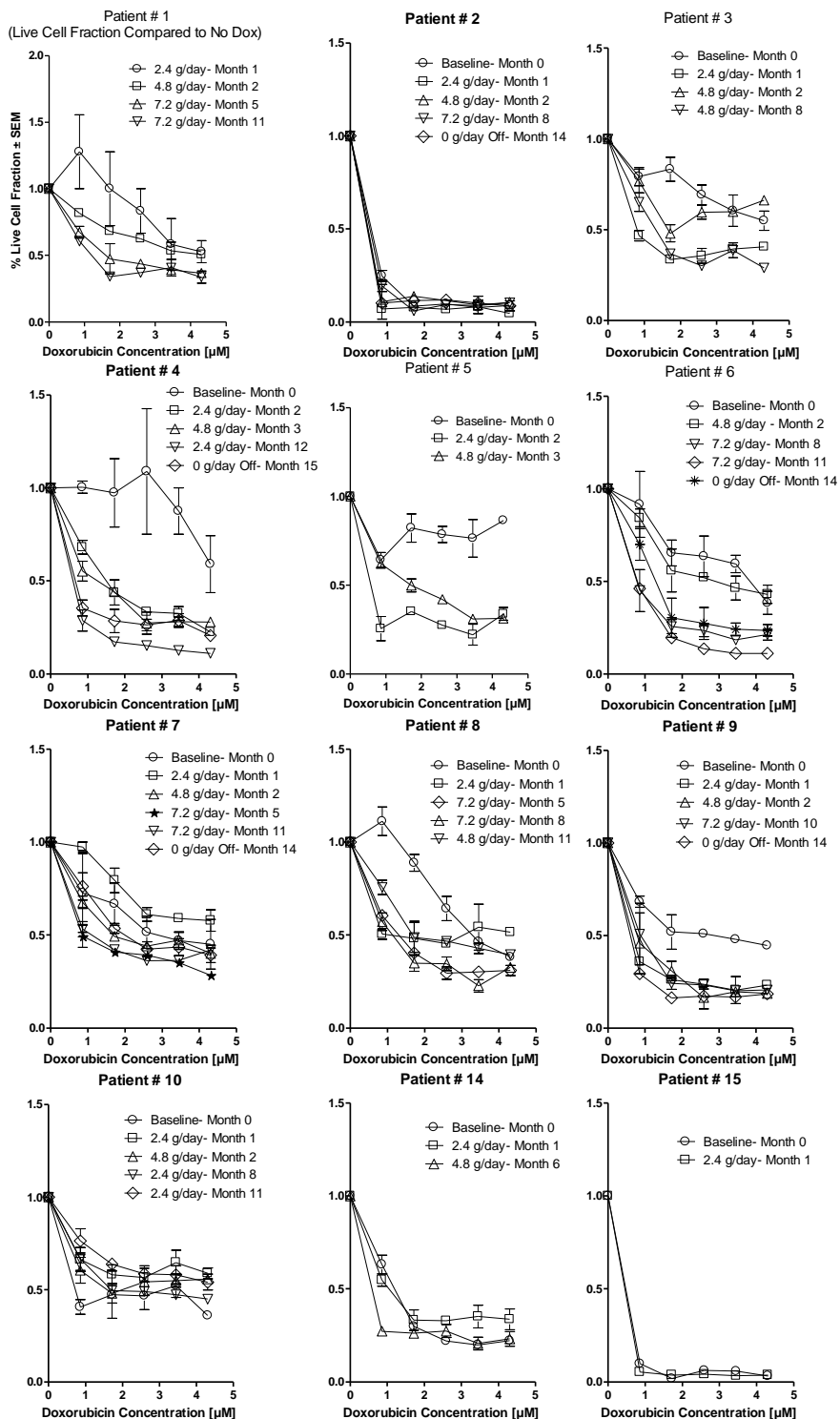


Figure 2.4. Chemo-sensitivity of Lymphocytes to Doxorubicin During Omega-3 Intervention.

The chemo-sensitivity (fraction of cells [compared to No doxorubicin] alive after each dose of doxorubicin treatment) of malignant lymphocytes to doxorubicin of 12 patients on omega-3 supplementation are shown. Consumption of omega-3 increased sensitivity of lymphocytes of most patients with higher NF κ B activity (1, 3, 4, 5) to doxorubicin *in vitro*. Discontinued omega-3 consumption decreased doxorubicin sensitivity of the lymphocytes *in vitro* as seen in patients 4, 6, 7 and 8. All Y-axis are the same as for patient # 1.

mRNA expression in lymphocytes

Since omega-3 altered activation of NFκB, sensitivity to doxorubicin and potentially slowed cancer progression, there should be changes in gene expression in the patient lymphocytes following consumption of omega-3. Expression profiling of mRNA from lymphocytes collected at baseline and following consumption of the highest level of omega-3 indicated:

1. In patients with lower initial NFκB activation, consumption of omega-3 did not significantly change NFκB activation and did not significantly change mRNA abundance of any genes (data not shown).
2. In patients with higher initial NFκB activation, consumption of omega-3 significantly decreased NFκB activation and significantly decreased mRNA abundance of 31 identified genes. Genes down-regulated by omega-3 are shown in Table 2.4. Only the sixteen genes with known function are illustrated. Three of these genes are known to be NFκB responsive genes.

Table 2. 4. Differential mRNA Expression in Lymphocytes in Patients with Higher Initial NFκB Activation

| Number | Gene | Description | Fold Change |
|--------|--|---|-------------|
| 1 | JMJD1C jumonji domain containing 1C [Homo sapiens] | Probable JmjC domain-containing histone demethylation protein 2C. Known target of NFκB. | 0.2018486 |
| 2 | RIF1 | Included in pluripotency of stem cells, response to double-strand breaks, and breast cancer development. Inhibition of RIF1 has been shown to sensitize cancer cells to camptothecin or staurosporine treatment. | 0.2184341 |
| 3 | ZNF644 | Metal ion binding, regulation of transcription. Known target of NFκB. | 0.2339526 |
| 4 | CHD9 | Chromatin remodeling transcriptional regulation of mesenchymal cells. | 0.2407181 |
| 5 | TPR | Directly binds Mad1 and Mad2. Important for the Mad1-Mad2-mediated spindle checkpoint. Translocated promotor region (to activated MET oncogene). | 0.2420173 |
| 6 | OSBPL8 (Oxysterol binding protein-like 8) | Encodes a member of the oxysterol-binding protein (OSBP) family, a group of intracellular lipid receptors. | 0.2487205 |
| 7 | KIAA1109 | Thought to function in the regulation of epithelial growth differentiation and in tumor development. | 0.2579169 |
| 8 | BRWD1 (Bromodomain and WD repeat domain containing 1) | Thought to facilitate formation of heterotrimeric or multiprotein complexes. Involved in variety of processes, including cell cycle progression, signal transduction, apoptosis and gene regulation. | 0.2635655 |
| 9 | MKL (myocardin-like protein 2) | Encodes myocardin-related transcription factor B in a megakaryoblastic leukemia gene family, shown to be involved in cancer cell invasion and motility. | 0.2762535 |
| 10 | PCM1 (Pericentriolar material 1) | Chromosomal aberrations involving this gene are associated with papillary thyroid carcinomas and a variety of hematological malignancies. | 0.2854771 |
| 11 | ITSN2 (Human Intersectin 2) | Thought to regulate formation of clathrin-coated vesicles and function in the induction of T cell receptor endocytosis. | 0.3021278 |
| 12 | ASH1L | Mammalian ASH1L is a histone methyltransferase that occupies the transcribed region of active genes. | 0.3140698 |
| 13 | PKN2 (Protein kinase N2) | Protein kinase C-related kinase-2 (PRK2) as an HDAC5-interacting protein. Evidence suggests that the unique phospho-acceptor cooperates with the 14-3-3 target sites to impair HDAC nuclear import. | 0.3235345 |
| 14 | SLC12A1 (Solute carrier family 12) | This gene encodes a kidney-specific sodium-potassium-chloride cotransporter. | 0.3310252 |
| 15 | CASC5 (Cancer susceptibility candidate 5) | Involved in chromosomal translocations that subsequently form chimeric oncoproteins BCR-ABL, TEL-ABL and MLL-AF15q14 in human leukaemia. Interact with the tumor suppressor pRb protein.Expressed in various human tumour cell lines and primary tumours. | 0.3414733 |
| 16 | PTGS2 (prostaglandin synthase 2, COX 2) | Linked to promotion of a variety of cancers via increased production of inflammatory eicosanoid prostaglandin E2. Known target of NFκB. | 0.4050415 |

Table 2.4. Differential mRNA Expression in Lymphocytes in Patients with Higher Initial NFκB Activation. Differentially regulated genes were identified by SAM analysis (false discovery rate = 10%, fold difference at least 1.5). Shown are results of repeated analyses of mRNA from 5 patients at highest omega-3 dose verses baseline (no omega 3 supplement). A fold change of less than 1 indicates that omega-3 consumption decreased expression of the gene to the indicated fraction compared to baseline expression of the gene.

DISCUSSION

Chronic lymphocytic leukemia is a disease that usually occurs in older people and in early stages, CLL does not require treatment. If the time to progression could be extended or if therapy were more effective then clinical benefit would be realized. Evidence has suggested that NFκB may play a key role in controlling apoptosis and disease progression in CLL (Braun et al., 2006; Cilloni et al., 2007; Endo et al., 2007) . NFκB is currently considered an important target for the treatment of hematological malignancies, especially CLL (Braun et al., 2006; Cilloni et al., 2007; Cilloni et al., 2006; Endo et al., 2007; Hewamana et al., 2009). In our animal studies with other types of cancers, we have shown that omega-3 suppressed the activation of NFκB (Hardman, 2002) and increased the effectiveness of several chemotherapies including doxorubicin (Hardman, Avula, Fernandes, & Cameron, 2001; Hardman, Moyer, & Cameron, 2000) . It was not known if this benefit would translate to humans. This pilot study was primarily designed to determine whether omega-3 would reduce NFκB activation in the lymphocytes of patients with early (RAI stage 0-1) CLL. Secondary objectives were to 1) evaluate the safety of consuming an omega-3 supplement, 2) determine circulating fatty acid concentrations in the plasma, 3) determine if omega-3 could increase the sensitivity of patient's lymphocytes to doxorubicin *in vitro* and 4) whether omega-3 might modify gene expression in the tumor cells (lymphocytes) of humans.

Our patient characteristics indicated a range of prognoses from good to poor based on differences in patient staging, cytogenetics, and phenotypes. For example, deletion of 13q14.3 is associated with good prognosis; trisomy 12 is associated with intermediate prognosis; CD38 positive lymphocytes, high levels of β2-microglobulin (mg/L) > 2, and/or deletion of 17p13.1 are associated with poor prognosis (Doubek et al., 2011; Mougalian & O'Brien, 2011) . Mutation

status of the IgVH chain was not evaluated as this is not performed during routine clinical practice at this institution.

Chronic lymphocytic leukemia cells have been demonstrated to have high NFκB activity. Our results appeared to indicate two populations of patients, those with higher initial NFκB activation (greater than the median) and those with lower initial NFκB activation (lower than the median). Hewamana *et al.* indicated that increased WBC counts significantly correlated with increased Rel A, a subunit of NFκB which contains the transcription activation domains, DNA binding (Hewamana *et al.*, 2008). Similarly, we found a positive correlation between WBC counts and NFκB activation (r^2 : 0.309, p : 0.0476, Appendix, Supplementary Figure 2.2). Data on absolute lymphocyte counts indicated that the ‘lower initial activation’ patients had significantly lower absolute lymphocyte counts than patients exhibiting ‘higher initial activation’ of NFκB at baseline. Patient staging indicated that 3 of 7 patients with higher initial NFκB activity had a Rai Stage of 1 (remainder had Rai Stage 0), whereas only 1 of 6 patients with lower initial NFκB activity had a Rai Stage of 1 (remainder had Rai Stage 0). The trend in patient staging, correlation between WBC count and NFκB activation, statistical differences observed at baseline for absolute lymphocyte counts, and differences in initial NFκB activity suggest that NFκB activation may be related to progression of the disease. Further studies will be needed to determine if NFκB activation correlates with progression of the disease and if suppressing NFκB activation will slow further progression.

Our results indicate that consumption of an n-3 supplement, predominantly composed of EPA and DHA, increased the amount of n-3 in the plasma of subjects. Although the subjects had early stage CLL (Rai Stages 0-1), we could not determine a reason, *a priori*, that would indicate that the fatty acid concentrations in their plasma should be different from that of persons without

CLL. Interestingly, despite plasma concentrations of EPA and DHA being highest following consumption of 7.2 g of n-3/per day for a month or more, these concentrations did not differ statistically from that following consumption of 4.8 g of n-3/day (Mean [μM] \pm SEM: 352.0 ± 15.98 vs 302.5 ± 14.95 ; p: 0.192 and 202.2 ± 14.43 vs 205.2 ± 13.34 ; p: 1.00, respectively (Table 2.3)). This suggests that plasma concentrations of EPA and DHA reached a steady state and that a steady state plasma concentration of EPA or DHA can be achieved at a lower dose that is more easily consumed. This is an important consideration for future clinical trials. A lower dose of supplement would increase compliance rates and decrease side effects associated with n-3 consumption.

Discontinuation of n-3 consumption resulted in a reduction of EPA toward values observed at baseline. Although DHA displayed a similar tendency for reduction toward baseline values, levels of DHA 3 months after discontinuation of n-3 consumption remained significantly higher than baseline values (Mean [μM] \pm SEM: 149.2 ± 6.54 vs 69.4 ± 9.41 ; p: <0.001 (Table 2.3)). This indicates that the retention time of DHA in the plasma of patients consuming n-3 is greater than EPA. It is important to note that plasma levels of AA decreased following n-3 consumption and subsequently increased toward values observed at baseline after discontinuation of n-3. It is not known if this is due to changes in the diet or if the increase in AA is as a result of reduced bioavailability of n-3.

These data demonstrate that in patients with early stage CLL, consumption of an omega-3 fatty acid supplement had minimal side effects and strongly suppressed highly activated NF κ B. A dose of 7.2 g of n-3/day suppressed NF κ B activity to nearly normal levels in all patients. This biomarker would be expected to correlate with clinical benefit, perhaps extending the time to requiring therapy. A longer randomized trial will be needed to determine if our intervention

slows disease progression. In patients unable or unwilling to take 7.2 g of n-3 per day, over time either 4.8 g or 2.4 g of n-3 per day also suppressed NFκB to nearly normal levels. This suggests that a maximum possible dose may not be required to achieve benefit from omega-3 but that a lower dose, with time, could provide benefit. This idea is further supported by the fact that plasma levels of n-3 after 7.2 g of n-3 per day did not differ statistically from 4.8 g of n-3 per day (Table 2.3).

These data also demonstrate that patients with lower initial NFκB activity did not have NFκB activity decreased below baseline levels while being supplemented with omega-3. A normal level of NFκB activation is needed for normal immune response and cellular responses to other stress.

Based on our data, it can be concluded that consumption of omega-3 fatty acids decreased the activity of NFκB, a key target for slowing cancer progression, and would potentially slow the progression of the disease. A larger randomized clinical trial will be needed to determine if consumption of omega-3 fatty acids can slow progression of the disease.

Omega-3 fatty acids have consistently been shown to enhance the sensitivity of various cancers to doxorubicin *in vitro* and *in vivo* (Bougnoux et al., 1999; Hardman, 2002; Hardman et al., 2000). Doxorubicin is a previously used treatment option for CLL (Keating, 2010) and is an active drug in this disease as part of the CHOP (cyclophosphamide, doxorubicin, oncovin and prednisone) regimen (Leporrier et al., 2001). Although doxorubicin has been replaced as the standard by other drugs such as bendamustine and fludarabine, we wanted to test the chemosensitivity of isolated lymphocytes to doxorubicin following inhibition of NFκB activation as this system was already in place. Omega-3 consumption was shown to increase sensitivity of malignant lymphocytes to doxorubicin while discontinued consumption of omega-3 resulted in

decreased sensitivity to doxorubicin. Our data illustrates that increased sensitivity of malignant lymphocytes to doxorubicin following n-3 consumption was most profound in patients with higher initial NFκB activity. Little effect on chemo-sensitivity following n-3 consumption was observed in patients with lower initial NFκB activity. Increased sensitivity of malignant lymphocytes from patients with higher initial NFκB activity to doxorubicin suggests that high NFκB activity is associated with chemoresistance. Numerous preclinical studies have shown that increased NFκB activity increases chemoresistance (Li & Sethi, 2010). We are currently preparing to conduct an additional clinical study aimed to determine if n-3 can increase the sensitivity of lymphocytes from patients with CLL to anti-cancer drugs fludarabine, chlorambucil or vincristine *in vitro* and whether enhanced sensitivity is through the inhibition of NFκB activation. Increasing the efficacy of the chemotherapeutic agent would be clinically beneficial if patients progress to requiring therapy.

In patients with higher initial NFκB activation, consumption of the omega-3 supplement significantly suppressed expression of 31 identified genes. In this small population, no genes had increased expression due to omega 3 consumption. The list of altered genes that have a known function is included in Table 2.4. Of these 31 genes, JMJD1C, ZNF644 and PTGS2 are known to be regulated by NFκB. Functions of some of these genes of particular interest are discussed below. (Numbers in brackets refer to number in first column of Table 2.4.)

Several chromatin conformation related proteins were identified including: JMJD1C [1] which contains histone demethylation protein 2C (Katoh & Katoh, 2007). CHD9 [4] is a chromatin remodeling protein (Marom, Shur, Hager, & Benayahu, 2006; Shur, Socher, & Benayahu, 2006). ASH1L [12] is a histone methyl transferase (An, Yeo, Jeon, & Song, 2011) and PKN2 [13] impairs histone deacetylase import (Harrison et al., 2010). Chromatin remodeling

is being recognized as an important mechanism by which dietary components may alter gene expression and influence cancer growth or progression.

Several down-regulated genes were identified as important in cancer development. RIF1 [2] is an embryonic stem cell marker. Inhibition of RIF1 has been shown to sensitize cancer cells to some chemotherapies (Park et al., 2010). KIAA1109 [7] is thought to function in epithelial growth and differentiation and in tumor development (Zhernakova et al., 2007). BRWD1 [8] is an Oct4 (stem cell marker) interacting protein, its family members are involved in cell cycle progression, signal transduction, apoptosis and gene regulation (Pardo et al., 2010). Chromosomal aberrations involving PCM1 [10] are associated with thyroid carcinomas and a variety of hematological malignancies (Hoeller, Walz, Reiter, Dirnhofer, & Tzankov, 2011). CASC5 (15q14) is involved in chromosomal translocations that form oncoproteins in human leukemias (Kuefer et al., 2003) and in increased cell proliferation of some cancers (Bogdanov & Takimoto, 2008; Takimoto et al., 2002).

PTGS2 (prostaglandin synthase 2, COX-2 [16]), a known target of NF κ B, has been linked to promotion of a variety of cancers possibly via increasing the production of inflammation producing prostaglandin E2 (Hardman, 2002; Prueitt et al., 2007). Suppression of COX-2 activity has been proposed as a mechanism to reduce cancer growth and resistance to chemotherapy.

In conclusion, omega-3 consumption increased circulating plasma levels of n-3 while decreasing n-6, suppressed NF κ B activation in patients with early stage (Rai Stage 0-1) CLL to near normal levels and increased chemo-sensitivity of malignant lymphocytes to doxorubicin *in vitro*. Suppression of NF κ B activity has been proposed as a drug development target to slow progression of cancers. The mRNA expression of multiple genes related to cancer promotion or

cancer aggressiveness were reduced in malignant lymphocytes following consumption of omega-3. Much work should be done to validate that reduced mRNA correlates with decreased expression of the protein and the clinical relevance. Since omega-3 was safe to consume and did not have detrimental side effects, a definitive randomized clinical trial should be initiated to determine whether there is clinical benefit for consumption of omega-3 to slowing progression of chronic lymphocytic leukemia. In agreement with animal studies, *in vitro* doxorubicin sensitivity assays suggest that when used in combination with chemotherapeutics, omega-3 fatty acids may increase the efficacy of some chemotherapeutic drugs for patients who require therapy. Current studies are underway to determine if omega-3 can enhance the sensitivity of lymphocytes from patients with CLL to anti-cancer drugs fludarabine, chlorambucil or vincristine. Plasma concentrations of fatty acids analyzed following consumption of n-3 and discontinued consumption of n-3 provides insight into doses of n-3 which can be used in future clinical trials.

DECLARATION OF INTEREST

The authors declare no competing financial interests. The authors alone are responsible for the content and writing of the paper.

CONTRIBUTIONS

J.F. Fahrman wrote the manuscript, performed the lymphocyte isolation, NFκB Activity assay, *in vitro* doxorubicin sensitivity assay, and GC analysis on plasma samples and all analyzed data. O.F Ballester designed research, interpreted data, and recruited patients into study. G. Ballester recruited patients into study and organized platelet function data. T.R. Witte performed *in vitro* doxorubicin sensitivity assay, lymphocyte isolation and provided key insight into carbon response and FID response corrections. A.J. Salazar performed *in vitro* doxorubicin sensitivity assay and lymphocyte isolation. G. Ion performed RNA extraction, and provided

critical reagents and internal standards. B. Kordusky performed GC analysis of plasma samples and analyzed results. K. Cowen performed GC analysis of plasma samples and analyzed results. D.A. Primerano and G. Boskovic designed and performed expression profiling experiments. J. Denvir performed statistical analysis on microarray data. W.E Hardman designed research, received grant funding, interpreted data, critically analyzed the manuscript and performed final approval of manuscript.

