Clemson University
TigerPrints

All Dissertations | Dissertations

8-2011

Anticancer Effects of Red Raspberries on Immune Cells and Blood Parameters

Jannet Romero
Clemson University, jromero@clemson.edu

Follow this and additional works at: https://tigerprints.clemson.edu/all_dissertations

Part of the Medical Immunology Commons

Recommended Citation

Romero, Jannet, "Anticancer Effects of Red Raspberries on Immune Cells and Blood Parameters" (2011). *All Dissertations*. 818.
https://tigerprints.clemson.edu/all_dissertations/818

This Dissertation is brought to you for free and open access by the Dissertations at TigerPrints. It has been accepted for inclusion in All Dissertations by an authorized administrator of TigerPrints. For more information, please contact kokeefe@clemson.edu.

ANTICANCER EFFECTS OF RED RASPBERRIES ON IMMUNE CELLS AND BLOOD PARAMETERS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Microbiology

---

by
Jannet Katherine Romero
August 2011

Accepted by:
Dr. Lyndon L. Larcom, Committee Chair
Dr. Alfred Wheeler
Dr. Julia A. Eggert
Dr. Jeremy Tzeng

ABSTRACT

There is an abundance of research correlating diets rich in fruits and vegetables to the reduction or prevention of chronic diseases such as cancer and cardiovascular disease. Plants extracts have also been claimed to possess antiviral, antibacterial, and immunological properties. This study focused on the *in vitro* and *in vivo* effects of a water extract from lyophilized Meeker red raspberries on tumor cell viability and immune parameters. A large number of studies have demonstrated the cytotoxic effect of different fruit juices and extracts against tumor cell lines *in vitro*. However, studies also show that the blood plasma levels of berry phytochemicals are several orders of magnitude lower than the levels which have activity *in vitro*. This study was undertaken as an attempt to reconcile these apparently contradictory observations and test the value of a novel system for detecting the effect of berry consumption in humans. Subjects who consumed berries donate blood which is tested *in vitro*. An extract of Meeker red raspberries was tested for its activity against 5 tumor cell lines. Berries from the same lot were consumed by test subjects and their plasma and white blood cells were tested for changes in several immune parameters.

The extract exhibited a potent cytotoxic effect on a variety of cancer cells *in vitro*; including cells from gastric, prostate, colon, and breast cancers. By comparison to an ascorbic acid control, it could be determined that the cytotoxicity of the raspberry extract was not solely attributable to pH or antioxidant effects.

The goal of the present study was to evaluate the effects of lyophilized red raspberry consumption on *in vivo* and *in vitro* immune parameters, including immune cell

proliferation, plasma MMP-9 concentrations, and cytotoxicity toward human tumor cell lines.

Sixteen healthy volunteers participated in the 3.5 day study. Venous blood samples were collected at baseline and after the last serving of berries. Although much variability was observed among participants, our results suggest that raspberry phytochemicals might augment immune function and affect both the innate and adaptive immune responses in some individuals, but have minimal effects in others.

In several of the participants, the levels of one or more subsets of leukocytes changed after berry intake. In five donors mitogen-induced T lymphocyte proliferation increased and in five it decreased. An inverse relationship was observed between T lymphocyte mitogen stimulation and the change in leukocyte levels. There was an increase in resting peripheral blood mononuclear cell metabolism, suggesting an *in vivo* priming or proliferative effect from the berry phytochemicals. Changes in plasma levels of MMP-9 correlated with changes in leukocyte levels.

In vitro plasma tumoricidal activity increased for all participants. An increase in peripheral blood mononuclear cell cytotoxicity was also observed in some donors. The results demonstrate that consumption of raspberry phytochemicals can affect immune parameters measured *in vitro* and may affect responses of the host to pathogenic challenge.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. Lyndon Larcom, an educator and scientist, whose integrity and love for science has been imparted into many of his students. I thank him for his dedication and invaluable guidance and for teaching me to think like a scientist. I also thank my committee members, Dr. Julie Eggert, Dr. Alfred Wheeler and Dr. Jeremy Tzeng for their guidance, encouragement and long-time support as I pursued my educational goals. I am indebted to Dr. Rosanne Pruitt and the Nursing Division for their support of my research efforts as well as to those who participated in this study and generously contributed their own blood samples for my research. This appreciation is extended to the laboratory staff at Redfern Health Center who assisted in the collection of these samples.