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REFERENCES

- An, S., Yeo, K. J., Jeon, Y. H., & Song, J. J. (2011). Crystal structure of the human histone methyltransferase ASH1L catalytic domain and its implications for the regulatory mechanism. *J Biol Chem*, 286(10), 8369-8374. doi: 10.1074/jbc.M110.203380
- Balkwill, F., & Coussens, L. M. (2004). Cancer: an inflammatory link. *Nature*, 431(7007), 405-406. doi: 10.1038/431405a
- Bogdanov, K. V., & Takimoto, M. (2008). [The involvement of c-Abl and D40 (AF15q14/CASC5) proteins in the regulation of cell proliferation and cancer]. *Tsitologiya*, 50(7), 590-596.
- Bougnoux, P., Germain, E., Chajes, V., Hubert, B., Lhuillery, C., Le Floch, O., . . . Calais, G. (1999). Cytotoxic drugs efficacy correlates with adipose tissue docosahexaenoic acid level in locally advanced breast carcinoma. *Br J Cancer*, 79(11-12), 1765-1769. doi: 10.1038/sj.bjc.6690281
- Braun, T., Carvalho, G., Fabre, C., Grosjean, J., Fenaux, P., & Kroemer, G. (2006). Targeting NF-kappaB in hematologic malignancies. *Cell Death Differ*, 13(5), 748-758. doi: 10.1038/sj.cdd.4401874
- Burns, C. P., Halabi, S., Clamon, G. H., Hars, V., Wagner, B. A., Hohl, R. J., . . . Paskett, E. (1999). Phase I clinical study of fish oil fatty acid capsules for patients with cancer cachexia: cancer and leukemia group B study 9473. *Clin Cancer Res*, 5(12), 3942-3947.
- Cilloni, D., Martinelli, G., Messa, F., Baccarani, M., & Saglio, G. (2007). Nuclear factor kB as a target for new drug development in myeloid malignancies. *Haematologica*, 92(9), 1224-1229.
- Cilloni, D., Messa, F., Arruga, F., Defilippi, I., Morotti, A., Messa, E., . . . Saglio, G. (2006). The NF-kappaB pathway blockade by the IKK inhibitor PS1145 can overcome imatinib resistance. *Leukemia*, 20(1), 61-67. doi: 10.1038/sj.leu.2403998
- Clevers, H. (2004). At the crossroads of inflammation and cancer. *Cell*, 118(6), 671-674. doi: 10.1016/j.cell.2004.09.005
- Darnell, J. E., Jr. (2002). Transcription factors as targets for cancer therapy. *Nat Rev Cancer*, 2(10), 740-749. doi: 10.1038/nrc906
- Ditsworth, D., & Zong, W. X. (2004). NF-kappaB: key mediator of inflammation-associated cancer. *Cancer Biol Ther*, 3(12), 1214-1216.
- Doubek, M., Mayer, J., Obrtlíkova, P., Smolej, L., Cmunt, E., Schwarz, J., . . . Trnėny, M. (2011). Modern and conventional prognostic markers of chronic lymphocytic leukaemia

- in the everyday haematological practice. *Eur J Haematol*, 87(2), 130-137. doi: 10.1111/j.1600-0609.2011.01639.x
- Endo, T., Nishio, M., Enzler, T., Cottam, H. B., Fukuda, T., James, D. F., . . . Kipps, T. J. (2007). BAFF and APRIL support chronic lymphocytic leukemia B-cell survival through activation of the canonical NF-kappaB pathway. *Blood*, 109(2), 703-710. doi: 10.1182/blood-2006-06-027755
- Hardman, W. E. (2002). Omega-3 fatty acids to augment cancer therapy. *J Nutr*, 132(11 Suppl), 3508S-3512S.
- Hardman, W. E., Avula, C. P., Fernandes, G., & Cameron, I. L. (2001). Three percent dietary fish oil concentrate increased efficacy of doxorubicin against MDA-MB 231 breast cancer xenografts. *Clin Cancer Res*, 7(7), 2041-2049.
- Hardman, W. E., Moyer, M. P., & Cameron, I. L. (2000). Dietary fish oil sensitizes A549 lung xenografts to doxorubicin chemotherapy. *Cancer Lett*, 151(2), 145-151.
- Harper, C. R., Edwards, M. J., DeFilippis, A. P., & Jacobson, T. A. (2006). Flaxseed oil increases the plasma concentrations of cardioprotective (n-3) fatty acids in humans. *J Nutr*, 136(1), 83-87.
- Harrison, B. C., Huynh, K., Lundgaard, G. L., Helmke, S. M., Perryman, M. B., & McKinsey, T. A. (2010). Protein kinase C-related kinase targets nuclear localization signals in a subset of class IIa histone deacetylases. *FEBS Lett*, 584(6), 1103-1110. doi: 10.1016/j.febslet.2010.02.057
- Hewamana, S., Alghazal, S., Lin, T. T., Clement, M., Jenkins, C., Guzman, M. L., . . . Pepper, C. (2008). The NF-kappaB subunit Rel A is associated with in vitro survival and clinical disease progression in chronic lymphocytic leukemia and represents a promising therapeutic target. *Blood*, 111(9), 4681-4689. doi: 10.1182/blood-2007-11-125278
- Hewamana, S., Lin, T. T., Rowntree, C., Karunanithi, K., Pratt, G., Hills, R., . . . Pepper, C. (2009). Rel a is an independent biomarker of clinical outcome in chronic lymphocytic leukemia. *J Clin Oncol*, 27(5), 763-769. doi: 10.1200/jco.2008.19.1114
- Hoeller, S., Walz, C., Reiter, A., Dirnhofer, S., & Tzankov, A. (2011). PCM1-JAK2-fusion: a potential treatment target in myelodysplastic-myeloproliferative and other hemato-lymphoid neoplasms. *Expert Opin Ther Targets*, 15(1), 53-62. doi: 10.1517/14728222.2011.538683
- Huang Yieru, Ou Qingyu, Yu Weile. (1990). Characteristics of Flame Ionization Detection for the Quantitative Analysis of Complex Organic Mixtures. *Analytical Chemistry*, 62, 2063-2064.

- Hyde, C. A., & Missailidis, S. (2009). Inhibition of arachidonic acid metabolism and its implication on cell proliferation and tumour-angiogenesis. *Int Immunopharmacol*, 9(6), 701-715. doi: 10.1016/j.intimp.2009.02.003
- Katoh, M., & Katoh, M. (2007). Comparative integromics on JMJD1C gene encoding histone demethylase: conserved POU5F1 binding site elucidating mechanism of JMJD1C expression in undifferentiated ES cells and diffuse-type gastric cancer. *Int J Oncol*, 31(1), 219-223.
- Keating, G. M. (2010). Rituximab: a review of its use in chronic lymphocytic leukaemia, low-grade or follicular lymphoma and diffuse large B-cell lymphoma. *Drugs*, 70(11), 1445-1476. doi: 10.2165/11201110-000000000-00000
- Kuefer, M. U., Chinwalla, V., Zeleznik-Le, N. J., Behm, F. G., Naeve, C. W., Rakestraw, K. M., . . . Morris, S. W. (2003). Characterization of the MLL partner gene AF15q14 involved in t(11;15)(q23;q14). *Oncogene*, 22(9), 1418-1424. doi: 10.1038/sj.onc.1206272
- Leporrier, M., Chevret, S., Cazin, B., Boudjerra, N., Feugier, P., Desablens, B., . . . Chastang, C. (2001). Randomized comparison of fludarabine, CAP, and ChOP in 938 previously untreated stage B and C chronic lymphocytic leukemia patients. *Blood*, 98(8), 2319-2325.
- Li, F., & Sethi, G. (2010). Targeting transcription factor NF-kappaB to overcome chemoresistance and radioresistance in cancer therapy. *Biochim Biophys Acta*, 1805(2), 167-180. doi: 10.1016/j.bbcan.2010.01.002
- Marom, R., Shur, I., Hager, G. L., & Benayahu, D. (2006). Expression and regulation of CReMM, a chromodomain helicase-DNA-binding (CHD), in marrow stroma derived osteoprogenitors. *J Cell Physiol*, 207(3), 628-635. doi: 10.1002/jcp.20611
- Mougalian, S. S., & O'Brien, S. (2011). Adverse prognostic features in chronic lymphocytic leukemia. *Oncology (Williston Park)*, 25(8), 692-696, 699.
- Munzert, G., Kirchner, D., Stobbe, H., Bergmann, L., Schmid, R. M., Dohner, H., & Heimpel, H. (2002). Tumor necrosis factor receptor-associated factor 1 gene overexpression in B-cell chronic lymphocytic leukemia: analysis of NF-kappa B/Rel-regulated inhibitors of apoptosis. *Blood*, 100(10), 3749-3756. doi: 10.1182/blood.V100.10.3749
- Narayanan, N. K., Narayanan, B. A., & Reddy, B. S. (2005). A combination of docosahexaenoic acid and celecoxib prevents prostate cancer cell growth in vitro and is associated with modulation of nuclear factor-kappaB, and steroid hormone receptors. *Int J Oncol*, 26(3), 785-792.
- Pardo, M., Lang, B., Yu, L., Prosser, H., Bradley, A., Babu, M. M., & Choudhary, J. (2010). An expanded Oct4 interaction network: implications for stem cell biology, development, and disease. *Cell Stem Cell*, 6(4), 382-395. doi: 10.1016/j.stem.2010.03.004

- Park, J. T., Chen, X., Trope, C. G., Davidson, B., Shih Ie, M., & Wang, T. L. (2010). Notch3 overexpression is related to the recurrence of ovarian cancer and confers resistance to carboplatin. *Am J Pathol*, *177*(3), 1087-1094. doi: 10.2353/ajpath.2010.100316
- Prueitt, R. L., Boersma, B. J., Howe, T. M., Goodman, J. E., Thomas, D. D., Ying, L., . . . Ambs, S. (2007). Inflammation and IGF-I activate the Akt pathway in breast cancer. *Int J Cancer*, *120*(4), 796-805. doi: 10.1002/ijc.22336
- Robak, T. (2007). Recent progress in the management of chronic lymphocytic leukemia. *Cancer Treat Rev*, *33*(8), 710-728. doi: 10.1016/j.ctrv.2007.08.003
- Ross, J. A., Maingay, J. P., Fearon, K. C., Sangster, K., & Powell, J. J. (2003). Eicosapentaenoic acid perturbs signalling via the NFkappaB transcriptional pathway in pancreatic tumour cells. *Int J Oncol*, *23*(6), 1733-1738.
- Shanafelt, T. D., & Call, T. G. (2004). Current approach to diagnosis and management of chronic lymphocytic leukemia. *Mayo Clin Proc*, *79*(3), 388-398. doi: 10.4065/79.3.388
- Shantha, N. C., & Ackman, R. G. (1990). Nervonic acid versus tricosanoic acid as internal standards in quantitative gas chromatographic analyses of fish oil longer-chain n-3 polyunsaturated fatty acid methyl esters. *J Chromatogr*, *533*, 1-10.
- Shur, I., Socher, R., & Benayahu, D. (2006). In vivo association of CREMM/CHD9 with promoters in osteogenic cells. *J Cell Physiol*, *207*(2), 374-378. doi: 10.1002/jcp.20586
- Silber, R., Degar, B., Costin, D., Newcomb, E. W., Mani, M., Rosenberg, C. R., . . . Potmesil, M. (1994). Chemosensitivity of lymphocytes from patients with B-cell chronic lymphocytic leukemia to chlorambucil, fludarabine, and camptothecin analogs. *Blood*, *84*(10), 3440-3446.
- Simopoulos, A. P. (1991). Omega-3 fatty acids in health and disease and in growth and development. *Am J Clin Nutr*, *54*(3), 438-463.
- Simopoulos, A. P. (2000). Human requirement for N-3 polyunsaturated fatty acids. *Poult Sci*, *79*(7), 961-970.
- Simopoulos, A. P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother*, *56*(8), 365-379.
- Syed, H. A., & Threadgill, D. W. (2006). Enhanced oligonucleotide microarray labeling and hybridization. *Biotechniques*, *41*(6), 685-686.
- Takimoto, M., Wei, G., Dosaka-Akita, H., Mao, P., Kondo, S., Sakuragi, N., . . . Kuzumaki, N. (2002). Frequent expression of new cancer/testis gene D40/AF15q14 in lung cancers of smokers. *Br J Cancer*, *86*(11), 1757-1762. doi: 10.1038/sj.bjc.6600328

Tusher, V. G., Tibshirani, R., & Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A*, 98(9), 5116-5121. doi: 10.1073/pnas.091062498

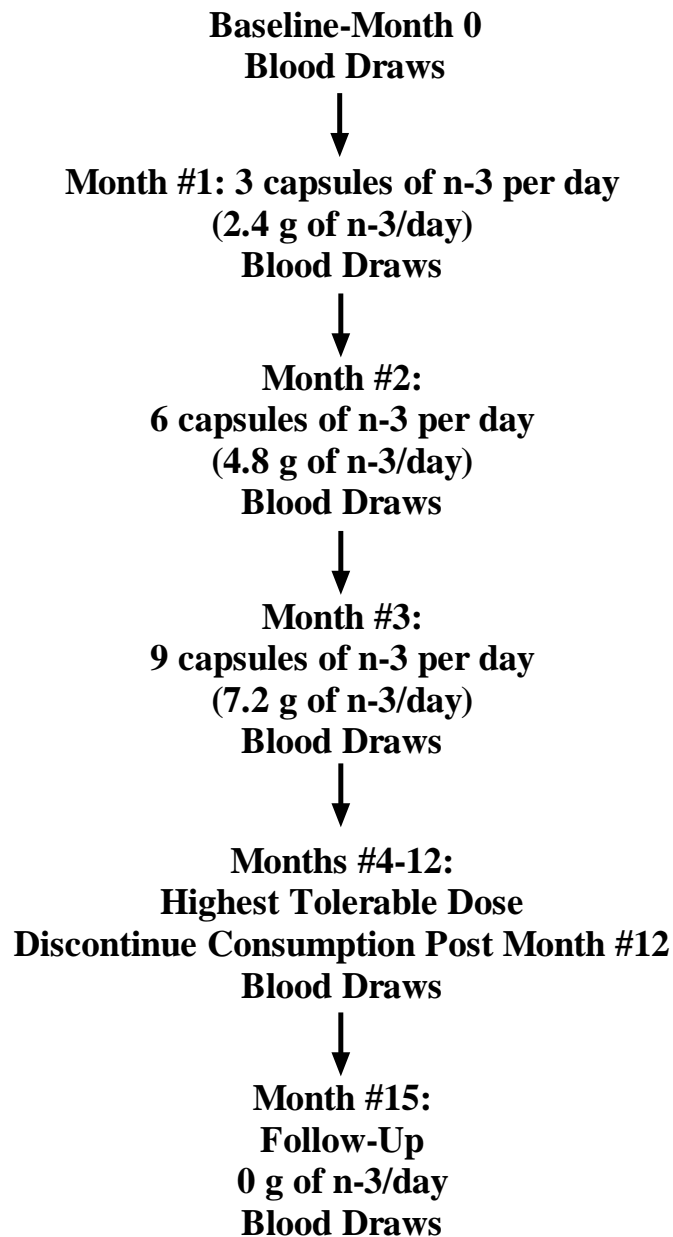
William, W.Christie. (1989). Gas Chromatographic Analysis of Fatty Acid Derivatives *Gas Chromatography and Lipids*: P.J. Barnes & Associates (The Oily Press Ltd).

Witte, T. R., Salazar, A. J., Ballester, O. F., & Hardman, W. E. (2010). RBC and WBC fatty acid composition following consumption of an omega 3 supplement: lessons for future clinical trials. *Lipids Health Dis*, 9, 31. doi: 10.1186/1476-511X-9-31

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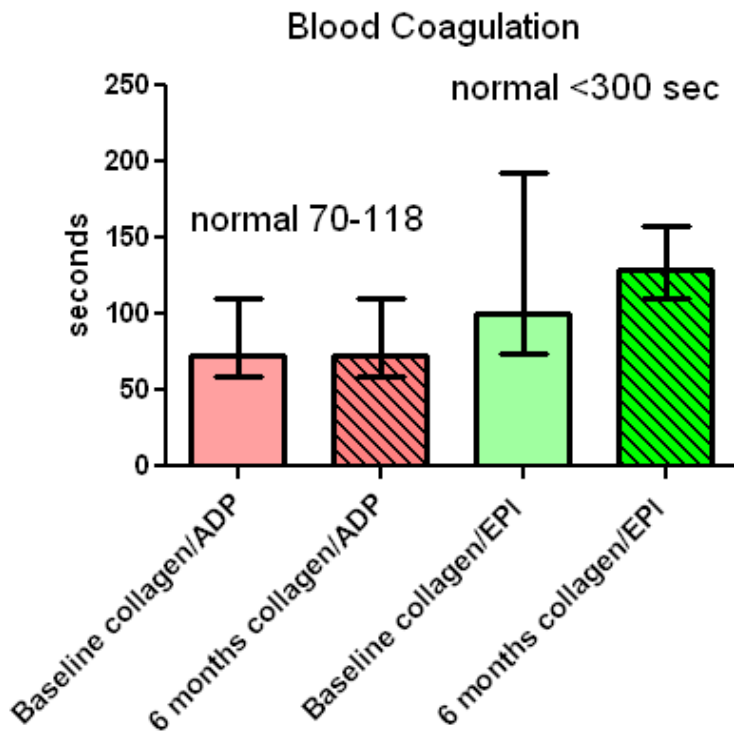
Zhernakova, A., Alizadeh, B. Z., Bevova, M., van Leeuwen, M. A., Coenen, M. J., Franke, B., . . . Wijnenga, C. (2007). Novel association in chromosome 4q27 region with rheumatoid arthritis and confirmation of type 1 diabetes point to a general risk locus for autoimmune diseases. *Am J Hum Genet*, 81(6), 1284-1288. doi: 10.1086/522037

APPENDIX



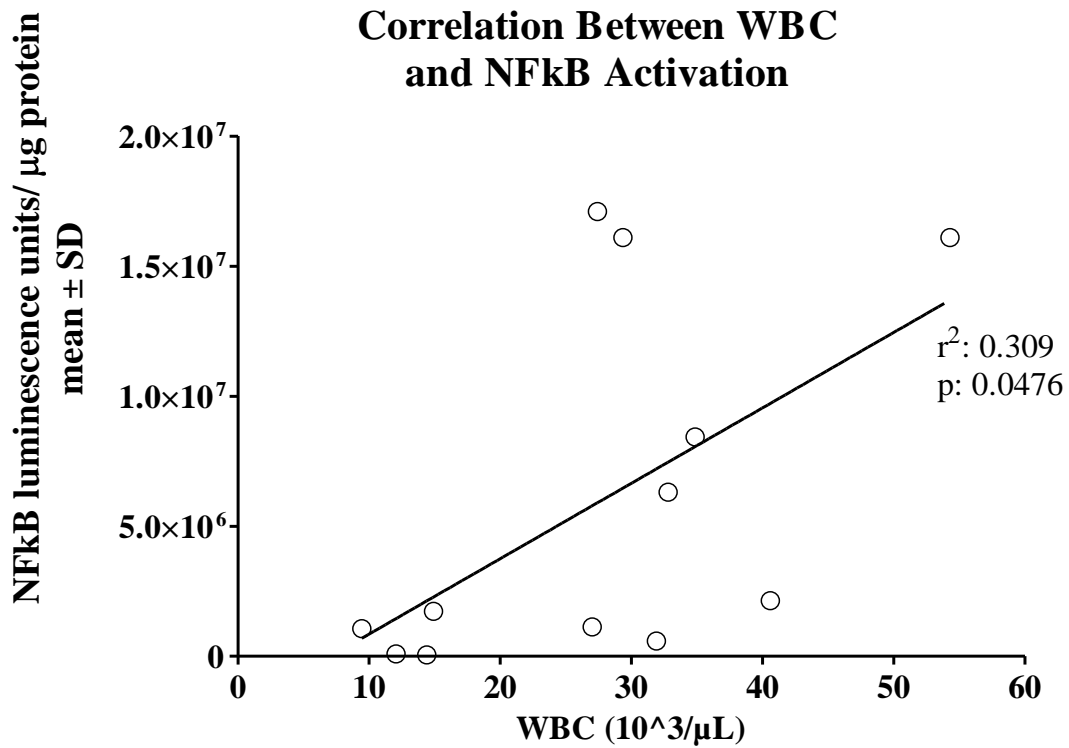
Supplementary Figure 2. 1. Omega-3 Dosing Schedule.

Each capsule of omega-3 contained 400mg EPA, 300mg DHA and 100-150mg other omega-3. Thus, consumption of 3, 6, or 9 capsules/day yielded 2.4 g, 4.8 g, or 7.2 g/day of n-3, respectively



Supplementary Figure 2. 2. Effects of Omega-3 on Blood Coagulation.

Blood coagulation times for collagen/ADP and collagen/EPI (epinephrine) on omega-3 intervention are shown. No difference was observed in collagen/ADP, a measure of platelet function, coagulation when omega-3 was introduced. A slight increase was observed in collagen/EPI, a measure of clotting time, when omega-3 was introduced but this increase was not statistically significant and was within normal parameters.



Supplementary Figure 2. 3. Correlation Between WBC Counts and NFκB Activation.

Correlation between WBC counts and NFκB activation is shown. A significant positive correlation was found between WBC counts and NFκB activation. Correlation coefficients were calculated by least squares linear regression.

CHAPTER 3: OMEGA-3 FATTY ACIDS INCREASE THE CHEMO-SENSITIVITY OF B-CELL-DERIVED CELL LINES EHEB AND MEC-2 AND B-PLL-DERIVED CELL LINE JVM-2 TO ANTI-CANCER DRUGS DOXORUBICIN, VINCRISTINE AND FLUDARABINE

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ABSTRACT

B-Cell chronic lymphocytic leukemia (CLL) is the most common form of leukemia in the United States. Clinical treatment of CLL is often limited due to drug resistance and severe therapy-induced toxicities. We hypothesized that the omega-3 (n-3) fatty acids, eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA), would increase the sensitivity of malignant B-lymphocytes to anti-cancer drugs doxorubicin, vincristine and/or fludarabine *in vitro* and that increased sensitivity is achieved by alterations in cell-cycle progression leading to growth inhibition and/or enhanced cell death. We further postulate that enhanced sensitivity is dependent on the formation of lipid peroxides and to the generation of reactive oxygen species (ROS).

In the present study, B-CLL-derived leukemic cell lines EHEB and MEC-2 and the B-Prolymphocytic leukemic-derived (PLL) cell line JVM-2 were tested for *in vitro* sensitivity against doxorubicin, vincristine or fludarabine in the presence or absence of vehicle, arachidonic acid (omega 6), EPA or DHA. Cell cycle analysis and Annexin-V assays were performed to determine cell cycle progression and % apoptotic cells, respectively. Assays for malondialdehyde, a measure of lipid peroxidation, and DCF fluorescence assays, a measure of intracellular ROS, were performed to determine if enhanced sensitivity of cells to the drugs by n-3 was dependent on the formation of ROS.

Our results indicated that: 1) EPA and DHA differentially sensitized B-leukemic cell lines EHEB, JVM-2 and MEC-2 to doxorubicin, vincristine and fludarabine *in vitro*; 2) n-3 alone and with drug treatment increased cell death and induced G2/M arrest in a cell-type specific manner; 3) lipid peroxidation increased in the presence of n-3; 4) there was higher lipid peroxidation in MEC-2 cells in presence of DHA and doxorubicin than with either alone; 5) n-3

increased generation of ROS in MEC-2, and 6) the addition of vitamin-E abrogated the increase in ROS generation and chemo-sensitivity of MEC-2 to doxorubicin by DHA.

N-3's are promising chemo-sensitizing agents for the treatment of CLL. Selective enhancement of chemo-sensitivity of EHEB, JVM-2 and MEC-2 to drugs by n-3 that is not dependent on increased lipid peroxidation and ROS generation indicates alternative mechanisms by which n-3 enhances chemo-sensitivity.

INTRODUCTION

B-Cell chronic lymphocytic leukemia (CLL) is the most common form of leukemia in the United States (Riches, Ramsay, & Gribben, 2011). CLL is a disease of the elderly with two thirds of patients being over 65 years of age at time of diagnosis (Riches et al., 2011). CLL remains largely incurable outside of allogeneic transplantation (Riches et al., 2011). Despite the success of current treatments such as fludarabine, many patients develop drug resistance and disease relapse (Silber et al., 1994). As such, clinical treatment of CLL is often hindered by drug resistance and the non-selectivity of most drugs (Schriever & Huhn, 2003). Additionally, treatment options for CLL patients who require aggressive treatment are limited due to significant side-effect profiles which are often too toxic for the elderly or those with comorbidities (Riches et al., 2011). Given the age group of patients diagnosed with CLL, a therapeutic intervention that can increase the sensitivity of CLL cells to chemotherapy without causing additional adverse effects would be clinically beneficial.

Omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) are essential fatty acids (FAs) which must be obtained from diet. Long chain omega-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) are primarily found in fish oils (Simopoulos, 1991). The omega-6 fatty acid, arachidonic acid (AA), is primarily found in the meat of animals that consumed corn or soybeans. The ratio of omega-3 FAs to omega-6 FAs in the average western diet is heavily weighted in favor of omega-6 (Simopoulos, 2000, 2003). Omega-3 fatty acids have consistently been shown to enhance sensitivity of various solid tumor cells to chemotherapy *in vitro* (Germain, Chajes, Cognault, Lhuillery, & Bougnoux, 1998; Kinsella & Black, 1993) and *in vivo* (Borgeson, Pardini, Pardini, & Reitz, 1989; Hardman, Avula, Fernandes, & Cameron,

2001; Shao, Pardini, & Pardini, 1997). However, it has not been shown whether n-3 can enhance the sensitivity of CLL to anti-cancer drugs.