I dedicate this work to my friends and family who never wavered in their love, support, or faith in me. To my children, who never ceased to encourage me, and to my husband, without whom I would never have finished the race.

TABLE OF CONTENTS

Page

TITLE PAGE i

ABSTRACT ii

ACKNOWLEDGEMENTS iv

LIST OF FIGURES viii

CHAPTER

1. LITERATURE REVIEW 1

Antioxidant Effects of Polyphenols 4
Antiproliferative Effects of Antioxidants on Normal and Tumor Cells *in vitro* 6
Apoptosis 9
Anti-inflammatory Effect of Polyphenols 11
Anti-angiogenic Effect of Polyphenols 12
Inhibition of Carcinogen-induced DNA Adducts and Tumors in Animal Models 13
DNA Repair 17
Anti-invasive Effect of Polyphenols 19
Bioavailability of Polyphenols 22
Antimicrobial Effects of Polyphenols 24
Immune Effects of Polyphenols 28

2. MATERIALS AND METHODS 38

Effect of Red Raspberry Extract on Cancer Cells *in vitro* 38
Effect of Ascorbic Acid on Cancer Cells *in vitro* 40
In Vivo Red Raspberry Study 41
Mononuclear Cell Isolation 43
PHA Assay 44
MMP-9 Immunoassay 44
Plasma Assay 46
PBMC Cytotoxicity Assay 47
Statistical Analysis 48

3. RESULTS 49

Effects of Red Raspberry Extract and Ascorbic Acid on Cancer Cell Proliferation *in vitro* 49
Effects of Red Raspberries on Immune Parameters *in vivo* 50
Complete Blood Counts 50
Effect of Raspberry Intake on Mitogen Responsiveness................................ 53
Effect of Raspberry Intake on Plasma Levels of MMP-9 56
Effect of Raspberry Phytochemicals on Plasma Cytotoxicity *in vitro*... 62
Effect of Raspberry Intake on PBMC Cytotoxicity *in vitro*........................... 66

4. DISCUSSION 80

APPENDICES 101
A. *In Vivo* Red Raspberry Study Questionnaire... 102
B: Complete Blood Counts of Study Participants... 104
C. Percent Decrease in Donor PBMCs Post-Raspberry Consumption..................... 134

LITERATURE CITED.. 135

LIST OF TABLES

Table Page

2.1 Cell Line Descriptions 48

2.2 Demographic Profiles of Study Participants 52

3.3 Effect of Raspberry Extract and Ascorbic Acid on Cell Proliferation *in vitro* 62

3.4 Proliferative Effect of Red Raspberries on Subpopulations of White Blood Cells *in vivo* 64

3.5 Effect of Red Raspberries on the Relative WBC Differential Counts *in vivo* 65

3.6 Effect of Red Raspberries on Mitogen Responsiveness 70

3.7 Effect of Red Raspberries on Total MMP-9 Plasma Levels 73

3.8 Donors with Increased MMP-9 SIs, PBMC Control SIs, and PBMC Counts Post-berry Consumption 75

3.9 Cytotoxic Effect of Pre- and Post-Raspberry Plasma on AGS and LNCaP Tumor Cell Growth at 72 Hours 79

3.10 Cytotoxic Effect of Pre- and Post-Raspberry Plasma on HCC 1500, LoVo, and K-562 Tumor Cell Growth at 72 Hours 80

3.11 Donors with Increased Post-Raspberry Plasma Cytotoxicity 81

3.12 Average Change in Post-Raspberry Plasma Cytotoxicity in Donor Groups 81

3.13 PBMC Treatment of AGS, LoVo, LNCaP, and HCC 1500 Tumor Cells at 72 Hours 87

3.14 PBMC Treatment of AGS and LNCaP Tumor Cells at 4 Hours 88

3.15 PBMC Treatment of HCC 1500 and K-562 Tumor Cells at 4 Hours 89

LIST OF FIGURES

Figure Page

2.1 MMP-9 Standard Curve 55

3.2 Dose Effectiveness of Red Raspberry Extract and Ascorbic Acid on Cell Line Viability 61

3.3 Group 1 Mitogen-Induced Stimulation of T Lymphocytes Pre- and Post-Raspberry Consumption 67

3.4 Group 2 Mitogen-Induced Stimulation of T Lymphocytes Pre- and Post-Raspberry Consumption 68

3.5 Group 3 Mitogen-Induced Stimulation of T Lymphocytes Pre- and Post-Raspberry Consumption 69

3.6 Total MMP-9 Levels in Donor Plasmas Pre- and Post-Raspberry Consumption 74

3.7 Donors with Increased MMP-9 Levels, Absolute Lymphocyte Counts, and Resting PBMC Activities Post-Raspberry Consumption 74