Previous studies performed by our group have shown that consumption of an omega-3 supplement, predominantly composed of EPA and DHA, increased the sensitivity of malignant B lymphocytes isolated from patients with early CLL (RAI stages 0, 1) to doxorubicin in an *in vitro* assay (Fahrman et al., 2013). These findings prompted us to further evaluate the potential use of omega-3 as a chemo-sensitizing agent for the treatment of CLL. The primary objective of this study was to determine whether EPA and/or DHA could increase the sensitivity of malignant B-lymphocytes to doxorubicin, vincristine and/or fludarabine *in vitro*. Secondary objectives were to elucidate potential mechanism(s) by which n-3 enhance chemo-sensitivity. We hypothesized that EPA and/or DHA would increase the sensitivity of malignant B-lymphocytes to doxorubicin, vincristine and fludarabine *in vitro* and that enhanced sensitivity is mediated by alterations in cell cycle progression leading to enhanced growth inhibition and/or enhanced cell death. We further postulate that increased chemo-sensitivity is dependent, in part, on the formation of lipid peroxides, and the generation of reactive oxygen species (ROS).

In this study we assayed for: 1) fatty acid lipid composition, 2) *in vitro* sensitivity of B-CLL-derived cell lines EHEB, and MEC-2 and B-Prolymphocytic-derived (PLL) cell line JVM-2 against doxorubicin, vincristine and fludarabine in the presence of vehicle (no added FA), AA, EPA or DHA, 3) % of apoptotic cells, 4) cell cycle distribution, 5) generation of intracellular reactive oxygen species (ROS), and 6) levels of lipid peroxidation.

METHODS

Chemicals

Ninety-five percent pure doxorubicin hydrochloride (Fisher Scientific), and 2-fluoroadenine-9- β -D-arabinofuranoside (Sigma Aldrich) were dissolved in dimethyl sulfoxide (DMSO) to stock solutions and diluted to the working concentrations in cell type specific culture media. Vincristine sulfate salt (Sigma Aldrich) was dissolved in ddH₂O to stock solutions and diluted to the working concentrations in cell type specific culture media. Vitamin E (α -tocopherol) (Sigma Aldrich) was dissolved in ethanol to stock solutions and diluted to working concentrations in the cell type specific media.

Cell Lines

EHEB (B-CLL), JVM-2 and MEC-2 (B-Prolymphocytic Leukemia) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Cells were grown in 1640 RPMI (ATCC) (EHEB and JVM-2) or Iscove's Modified Dulbecco's Medium (HyClone, Thermo Scientific) (MEC-2) supplemented with 10% Fetal Bovine Serum (HyClone, Thermo Scientific), 100 units/mL penicillin and 0.1 mg/mL streptomycin. All cell lines were grown in humidified incubator at 37⁰C and 5% CO₂.

Fatty Acid Treatments

Stock solutions of 100 mM eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or arachidonic acid (AA) (Cayman Chemical) in ethanol were made and diluted to the working concentrations in cell type specific culture media. Fatty acid concentrations used represent the concentration of each fatty acid that alone did not induce significant cytotoxicity. One million five hundred thousand (1.5×10^6) cells per well were seeded in a 6 well plate (Santa Cruz Biotechnology, Inc) and treated with vehicle (ethanol only), AA, EPA or DHA at the stated

concentration for 72 hours. Cells were treated for 72 hours with FA to allow adequate time for FA incorporation and to allow for any FA-dependent changes in cellular function. Chosen concentrations of FA are clinically achievable (Fahrman et al., 2013). Post 72 hours, cells were counted with a hemocytometer and prepared for the assays below.

Lipid Composition

Fatty acid composition was assessed by gas chromatography according to our routine techniques (Witte, Salazar, Ballester, & Hardman, 2010). Post 72 hours, cells were washed twice with 1X PBS. Cells were subsequently homogenized in distilled water with 0.1% BHT to prevent fatty acid oxidation. Lipids were extracted with chloroform/methanol, and then methylated. Methylated lipids were separated and identified using gas chromatography as previously published (Witte et al., 2010). Fatty acid methyl ester standards (Nu-Chek-Prep, Elysian, MN) were used for peak identification.

Sensitivity Trials

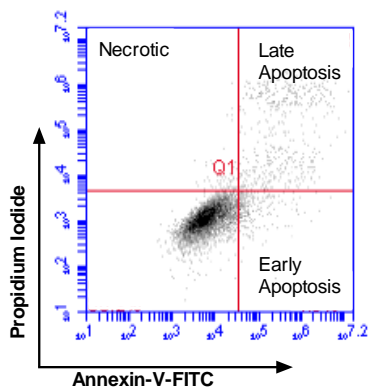
Cell counts were performed and viability was determined by Trypan Blue Exclusion assay following 72 hour fatty acid treatments (FA). Approximately 1×10^5 living cells/well were seeded in triplicate into a round bottom 96 well plate (CELLSTAR, Greiner Bio One International AG). Cells were subsequently treated with culture media containing DMSO, H₂O (solvent controls), doxorubicin (0–7.5 μ M), vincristine (0–250nM) or fludarabine (0–50 μ M) without FA for 20 hours (doxorubicin) or 24 hours (vincristine, fludarabine). Cells were treated in the presence or absence of 50 μ M vitamin E alone and in combination with doxorubicin (1.5 μ M) after 72 hour FA pre-treatment for Vitamin E rescue trials. Cell viability was determined using colorimetric MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (CALBIOCHEM, EMD MILLIPORE) assay. Cell viability was assessed by measuring

the intensity of precipitate formed, relative to control specimens. Absorption was measured using a SpectraMax M2 (Molecular Devices, Sunnyvale, CA) spectrophotometer at 570 nm. All measurements obtained from the MTT assay following treatment with the anti-cancer drugs in the presence of vehicle, AA, EPA or DHA were compared to cells treated with the vehicle or FA alone. MTT assays were performed in technical and biological triplicate.

Measurement of Apoptosis by Annexin-V/Propidium Iodide Dual Stain

Apoptosis was measured by dual stain immunofluorescence flow cytometry. Briefly, post 72 hour FA treatments, cell counts were performed and approximately 5×10^5 cells were treated in the presence of DMSO, H₂O (solvent controls), doxorubicin (1.5 μ M), vincristine (100nM) or fludarabine (40 μ M) as previously described under sensitivity trials. Cells were washed twice with cold 1X PBS and subsequently incubated for 15 minutes in the dark in 100 μ L of Annexin-V binding buffer (0.01 M HEPES, 0.14 M NaCl, 2.5 mM CaCl₂), 1 μ L Annexin-V Alexa Fluor 488-Conjugate (Invitrogen), and 10 μ g/mL propidium iodide (Sigma Aldrich). Cells were analyzed using an Accuri Flow Cytometer. ‘Early’ Apoptosis was defined as cells positive for Annexin-V-FITC only. ‘Late’ Apoptosis was defined as cells positive for Annexin-V-FITC and Propidium Iodide (PI). ‘Necrotic’ was defined as cells positive for PI only (Equation 2). Total% cell death was defined as the sum population of cells in early apoptosis, late apoptosis and necrosis.

Annexin-V assays were performed in biological triplicate.

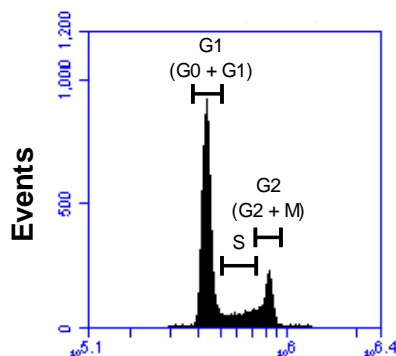


Equation 2: Total % Cell Death

$$\text{Total \% Cell Death} = (\% \text{ Necrotic}) + (\% \text{ Late Apoptosis}) + (\% \text{ Early Apoptosis})$$

Cell Cycle Analysis

Post 72 hour FA treatments, cells were counted and approximately 1×10^6 cells were treated in the presence of DMSO, H₂O (solvent controls), doxorubicin (1.5 μ M), vincristine (100nM) or fludarabine (40 μ M) as previously described under sensitivity trials. Cells were subsequently washed twice with cold 1X PBS and resuspended in DNA staining buffer containing 0.2% Triton X-100, 0.2% Na₃-Citrate, 30 μ g/mL RNase and 20 μ g/mL propidium iodide (Sigma Aldrich) or DNA staining buffer without propidium iodide to serve as negative controls. Cells were incubated for 30 minutes in the dark at room temperature and subsequently analyzed using an Accuri Flow Cytometer. Cell cycle analyses were performed in biological triplicate. Calculation of G1/G2 ratio is described in Equation 3.



Equation 3: G1/G2 Ratio

$$\text{G1/G2 Ratio} = (\# \text{ of events in G0 + G1}) / (\# \text{ of events in G2 + M})$$

Lipid Peroxidation

Lipid peroxidation was measured by means of thiobarbituric acid reactive substances (TBARS) assay. Briefly, approximately 1 to 1.5×10^6 cells were collected in 600 μ L 1X PBS post 72 hour fatty acid treatments as described under fatty acid treatments and after doxorubicin (1.5 μ M) or vincristine (100nM) or fludarabine (40 μ M) alone or in combination with 50 μ M vitamin E (doxorubicin only). Cells were sonicated 2X on 10 second intervals at 40 V setting

over ice using a Fisher Scientific Sonic Dismembrator. TBARS assay (Cayman Chemical), in biological triplicate, was performed according to protocol and reported as ng of malondialdehyde/ μg of protein.

Intracellular ROS Generation

Levels of intracellular ROS were determined using 5-(and-6-)chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H₂DCFDA) (Invitrogen). Briefly, post 72 hour fatty acid treatments, one hundred thousand (10^5) live cells were seeded in triplicate into a round bottom 96 well plate (CELLSTAR, Greiner Bio One International AG). Cells were washed twice with 1X Dulbecco's PBS (DPBS) (GIBCO, Invitrogen) and incubated for 60 minutes in the presence or absence of 10 μM CM-H₂DCFDA in Dulbecco's Modified Eagles Medium (DMEM, Thermo Scientific) (SIGMA) without FBS containing 100 units/mL penicillin, 0.1 mg/mL streptomycin. Post 60 minute incubation, cells were washed 2X with 1X DPBS and treated in the presence or absence of DMSO, doxorubicin (1.5 μM), or fludarabine (40 μM) where after cell suspensions were transferred to a 96 well flat bottom black well plate (VWR International, LLC). Fluorescence was measured every 10 minutes for 2 hours using SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA) at 480 nm excitation 530 nm emission. Assays for intracellular ROS generation were performed in technical triplicates and biological duplicates.

Statistical Analysis

Prism[®] software (Graphpad, Inc., La Jolla, CA) was used for statistical analysis of numeric data by Multiple Comparison using appropriate Post-Hoc Test and for linear regression analyses. Prism[®] software (Graphpad, Inc., La Jolla, CA) was used for preparation of graphs.

Statistical significance on Annexin-V assays is based on total% cell death as described in Equation 2.

RESULTS

N-3 and N-6 fatty Acids Induce Cell Death

Figures 3.1A-C illustrates the % alive cells \pm SEM of EHEB, JVM-2 and MEC-2 following treatment with vehicle, or increasing concentrations of AA, EPA and DHA. Cell viability was assessed by Trypan Blue Exclusion assay following treatment for 72 hours. Treatment with AA, EPA or DHA induced dose-responsive reductions in cell viability as compared to vehicle in all three cell lines. We wanted to determine the chemo-sensitizing effects of FA following treatment with concentrations of FA that alone did not induce significant cytotoxicity. Thus, we chose to use concentrations of AA at 25 μ M, 35 μ M and 25 μ M, EPA at 50 μ M (all cell lines) and DHA at 75 μ M, 50 μ M and 50 μ M for EHEB, JVM-2 and MEC-2, respectively. The chosen FA concentrations used in this study are clinically achievable (Fahrman et al., 2013). Gas chromatography post 72 hour of FA treatment validated FA incorporation in all cells (Figure 3.2).

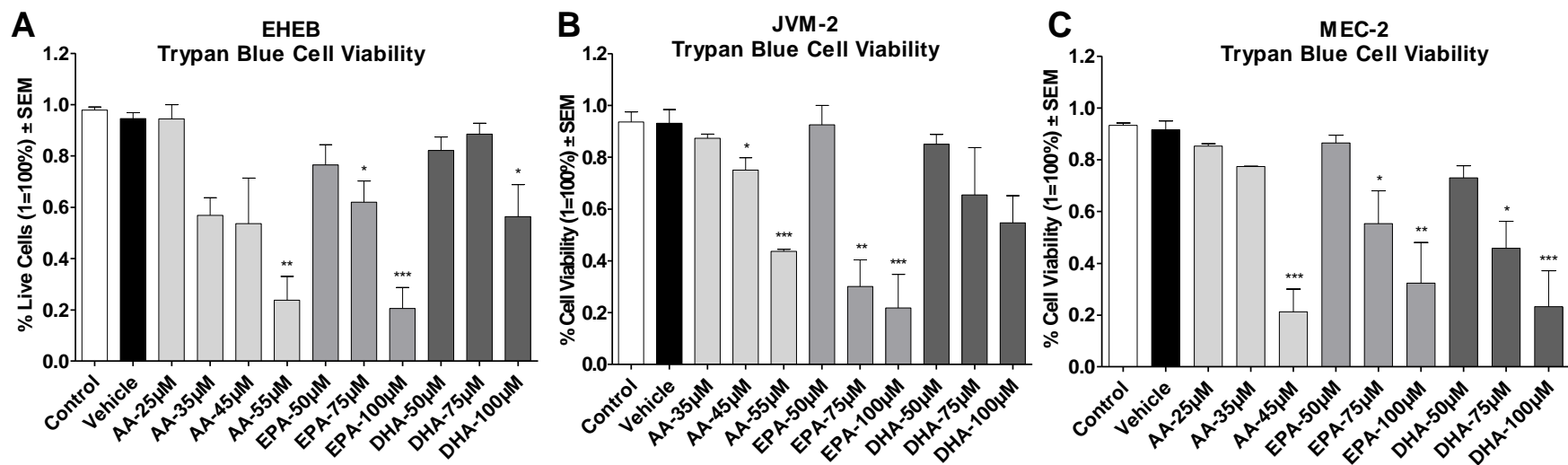


Figure 3. 1. Determination of optimal FA concentrations.

Figures 3.1A-C illustrates the mean % live cells \pm SEM of EHEB, JVM-2 and MEC-2 following 72 hour treatment with vehicle, or increasing concentrations of AA, EPA or DHA. Percent (%) live cells was determined after 72 hour treatments by trypan blue exclusion assay. **Figure 3.1A** illustrates the mean % live cells \pm SEM of EHEB following treatment with vehicle, or increasing concentrations of AA, EPA or DHA. Significant reductions in % live cells were observed at 55µM AA, 75µM and 100µM EPA, and 100µM DHA as compared to vehicle. Although not statistically significant, concentrations of AA35µM and AA45µM indicated viabilities of 57% and 54%. FA concentrations of AA25µM, EPA50µM and DHA75µM were chosen for the remainder of the study. **Figure 3.1B** illustrates the mean % live cells \pm SEM of JVM-2 following treatment with vehicle, or increasing concentrations of AA, EPA or DHA. Significant reductions in % live cells were observed at 45µM and 55µM AA, and 75µM and 100µM EPA as compared to vehicle. Although not statistically significant, concentrations of DHA75µM and DHA100µM indicated cell viabilities of 65% and 55%. FA concentrations of AA35µM, EPA50µM and DHA50µM were chosen for the remainder of the study. **Figure 3.1C** illustrates the mean % live cells \pm SEM of MEC-2 following treatment with vehicle, or increasing concentrations of AA, EPA or DHA. Significant reductions in % live cells were observed at 45µM AA, 75µM and 100µM EPA and 75µM and 100µM DHA as compared to vehicle. FA concentrations of AA25µM, EPA50µM and DHA50µM were chosen for the remainder of the study. Statistical significant was determined by Multiple Comparison Test using Tukey's correction. Abbreviations: AA- arachidonic acid, EPA- eicosapentaenoic acid, DHA- docosahexaenoic acid, $\alpha=0.05$, * <0.05, **<0.01, ***<0.001

MEC-2 Fatty Acid Lipid Composition

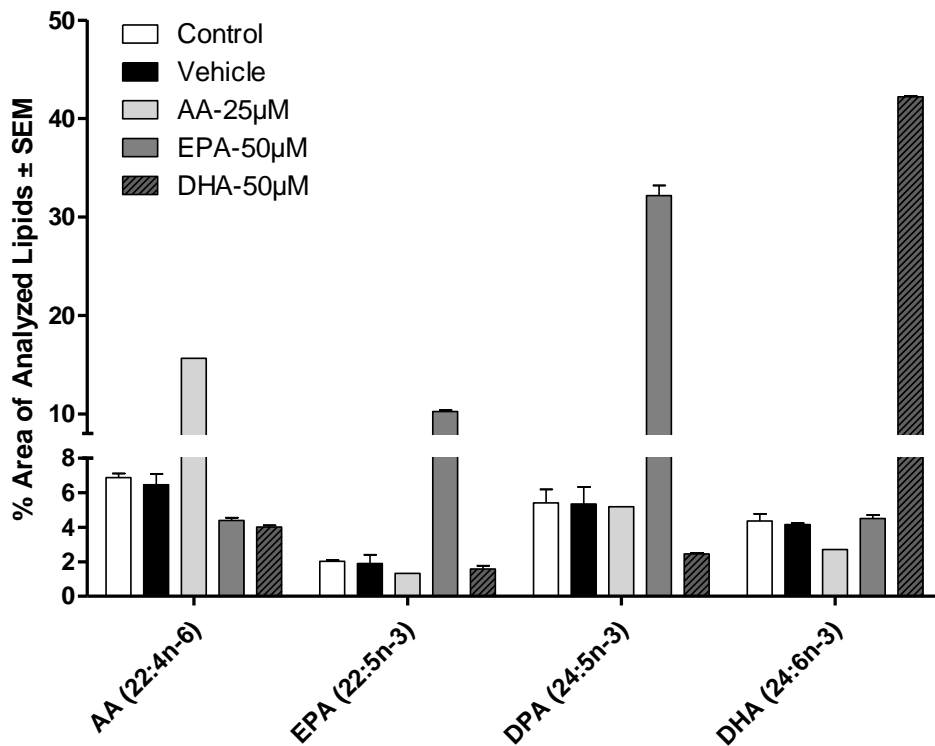


Figure 3. 2. Validation of FA incorporation.

Figure 3.2 illustrates the percentage (%) \pm SEM of analyzed fatty acids in MEC-2 following 72 hour treatments with vehicle, 25µM AA, 50µM EPA or 50µM DHA. Ten (10) fatty acids were analyzed, only % of AA, EPA, DPA and DHA are shown. B-Leukemic cell lines EHEB and JVM-2 indicated similar results (data not shown). Abbreviations: AA-arachidonic acid; EPA- eicosapentaenoic acid; DPA- docosapentaenoic acid; DHA- docosahexaenoic acid; (X:Yn-3/6): X denotes number of carbon atoms, Y denotes number of unsaturations, n-3: omega 3, n-6: omega 6.

EPA and DHA Differentially Sensitize Malignant B-lymphocytes to Doxorubicin, Vincristine and Fludarabine *In Vitro*

In our study, we wanted to determine whether FA pre-treatment would synergistically increase the cytotoxic effects (greater effect than the sum effect due to FA and drug individually) of anti-cancer drugs doxorubicin, vincristine or fludarabine on three different B-leukemic cells. Thus, all measurements obtained from the MTT assay following treatment with the anti-cancer drug in the presence of vehicle, or FA were compared to cells treated with vehicle or FA only.

Figure 3.3A illustrates the *in vitro* sensitivity of EHEB to doxorubicin (0–7.5 μM) in the presence or absence of vehicle, AA, EPA or DHA. Compared to vehicle, cell viability was significantly reduced in cells pre-treated with either EPA or DHA but not with AA when treated with doxorubicin.

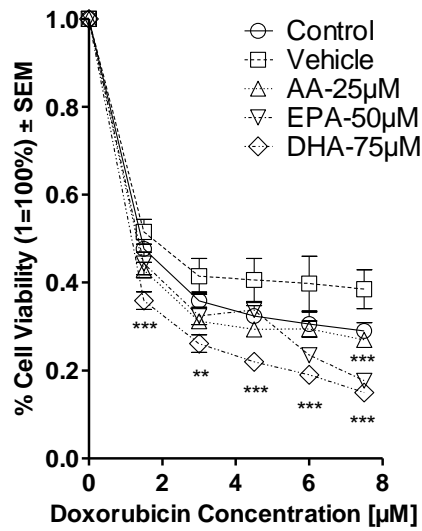
Figure 3.3B illustrates the *in vitro* sensitivity of EHEB to fludarabine (0–50 μM) in the presence or absence of vehicle, AA, EPA or DHA. Compared to vehicle, cell viability was significantly reduced in cells pre-treated with EPA when treated with fludarabine (30 μM and 40 μM). However, there was no difference in the sensitivity of EHEB to fludarabine when cells were pre-treated with either AA or DHA. It is interesting to note that AA pretreatment had a non-significant slightly protective effect on EHEB cells treated with fludarabine.

Compared to vehicle, pre-treatment with AA, EPA or DHA did not significantly change the sensitivity of EHEB to vincristine (Appendix, Supplementary Figure 3.1).

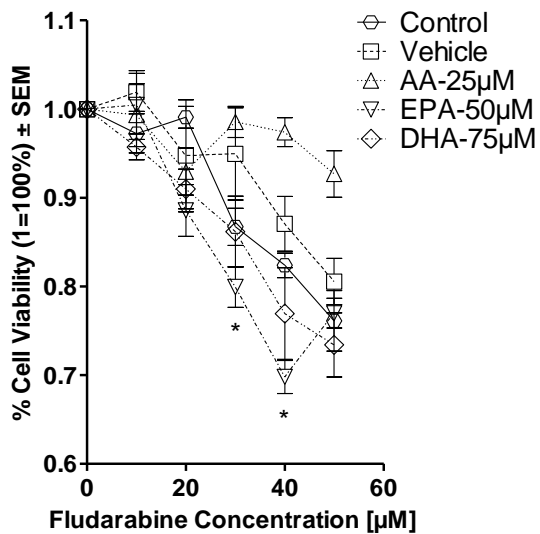
In our model, an increase in chemo-sensitivity of cells to the drug by FA can be mediated by both enhanced cell death and/or enhanced growth-inhibition. To determine whether the decreases in cell viability seen in the MTT assays were a result of enhanced cell death or of growth-inhibition we performed an Annexin-V assay.

Figure 3.3C illustrates the % dead EHEB cells (\pm SEM) in the presence or absence of vehicle, AA, EPA, or DHA alone and after treatment with doxorubicin (1.5 μ M) or fludarabine (40 μ M). The concentration of doxorubicin (1.5 μ M) was chosen as this concentration induced a significant difference in cell viability between FA and vehicle pre-treated cells and because this concentration is clinically achievable (Muller et al., 1993). The concentration of fludarabine (40 μ M) was chosen as this concentration induced the greatest significant difference in cell viability between EPA and vehicle pre-treated cells (Figure 3.3B); however, this concentration is ~5-10 times greater than the peak plasma concentration of fludarabine (Foran et al., 1999). Compared to vehicle, cells pre-treated with DHA, but without drug, had significantly higher cell death. The addition of doxorubicin or fludarabine to DHA pre-treated cells significantly increased cell death as compared to vehicle and drug treatment. Cell death was mediated predominately through apoptosis (Figure 3.3C). In cells treated with doxorubicin or fludarabine, pre-treatment with either AA or EPA did not increase cell death as compared to vehicle. Figure 3.3D displays a graphical 2D representation of Annexin-V/PI plots of EHEB cells pre-treated with either vehicle or DHA and following treatment with doxorubicin or fludarabine.