3.8 Cytotoxic Effect of Pre- and Post-Raspberry Plasma on AGS, LNCaP, and HCC 1500 Tumor Cell Growth at 72 Hours 77

3.9 Cytotoxic Effect of Pre- and Post-Raspberry Plasma on LoVo and K-562 Tumor Cell Growth at 72 Hours 78

3.10 PBMC Treatment of AGS, LoVo, LNCaP, and HCC 1500 Tumor Cells at 72 Hours 84

3.11 PBMC Treatment of AGS and LNCaP Tumor Cells at 4 Hours 85

3.12 PBMC Treatment of HCC 1500 and K-562 Tumor Cells at 4 Hours...86

1. LITERATURE REVIEW

Today there is a plethora of information correlating diets rich in fruits and vegetables to the reduction or prevention of chronic diseases such as cardiovascular and neurodegenerative diseases, diabetes, obesity, and certain cancers. Worldwide, chronic diseases are increasing rapidly. According to the World Health Organization (WHO), in 2001 chronic diseases contributed approximately 60% of the 56.5 million total reported deaths in the world and approximately 46% of the global burden of disease. By 2020 chronic diseases are expected to account for 57% of the global burden (1). They further report that low fruit and vegetable intake is among the top 10 risk factors for global mortality; however, sufficient intake of fruits and vegetables could reduce the risk for chronic disease and save up to 2.7 million lives annually (2).

The disease-prevention properties of fruits and vegetables are attributed to the biological activities of the dietary fiber, vitamins, minerals and phytochemicals in the plants, however many studies suggest the protective effects of fruits and vegetables against chronic diseases are due in large part to the phytochemical content of the plants (3). Phytochemicals are the bioactive, non-nutritive components of fruits and vegetables and can vary widely among plants and between plants of the same species. Phytochemicals are split into groups such as carotenoids, alkaloids and polyphenols. Carotenoids are found in fruits and vegetables ranging in color from pale yellow through orange to deep red and are efficient free radical scavengers. Alkaloids are plant compounds which mostly contain nitrogen atoms and invoke a bitter taste when ingested. Many alkaloids have pharmacological effects and are used in medications such as

morphine and quinine. Polyphenols are complex plant components characterized by the presence of multiple phenol rings which are the source of their biological activity. They are subdivided into three categories: lignin, hydrolysable tannins and flavonoids, with the flavonoids being the most diverse category of polyphenols (4). To date, over 8,000 structural variants of polyphenols (5) and 7,000 varieties of naturally occurring flavonoids (4) have been identified. Flavonoids are further divided into flavones, flavonols, anthocyanidins, isoflavones and procyanidins (condensed tannins) (4). Experimentally, these phenolic compounds have been shown to exert a wide range of biological activities in both *in vitro* and *in vivo* systems, including antioxidant, anti-inflammatory and anticarcinogenic properties. In addition, polyphenols have been reported to have immunological, antiviral, antibacterial and estrogenic properties as well as antiproliferative and cytotoxic effects in human and animal tumor cell lines (3, 6).

Of particular interest to this laboratory are the polyphenolic compounds found in red raspberries (*Rubus idaeus*) and their *in vitro* and *in vivo* effects upon biological systems. Raspberries contain a wide range of bioactive phytochemicals and are rich in vitamin C and total phenolics (7), including flavonoids, anthocyanins and ellagitannins. It is the flavonoids and anthocyanin pigments that give raspberries their characteristic color. Many of the health benefits derived from raspberry consumption are due to their content of polyphenols, which are responsible for many of the biological activities of these berries, including antioxidant, anti-inflammatory and anticancer properties (8).

A number of studies have been performed to assess and compare the total antioxidant capacity of fruits and vegetables that might contribute to health benefits in

humans. Different methods are used to assess the antioxidant activity of fruits and vegetables. Commonly performed assays include ORAC (oxygen radical absorption capacity), TRAP (total radical-trapping antioxidant parameter), FRAP (ferric reducing ability of plasma) and ABTS (2,2'-azinobis cation radical). Of these assays, raspberries place very high on the ranking of antioxidant fruits and vegetables and are considered among the richest sources of dietary antioxidants available (9). The antioxidant property of raspberries has been attributed to their content of vitamin C, anthocyanins and ellagitannins. Vitamin C contributes about 20% of the total antioxidant capacity, anthocyanins about 25% and the largest contributor to antioxidant capacity is made by the ellagitannins at more than 50% (9). Ellagitannins are an uncommon phytochemical found in only a limited number of berry species, including cloudberry and raspberry and to a limited extent in strawberries.

Berry bioactives, including raspberry, have many roles in cancer prevention according to Stoner *et al* (10). Laboratory studies show berry bioactives protect against oxidative DNA damage by direct scavenging of reactive oxygen species (ROS), often considered a first line of defense against the multistage process of carcinogenesis. Berry bioactives are also effective in inhibiting the formation of carcinogen-induced DNA adducts, enhancement of DNA repair and inhibition of carcinogen-induced tumorigenesis in animal models. In addition, berry bioactives modulate signaling pathways involved with cellular proliferation, apoptosis, inflammation, angiogenesis and cell cycle arrest (10).

Antioxidant Effects of Polyphenols

A large number of chronic diseases, including many types of cancers, are caused in part from reactive oxygen species (ROS) that are generated during normal cellular metabolism. ROS are also produced from exogenous sources such as environmental toxins, carcinogens and ionizing radiation (10). Oxygen free radicals such as superoxide ($O_2^{\cdot -}$), singlet oxygen (O_2), peroxide (ROO^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and nitric oxide (NO^-) are highly reactive and can damage cellular components, including DNA, RNA, proteins, and carbohydrates. Unrepaired oxidative damage to DNA can result in mutations that can potentially lead to cell transformation and carcinogenesis. Damage to cell membrane lipids can also occur and lead to swelling and cell death. Free radicals released from damaged cells attract inflammatory mediators that contribute to a general inflammatory response, leading to further cell and tissue damage (11).

A number of effective defense mechanisms are present in humans to protect them from the damaging effects caused by reactive oxygen species. Enzymes such as superoxide dismutase, catalase and glutathione peroxidase function as antioxidants *in vivo*. Nonenzymatic molecules such as ascorbic acid (vitamin C), glutathione and α-tocopherol (vitamin E) also possess antioxidant properties to inactivate free radicals. However, when cellular or tissue injury occurs there is an increased production of ROS that results in consumption and depletion of the endogenous antioxidants (11). This can result in oxidative damage to numerous cellular components and may serve as an initiating event in many human diseases.

Most of the protective effects of various phytochemicals are attributed to their ability to scavenge ROS. Dietary supplementation of antioxidants may produce an additive effect with endogenous scavenging compounds (11), helping to minimize or eliminate cellular damage from ROS. Plant phytochemicals, especially the flavonoids, have been shown to prevent cellular injury by several mechanisms. Flavonoids can directly scavenge free radicals. This is achieved when flavonoids are oxidized by the ROS, resulting in more stable, less reactive molecules. Some flavonoids are able to chelate bivalent metals such as iron and copper to make them unavailable for redox cycling reactions (12). Another mechanism is through inhibition of pro-oxidant enzymes such as inducible nitric oxide synthase (iNOS). Nitric oxide, produced by endothelial cells and macrophages, is important in maintaining the dilation of blood vessels. Homeostatic levels of nitric oxide are maintained by constitutive nitric oxide synthase, however, during inflammation much higher concentrations are produced by iNOS in macrophages. This is able to cause oxidative injury by increasing production of nitric oxide and superoxide anions by the activated macrophages. The increased levels of nitric oxide react with free radicals and produce the highly damaging peroxynitrite, resulting in irreversible damage to cell membranes. Flavonoids can intercept and scavenge free radicals before they react with nitric oxide (11), or nitric oxide can be directly scavenged by certain flavonoids (13), resulting in less damage to the cells and surrounding tissues. Various flavonoids have the ability to inhibit other pro-oxidant enzymes such as lipoxygenase, cyclooxygenase (COX-2) and xanthine oxidase, inflammatory mediators implicated in the pathogenesis of chronic inflammatory diseases (14).