A EHEB
Live Cell Fraction (compared to no Doxorubicin)



B EHEB
Live Cell Fraction (compared to no Fludarabine)



C EHEB
Total Cell Death
(+ 1.5µM Doxorubicin) (+ 40µM Fludarabine)

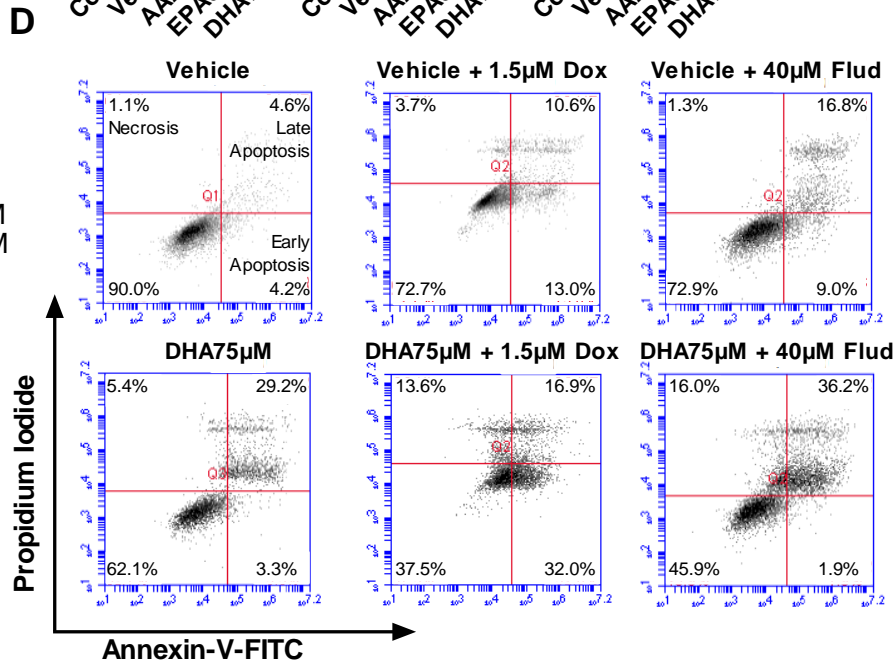
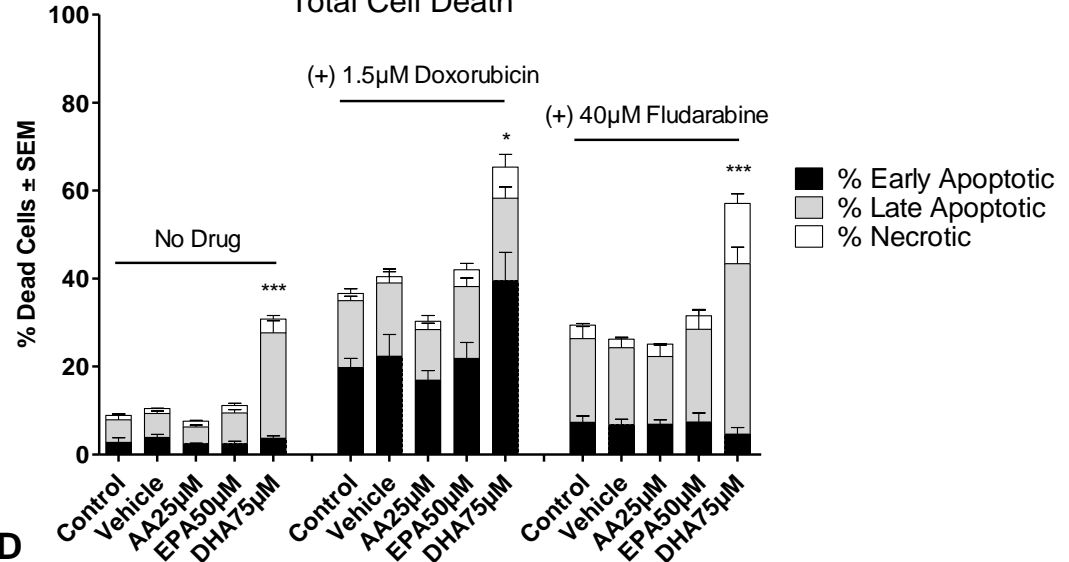


Figure 3. 3. In vitro sensitivity of EHEB following treatment with doxorubicin or fludarabine in the presence and absence vehicle, AA, EPA or DHA.

Figure 3.3A illustrates the % cell viability \pm SEM of EHEB to doxorubicin (0-7.5 μ M) in the presence or absence of vehicle, AA25 μ M, EPA50 μ M or DHA75 μ M. Cells pre-treated with either EPA or DHA had significantly greater decreases in cell viability as compared to vehicle when treatment with doxorubicin. **Figure 3.3B** illustrates the % cell viability \pm SEM of EHEB to fludarabine (0-50 μ M) in the presence or absence of vehicle, AA25 μ M, EPA50 μ M or DHA75 μ M. Pre-treatment of cells with EPA had significantly greater reductions in cell viability as compared to vehicle when treated with 30 μ M and 40 μ M fludarabine. **Figure 3.3C** illustrates the % dead cells \pm SEM of EHEB following pre-treatment with vehicle, AA25 μ M, EPA50 μ M or DHA75 μ M alone and following treatment with 1.5 μ M doxorubicin or 40 μ M fludarabine. The % cell death was determined by annexin-V/propidium iodide dual stain with flow cytometry. Pre-treatment with DHA alone induced significantly greater cell death as compared to vehicle. Compared to vehicle, cells pre-treated with DHA had significantly higher cell death when treated with doxorubicin or fludarabine. **Figure 3.3D** provides 2D graphical representations of Annexin-V/PI Plots. 'Early' Apoptosis was defined as cells positive for Annexin-V-FITC only. 'Late' Apoptosis was defined as cells positive for Annexin-V-FITC and PI. 'Necrotic' was defined as cells positive for PI only. Statistical significance was determined by Multiple Comparison Test with Dunnet's correction (**Figures 3.3A and B**) or Tukey's correction (**Figure 3.3C**). $\alpha = 0.05$, * <0.05, **<0.01, ***< 0.001.

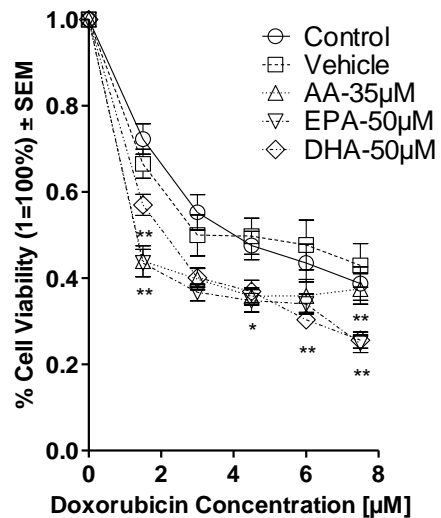
Figure 3.4A illustrates the *in vitro* sensitivity of JVM-2 to doxorubicin (0–7.5 μ M) in the presence or absence of vehicle, AA, EPA or DHA. Compared to vehicle, all FA pre-treatment significantly decreased cell viability due to doxorubicin treatment.

Figure 3.4B illustrates the *in vitro* sensitivity of JVM-2 to vincristine (0-250nM) in the presence or absence of vehicle, AA, EPA or DHA. Compared to vehicle, only DHA pre-treatment significantly decreased cell viability due to vincristine treatment.

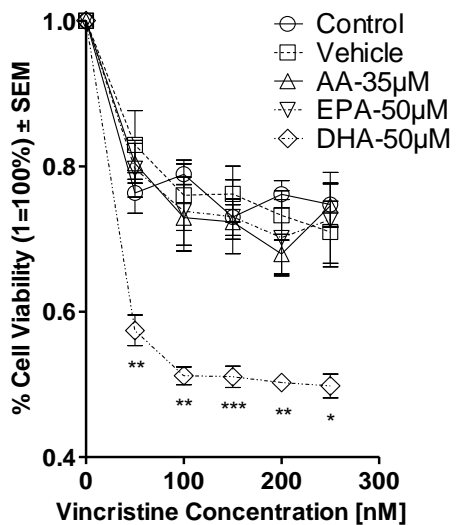
Pre-treatment with AA, EPA or DHA did not induce any significant differences in the sensitivity of JVM-2 cells to fludarabine (Appendix, Supplementary Figure 3.2).

Figure 3.4C illustrates the % dead JVM-2 cells (\pm SEM) in the presence or absence of vehicle, AA, EPA, or DHA alone and following treatment with doxorubicin (1.5 μ M) or vincristine (100nM). The concentration of vincristine (100nM) was chosen as this concentration induced a significant difference in cell viability between DHA and vehicle pre-treated cells and because this concentration is clinically achievable ([Guilhaumou et al., 2011](#)). Compared to vehicle, pre-treatment with DHA alone induced significant cell death. Pre-treatment with DHA significantly increased cell death due to either doxorubicin or vincristine treatment. Figure 3.4D displays a graphical 2D representation of Annexin-V/PI plots of JVM-2 cells pre-treated with either vehicle or DHA and following treatment with doxorubicin or vincristine.

A JVM-2
Live Cell Fraction (Compared to no Doxorubicin)



B JVM-2
Live Cell Fraction (Compared to no Vincristine)



C JVM-2
Total Cell Death

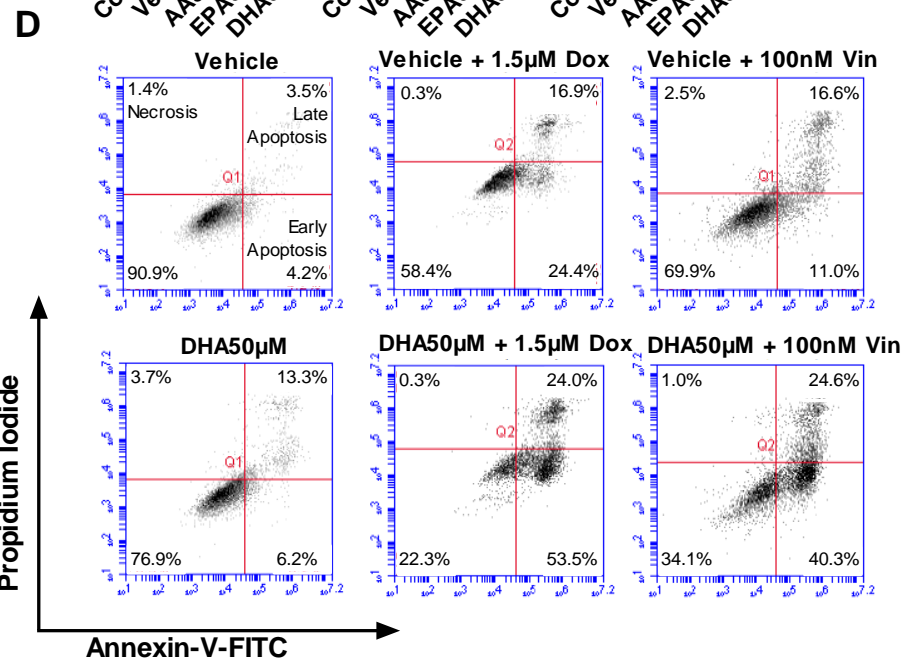
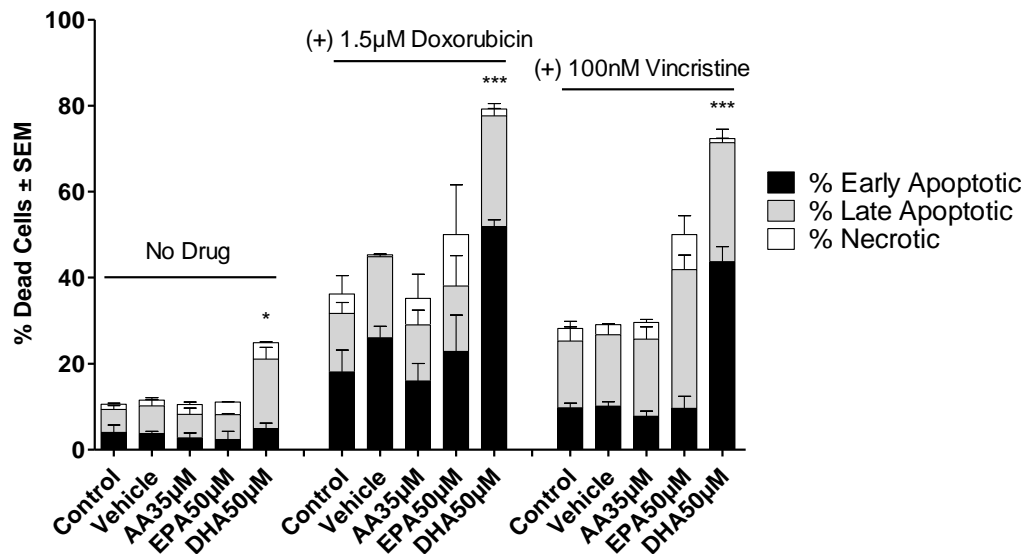


Figure 3.4. In vitro sensitivity of JVM-2 following treatment with doxorubicin or vincristine in the presence and absence vehicle, AA, EPA or DHA.

Figure 3.4A illustrates the % cell viability \pm SEM of JVM-2 to doxorubicin (0-7.5 μ M) in the presence or absence of vehicle, AA35 μ M, EPA50 μ M or DHA50 μ M. Cells pre-treated with AA, EPA or DHA induced significantly greater decreases in cell viability as compared to vehicle when treated with doxorubicin. **Figure 3.4B** illustrates the % cell viability \pm SEM of JVM-2 to vincristine (0-250nM) in the presence or absence of vehicle, AA35 μ M, EPA50 μ M or DHA50 μ M. Compared to vehicle, only cells pre-treated with DHA had significantly greater reductions in cell viability when treatment with vincristine. **Figure 3.4C** illustrates the % dead cells \pm SEM of JVM-2 following pre-treatment with vehicle, AA35 μ M, EPA50 μ M or DHA75 μ M alone and following treatment with 1.5 μ M doxorubicin or 100nM vincristine. Pre-treatment with DHA alone induced significantly greater cell death as compared to vehicle. Compared to vehicle, Cells pre-treated with DHA had significantly greater cell death when treated with doxorubicin or vincristine. **Figure 3.4D** provides 2D graphical representations of Annexin-V/PI Plots. 'Early' Apoptosis was defined as cells positive for Annexin-V-FITC only. 'Late' Apoptosis was defined as cells positive for Annexin-V-FITC and PI. 'Necrotic' was defined as cells positive for PI only. Statistical significance was determined by Multiple Comparison Test with Dunnet's correction (**Figures 3.4A and B**) or Tukey's correction (**Figure 3.4C**). $\alpha = 0.05$, * <0.05, **<0.01, ***< 0.001.

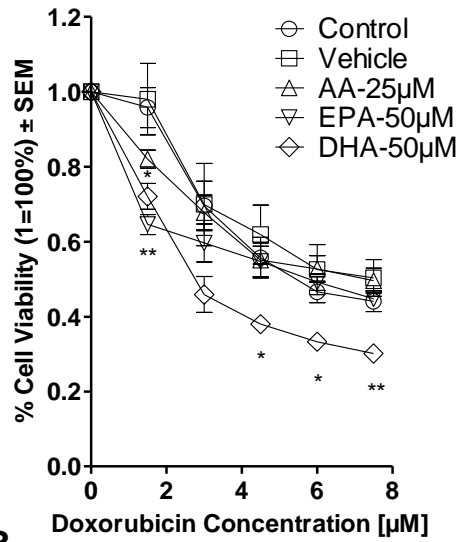
Figure 3.5A illustrates the *in vitro* sensitivity of MEC-2 to doxorubicin (0–7.5 μ M) in the presence or absence of vehicle, AA, EPA or DHA. Compared to vehicle, pre-treatment with either EPA or DHA significantly decreased cell viability due to doxorubicin treatment.

Figure 3.5B illustrates the *in vitro* sensitivity of MEC-2 to vincristine (0-250nM) in the presence or absence of vehicle, AA, EPA or DHA. Compared to vehicle, pre-treatment with either EPA or DHA significantly decreased viability of cells treated with vincristine.

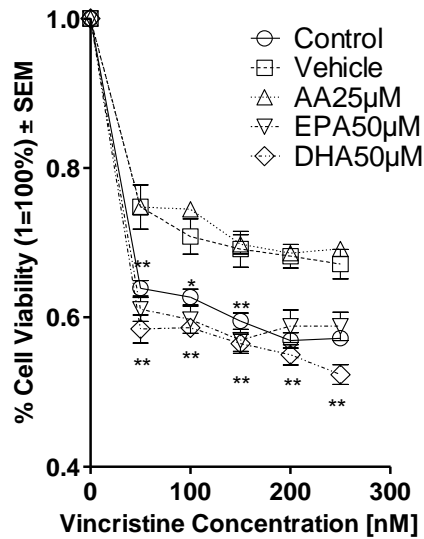
Pre-treatment of cells with AA, EPA or DHA did not increase the sensitivity of MEC-2 to fludarabine (Appendix, Supplementary Figure 3.3).

Figure 3.5C illustrates the % dead cells \pm SEM of MEC-2 in the presence or absence of vehicle, AA, EPA, or DHA alone and following treatment with doxorubicin (1.5 μ M) or vincristine (100nM). Compared to vehicle, cells pre-treated with DHA alone had significantly higher cell death; whereas cells pre-treated with either AA or EPA had significantly less cell death. The addition of doxorubicin or vincristine to DHA pre-treated cells induced higher cell death as compared to vehicle; however, this was only significant when compared to AA pre-treated cells. Figure 3.5D displays a graphical 2D representation of Annexin-V/PI plots of MEC-2 cells pre-treated with either vehicle or DHA and following treatment with doxorubicin or vincristine.

A MEC-2
Live Cell Fraction (compared to no Doxorubicin)



B MEC-2
Live Cell Fraction (compared to no Vincristine)



C MEC-2
Annexin-V/Propidium Iodide Duel Stain
(+) 100nM Vincristine

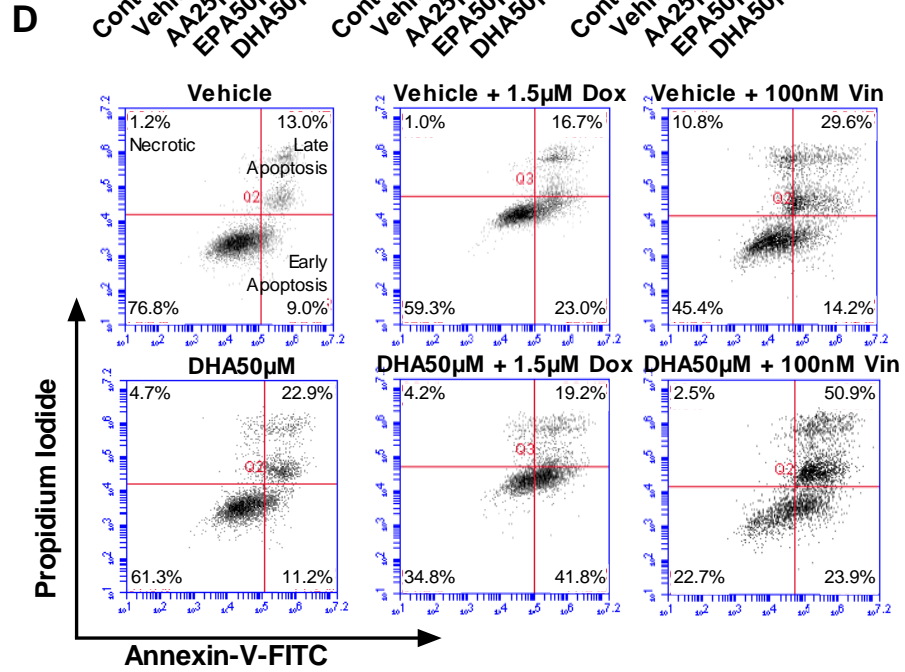
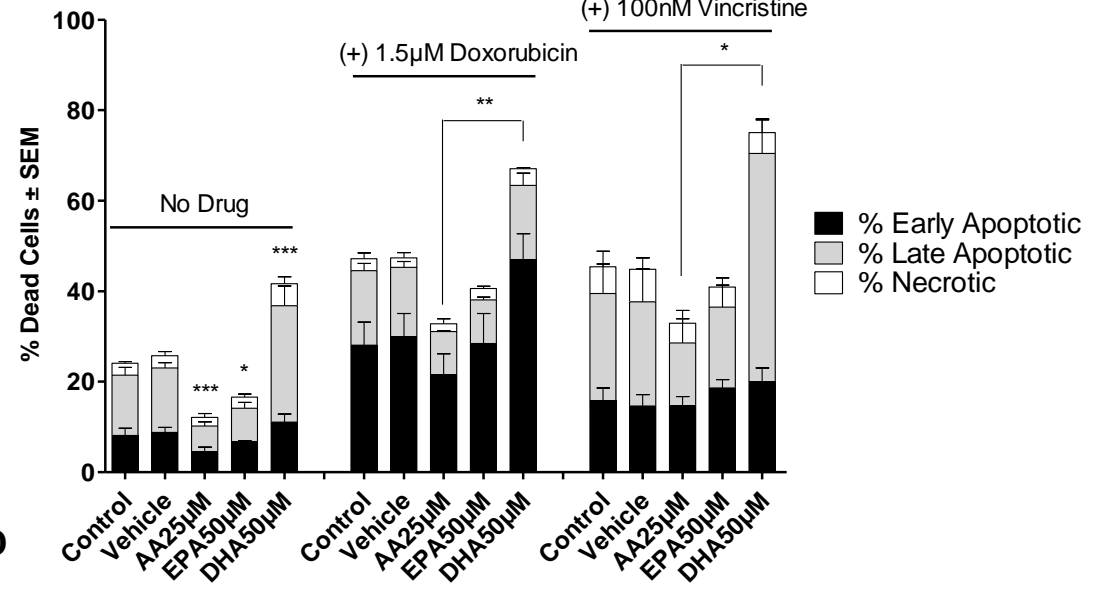


Figure 3. 5. In vitro sensitivity of MEC-2 following treatment with doxorubicin or vincristine in the presence and absence vehicle, AA, EPA or DHA.

Figure 3.5A illustrates the % cell viability \pm SEM of MEC-2 to doxorubicin (0-7.5 μ M) in the presence or absence of vehicle, AA25 μ M, EPA50 μ M or DHA50 μ M. Cells pre-treated with either EPA or DHA had significantly greater decreases in cell viability as compared to vehicle when treated with doxorubicin. **Figure 3.5B** illustrates the % cell viability \pm SEM of MEC-2 to vincristine (0-250nM) in the presence or absence of vehicle, AA25 μ M, EPA50 μ M or DHA50 μ M. Cells pre-treated with either EPA or DHA had significantly greater decreases in cell viability as compared to vehicle following treatment with vincristine. **Figure 3.5C** illustrates the % dead cells \pm SEM of MEC-2 following pre-treatment with vehicle, AA25 μ M, EPA50 μ M or DHA50 μ M alone and following treatment with 1.5 μ M doxorubicin or 100nM vincristine. Compared to vehicle, pre-treatment DHA alone induced significantly greater cell death; whereas pre-treatment with either AA or EPA induced significantly lower cell death. Cells pre-treated with DHA had higher cell death, as compared to vehicle, when treated with doxorubicin or vincristine; however, this was only significant when compared against AA pre-treated cells. **Figure 3.5D** provides 2D graphical representations of Annexin-V/PI Plots. ‘Early’ Apoptosis was defined as cells positive for Annexin-V-FITC only. ‘Late’ Apoptosis was defined as cells positive for Annexin-V-FITC and PI. ‘Necrotic’ was defined as cells positive for PI only. Statistical significance was determined by Multiple Comparison Test with Dunnet’s correction (**Figures 3.5A and B**) or Tukey’s correction (**Figure 3.5C**). $\alpha = 0.05$, * <0.05, **<0.01, ***< 0.001.