Antiproliferative Effects of Antioxidants on Normal and Tumor Cells *in vitro*

Although berry phenolics are best known for their antioxidant activity, it has been firmly established from *in vitro* studies that berry phytochemicals have an antiproliferative effect on multiple cancer cell types. Individual polyphenols such as anthocyanins, ellagitannins and ellagic acid have been shown to inhibit cancer cell proliferation *in vitro* and *in vivo*. One study examined four varieties of fresh raspberries to measure their antioxidant and antiproliferative activities as well as determine the total amount of phenolics and flavonoids for each variety. Interestingly, they found the color of the raspberry juice correlated well to the total phenolic, flavonoid and anthocyanin content of each berry - the darker the color, the higher the content of total phenolics in the berry juice. Results of the antioxidant activity of the raspberries also correlated with berry color - the darker the berry the greater the antioxidant activity. Although the antioxidant activity of the raspberry was directly related to the total amount of phenolics and flavonoids, no relationship was established between the antiproliferative activity and the total amount of phenolic compounds in the berries. $HepG_2$ human liver cancer cells were treated with various concentrations (1 – 50 mg/mL) of raspberry extracts to determine the antiproliferative activity of each extract. While $HepG_2$ cell proliferation was inhibited in a dose-dependent manner in all varieties of raspberry extracts, there was no relationship between the antiproliferative activity and total amount of phenolics/flavonoids found in each berry (15). Olsson *et al.* performed a similar study to determine if a correlation exists between the antiproliferative activity of fruit extracts and antioxidant levels. They treated HT29 colon cancer cells and MCF-7 breast cancer cells

with ten different extracts of fruits and berries, including raspberries, and all extracts decreased proliferation to varying degrees in both cell types in a concentration-dependent manner. They also reported great differences in the content and composition of the antioxidants in the extracts. Vitamin C varied almost 100-fold and total carotenoid content varied almost 150-fold among the species. There was also a large difference in the composition of polyphenols among the fruits. The results of their experiments showed a correlation between inhibition of cancer cell proliferation and the content of vitamin C and levels of certain carotenoids in the fruits. The same inhibition effect on proliferation in HT29 and MCF-7 cells was not observed in cells treated with ascorbic acid alone. These investigators concluded a correlation exists between the inhibition of cancer cell proliferation and the vitamin C content and certain carotenoids that act synergistically with the polyphenols to inhibit cancer cell growth (16). On the other hand, individual polyphenols such as anthocyanins, ellagitannins and ellagic acid have been shown to inhibit cancer cell proliferation *in vitro*. A study conducted by McDougall *et al*. ranked the antiproliferative effectiveness of a range of fruit extracts rich in polyphenols but devoid of vitamin C, carotenoids, sugars and organic acids. Human cervical cancer (HeLa) cells and human colon cancer (CaCo-2) cells were treated with fruit extracts at 25, 50 and 75 μg/mL and assayed for cell viability after 72 hours. The berry extracts from the *Rubus* family (raspberry, arctic bramble and cloudberry) as well as strawberry and lingonberry were effective in preventing the proliferation of these two cell types, reducing viability to ≤50% of the control at 50 μg/mL. In this study as well as others on polyphenolic compositions for the most effective berry types, they suggest that