N-3 Alone and in Combination with Anti-cancer Drugs Induce G2/M Arrest

We wanted to determine whether increased chemo-sensitivity by FA was also associated with enhanced growth-inhibition; thus, we performed a cell-cycle analysis. Inhibition of cell-cycle progression leads to growth-inhibition (reduced proliferation).

Table 3.1 illustrates the mean G1/G2 ratio \pm SEM of all three cell lines in the presence of vehicle, AA, EPA or DHA alone and following treatment with 1.5 μ M doxorubicin (EHEB, JVM-2, MEC-2), 40 μ M fludarabine (EHEB), or 100nM vincristine (JVM-2, MEC-2). Cell cycle analysis was not performed on EHEB following treatment with vincristine or on JVM-2 and MEC-2 following treatment with fludarabine as there were no significant differences in the *in vitro* sensitivity of these cell lines to these drugs in the presence AA, EPA or DHA as compared to vehicle.

| Table 3. 1. Cell Cycle Analysis: G1/G2 Ratio | | | | | | | | | |
|---|---------------|---------------|---------|--------------|---------|---------------|---------|---------------|---------|
| EHEB | | | | | | | | | |
| | Control ± SEM | Vehicle ± SEM | p-value | AA25µM ± SEM | p-value | EPA50µM ± SEM | p-value | DHA75µM ± SEM | p-value |
| (-) Drugs | 4.1 ± 0.09 | 4.3 ± 0.04 | NS | 3.7 ± 0.8 | NS | 4.0 ± 0.50 | NS | 4.2 ± 0.20 | NS |
| (+) 1.5µM Dox | 9.2 ± 0.21 | 9.0 ± 0.27 | NS | 9.5 ± 0.32 | NS | 8.9 ± 0.72 | NS | 5.8 ± 0.77 | <0.05 |
| (+) 40µM Flud | 13.3 ± 0.21 | 15.5 ± 0.04 | NS | 13.5 ± 0.19 | NS | 11.6 ± 2.08 | NS | 4.9 ± 0.22 | <0.01 |
| JVM-2 | | | | | | | | | |
| | Control ± SEM | Vehicle ± SEM | p-value | AA35µM ± SEM | p-value | EPA50µM ± SEM | p-value | DHA50µM ± SEM | p-value |
| (-) Drugs | 2.4 ± 0.14 | 2.4 ± 0.04 | NS | 2.2 ± 0.06 | NS | 1.8 ± 0.12 | NS | 1.8 ± 0.09 | <0.05 |
| (+) 1.5µM Dox | 2.9 ± 0.16 | 3.1 ± 0.18 | NS | 2.6 ± 0.00 | NS | 2.6 ± 0.07 | NS | 2.18 ± 0.23 | <0.05 |
| (+) 100nM Vin | 1.2 ± 0.00 | 1.2 ± 0.02 | NS | 0.9 ± 0.00 | <0.05 | 0.8 ± 0.01 | <0.01 | 0.9 ± 0.06 | <0.05 |
| MEC-2 | | | | | | | | | |
| | Control ± SEM | Vehicle ± SEM | p-value | AA25µM ± SEM | p-value | EPA50µM ± SEM | p-value | DHA50µM ± SEM | p-value |
| (-) Drugs | 5.2 ± 0.29 | 4.8 ± 0.10 | NS | 4.9 ± 0.37 | NS | 3.6 ± 0.14 | <0.05 | 2.8 ± 0.28 | < 0.001 |
| (+) 1.5µM Dox | 3.0 ± 0.11 | 2.8 ± 0.23 | NS | 1.5 ± 0.08 | <0.001 | 1.12 ± 0.10 | <0.001 | 1.1 ± 0.15 | <0.001 |
| (+) 100nM Vin | 1.2 ± 0.07 | 1.4 ± 0.19 | NS | 0.7 ± 0.06 | <0.05 | 0.6 ± 0.04 | <0.01 | 0.9 ± 0.18 | NS |

Table 3.1. Pre-treatment with FAs alone and following treatment with doxorubicin, vincristine or fludarabine induce G2/M arrest. Table 3.1

illustrates the G1/G2 ratios ± SEM of EHEB, JVM-2 and MEC-2 following treatment with 1.5µM doxorubicin, 100nM vincristine or 40µM fludarabine in the presence or absence of vehicle, AA, EPA or DHA. Treatment with DHA alone indicated significantly lower ratios of G1/G2, indicative of G2/M arrest, as compared to vehicle in all three cell lines. Treatment with EPA indicated a significantly lower G1/G2 ratio as compared to vehicle in MEC-2. Pre-treatment with AA, EPA or DHA indicated significantly lower G1/G2 ratios as compared to vehicle following treatment with doxorubicin or vincristine. Only pre-treatment with DHA indicated a significantly lower G1/G2 ratio as compared to vehicle following treatment with fludarabine in EHEB. Statistical significance was determined by Multiple Comparison Test using Tukey's correction. Abbreviations: Dox: doxorubicin, Vin: Vincristine, Flud: Fludarabine. α = 0.05, * <0.05, **<0.01, ***< 0.001.

FA treatment alone: A significantly lower G1/G2 ratio indicates G2/M arrest. Treatment with EPA alone induced a significantly lower G1/G2 ratio, as compared to vehicle in MEC-2 ($p < 0.05$). Treatment with DHA alone induced a significantly lower G1/G2 ratio as compared to vehicle in JVM-2 and MEC-2 ($p < 0.05$ and < 0.001 ; respectively). Treatment with AA alone did not induce any significant differences in the G1/G2 ratio as compared to vehicle in EHEB, JVM-2 or MEC-2.

FA plus drug treatment: Cells pre-treated with AA had significantly lower G1/G2 ratios as compared to vehicle when treated with doxorubicin ($p < 0.001$ in MEC-2) or vincristine ($p < 0.05$ and < 0.05 in JVM-2 and MEC-2, respectively).

Cells pre-treated with EPA had significantly lower G1/G2 ratios as compared to vehicle when treated with doxorubicin ($p < 0.001$ in MEC-2) or vincristine ($p < 0.01$ in JVM-2 and MEC-2). Cells pre-treated with DHA had significantly lower G1/G2 ratios as compared to vehicle when treated with doxorubicin ($p < 0.05$, < 0.05 , and < 0.001 in EHEB, JVM-2 and MEC-2, respectively), fludarabine ($p < 0.01$ in EHEB), or vincristine ($p < 0.05$ and < 0.001 in JVM-2 and MEC-2, respectively).

N-3 Increases Generation of Intracellular ROS

To investigate ROS production in response to AA, EPA or DHA alone and following treatment with doxorubicin or fludarabine we used a CM-H₂DCFDA probe.

Figure 3.6A illustrates mean relative fluorescence units (RFU) \pm SEM across time for MEC-2. Of the 3 cell types, ROS were increased only in MEC-2 cells due to pre-treatment with either EPA or DHA. Linear regression analysis indicated that the rate of increase in ROS was significantly greater in DHA pre-treated MEC-2 cells than in vehicle pre-treated cells (Mean slope (RFU)/min: 0.683 versus 0.267, $p < 0.01$). Similarly, the rate of increase in ROS was

greater in the presence of EPA than in vehicle treated MEC-2 cells (Mean slope (RFU)/min: 0.483 versus 0.267, p: 0.08); however this was not statistically significant. Pre-treatment with AA did not induce any differences in the levels of ROS as compared to vehicle.

N-3 Increases Lipid Peroxidation

To investigate the formation of lipid peroxides in response to AA, EPA or DHA treatment alone and with doxorubicin, fludarabine or vincristine, levels of thiobarbituric acid reactive substances (TBARS, byproducts of lipid peroxidation) were evaluated and compared to a malondialdehyde (MDA, (a TBARS)) standard curve. Figure 3.6B illustrates the mean ng MDA/ μ g of protein \pm SEM of MEC-2 treated in the presence of vehicle, AA, EPA or DHA alone and following treatment with 1.5 μ M doxorubicin or 100nM vincristine. Pre-treatment of MEC-2 cells with DHA alone induced significantly higher levels of TBARS than did vehicle. Only DHA pre-treatment induced significantly higher levels of TBARS in doxorubicin treated cells. Cells pre-treated with either EPA or DHA had significantly lower levels of TBARS when treated with vincristine as compared to the FA alone. Analysis of TBARS levels following treatment with fludarabine were not performed as no statistical differences were found in the *in vitro* sensitivity trials. EHEB and JVM-2 had similar trends in levels of TBARS following n-3 pre-treatment as observed in MEC-2; however, the drug treatment did not induce any significant differences as compared to vehicle or n-3 alone (Appendix, Supplementary Figure 3.4).

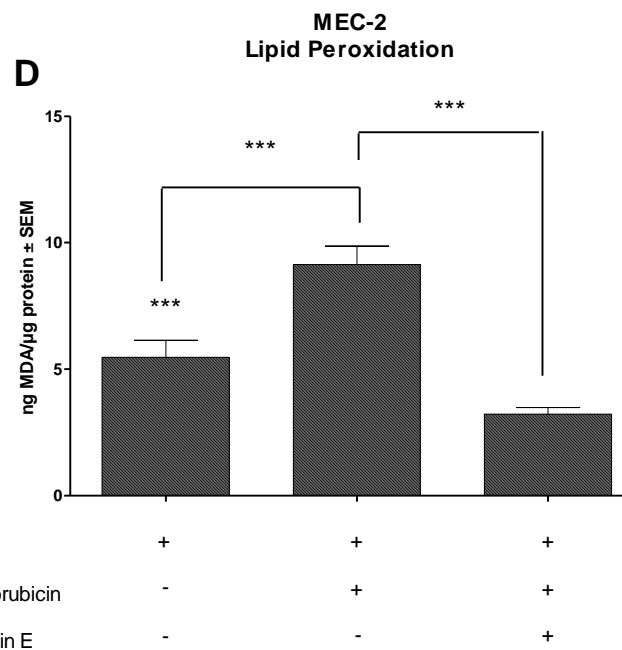
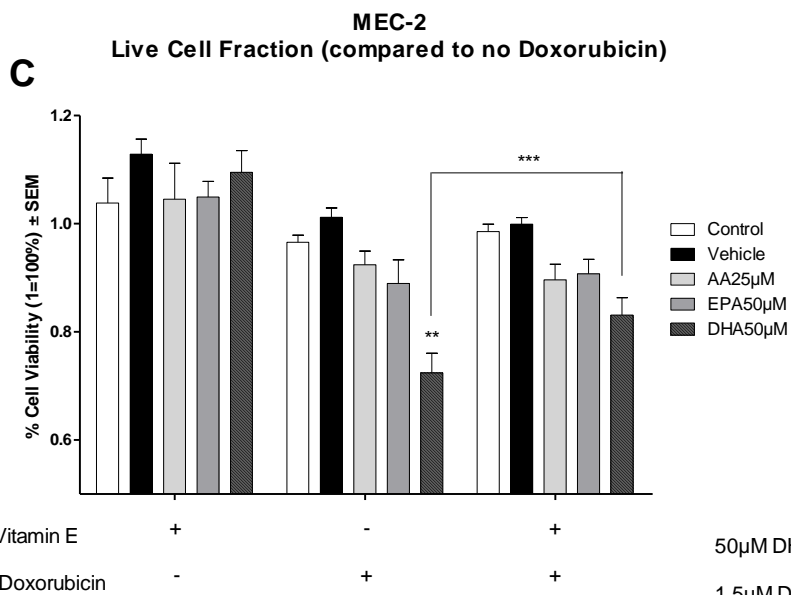
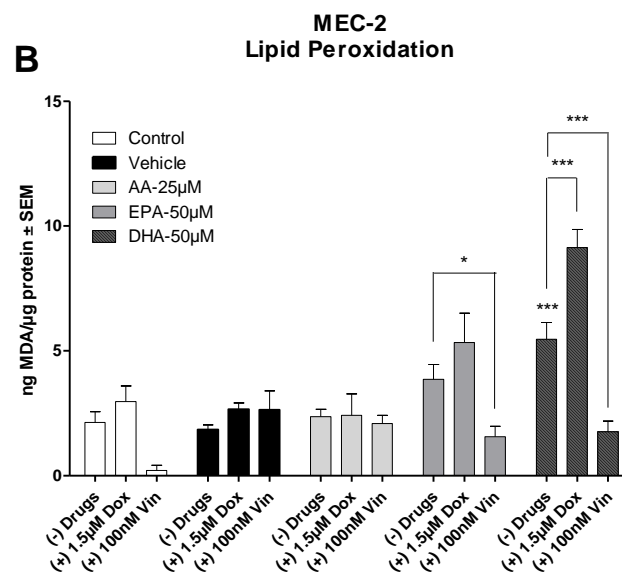
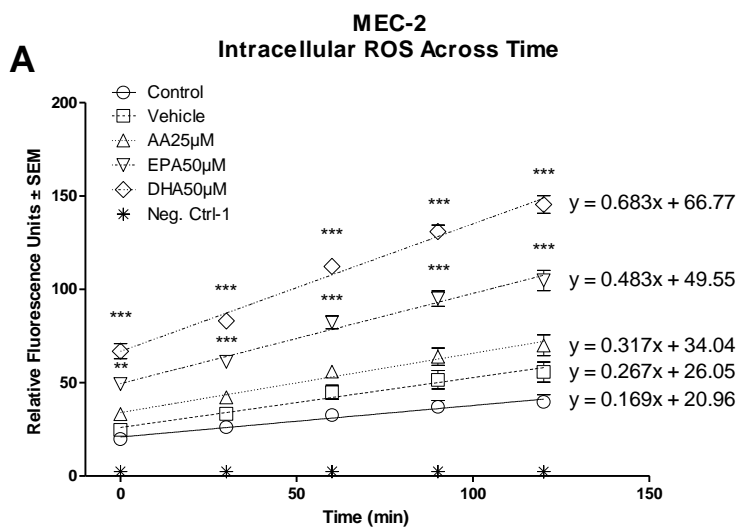


Figure 3. 6. Chemo-sensitizing capability of DHA is mediated through generation of intracellular ROS and formation of lipid peroxides.

Figure 3.6A illustrates mean relative fluorescence units \pm SEM of MEC-2 over the course of 2 hours following 72 hour pre-treatment with vehicle, AA25 μ M, EPA50 μ M or DHA50 μ M. Intracellular ROS was determined using 5-(and-6-)chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H₂DCFDA) dye. Treatment with either EPA or DHA alone induced significant increases in ROS generation. Linear regression analysis indicated a greater slope following treatment with EPA or DHA versus vehicle (0.483 and 0.683 versus 0.267) indicating an increased generation of ROS across time. The addition of doxorubicin did not significantly alter levels of ROS as compared to vehicle or FA alone (Appendix, Supplementary Figure 3.5). **Figure 3.6B** illustrates the mean ng MDA/ μ g protein \pm SEM in the presence or absence of vehicle, AA25 μ M, EPA50 μ M or DHA50 μ M alone and following treatment with doxorubicin (1.5 μ M) or vincristine (100nM). Pre-treatment with DHA alone induced significantly higher levels of MDA as compared to vehicle. The addition of doxorubicin to DHA pre-treated cells induced significantly higher levels of MDA versus either alone. The addition of vincristine to either EPA or DHA pre-treated cells induced significantly lower levels of MDA as compared to n-3 or drug alone. **Figure 3.6C** illustrates the % cell viability \pm SEM of MEC-2 following treatment with 1.5 μ M doxorubicin and 50 μ M vitamin E alone and in combination in the presence or absence of vehicle, AA25 μ M, EPA50 μ M or DHA50 μ M. Compared to vehicle, cells pre-treated with DHA had significantly greater reductions in cell viability when treated with doxorubicin. Co-treatment of DHA pre-treated cells with doxorubicin and vitamin E abrogated the enhanced sensitivity of MEC-2 to doxorubicin by DHA. **Figure 3.6D** illustrates the mean ng MDA/ μ g protein \pm SEM of MEC-2 following pre-treatment with DHA alone and after treatment with 1.5 μ M doxorubicin alone and in combination with 50 μ M vitamin E. Co-treatment with doxorubicin and vitamin E in DHA pre-treated cells indicated significantly lower levels of MDA versus doxorubicin alone. Statistical significance was determined by Multiple Comparison Test with Tukey's correction. Abbreviations: MDA: malondialdehyde, $\alpha = 0.05$, * <0.05, **<0.01, ***< 0.001.

Vitamin E Abrogates Enhanced Sensitization of MEC-2 to Doxorubicin by DHA

To validate that the ability of n-3 to sensitize malignant B-lymphocytes to doxorubicin is dependent on the formation of toxic lipid peroxides, we tested the *in vitro* sensitivity of MEC-2 in the presence of vehicle, AA, EPA or DHA alone and following treatment with 1.5 μ M doxorubicin alone or in combination with 50 μ M vitamin E. Figure 3.6C illustrates the *in vitro* sensitivity of MEC-2 to 1.5 μ M doxorubicin and 50 μ M vitamin E alone and in combination in the presence or absence of vehicle, AA, EPA or DHA. Compared to vehicle, DHA pre-treated cells had significantly greater reductions in viability when treated with doxorubicin. The addition of vitamin E abrogated the enhanced sensitization of MEC-2 to doxorubicin by DHA. In agreement with the *in vitro* sensitivity trial, co-treatment of DHA pre-treated cells with doxorubicin and vitamin E induced significant reductions in the levels of TBARs as compared to doxorubicin alone (Figure 3.6D).

DISCUSSION

Chronic lymphocytic leukemia is the most common form of adult leukemia in the western world (Riches et al., 2011). Clinical treatment of CLL is often limited due to drug resistance and severe toxicities associated with chemotherapy (Riches et al., 2011; Schriever & Huhn, 2003; Silber et al., 1994). A therapeutic intervention that could enhance the sensitivity of CLL cells to anti-cancer drugs without causing additional adverse effects would be clinically beneficial.

Omega-3 fatty acids have consistently been shown to enhance the sensitivity of various solid tumor cells to chemotherapy *in vitro* (Germain et al., 1998; Kinsella & Black, 1993) and *in vivo* (Borgeson et al., 1989; Hardman et al., 2001; Shao et al., 1997). However, this has not been

shown in CLL. Previous results from our group indicated that consumption of an n-3 supplement enhanced the sensitivity of lymphocytes isolated from patients with early stage (Rai 0,1) CLL to doxorubicin in an *in vitro* assay (Fahrman et al., 2013). These findings prompted us to further evaluate the potential use of n-3 as chemo-sensitizing agents for the treatment of CLL.

The primary purpose of this study was to illustrate that pre-treatment of B-CLL-and B-PLL-derived cells with n-3 increases the sensitivity of cells to actively used chemotherapeutic drugs: doxorubicin and vincristine, components of the CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) regimen (Leporrier et al., 2001; Van der Jagt et al., 2012), or fludarabine, a commonly used first-line treatment option for CLL (Riches et al., 2011; Silber et al., 1994). Rather than testing combination therapies, we evaluated the ability of n-3 to enhance the sensitivity of malignant B-lymphocytes to single-arm treatments. Secondary objectives were to elucidate potential mechanism(s) by which n-3 enhanced chemo-sensitivity.

Although designated as a single disease, CLL is characterized by biological and clinical heterogeneity. For these reasons, we particularly wanted to demonstrate that the chemo-sensitizing effects of n-3 were not limited to one specific cell sub-type (cell line). Rather we wanted to demonstrate that the chemo-sensitizing effects of n-3 would be seen in multiple cell-types. Thus, each cell line could be viewed as a distinct case of CLL.

For the purposes of this study, we used the highest concentrations FAs that alone did not induce significant cytotoxicity (Figures 3.1A-C). Our results indicated that clinically achievable concentrations of EPA and DHA generally, but not equally, sensitized the B-leukemic cells to the drugs. Only JVM-2 cells were sensitized to doxorubicin (1.5 μ M) when cells were pre-treated with AA (Figure 3.4A), indicating that the chemo-sensitizing capabilities of FAs are more likely to be found amongst n-3 fatty acids than n-6 fatty acids. This is an important consideration. The

western diet is heavily favored towards n-6 FA with little to no n-3 FA intake (Simopoulos, 2000, 2002). Omega-3 and n-6 FAs compete with each other for incorporation into the cell (Simopoulos, 2000, 2002). The addition of n-3 as an augment to therapy may, therefore, provide clinical benefit to the patient receiving therapy. We are currently conducting a clinical trial to determine if we will see the same chemo-sensitizing capabilities of n-3 on lymphocytes isolated from patients with CLL.

We have illustrated that pre-treatment with n-3 increased the sensitivity of B-CLL- and B-PLL-derived cells to three actively used chemo-therapeutic drugs. While doxorubicin, vincristine and fludarabine have different mechanisms by which they exert their cytotoxic effects, all three drugs can induce cell death and/or growth-inhibition. Thus, increasing the sensitivity of cells to the drug is not a function limited to increased cell death, but can also be mediated through increased growth-inhibition (reduced proliferation). Both cell death and/or growth-inhibition leads to a decrease in numbers of viable cells in culture. For these reasons, we performed Annexin-V assays, as a measure of cellular death, and cell cycle analyses, as an indirect measure of growth (proliferation). Increased cell death and/or increased growth-inhibition are clinically relevant and would provide benefit to the patient.

Collectively, our results indicated that pre-treatment with DHA, as compared to vehicle, enhanced cell death due to doxorubicin in all three cell lines, vincristine in two (JVM-2 and MEC-2) of the three cell lines, and fludarabine in one (EHEB) of the three cell lines. Increased cell death is clinically beneficial and would improve the outcome of the patient receiving therapy.

Noteworthy, MEC-2, which harbors a p53 mutation, showed enhanced cell death due to vincristine or doxorubicin when pre-treated with DHA as compared to vehicle. This is an

important observation. The loss of short arm p13 of chromosome 17, which disrupts the p53 tumor suppressor gene, is found in approximately 5-10% of all CLL patients and is associated with particularly poor prognosis and chemorefractoriness (Gaidano, Foa, & Dalla-Favera, 2012). N-3 may provide a beneficial augment to the treatment of chemorefractory CLL patients.

We performed cell-cycle analyses to determine whether increased chemo-sensitivity by FA was associated with enhanced growth-inhibition. Previous studies have demonstrated that n-3 treatment alone can induce cell cycle arrest at the G2/M phase (Dekoj et al., 2007). Vincristine is a mitotic inhibitor known to induce cell cycle arrest at the M phase (Casado et al., 2009). Similarly, studies have indicated that malignant cells in G2/M arrest are more sensitive to doxorubicin than normal cells (Ling, el-Naggar, Priebe, & Perez-Soler, 1996; Tyagi, Singh, Agarwal, Chan, & Agarwal, 2002). For these reasons, we were particularly interested in the ratio of cells in G1 (G0 + G1) and G2 (G2 + M) phases. An increase in the population of cells in the G2 (G2 + M) phase is indicative of G2/M arrest. Thus, an increase in G2 (G2 + M) would result in a lower population of cells in G1 (G0 + G1) and a lower G1/G2 ratio. A decrease in the G1/G2 ratio (indicative of G2/M arrest) would be expected to result in growth-inhibition (reduced proliferation).

Our results illustrate that cells pre-treated with n-3, but without drug, had significantly greater G2/M arrest, indicated by a lower G1/G2 ratio, as compared to vehicle pre-treated cells (Table 3.1). This demonstrates that n-3 by themselves can potentially slow the growth of malignant B-lymphocytes. This is of considerable interest as we had previously shown that consumption of n-3 decreased the activity of nuclear factor kappa B (NFκB) in isolated lymphocytes of patients with early stage CLL and would be expected to slow the progression of the disease (Fahrman et al., 2013). Studies have shown that inhibition of NFκB activation leads

to cell cycle arrest at the G2/M phase aiding to both growth-inhibition and cell death (Zhang, Ruan, et al., 2012; Zhang, Tao, et al., 2012). Future studies will be aimed in determining if n-3 can slow the progression and growth of CLL and whether growth-inhibition is mediated through suppression of NF κ B activation and G2/M arrest. Slowing the progression of CLL by n-3 FAs could be a therapeutic choice in patients for whom standard chemotherapy is not an option.

The addition of doxorubicin to FA pre-treated cells induced significantly greater G2/M arrest than when cells were not treated with n-3 prior to doxorubicin (Table 3.1). It is interesting to note that cells pre-treated with either EPA (MEC-2) or DHA (EHEB, JVM-2 and MEC-2) which had significantly greater G2/M arrest due to doxorubicin also showed increased chemo-sensitivity to doxorubicin than did cells pre-treated with vehicle (Figures 3.3A, 3.4A and 3.5A). This suggests that n-3 plus doxorubicin induced greater growth-inhibition than doxorubicin alone. This notion is supported by other investigators who have shown that cells in G2/M arrest are more sensitive to doxorubicin as compared to normal cells (Ling et al., 1996; Tyagi et al., 2002) and, importantly, that enhanced sensitivity of cells in G2/M arrest to doxorubicin was mediated through both growth-inhibition and apoptosis (Tyagi et al., 2002).

Similarly, the addition of vincristine (JVM-2 and MEC-2) or fludarabine (EHEB) to cells pre-treated with certain FAs (all FAs in JVM-2, AA and EPA in MEC-2, DHA in EHEB) had significantly greater G2/M arrest as compared to vehicle pre-treated cells (Table 3.1). However, there was no association between the increase in chemo-sensitivity of cells to vincristine (JVM-2 and MEC-2) or fludarabine (EHEB) by FA and the increase in G2/M arrest.

Numerous pre-clinical studies have demonstrated that enhanced chemo-sensitization by n-3, particularly DHA, was dependent on the formation of toxic lipid peroxides and generation of ROS (Begin, Ells, Das, & Horrobin, 1986; Begin, Ells, & Horrobin, 1988; Das & Madhavi,

2011; Germain et al., 1998; Hardman et al., 2001; Kang et al., 2010). We wanted to determine whether the increase in chemo-sensitivity of cells to the anti-cancer drugs by FA was dependent on the induction of oxidative stress. Our results illustrate that n-3 induced significantly higher levels of intracellular ROS than did vehicle in MEC-2 cells (Figure 3.6A). Linear regression analysis indicated an increased rate of ROS generation in the presence of either EPA or DHA as compared to vehicle. However, this effect was not enhanced by the addition of any of the anti-cancer drugs. Results also illustrate that treatment with n-3 alone induced higher levels of TBARS, (products of lipid peroxidation), as compared to vehicle in all three cell lines (only MEC-2 is shown, Figure 3.6B). Only MEC-2 had significantly higher levels of TBARS following treatment with doxorubicin in cells pre-treated with DHA as compared to cells treated with DHA or doxorubicin alone (Figure 3.6B). The addition of vitamin E, a fat soluble anti-oxidant, abrogated the enhanced sensitivity of MEC-2 to doxorubicin by DHA (Figure 3.6C) and decreased the levels of TBARS (Figure 3.6D). The fact that enhanced sensitivity of MEC-2 to doxorubicin by DHA and increased formation of TBARS was abrogated by vitamin E supports the notion that enhanced chemo-sensitivity by DHA is, in part, dependent on the formation of lipid peroxides.

In conclusion, EPA and DHA differentially sensitized B-leukemic cell lines EHEB, JVM-2 and MEC-2 to doxorubicin, vincristine and fludarabine *in vitro*. Enhanced chemo-sensitivity is likely mediated through both increased cellular death as well as growth-inhibition. Our results have shown that enhanced sensitivity is also, in part, dependent on the formation of toxic lipid peroxides. Additional work should be done to elucidate the mechanisms by which n-3 increase chemo-sensitivity. Supplementation of the diet with n-3 fatty acids provides a promising non-toxic approach to not only sensitize CLL cells to anti-cancer drugs but may have

independent therapeutic benefit. Importantly, the chemo-sensitizing effects of n-3 do not appear to be limited to a specific cell-type or a specific drug. Increased chemo-sensitivity is clinically beneficial and would be expected to increase drug efficacy, and potentially reduce drug dosage resulting in decreased drug-induced toxicities.

DECLARATION OF INTEREST

The authors declare that they have no competing interests.

CONTRIBUTIONS

JFF wrote the manuscript, performed all assays and cell culture experiments, analyzed and interpreted data and designed research and received funding with WEH as mentor. WEH critically analyzed the manuscript and performed final approval of manuscript. All authors read and approved the final manuscript.

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REFERENCES

- Begin, M. E., Ells, G., Das, U. N., & Horrobin, D. F. (1986). Differential killing of human carcinoma cells supplemented with n-3 and n-6 polyunsaturated fatty acids. *J Natl Cancer Inst*, 77(5), 1053-1062.
- Begin, M. E., Ells, G., & Horrobin, D. F. (1988). Polyunsaturated fatty acid-induced cytotoxicity against tumor cells and its relationship to lipid peroxidation. *J Natl Cancer Inst*, 80(3), 188-194.
- Borgeson, C. E., Pardini, L., Pardini, R. S., & Reitz, R. C. (1989). Effects of dietary fish oil on human mammary carcinoma and on lipid-metabolizing enzymes. *Lipids*, 24(4), 290-295.
- Casado, P., Prado, M. A., Zuazua-Villar, P., Del Valle, E., Artime, N., Cabal-Hierro, L., . . . Ramos, S. (2009). Microtubule interfering agents and KSP inhibitors induce the phosphorylation of the nuclear protein p54(nrb), an event linked to G2/M arrest. *J Proteomics*, 71(6), 592-600. doi: 10.1016/j.jprot.2008.09.001
- S1874-3919(08)00137-1 [pii]
- Das, U. N., & Madhavi, N. (2011). Effect of polyunsaturated fatty acids on drug-sensitive and resistant tumor cells in vitro. *Lipids Health Dis*, 10, 159. doi: 10.1186/1476-511X-10-159
- 1476-511X-10-159 [pii]
- Dekoj, T., Lee, S., Desai, S., Trevino, J., Babcock, T. A., Helton, W. S., & Espat, N. J. (2007). G2/M cell-cycle arrest and apoptosis by n-3 fatty acids in a pancreatic cancer model. *J Surg Res*, 139(1), 106-112. doi: S0022-4804(06)00559-2 [pii]
- 10.1016/j.jss.2006.10.024
- Fahrman, J. F., Ballester, O. F., Ballester, G., Witte, T. R., Salazar, A. J., Kordusky, B., . . . Hardman, W. E. (2013). Inhibition of Nuclear Factor Kappa B activation in Early-Stage Chronic Lymphocytic Leukemia by Omega-3 Fatty Acids. *Cancer Invest*, 31(1), 29-43. doi: 10.3109/07357907.2012.743553
- Foran, J. M., Oscier, D., Orchard, J., Johnson, S. A., Tighe, M., Cullen, M. H., . . . Lister, T. A. (1999). Pharmacokinetic study of single doses of oral fludarabine phosphate in patients with "low-grade" non-Hodgkin's lymphoma and B-cell chronic lymphocytic leukemia. *J Clin Oncol*, 17(5), 1574-1579.
- Gaidano, G., Foa, R., & Dalla-Favera, R. (2012). Molecular pathogenesis of chronic lymphocytic leukemia. *J Clin Invest*, 122(10), 3432-3438. doi: 10.1172/JCI64101
- 64101 [pii]

- Germain, E., Chajes, V., Cognault, S., Lhuillery, C., & Bougnoux, P. (1998). Enhancement of doxorubicin cytotoxicity by polyunsaturated fatty acids in the human breast tumor cell line MDA-MB-231: relationship to lipid peroxidation. *Int J Cancer*, *75*(4), 578-583. doi: 10.1002/(SICI)1097-0215(19980209)75:4<578::AID-IJC14>3.0.CO;2-5 [pii]
- Guilhaumou, R., Solas, C., Bourgarel-Rey, V., Quaranta, S., Rome, A., Simon, N., . . . Andre, N. (2011). Impact of plasma and intracellular exposure and CYP3A4, CYP3A5, and ABCB1 genetic polymorphisms on vincristine-induced neurotoxicity. *Cancer Chemother Pharmacol*, *68*(6), 1633-1638. doi: 10.1007/s00280-011-1745-2
- Hardman, W. E., Avula, C. P., Fernandes, G., & Cameron, I. L. (2001). Three percent dietary fish oil concentrate increased efficacy of doxorubicin against MDA-MB 231 breast cancer xenografts. *Clin Cancer Res*, *7*(7), 2041-2049.
- Kang, K. S., Wang, P., Yamabe, N., Fukui, M., Jay, T., & Zhu, B. T. (2010). Docosahexaenoic acid induces apoptosis in MCF-7 cells in vitro and in vivo via reactive oxygen species formation and caspase 8 activation. *PLoS One*, *5*(4), e10296. doi: 10.1371/journal.pone.0010296
- Kinsella, J. E., & Black, J. M. (1993). Effects of polyunsaturated fatty acids on the efficacy of antineoplastic agents toward L5178Y lymphoma cells. *Biochem Pharmacol*, *45*(9), 1881-1887. doi: 0006-2952(93)90447-5 [pii]
- Leporrier, M., Chevret, S., Cazin, B., Boudjerra, N., Feugier, P., Desablens, B., . . . Chastang, C. (2001). Randomized comparison of fludarabine, CAP, and ChOP in 938 previously untreated stage B and C chronic lymphocytic leukemia patients. *Blood*, *98*(8), 2319-2325.
- Ling, Y. H., el-Naggar, A. K., Priebe, W., & Perez-Soler, R. (1996). Cell cycle-dependent cytotoxicity, G2/M phase arrest, and disruption of p34cdc2/cyclin B1 activity induced by doxorubicin in synchronized P388 cells. *Mol Pharmacol*, *49*(5), 832-841.
- Muller, C., Chatelut, E., Gualano, V., De Forni, M., Huguet, F., Attal, M., . . . Laurent, G. (1993). Cellular pharmacokinetics of doxorubicin in patients with chronic lymphocytic leukemia: comparison of bolus administration and continuous infusion. *Cancer Chemother Pharmacol*, *32*(5), 379-384.
- Riches, J. C., Ramsay, A. G., & Gribben, J. G. (2011). Chronic lymphocytic leukemia: an update on biology and treatment. *Curr Oncol Rep*, *13*(5), 379-385. doi: 10.1007/s11912-011-0188-6
- Schriever, F., & Huhn, D. (2003). New directions in the diagnosis and treatment of chronic lymphocytic leukaemia. *Drugs*, *63*(10), 953-969. doi: 63103 [pii]
- Shao, Y., Pardini, L., & Pardini, R. S. (1997). Intervention of transplantable human mammary carcinoma MX-1 chemotherapy with dietary menhaden oil in athymic mice: increased

therapeutic effects and decreased toxicity of cyclophosphamide. *Nutr Cancer*, 28(1), 63-73. doi: 10.1080/01635589709514554

Silber, R., Degar, B., Costin, D., Newcomb, E. W., Mani, M., Rosenberg, C. R., . . . Potmesil, M. (1994). Chemosensitivity of lymphocytes from patients with B-cell chronic lymphocytic leukemia to chlorambucil, fludarabine, and camptothecin analogs. *Blood*, 84(10), 3440-3446.

Simopoulos, A. P. (1991). Omega-3 fatty acids in health and disease and in growth and development. *Am J Clin Nutr*, 54(3), 438-463.

Simopoulos, A. P. (2000). Human requirement for N-3 polyunsaturated fatty acids. *Poult Sci*, 79(7), 961-970.

Simopoulos, A. P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother*, 56(8), 365-379.

Simopoulos, A. P. (2003). Importance of the ratio of omega-6/omega-3 essential fatty acids: evolutionary aspects. *World Rev Nutr Diet*, 92, 1-22.

Tyagi, A. K., Singh, R. P., Agarwal, C., Chan, D. C., & Agarwal, R. (2002). Silibinin strongly synergizes human prostate carcinoma DU145 cells to doxorubicin-induced growth inhibition, G2-M arrest, and apoptosis. *Clin Cancer Res*, 8(11), 3512-3519.

Van der Jagt, R., Laneuville, P., Macdonald, D., Stewart, D., Christofides, A., & Sehn, L. H. (2012). A Canadian perspective on bendamustine for the treatment of chronic lymphocytic leukemia and non-Hodgkin lymphoma. *Curr Oncol*, 19(3), 160-168. doi: 10.3747/co.19.1064

conc-19-160 [pii]

Witte, T. R., Salazar, A. J., Ballester, O. F., & Hardman, W. E. (2010). RBC and WBC fatty acid composition following consumption of an omega 3 supplement: lessons for future clinical trials. *Lipids Health Dis*, 9, 31. doi: 10.1186/1476-511X-9-31

1476-511X-9-31 [pii]

Zhang, L., Ruan, J., Yan, L., Li, W., Wu, Y., Tao, L., . . . Lu, Y. (2012). Xanthatin induces cell cycle arrest at G2/M checkpoint and apoptosis via disrupting NF-kappaB pathway in A549 non-small-cell lung cancer cells. *Molecules*, 17(4), 3736-3750. doi: 10.3390/molecules17043736

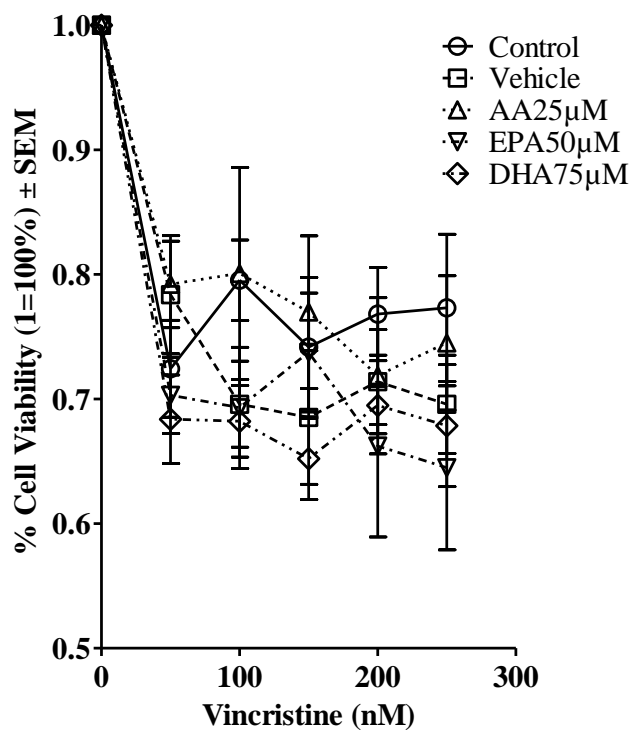
molecules17043736 [pii]

Zhang, L., Tao, L., Ruan, J., Li, W., Wu, Y., Yan, L., . . . Lu, Y. (2012). Xanthatin induces G2/M cell cycle arrest and apoptosis in human gastric carcinoma MKN-45 cells. *Planta Med*, 78(9), 890-895. doi: 10.1055/s-0031-1298481

APPENDIX

EHEB

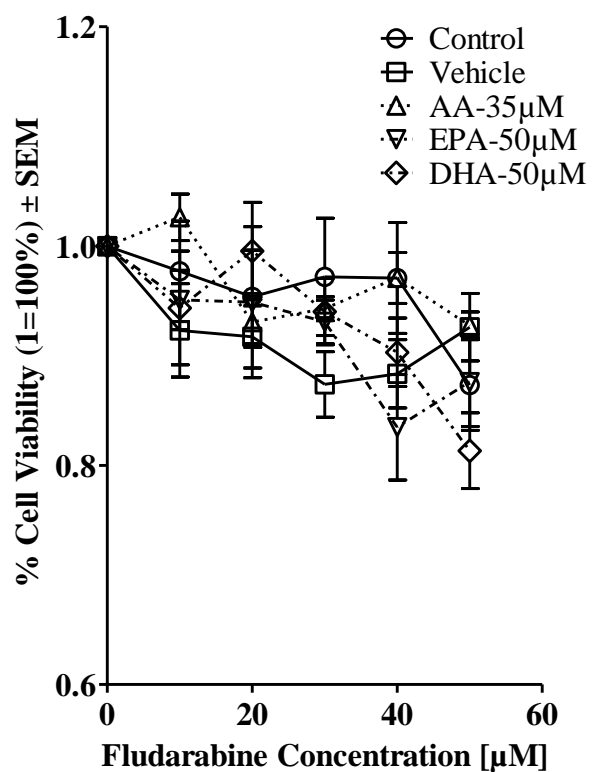
Live Cell Fraction (compared to no Vincristine)



Supplementary Figure 3. 1. *In Vitro* sensitivity of EHEB following treatment with vincristine in the presence or absence of vehicle, AA, EPA or DHA.

The % cell viability \pm SEM of EHEB to vincristine (0-250nM) in the presence or absence of vehicle, AA25µM, EPA50µM or DHA75µM is shown. Pre-treatment with AA, EPA or DHA did not induce any significant differences in the sensitivity of EHEB cells to vincristine.

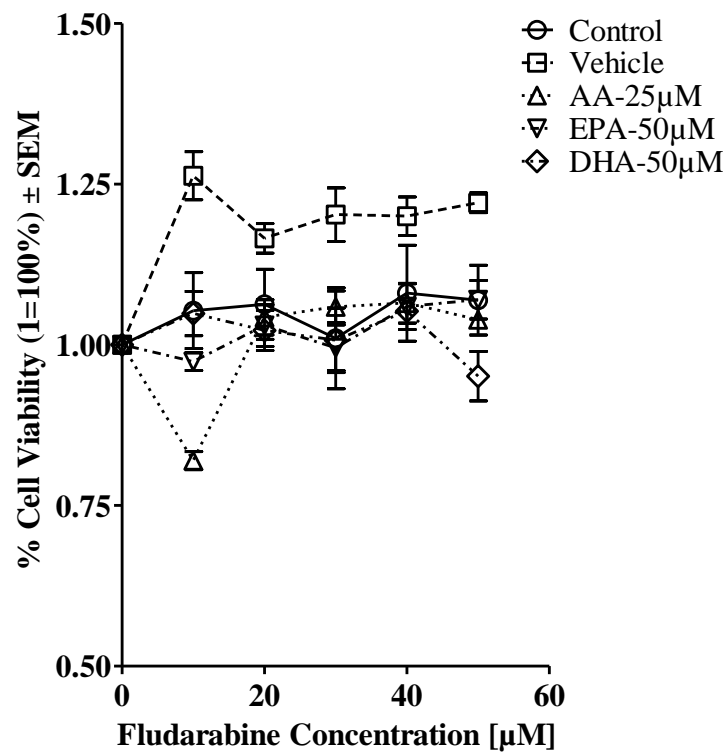
JVM-2
Live Cell Fraction (Compared to no Fludarabine)



Supplementary Figure 3. 2. *In Vitro* sensitivity of JVM-2 following treatment with fludarabine in the presence or absence of vehicle, AA, EPA or DHA.

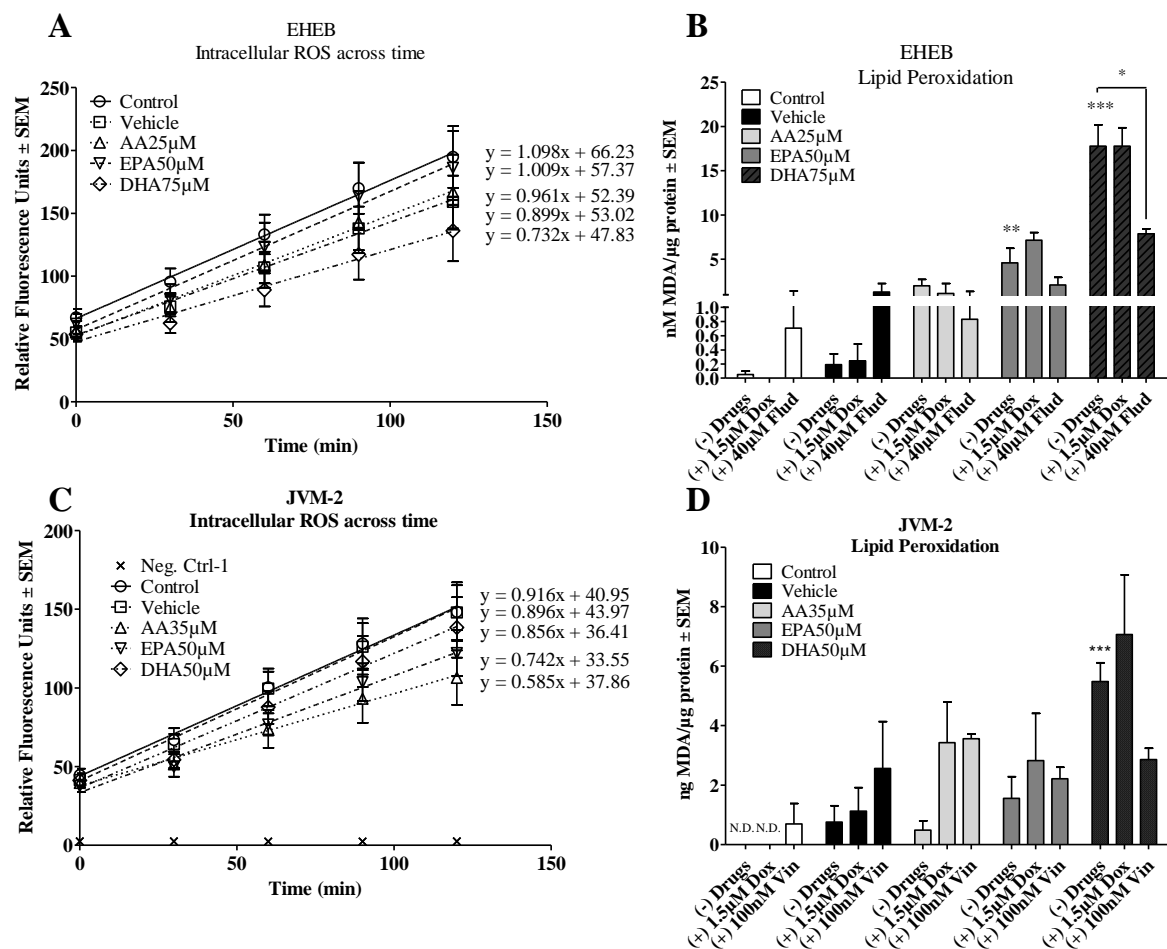
The % cell viability \pm SEM of JVM-2 to fludarabine (0-40 μ M) in the presence or absence of vehicle, AA35 μ M, EPA50 μ M or DHA50 μ M is shown. Pre-treatment with AA, EPA or DHA did not induce any significant differences in the sensitivity of JVM-2 cells to fludarabine.

MEC-2
Live Cell Fraction (compared to no Fludarabine)



Supplementary Figure 3. 3. *In Vitro* sensitivity of MEC-2 following treatment with fludarabine in the presence or absence of vehicle, AA, EPA or DHA.