the effectiveness of the *Rubus* family (raspberry, arctic bramble and cloudberry) as well as strawberry could be due to their high content of ellagitannins, which have been shown to be effective antiproliferative agents (17). Similar antiproliferative activities were observed in this laboratory by God from red raspberry extract treatment on AGS (gastric), LoVo (colon) and MCF-7 (breast) cancer cell lines. Following a 48 hour treatment in 5%, 7.5% or 10% extract, all three cell lines showed significant growth inhibition, with AGS and LoVo exhibiting the greatest sensitivity. God also examined the effects of pH and antioxidant activity on cell killing to determine their contribution to the potent cytotoxicity observed in the berry-treated samples. Cell lines were treated with solutions of hydrochloric acid (HCl) and ascorbic acid (antioxidant) that were adjusted to the same pH as the raspberry extract. The HCl treatment had no effect on the survival of any of the cell lines, suggesting pH was not a factor in cell killing. Although ascorbic acid treatment inhibited the growth of all three cell lines, MCF-7 was the most sensitive and showed no significant difference in cytotoxicity by raspberry extract and ascorbic acid. However, there was a significantly higher level of inhibition in AGS and LoVo treated with raspberry extract compared to ascorbic acid treatment. God concluded that the potent cytotoxic effect observed from red raspberry extract treatment was not attributed solely to its antioxidant capacity or to changes in pH (18). Other investigations have compared the antiproliferative effects of anthocyanins on normal versus cancer cells and found that they selectively inhibit growth of the cancer cells with little or no effect on the growth of normal cells. Stoner *et al.* studied the effect of black raspberries on a highly tumorigenic rat esophageal epithelial cell line (RE-149-DHD) with its low tumorigenic

precursor line (RE-149). He found that the uptake of the three anthocyanins in the black raspberries into the highly tumorigenic line (RE-149-DHD) exceeded the uptake in the precursor line (RE-149) by 100-fold. Additionally, one of the anthocyanins (cyanidin-3-rutinoside) remained at steady-state levels in RE-149-DHD cells for up to 12 hours while it became undetectable in RE-149 cells after 2 hours. Stoner also reported black raspberry treatment stimulated apoptosis in RE-149-DHD but not in the precursor line RE-149 (10).

Apoptosis

In addition to the antiproliferative activity of berry bioactives, their effect on apoptosis in human cancer cell lines has also been reported. Among berry phenolics, anthocyanins have been shown to play a major role in the induction of apoptosis (8). Because apoptosis is often dysregulated in cancer cells, berry extracts capable of inducing apoptosis or cell cycle arrest in pre-malignant and malignant cells could potentially be used as chemopreventive or chemotherapeutic agents. Seeram's laboratory investigated the ability of six berry extracts (blackberry, black raspberry, blueberry, cranberry, red raspberry and strawberry) to stimulate apoptosis of the COX-2 expressing colon cancer cell line HT-29. HT-29 expresses the COX-2 (cyclooxygenase-2) enzyme, an inflammatory protein associated with chronic inflammation and cancer. Of the six extracts tested, two showed significant pro-apoptotic activity at the highest dose tested (200 µg/mL). Black raspberry extract induced apoptosis 3-fold over control cells and strawberry extract induced apoptosis 2.8-fold over controls. Red raspberry exhibited a 1.7-fold increase whereas cranberry had no pro-apoptotic activity against HT-29 cells.

(The investigator noted that the test concentrations of the berry extracts far exceeded the levels of phenolics and/or their metabolites that are physiologically achievable based on current knowledge of polyphenol bioavailability) (19). Another study demonstrated apoptosis in a human lung cancer cell line NCI-H209 following treatment with quercetin, a flavonol found in several varieties of berries, including raspberries. In contrast to Seeram's treatment conditions, quercetin was effective *in vitro* at doses that are pharmacologically attainable *in vivo* (10). Following treatment with quercetin (0 – 10 μM) NCI-H209 cell viability decreased in a dose- and time-dependent manner. Cell cycle analysis showed a significant increase in the percentage of cells in G_2/M phase and subG_0/G_1 phase, indicating cell cycle arrest and apoptosis. They found that quercetin increased the expression of proteins responsible for cell cycle arrest (cyclin B, WEE1, Cdc25C-ser-216-p and p21) and induced apoptosis through activation of the caspase-3 cascade, key components of the apoptotic pathway (20). God also examined caspase activity to determine if cytotoxicity was due to raspberry extract-induced apoptosis. The results of his study indicated there were no significant differences between the caspase activities observed for the treated and untreated cells, suggesting the cytotoxic effects from red raspberry extract were not attributed to caspase-dependent apoptosis (unpublished data, 18). Feng *et al*. demonstrated that cyanidin-3-rutinoside (C-3-R), an anthocyanin derived from black raspberries, selectively induced apoptosis in HL-60 leukemic cells by promoting oxidative stress, but showed no pro-oxidant activity in normal human peripheral blood mononuclear cells (PBMC). Treatment of cells with C-3-R induced a significant amount of apoptosis in HL-60 cells in a time- and dose-

dependent manner (caspase-3 and caspase-9 activity) but had little toxicity against the normal PBMCs. They found that there were increased levels of H_2O_2 that accumulated in the HL-60 cells but were reduced in the PBMCs, suggesting that C-3-R could have a radical scavenging effect in the normal cells. They concluded from their observations that C-3-R selectively causes ROS accumulation in leukemic cells and that the increased oxidative stress is sufficient to activate the downstream apoptotic events observed in this study (21).