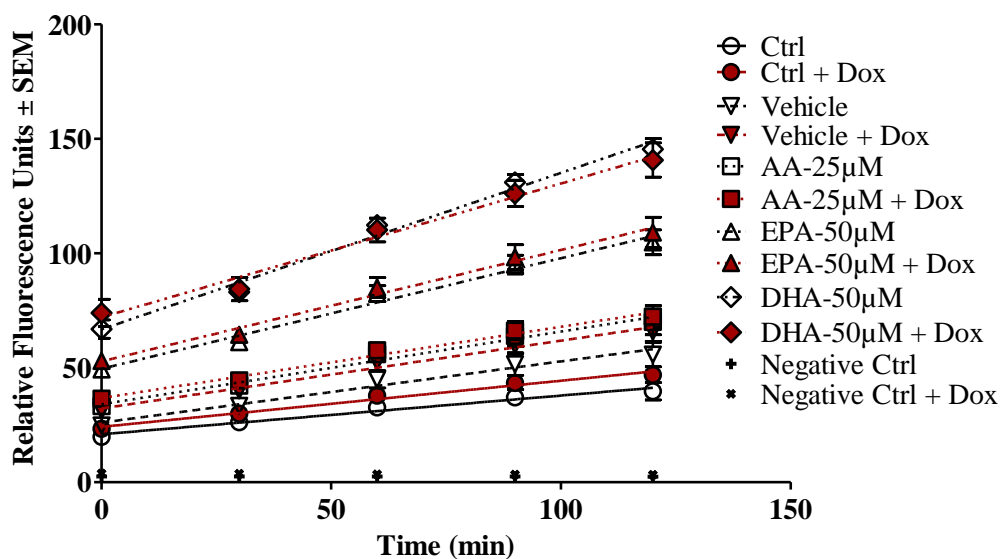
The % cell viability \pm SEM of MEC-2 to fludarabine (0-40µM) in the presence or absence of vehicle, AA25µM, EPA50µM or DHA50µM is shown. Pre-treatment with AA, EPA or DHA did not induce any significant differences in the sensitivity of MEC-2 cells to fludarabine.



Supplementary Figure 3.4. Effects of FA pre-treatment alone and followed by treatment with anti-cancer drugs on the formation of lipid peroxides and generation of ROS in EHEB and JVM-2.

Supplementary Figure 3.4A illustrates mean relative fluorescence units \pm SEM of EHEB over the course of 2 hours following 72 hour pre-treatment with vehicle, AA25 μ M, EPA50 μ M or DHA75 μ M. Intracellular ROS was determined using 5-(and-6-)chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H₂DCFDA) dye. Compared to vehicle, pre-treatment with FA did not induce any significant changes in the levels or generation of ROS. **Supplementary Figure 3.4B** illustrates the mean ng MDA/ μ g protein \pm SEM of EHEB in the presence or absence of vehicle, AA25 μ M, EPA50 μ M or DHA75 μ M alone and following treatment with doxorubicin (1.5 μ M) or fludarabine (40 μ M). Pre-treatment with either EPA or DHA alone induced significantly higher levels of MDA as compared to vehicle. The addition of doxorubicin to FA pre-treated cells did not induced any significantly changes in the levels of MDA versus either alone. The addition of fludarabine to DHA pre-treated cells induced significantly lower levels of MDA as compared to DHA or drug alone. **Supplementary Figure 3.4C** illustrates mean relative fluorescence units \pm SEM of JVM-2 over the course of 2 hours following 72 hour pre-treatment with vehicle, AA35 μ M, EPA50 μ M or DHA50 μ M. Compared to vehicle, pre-treatment with FA did not induce any significant changes in the levels or generation of ROS. **Supplementary Figure 3.4D** illustrates the mean ng MDA/ μ g protein \pm SEM of JVM-2 in the presence or absence of vehicle, AA35 μ M, EPA50 μ M or DHA50 μ M alone and following treatment with doxorubicin (1.5 μ M) or vincristine (100nM). Pre-treatment with DHA alone induced significantly higher levels of MDA as compared to vehicle. The addition of either doxorubicin or vincristine to FA pre-treated cells did not induce any significant differences in levels of MDA as compared to FA alone. Statistical significance was determined by Multiple Comparison Test with Dunnet's correction. Abbreviations: MDA: malondialdehyde, $\alpha = 0.05$, * <0.05, **<0.01, ***< 0.001.; N.D.: no detect.

MEC-2
Intracellular ROS across time



Supplementary Figure 3. 5. The addition of doxorubicin to FA pre-treated MEC-2 cells does not increase the generation of ROS versus either alone.

Mean relative fluorescence units \pm SEM of MEC-2 in the presence or absence of AA25µM, EPA50µM or DHA50µM alone and following treatment with 1.5µM doxorubicin is shown. The addition of doxorubicin to FA pre-treated cells did not increase the levels or generation of ROS versus FA alone. Abbrev. Dox: doxorubicin.

CHAPTER 4: DISCUSSIONS AND CONCLUSIONS

The body of work performed and compiled in the previous chapters highlights the potential applications of n-3 as therapeutic options (both as adjuvants and individual treatments) for the treatment of CLL. In this context, it is important to remember the over-arching goal of these projects. It should be recalled that CLL is the most common form of adult leukemia in the western world (Gaidano, Foa, & Dalla-Favera, 2012). CLL is a disease of the elderly with a median age range of 60-68 years (Shanafelt & Call, 2004). CLL is typically diagnosed in the asymptomatic stages where “watchful waiting” remains the primary course of treatment (Shanafelt & Call, 2004). However, approximately 50% of these patients will progress to symptomatic stage and require therapy (Shanafelt & Call, 2004). Although numerous therapies exist for CLL, most are undermined due to progressive drug resistance which can manifest during the course of treatment and severe drug-induced toxicities which are often too toxic for the elderly or those with co-morbidities (Shanafelt & Call, 2004). Therefore, a non-toxic therapeutic intervention which could slow the progression of the disease or enhance the chemo-sensitivity of malignant cells to chemotherapy would be clinically beneficial. Slowing progression of the early stages of the disease would provide improved quality of life and clinical benefit. Increased chemo-sensitivity for individuals who require therapy would increase drug efficacy and potentially reduce drug dosage, thereby reducing drug-induced toxicities. Similarly, increased tolerability of therapy regimens may also increase the number of chemotherapy cycles a patient can tolerate, thereby increasing the likelihood of receiving a response.

Several studies have demonstrated that NF κ B can promote oncogenesis by virtue of its ability to regulate the expression of a wide array of genes that modulate apoptosis, proliferation, inflammation, tumor metastasis, and angiogenesis (Clevers, 2004). Constitutively active NF κ B

has been observed in various solid tumors as well as hematological malignancies (Braun et al., 2006; Darnell, 2002; Ditsworth & Zong, 2004) and has been linked to aggressive tumor growth and resistance to both chemotherapy and radiotherapy (Li & Sethi, 2010). Consistent with these thoughts, NF κ B has been found to be constitutively active in CLL cells (Hewamana, Alghazal, et al., 2008; Hewamana, Lin, et al., 2008; Hewamana et al., 2009). Studies by Hewamana et al. indicated that increased binding of Rel A, a subunit of NF κ B which contains the transactivation domain, was positively correlated with WBC counts, a measure of tumor burden, and lymphocyte doubling time, a measure of tumor kinetics (Hewamana, Alghazal, et al., 2008). Furthermore, Hewamana et al. also demonstrated that Rel A DNA binding was strongly associated with Binet staging, predictive of time to initial treatment and subsequent treatment, and predictive of overall survival (Hewamana et al., 2009). Similarly, we found that NF κ B activation was positively correlated with WBC counts (Appendix, Supplementary Figure 2.2). Additionally, we found that patients who displayed ‘lower initial NF κ B activation’ had significantly lower absolute lymphocyte counts (Mean \pm SEM: $11.9 \pm 3.2 \times 10^3$ lymphocytes/ μ l vs $28.8 \pm 4.0 \times 10^3$ lymphocytes/ μ l; p: 0.009; Table 2.2) and tended to be at lower Rai stage (stage 0, 84% vs 58%, not statistically different by Fisher exact test) than patients exhibiting ‘higher initial NF κ B activation’ at baseline. Collectively, our results support the observations of Hewamana et al. and indicate the prognostic value of NF κ B activity.

Rel A DNA binding has also been shown to be negatively correlated with induction of spontaneous apoptosis *in vitro* suggesting that NF κ B is an important mediator of survival (Hewamana, Alghazal, et al., 2008). This is supported by the fact that inhibition of NF κ B activation, through the use of an irreversible-IKK inhibitor (LC-1), induced dose-responsive increases in apoptosis (Hewamana, Lin, et al., 2008). The addition of LC-1 was also able to

overcome the cytoprotective effects of CD40L and IL-4, upstream agonists of NF κ B activation, providing further support of the importance of NF κ B in CLL (Hewamana, Lin, et al., 2008). Inhibition of NF κ B activation by LC-1 was also shown to exhibit strong synergy with fludarabine, a commonly used first-line treatment in CLL (Riches, Ramsay, & Gribben, 2011; Silber et al., 1994), in both good prognostic CLL patients who were fludarabine-sensitive and poor prognostic CLL patients who displayed chemorefractoriness toward fludarabine (Hewamana, Lin, et al., 2008). Given the ability of NF κ B to promote survival of CLL cells *in vitro* and aid in various biological processes such as proliferation, migration, inhibition of apoptosis and chemo-resistance, a therapeutic interventions which target NF κ B would be of considerable interest and clinically relevant.

In our initial study, we demonstrated that consumption of an n-3 supplement, predominately composed of EPA and DHA, suppressed activation of NF κ B in lymphocytes isolated from patients in the early stages of CLL (Figures 2.2. and 2.3). Gene expression profiles indicated that consumption of n-3 decreased the expression of 31 genes including genes known to be regulated by NF κ B (JMJD1C, ZNF644 and PTGS2; Table 2.4). Suppression of genes known to be regulated by NF κ B supports our findings that n-3 suppressed NF κ B activation. We also found that, in patients who were unwilling to take 7.2 g of n-3 per day, over time either 4.8 g or 2.4 g of n-3 per day also suppressed NF κ B to nearly normal levels. This provides an important piece of information as it indicates that a lower consumption of n-3 can provide equal benefit as a higher dose over a period of time. A lower dose would aid in study design allowing for increased compliancy rates and a decreased likelihood of adverse effects associated with n-3 consumption.

In line with this thought, despite plasma concentrations of EPA and DHA being highest following consumption of 7.2 g of n-3/per day for a month or more, these concentrations did not differ statistically from that following consumption of 4.8 g (6 capsules; composition: 2.4 g of EPA, 1.8 g of DHA, 0.6 g of other n-3) of n-3/day (Table 2.3). This suggests that plasma concentrations of EPA and DHA reached a state of saturation and that saturated concentrations of EPA or DHA can be achieved at lower concentrations. Our findings are supported by Yee et al. who indicated that a dose of ~2.5g/day EPA+DHA achieved the maximum possible elevation of concentrations in mammary tissue and blood in a supplementation study (Yee et al., 2010). Similarly, Arterburn et al. indicated that supplementation with 2g/day DHA resulted in plasma saturation (Arterburn, Hall, & Oken, 2006). Thus, consumption of a lower concentration of n-3 over time could achieve similar effects as those observed at higher concentrations supporting our findings that either 2.4 g or 4.8 g of n-3 per day can also suppress NFκB activation.

Evidence by our group and others (Hewamana, Alghazal, et al., 2008; Hewamana, Lin, et al., 2008; Hewamana et al., 2009) suggest that activated NFκB correlates with disease progression. Therefore, inhibition of NFκB activation would be expected to slow the progression of CLL. Our results justify expansion into a definitive randomized clinical trial aimed to determine whether consumption of n-3 will slow the progression of early stage CLL to symptomatic CLL and whether clinical benefit is correlated with suppression of NFκB activation. Given that CLL is a heterogeneous disease with a highly variable course of progression (Eichhorst, Dreyling, Robak, Montserrat, & Hallek, 2011; Gaidano et al., 2012); aim should be taken to target patients who are at risk of progressing quickly. This would allow for identification of potential clinical benefit in a short period of time, thereby preventing the use of long multi-year studies.

Although we have demonstrated that n-3 suppressed NFκB activation in lymphocytes from our patient population, potential benefit from n-3 is likely multi-factorial, a trait provided by the diversity of the various n-3 metabolites. In this sense, the application of metabolomics coupled with lipidomics and genomic studies should be explored. The application of these types of profiling studies may provide key insight into the functionality and potential benefits of n-3 consumption.

Increasing evidence suggests that leukaemogenesis can be explained by epigenetic alterations (Chen, Odenike, & Rowley, 2010; Galm, Herman, & Baylin, 2006). Epigenetic changes, which include DNA methylation, histone modifications and microRNAs, alter gene expression without changing DNA sequences. Histone modifications alter gene expression through changes in chromatin composition and conformation. Our results indicated that consumption of n-3 reduced mRNA abundance of multiple genes associated with chromatin remodeling (Table 2.3). These genes are JMJD1C (JmjC domain-containing histone demethylation protein 2C) (Kato & Katoh, 2007), CHD9 (a chromatin remodeling transcription regulator) (Marom, Shur, Hager, & Benayahu, 2006; Shur, Socher, & Benayahu, 2006), ASH1L (a histone methyltransferase) (An, Yeo, Jeon, & Song, 2011) and PKN2 (a histone deacetylase 5 interacting protein) (Harrison et al., 2010). Current studies are underway to determine whether n-3, EPA, induces changes in histone post-translational modifications (PTMs) in lymphocytes isolated from patients with CLL and how changes in PTMs correlate to changes in mRNA levels. The intent of this study is to provide novel insight into specific epigenetic changes induced by n-3 consumption and the genes that are influenced by those changes. Future studies will be aimed at determining whether epigenetic changes induced by n-3 in CLL cells will correlate with increased clinical benefit.

Our results also indicated that consumption of n-3 increased the sensitivity of lymphocytes from patients with early stage CLL to doxorubicin in an *in vitro* assay. These findings prompted us to further explore the chemo-sensitizing capabilities of n-3. In our second study, we evaluated whether n-3, EPA and/or DHA, would enhance the chemo-sensitivity of CLL-derived cell lines EHEB and MEC-2 and PLL-derived cell line JVM-2 to actively used chemo-therapeutic drugs doxorubicin, vincristine and/or fludarabine *in vitro*.

Results from these studies indicated that EPA and DHA generally, but not equally, sensitized B-leukemic cells to actively used chemotherapeutic drugs: doxorubicin and vincristine, components of the CHOP regime (Leporrier et al., 2001; Van der Jagt et al., 2012), or fludarabine, a commonly used first-line treatment option for CLL (Riches et al., 2011; Silber et al., 1994). Importantly, we demonstrated that the chemo-sensitizing capabilities of n-3 were not specific towards one specific cell sub-type, but rather, multiple sub-types. This is a desirable trait given the vast heterogeneity that exists amongst CLL patients (Gaidano et al., 2012; Riches et al., 2011). Similarly, the fact that n-3 enhanced the chemo-sensitivity of B-leukemic cells to three different anti-cancer drugs, whose cytotoxic effects are mediated through different mechanisms of action, highlights the versatility and potential applicability of n-3 as adjuvant therapies.

Even though n-3 generally increased the chemo-sensitivity of B-leukemic cells to anti-cancer drugs, cells pre-treated with AA generally indicated no difference in chemo-sensitivity as compared to vehicle. Inversely, in EHEB, pre-treatment with AA appeared to protect against the cytotoxic effects of fludarabine; however, this was not statistically significant (Figure 3.3B). Collectively, this indicates that the sensitizing capacities of FAs are more likely to be found amongst the n-3 FA rather than the n-6 FA. This is an important consideration as the western diet

is heavily favored towards n-6 with little to no n-3 consumption (Simopoulos, 2008). N-3 and n-6 compete with each other for incorporation and metabolism, often times having antagonistic actions (Simopoulos, 2002). The addition of n-3 as an adjuvant to therapy may, therefore, provide clinical benefit to patients receiving therapy.

Results from our second study also indicated that enhanced chemo-sensitivity of B-leukemic cells to anti-cancer drugs by n-3, particularly DHA, was associated with an increase in G2/M arrest (indicative of growth-inhibition) (Table 3.1) and increased cell death (Figures 3.3C, 3.4C and 3.5C). Slowing the growth and/or increasing cell death of malignant cells is highly desirable and would provide clinical benefit.

Notably, MEC-2, which harbors a p53 mutation, showed enhanced cell death due to vincristine or doxorubicin when pre-treated with DHA as compared to vehicle. The loss of short arm p13 of chromosome 17, which disrupts the p53 tumor suppressor gene, is found in approximately 5-10% of all CLL patients and is associated with particularly poor prognosis and chemo-refractoriness (Gaidano et al., 2012). N-3 may provide a beneficial augment to the treatment of chemo-refractory CLL patients.

We have also demonstrated that enhanced chemo-sensitivity of MEC-2 cells to doxorubicin by DHA was, in part, mediated through generation of ROS and formation of lipid peroxides (Figure 3.6). Selective enhancement of chemo-sensitivity of EHEB, MEC-2 and JVM-2 to anti-cancer drugs by n-3 that was not dependent on increased lipid peroxidation and generation of ROS indicates alternative mechanism(s) through which n-3 enhance chemo-sensitivity.

Collectively, results from our initial study and subsequent study highlight the potential application of n-3 as therapeutic options for the treatment of CLL and provided key data to

support expansion into our current clinical trial. The primary aim of our current study is to determine whether n-3 EPA and/or DHA increase the chemo-sensitivity of lymphocytes isolated from patients with CLL who 1) have a WBC count ≥ 17 ($10^3/\mu\text{L}$) or have progressed to the point of requiring therapy but have not received therapy or 2) have relapsed post standard first line therapy and require second-line therapy but have not received second-line therapy to anti-cancer drugs fludarabine, vincristine or chlorambucil *in vitro*. Secondary objectives of this study are to 1) evaluate whether increased chemo-sensitivity of cells to anti-cancer drugs by n-3 is mediate, in part, through suppression of NF κ B activation and 2) demonstrate that increased chemo-sensitivity is selective against the malignant cells and not healthy cells. While the data obtained thus far is limited, the results are encouraging. The primary goal of this study is to provide the final data necessary for expansion into a definitive clinical trial aimed to determine whether consumption of n-3 will improve the efficacy of chemo-therapy in CLL patients who require treatment. Future studies will also be aimed at evaluating the safety of n-3 as an adjuvant therapy.

Whereas transitions to clinical studies are the primary focus of the studies highlighted in this thesis, further studies evaluating the mechanism(s) of actions through which n-3 induce growth-inhibitory effects, increased cell death and/or enhanced chemo-sensitivity are also areas of importance. Mechanistic studies will provide a further understanding of how n-3 function as therapeutic options. In this sense the following areas of research are of particular interest:

Studies have indicated that malignant cells in G2/M arrest are more sensitive to doxorubicin than normal cells (Ling, el-Naggar, Priebe, & Perez-Soler, 1996; Tyagi, Singh, Agarwal, Chan, & Agarwal, 2002). Similarly, vincristine is a mitotic inhibitor known to induce cell cycle arrest at the M phase (Casado et al., 2009). Studies have demonstrated that inhibition

of NFκB activation leads to cell cycle arrest at the G2/M phase aiding to both growth-inhibition and cell death (Zhang, Ruan, et al., 2012; Zhang, Tao, et al., 2012). Our initial study demonstrated that consumption of n-3 reduced activation of NFκB in lymphocytes of early stage CLL patients. In our second study, we demonstrated that n-3, particularly DHA, induced G2/M arrest alone and indicated greater G2/M arrest when used with anti-cancer drugs versus anti-cancer drugs alone. Future studies should be conducted to determine whether the ability of n-3 to induce arrest at G2/M is mediated through suppression of NFκB activation and how this proposed mechanism influences the chemo-sensitizing capabilities of n-3.

Multi-drug resistance remains one of the primary causes of suboptimal outcomes in cancer therapy and is a major target of intervention (Gottesman, Fojo, & Bates, 2002). P-glycoprotein (Pgp), encoded by MDR1 (ABCB1) gene, is an ABC transporter shown to confer resistance to certain chemotherapeutic drugs including anthracyclines and alkaloids (Gottesman et al., 2002; Leonard, Fojo, & Bates, 2003; Sonneveld et al., 1992). MDR1 has been shown to be overexpressed in CLL and correlate with increased Pgp activity (Consoli et al., 2002; Hoellein et al., 2010; Sparrow, Hall, Siregar, & Van der Weyden, 1993; Szendrei et al., 2008). Additionally, MDR1 was particularly found to be elevated in patients with advanced stages of the disease and correlated with clinical drug resistance (Hoellein et al., 2010; Sparrow et al., 1993; Szendrei et al., 2008). Similarly, multidrug-resistance-associated protein 1 (MRP1), another type of ABC transporter, has been shown to be increased in CLL (Hoellein et al., 2010) and confer drug resistance *in vitro* (Gottesman et al., 2002). MRP1 (ABCC1) expression, like MDR1, has also been negatively correlated with response to therapy and survival in clinical settings (Gottesman et al., 2002). MRP1's mode of action is primarily by transporting glutathione and glutathione-conjugated anti-cancer drugs (Gottesman et al., 2002; Keppler, 1999). MDR1 expression is

known to be regulated through COX-2 (Liu, Qu, & Tao, 2010; Sorokin, 2004) and NFκB (Wang, Liu, Zhang, Yu, & Wang, 2007; Zatelli et al., 2009). MRP1 expression has also been shown to be strongly correlated with COX-2 expression (Szczeraszek et al., 2009). Studies have illustrated that inhibition of COX-2 and NFκB lead to decreased expression of MDR1 mRNA and Pgp resulting in increased sensitivity of solid tumors and hematologic malignancies to chemotherapeutic drugs *in vitro* (Wang et al., 2007; Zatelli et al., 2009). Similarly, studies utilizing COX-2 inhibitors indicated decreased expression of MRP1 and enhanced cytotoxicity of anticancer drugs *in vitro* (Liu et al., 2010). We have demonstrated that consumption of n-3 suppressed activation of NFκB and reduced mRNA abundance of COX-2 in lymphocytes isolated from early stage CLL patients (Table 2.4). Future studies evaluating whether n-3 can suppress the expression and/or the efflux capabilities of MDR1/MRP1 and whether this is through suppression of NFκB activation and/or COX-2 are of considerable interest. Additionally, studies have demonstrated that n-3 can deplete glutathione pools (Merendino et al., 2003). Glutathione is a key component for the functional activity of MRP-mediated efflux (Gottesman et al., 2002; Keppler, 1999). Therefore, depletion of glutathione pools by n-3 may inhibit the efflux capabilities of MRPs, thereby increasing the efficacy of certain anti-cancer drugs. Future studies should be performed to explore whether n-3 can deplete glutathione pools and whether this will reduce the functional capabilities of MRPs and aid to increased anti-cancer drug efficacy. The potential application of n-3 to combat multi-drug resistance remains of considerable importance and warrants investigation.

In conclusion, the application of n-3 as therapeutic options for the treatment of CLL is very promising and presents several advantages over conventional therapy. N-3 are non-toxic (dose-dependent) and have the potential of slowing the growth of cancer, increasing the chemo-

sensitivity of malignant cells to chemo- and radio-therapy and reducing toxicities. Slowing the growth of cancer would prolong the time before treatment is required and improve the quality of life for the individual. Increased chemo-sensitivity would lead to increased drug efficacy and potential decreases in drug dosage thereby leading to reduced drug-induced toxicities. Similarly, reducing therapy-induced toxicities by n-3 can lead to potential increases in the amount of cycles a patient can tolerate, thereby increasing the likelihood of receiving a response. Therefore, the use of n-3 as therapeutic options for CLL warrants further attention and presents a novel therapeutic approach.