Anti-inflammatory Effect of Polyphenols

In addition to its carcinogenic effects, oxidative stress is also integral to the inflammatory response. Excessive inflammation has been linked to a number of chronic diseases in humans, including cardiovascular disease and cancer. Elevated levels of key inflammatory proteins such as nuclear factor-kappa B (NF-κ B), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) are often present in these conditions. It has been shown that plant phytochemicals, including flavonoids and anthocyanins, are able to inhibit these enzymes as well as other mediators (i.e. cytokines) of the inflammatory process, mainly by acting through NF-κ B and mitogen-activated protein kinase signaling (22). For example, Huang *et al*. treated mouse epidermal JB6 Cl 41 cells with 50 mg/mL black raspberry extract and benzo(*a*)pyrene-diol-epoxide (BPDE), a carcinogen and inducer of NF-κB gene transcription. Treatment with the anthocyanin-rich raspberry extract resulted in a down-regulation of BPDE-induced expression of NF-κ B (23). Likewise, Rodrigo *et al.* demonstrated a suppression of iNOS activity in five cell lines isolated from human oral squamous cell carcinoma tumors

treated with freeze-dried blackberries (10, 50 and 100 μg/mL) (24). Different studies have shown that flavonoids can modulate the NF-κB signaling pathway during inflammation. In addition, based upon *in vitro* studies in multiple cell types, the anti-inflammatory activity of flavonoids and anthocyanins has been attributed to their ability to inhibit the transcription and/or translation expression levels of the various inflammatory mediators involved in chronic inflammatory disorders (6).

Anti-angiogenic Effect of Polyphenols

Angiogenesis is a normal process involving the growth of new blood vessels from pre-existing vessels; however, it also plays a crucial role in tumor growth and cancer metastasis. Vascular endothelial growth factor (VEGF) has been shown to be a major contributor to angiogenesis, and VEGF expression is often elevated in developing tumors (26). The anti-angiogenic effects of multiple berry extracts on inducible VEGF have been evaluated in various studies. Bagchi and colleagues tested individual berry extracts (wild blueberry, bilberry, cranberry, elderberry, raspberry seed and strawberry) on human HaCaT keratinocytes and found that 50 μg/mL berry extract produced significant inhibition of H_2O_2- and TNF-α-induced VEGF expression by these cells. Wild blueberry extracts exhibited the greatest inhibitory effect in both the H_2O_2- and TNF-α-induced VEGF samples. Antioxidants such as GSPE (grape seed proanthocyanidin extract), with comparable ORAC, or α-tocopherol (vitamin E) tested under the same conditions did not inhibit inducible VEGF expression, suggesting that the observed effect of the berry extracts was not dependent on their antioxidant property alone. These investigators concluded that the flavonoid constituents of the berry extracts may have been responsible

for the effect on inducible VEGF expression and release (26). Rodrigo *et al*. also demonstrated an anti-angiogenic effect of black raspberry extract treatment on human oral squamous cell carcinoma cells (SCC) that produce high levels of VEGF protein. This study examined the effect of raspberry extract on VEGF gene transcription and translation and found the extract was inhibitory at both levels of protein synthesis. VEGF RNA transcript levels were measured following a 6-hour exposure to TNF-α or black raspberry extract and this resulted in reduced VEGF mRNA expression in three of the four extract-treated cell lines compared with the TNF-α- and untreated control samples. Raspberry extract-treated cells also showed a significant suppression of VEGF protein levels, although there were cell line-associated differences in protein levels (24).