REFERENCES

- An, S., Yeo, K. J., Jeon, Y. H., & Song, J. J. (2011). Crystal structure of the human histone methyltransferase ASH1L catalytic domain and its implications for the regulatory mechanism. *J Biol Chem*, 286(10), 8369-8374. doi: 10.1074/jbc.M110.203380
- Arterburn, L. M., Hall, E. B., & Oken, H. (2006). Distribution, interconversion, and dose response of n-3 fatty acids in humans. *Am J Clin Nutr*, 83(6 Suppl), 1467S-1476S.
- Braun, T., Carvalho, G., Fabre, C., Grosjean, J., Fenaux, P., & Kroemer, G. (2006). Targeting NF-kappaB in hematologic malignancies. *Cell Death Differ*, 13(5), 748-758. doi: 10.1038/sj.cdd.4401874
- Casado, P., Prado, M. A., Zuazua-Villar, P., Del Valle, E., Artime, N., Cabal-Hierro, L., . . . Ramos, S. (2009). Microtubule interfering agents and KSP inhibitors induce the phosphorylation of the nuclear protein p54(nrb), an event linked to G2/M arrest. *J Proteomics*, 71(6), 592-600. doi: 10.1016/j.jprot.2008.09.001
- S1874-3919(08)00137-1 [pii]
- Chen, J., Odenike, O., & Rowley, J. D. (2010). Leukaemogenesis: more than mutant genes. *Nat Rev Cancer*, 10(1), 23-36. doi: 10.1038/nrc2765
- Clevers, H. (2004). At the crossroads of inflammation and cancer. *Cell*, 118(6), 671-674. doi: 10.1016/j.cell.2004.09.005
- Consoli, U., Santonocito, A., Stagno, F., Fiumara, P., Privitera, A., Parisi, G., . . . Giustolisi, R. (2002). Multidrug resistance mechanisms in chronic lymphocytic leukaemia. *Br J Haematol*, 116(4), 774-780.
- Darnell, J. E., Jr. (2002). Transcription factors as targets for cancer therapy. *Nat Rev Cancer*, 2(10), 740-749. doi: 10.1038/nrc906
- Ditsworth, D., & Zong, W. X. (2004). NF-kappaB: key mediator of inflammation-associated cancer. *Cancer Biol Ther*, 3(12), 1214-1216.
- Eichhorst, B., Dreyling, M., Robak, T., Montserrat, E., & Hallek, M. (2011). Chronic lymphocytic leukemia: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*, 22 Suppl 6, vi50-54. doi: 10.1093/annonc/mdr377
- Gaidano, G., Foa, R., & Dalla-Favera, R. (2012). Molecular pathogenesis of chronic lymphocytic leukemia. *J Clin Invest*, 122(10), 3432-3438. doi: 10.1172/JCI64101
- 64101 [pii]

- Galm, O., Herman, J. G., & Baylin, S. B. (2006). The fundamental role of epigenetics in hematopoietic malignancies. *Blood Rev*, *20*(1), 1-13. doi: 10.1016/j.blre.2005.01.006
- Gottesman, M. M., Fojo, T., & Bates, S. E. (2002). Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer*, *2*(1), 48-58. doi: 10.1038/nrc706
- Harrison, B. C., Huynh, K., Lundgaard, G. L., Helmke, S. M., Perryman, M. B., & McKinsey, T. A. (2010). Protein kinase C-related kinase targets nuclear localization signals in a subset of class IIa histone deacetylases. *FEBS Lett*, *584*(6), 1103-1110. doi: 10.1016/j.febslet.2010.02.057
- Hewamana, S., Alghazal, S., Lin, T. T., Clement, M., Jenkins, C., Guzman, M. L., . . . Pepper, C. (2008). The NF-kappaB subunit Rel A is associated with in vitro survival and clinical disease progression in chronic lymphocytic leukemia and represents a promising therapeutic target. *Blood*, *111*(9), 4681-4689. doi: 10.1182/blood-2007-11-125278
- Hewamana, S., Lin, T. T., Jenkins, C., Burnett, A. K., Jordan, C. T., Fegan, C., . . . Pepper, C. (2008). The novel nuclear factor-kappaB inhibitor LC-1 is equipotent in poor prognostic subsets of chronic lymphocytic leukemia and shows strong synergy with fludarabine. *Clin Cancer Res*, *14*(24), 8102-8111. doi: 10.1158/1078-0432.ccr-08-1673
- Hewamana, S., Lin, T. T., Rowntree, C., Karunanithi, K., Pratt, G., Hills, R., . . . Pepper, C. (2009). Rel a is an independent biomarker of clinical outcome in chronic lymphocytic leukemia. *J Clin Oncol*, *27*(5), 763-769. doi: 10.1200/jco.2008.19.1114
- Hoellein, A., Decker, T., Bogner, C., Oelsner, M., Hauswald, S., Peschel, C., . . . Licht, T. (2010). Expression of multidrug resistance-associated ABC transporters in B-CLL is independent of ZAP70 status. *J Cancer Res Clin Oncol*, *136*(3), 403-410. doi: 10.1007/s00432-009-0670-9
- Katoh, M., & Katoh, M. (2007). Comparative integromics on JMJD1C gene encoding histone demethylase: conserved POU5F1 binding site elucidating mechanism of JMJD1C expression in undifferentiated ES cells and diffuse-type gastric cancer. *Int J Oncol*, *31*(1), 219-223.
- Keppler, D. (1999). Export pumps for glutathione S-conjugates. *Free Radic Biol Med*, *27*(9-10), 985-991.
- Leonard, G. D., Fojo, T., & Bates, S. E. (2003). The role of ABC transporters in clinical practice. *Oncologist*, *8*(5), 411-424.
- Leporrier, M., Chevret, S., Cazin, B., Boudjerra, N., Feugier, P., Desablens, B., . . . Chastang, C. (2001). Randomized comparison of fludarabine, CAP, and ChOP in 938 previously untreated stage B and C chronic lymphocytic leukemia patients. *Blood*, *98*(8), 2319-2325.

- Li, F., & Sethi, G. (2010). Targeting transcription factor NF-kappaB to overcome chemoresistance and radioresistance in cancer therapy. *Biochim Biophys Acta*, 1805(2), 167-180. doi: 10.1016/j.bbcan.2010.01.002
- Ling, Y. H., el-Naggar, A. K., Priebe, W., & Perez-Soler, R. (1996). Cell cycle-dependent cytotoxicity, G2/M phase arrest, and disruption of p34cdc2/cyclin B1 activity induced by doxorubicin in synchronized P388 cells. *Mol Pharmacol*, 49(5), 832-841.
- Liu, B., Qu, L., & Tao, H. (2010). Cyclo-oxygenase 2 up-regulates the effect of multidrug resistance. *Cell Biol Int*, 34(1), 21-25. doi: 10.1042/cbi20090129
- Marom, R., Shur, I., Hager, G. L., & Benayahu, D. (2006). Expression and regulation of CReMM, a chromodomain helicase-DNA-binding (CHD), in marrow stroma derived osteoprogenitors. *J Cell Physiol*, 207(3), 628-635. doi: 10.1002/jcp.20611
- Merendino, N., Molinari, R., Loppi, B., Pessina, G., M, D' Aquino, Tomassi, G., & Velottia, F. (2003). Induction of apoptosis in human pancreatic cancer cells by docosahexaenoic acid. *Ann N Y Acad Sci*, 1010, 361-364.
- Riches, J. C., Ramsay, A. G., & Gribben, J. G. (2011). Chronic lymphocytic leukemia: an update on biology and treatment. *Curr Oncol Rep*, 13(5), 379-385. doi: 10.1007/s11912-011-0188-6
- Shanafelt, T. D., & Call, T. G. (2004). Current approach to diagnosis and management of chronic lymphocytic leukemia. *Mayo Clin Proc*, 79(3), 388-398. doi: 10.4065/79.3.388
- Shur, I., Socher, R., & Benayahu, D. (2006). In vivo association of CReMM/CHD9 with promoters in osteogenic cells. *J Cell Physiol*, 207(2), 374-378. doi: 10.1002/jcp.20586
- Silber, R., Degar, B., Costin, D., Newcomb, E. W., Mani, M., Rosenberg, C. R., . . . Potmesil, M. (1994). Chemosensitivity of lymphocytes from patients with B-cell chronic lymphocytic leukemia to chlorambucil, fludarabine, and camptothecin analogs. *Blood*, 84(10), 3440-3446.
- Simopoulos, A. P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother*, 56(8), 365-379.
- Simopoulos, A. P. (2008). The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp Biol Med (Maywood)*, 233(6), 674-688. doi: 10.3181/0711-mr-311
- Sonneveld, P., Nooter, K., Burghouts, J. T., Herweijer, H., Adriaansen, H. J., & van Dongen, J. J. (1992). High expression of the *mdr3* multidrug-resistance gene in advanced-stage chronic lymphocytic leukemia. *Blood*, 79(6), 1496-1500.
- Sorokin, A. (2004). Cyclooxygenase-2: potential role in regulation of drug efflux and multidrug resistance phenotype. *Curr Pharm Des*, 10(6), 647-657.

Sparrow, R. L., Hall, F. J., Siregar, H., & Van der Weyden, M. B. (1993). Common expression of the multidrug resistance marker P-glycoprotein in B-cell chronic lymphocytic leukaemia and correlation with in vitro drug resistance. *Leuk Res*, 17(11), 941-947.

Szczuraszek, K., Materna, V., Halon, A., Mazur, G., Wrobel, T., Kuliczkowski, K., . . . Surowiak, P. (2009). Positive correlation between cyclooxygenase-2 and ABC-transporter expression in non-Hodgkin's lymphomas. *Oncol Rep*, 22(6), 1315-1323.

Szendrei, T., Magyarlaki, T., Kovacs, G., Nagy, A., Szomor, A., Molnar, L., . . . Losonczy, H. (2008). [Multidrug resistance in chronic lymphocytic leukemia]. *Orv Hetil*, 149(4), 161-167. doi: 10.1556/oh.2008.28203

Tyagi, A. K., Singh, R. P., Agarwal, C., Chan, D. C., & Agarwal, R. (2002). Silibinin strongly synergizes human prostate carcinoma DU145 cells to doxorubicin-induced growth inhibition, G2-M arrest, and apoptosis. *Clin Cancer Res*, 8(11), 3512-3519.

Van der Jagt, R., Laneuville, P., Macdonald, D., Stewart, D., Christofides, A., & Sehn, L. H. (2012). A Canadian perspective on bendamustine for the treatment of chronic lymphocytic leukemia and non-Hodgkin lymphoma. *Curr Oncol*, 19(3), 160-168. doi: 10.3747/co.19.1064

conc-19-160 [pii]

Wang, Y., Liu, X., Zhang, H. T., Yu, M., & Wang, H. (2007). [NF-kappaB regulating expression of *mdr1* gene and P-gp to reverse drug-resistance in leukemic cells]. *Zhongguo Shi Yan Xue Ye Xue Za Zhi*, 15(5), 950-954.

Yee, L. D., Lester, J. L., Cole, R. M., Richardson, J. R., Hsu, J. C., Li, Y., . . . Clinton, S. K. (2010). Omega-3 fatty acid supplements in women at high risk of breast cancer have dose-dependent effects on breast adipose tissue fatty acid composition. *Am J Clin Nutr*, 91(5), 1185-1194. doi: 10.3945/ajcn.2009.29036

Zatelli, M. C., Mole, D., Tagliati, F., Minoia, M., Ambrosio, M. R., & degli Uberti, E. (2009). Cyclo-oxygenase 2 modulates chemoresistance in breast cancer cells involving NF-kappaB. *Cell Oncol*, 31(6), 457-465. doi: 10.3233/clo-2009-0490

Zhang, L., Ruan, J., Yan, L., Li, W., Wu, Y., Tao, L., . . . Lu, Y. (2012). Xanthatin induces cell cycle arrest at G2/M checkpoint and apoptosis via disrupting NF-kappaB pathway in A549 non-small-cell lung cancer cells. *Molecules*, 17(4), 3736-3750. doi: 10.3390/molecules17043736

molecules17043736 [pii]

Zhang, L., Tao, L., Ruan, J., Li, W., Wu, Y., Yan, L., . . . Lu, Y. (2012). Xanthatin induces G2/M cell cycle arrest and apoptosis in human gastric carcinoma MKN-45 cells. *Planta Med*, 78(9), 890-895. doi: 10.1055/s-0031-1298481

APPENDIX 1

OFFICE OF RESEARCH INTEGRITY IRB APPROVAL



Office of Research Integrity

June 11, 2013

Johannes F. Fahrman
1205 Charleston Avenue, Apartment 2
Huntington, WV 25701

Dear Johannes:

This letter is in response to the submitted thesis abstract titled "OMEGA-3 Fatty Acids as Therapeutic Options for the Treatment of B-Cell Chronic Lymphocytic Leukemia." This study was reviewed and approved by the Marshall University Medical Institutional Review Board (IRB#1) under study #346687. If there are any changes or amendments to the study you will need to submit that information to IRB#1 for review and approval.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

A handwritten signature in blue ink that reads "Bruce F. Day". The signature is fluid and cursive, with the first name "Bruce" and last name "Day" clearly legible.

Bruce F. Day, ThD, CIP
Director

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EDUCATION

Marshall University · Huntington, WV (2009-present)

- Biomedical Sciences Graduate Program; GPA: 3.68
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- Bachelor of Science, Department of Biological Sciences
- Bachelor of Science, Department of Chemistry
- Graduated Cum Laude; GPA: 3.31

PEER-REVIEWED PUBLICATIONS

1. **Fahrmann JF**, Witte TR, Hardman WE. OMEGA-3 FATTY ACIDS AS THERAPY FOR CANCER. *Cutting edge therapies for cancer in the 21st century*. Bentham eBooks, Bentham Science Publishers Ltd. Editors: Pier Paolo Claudio, and Paraskevi Vogiatzi. *Completed, in formatting stages*.
2. Ray, K, **Fahrmann JF**, King H, Cook C, Crain C, Dawley B, Santanam N. Oxidative sensitive nociception involved in endometriosis associated pain. *Manuscript in progress*.
3. **Fahrmann JF**, Hardman WE. Omega 3 fatty acids increase the chemo-sensitivity of B-CLL-derived cell lines EHEB and MEC-2 and of B-PLL-derived cell line JVM-2 to anti-cancer drugs doxorubicin, vincristine and fludarabine. *Lipids in Health and Disease* (2013); 12(1): 36
4. **Fahrmann JF**, Ballester OF, Ballester G, Witte TR, Salazar AJ, Kordusky B, Cowen KG, Ion G, Primerano DA, Boskovic G, Denvir J, Hardman WE. Inhibition of Nuclear Factor Kappa B activation in Early Stage Chronic Lymphocytic Leukemia by Omega 3 Fatty Acids. *Cancer Investigations* (2013); 31(1): 29-43.

SELECTED ABSTRACTS (2008-present)

1. **Fahrmann JF** and W.E. Hardman. Omega-3 Fatty Acids Are Potential Chemo-sensitizing Agents for the Treatment of B-Cell Chronic Lymphocytic Leukemia. (2013) **Abstract**
2. **Fahrmann JF** and W.E. Hardman. Omega 3 Fatty Acids as Potential Chemo-sensitizing Agents in the Treatment of B-Cell Chronic Lymphocytic Leukemia. (2012) **Abstract**
3. Benjamin A. Kordusky, Kelsey G. Cowen, **Johannes F. Fahrmann**, Theodore R. Witte, W. Elaine Hardman. Quantification of the bioavailability of fatty acids in plasma of omega-3 supplemented early stage Chronic Lymphocytic Leukemia Patients. (2011) **Abstract**
4. Kelsey G. Cowen, Benjamin A. Kordusky, **Johannes F. Fahrmann**, Theodore R. Witte, W. Elaine Hardman. The Quantification of Fatty Acids in the Plasma and Mammary Tissue of Transgenic Mouse Model fed Omega-3 Rich Diets. (2011) **Abstract**
5. **Johannes F. Fahrmann**, Oscar F. Ballester, Gabriela Ballester, Theodore R. Witte, Alexander J. Salazar, Gabriela Ion, Donald A. Primerano, Goran Boskovic, James Denvir, W. Elaine Hardman.

Inhibition of Nuclear Factor Kappa B activation in Early Stage Chronic Lymphocytic Leukemia by Omega 3 Fatty Acids. (2010) **Abstract**.

6. **Johannes F. Fahrmann**, Courtney Crain, Carla Cook, Brenda L. Dawley, Holly King, Richard Egleton, Nalini Santanam. Oxidized Lipoproteins are the Alleged Pain Molecules in the Peritoneal Fluid of Women with Endometriosis. (2010) **Abstract**
7. **Johannes F. Fahrmann**, Rudolf Burcl. Characterization of Phthalocyanines and Phthalocyanine Derivatives via Theoretical Spectroscopy. (2009) **Abstract**
8. **Johannes F. Fahrmann**, N.A Wright, C.C Somerville. Can Bacteria Exchange Genes for Antibiotic Resistance While Trapped on Sand Bed Filters. (2008) **Abstract**

SELECTED ORAL AND POSTER PRESENTATIONS (2008-Present)

Presentations (Oral):

1. **Fahrmann JF**. The Imbalance that Persists: Application of Omega-3 Fatty Acids as Therapeutic Options for Cancers. **Medical Oncology Fellowship Program**, Marshall University School of Medicine, WV, 2013
2. **Fahrmann JF** and W.E. Hardman. Omega-3 Fatty Acids Are Potential Chemo-sensitizing Agents for the Treatment of B-Cell Chronic Lymphocytic Leukemia. **Research Day**, Marshall University School of Medicine, WV, 2013
3. **Fahrmann JF**, O.F. Ballester, G. Ballester, T.R. Witte, A.J. Salazar, B. Kordusky, K.G. Cowen, G. Ion, D.A. Primerano, G. Boskovic, J. Denvir and W.E. Hardman. Inhibition of Nuclear Factor Kappa B activation in Early Stage Chronic Lymphocytic Leukemia by Omega 3 Fatty Acids. **Experimental Biology Conference**, San Diego, CA, 2012
4. **Fahrmann JF** and W.E. Hardman. Omega 3 Fatty Acids as Potential Chemo-sensitizing Agents in the Treatment of B-Cell Chronic Lymphocytic Leukemia. **Research Day**, Marshall University School of Medicine, WV, 2012
5. **Fahrmann JF**, T.R. Witte, W. Patterson, G. Ion, J. Denvir, D.A. Primerano, G. Boskovic, P.T. Georgel and W.E. Hardman. Omega 3 Rich Maternal Diet Alters MicroRNA Expression in First Generation Mouse Pups. **Next Generation Sequencing & Bioinformatics Forum**, Marshall University, WV, 2011

Presentations (Poster):

1. **Fahrmann JF** and W.E. Hardman. Omega-3 Fatty Acids Are Potential Chemo-sensitizing Agents for the Treatment of B-Cell Chronic Lymphocytic Leukemia. **Experimental Biology Conference**, Boston, MA, 2013
2. **Fahrmann JF**, C. Crain, C. Cook, B.L. Dawley, H. King, R. Egleton and N. Santanam. Oxidized Lipoproteins are the Alleged Pain Molecules in the Peritoneal Fluid of Women with Endometriosis. **Annual American Society of Reproductive Medicine (ASRM) Conference**, Denver, CO, 2010
3. **Fahrmann JF** and R. Burcl. Characterization of Phthalocyanines and Phthalocyanine Derivatives via Theoretical Spectroscopy. **Annual American Chemistry Society (ACS) Convention**, Salt Lake City, Utah, 2009

4. **Fahrman JF** and R. Burel. Characterization of Phthalocyanines and Phthalocyanine Derivatives via Theoretical Spectroscopy. **Sigma Xi**, Marshall University, Huntington, WV, 2009
5. **Fahrman JF**, N.A. Wright and C.C. Somerville. Can Bacteria Exchange Genes for Antibiotic Resistance While Trapped on Sand Bed Filters. **Sigma Xi**, Marshall University, Huntington, WV, 2008

GRANTS

| | |
|-----------|--|
| 2012-2013 | WV-NASA SGC TITLE: Eicosapentaenoic Acid and Docosahexaenoic Acid as Potential Chemosensitizing Agents for the Treatment of Chronic Lymphocytic Leukemia. -\$12,000 |
| 2011-2012 | WV-NASA SGC TITLE: Omega 3 Fatty Acids and Chemosensitization of Malignant B Lymphocytes: A Potential Role of Reactive Oxygen Species.- \$12,000 |

PROFESSIONAL AFFILIATIONS

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|--------------|-------------------------------|
| 2013-present | American Society of Nutrition |
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TEACHING EXPERIENCE

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|-----------|--|
| 2013 | Biomedical Sciences Teaching Practicum, taught 4 hours of BMS 651 Cancer Biology Graduate Course |
| 2012 | Biomedical Sciences Teaching Practicum, taught 3 hours of the BMS 680 Graduate Course |
| 2005-2009 | Marshall University Principles of Chemistry Lab 217 Teaching Assistant |

STUDENTS MENTORED

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|--------------|--|
| 2013-present | Diana Dawley, Marshall University MD/PhD Student |
| 2013-present | Dylan Solise, Marshall University Medical Student |
| 2012-13 | Miranda Davis, Marshall University Forensics Student |
| 2011-12 | Benjamin Kordusky, West Virginia Wesleyan College (2 months) |
| 2011 | Kelsey G. Cowen, Cedarville University (2 months) |

HONORS & AWARDS

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| 2012 | “Best Research Performance” for the Academic Year, National Travel Award |
| 2011 | Honorary Member of Marshall University Award |
| 2011 | Successful Completion of Oral and Written Exam for Ph.D. Candidacy, Marshall University |
| 2010-2011 | Grand Club Top Fundraiser- Relay-For-Life, Marshall University |
| 2008 | NASA Scholarship Recipient |
| 2004-2008 | Promise Scholarship Recipient |
| 2004-2006 | Presidential Scholarship Recipient |

COMMITTEES

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|--------------|--|
| 2010-present | Biomedical Sciences Graduate Student Organization; Vice President (‘11-present); Secretary/Treasurer (‘10-‘11) |
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| 2005-present | Alpha Sigma Phi Fraternity; Alumni Counsel Committee; Philanthropy and Community Service Chairman ('12-present); Executive Board Member-At-Large ('06-'09); Philanthropy Chairman ('06-'09); Community Service Chairman ('06-'08); Recruitment Officer ('05-'07) |
| 2009-2010 | Relay-For-Life, Marshall University; President of Colleges Against Cancer |
| 2009-2010 | Relay-For-Life, Marshall University; Fundraising Chairman |

SERVICE

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| 2005-present | Actively participate in various community service events. Logged over 670 hours of individual community service hours. |
| 2013 | Supervised the planning for the first Teen Science Café in Huntington, WV. Partnered with The American Society for Biochemistry and Molecular Biology (ASBMB) to prepare a meeting with local high school students. Goal of the Science Café is to raise awareness and interest about biomedical research. |
| 2012 | Assisted in planning for the first Appalachian Regional Cell Conference (ARCC). ARCC was a student driven research conference held in October in Charleston, WV. |
| 2012 | Student mentor for incoming Ph.D. students |
| 2012 | Coordinated and managed "GRΣΣKS AGAINST CANCER" in conjunction with Relay-For-Life, and The Marshall University Greek Community. Generated \$17,582. Funds were donated to the American Cancer Society |
| 2011-2012 | Organized the Jared Box Project donation drive for the BMS Graduate Student Organization (GSO). Delivered toys to Cabell Huntington Hospital and St. Mary's Medical Center. Goal of the Jared Box Project is to provide childhood joy and happiness to children with chronic illnesses. |
| 2011-2012 | Organized the first Graduate Student Organization (GSO) scholarship to be given annually to BMS Ph.D. and Masters Students; on selection committee (2011, 2012) |
| 2011 | Spearheaded and managed "Stand Up to Cancer" in conjunction with the Huntington Funnybone Comedy Club. Generated over \$3000. Funds were donated to the American Cancer Society. |
| 2011 | Coordinated and managed the GSO Volleyball Tournament. Funds benefited the GSO Scholarship. |
| 2008-2011 | Coordinated and planned multiple fundraising events on behalf of Marshall University's Relay-For-Life; Relay-For-Life Rock Concert ('09-'11); Relay-For-Life Soccer Tournament ('08-'10); Relay-For-Life 5K Run ('09); Relay-For-Life Volleyball Tournament '09); Relay-For-Life Basketball Tournament ('09); Relay-For-Life Brazilian Jiu-Jitsu Tournament ('08) |