Inhibition of Carcinogen-induced DNA Adducts and Tumors in Animal Models

Berry bioactives have also been examined by various laboratories to determine their effectiveness in inhibiting or reducing the formation of DNA adducts from chemical carcinogens. Kresty *et al*. fed male rats diets containing either 5 or 10% lyophilized black raspberries (LBR) or 0.04% ellagic acid for 14 days followed by an injection of *N*-nitrosomethylbenzylamine (NMBA), an esophageal carcinogen. The esophageal DNA was isolated and O-methylguanine measured. O-methylguanine is an indicator of DNA damage. Rats fed the LBR had reduced levels of O-methylguanine adducts in the DNA of their esophagi. The 5 and 10% LBR treatment showed 73 and 80% inhibition respectively of DNA adduct formation and the ellagic acid produced 38% inhibition compared to the NMBA controls (27). Inhibition of oral cancer by dietary administration of LBRs was reported by Casto *et al*. They observed similar reductions in DNA adducts

in the cheek pouches of Syrian golden hamsters treated with 7,12-dimethylbenz(*a*)anthracene (DMBA). Hamsters were fed a diet of 5% LBR for 2 weeks before a topical application of DMBA was applied to the surface of the cheek pouch. DNA adducts in the berry-fed hamsters were reduced by 29 and 55% at 24 and 48 hours respectively (28). In contrast to these reports, Carlton *et al* found that diets containing 10% lyophilized strawberries were ineffective at reducing lung tumors in mice that were induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1 butanone (B(*a*)P). The mice were fed the strawberry diet before and during treatment with B(*a*)P and at 24 weeks there was no difference in lung tumor incidence or multiplicity compared to control mice (29).

Other laboratories focused on the role of anthocyanins in cancer prevention. These plant constituents are the most abundant flavonoids in fruits and vegetables, and epidemiologic studies suggest that diets rich in anthocyanins lower the risk of cardiovascular disease, diabetes, arthritis and cancer, due in part to their antioxidant and anti-inflammatory properties (30). Using *in vivo* animal model tumor systems, researchers have shown a reduction in esophageal, colon, skin and lung cancer in animals administered berry diets. In the azoxymethane (AOM)-induced model of colon cancer in F344 rats on 2.5, 5 and 10% LBR diets, Harris *et al*. found that these animals showed a significant decrease in total tumors (adenomas and adenocarcinomas) by 42, 45 and 71% respectively. They further reported significant reductions in urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG, an indicator of oxidative stress-induced DNA damage) levels by 73, 81 and 83% respectively, indicating that berries reduce ROS-induced DNA damage in animals (31). The chemopreventive properties of delphinidin, a major

anthocyanin, in skin tumor development in mice have also been described. Afaq *et al.* treated female SKH-1 hairless mice topically with delphinidin (1 mg/0.1 mL DMSO) pre- and post-UVB irradiation (180 mJ/cm^2) and monitored key UVB-induced biomarkers of skin cancer development. They found in both the pre- and post-UVB treatments that delphinidin inhibited apoptosis and markers of DNA damage such as cyclobutane pyrimidine dimers and 8-OHdG compared to the untreated control mice. According to Afaq, their results suggest that delphinidin inhibited UVB-mediated oxidative stress and reduced DNA damage, thereby protecting the cells from UVB-induced apoptosis (32). Although berry bioactives have shown anti-tumor activity in esophageal, oral and skin cancers in animal models, they have not been successful in other types of cancers (i.e. lung). Stoner suggests this may be due to berry components coming into direct contact with tissues where they can be absorbed in sufficient concentration to provide protection. In cancers other than those of the gastrointestinal tract (GI) and skin, the berry bioactives may fail to reach the tumor sites in sufficient amounts to be efficacious. This is supported from studies that show the absorption of anthocyanins and ellagitannins into the bloodstream of rodents and humans is minimal, and their plasma levels decline rapidly (10), making it unclear whether the *in vivo* concentrations are sufficient to provide anti-carcinogenic protection (25).

Human studies examining the anti-cancer effect of berry bioactives are limited and have yielded mixed results. A study conducted in the United Kingdom investigated the effects of cranberry juice consumption on plasma antioxidant activity and biomarkers of oxidative stress. Healthy female subjects ingested 750 mL/day of either cranberry

juice (anthocyanin-rich) or a placebo drink for 2 weeks. Fasted blood and urine were collected over 4 weeks and various parameters measured. The investigators reported similar results for both groups. Cranberry juice did not alter blood or cellular antioxidant status or biomarkers of lipid status pertinent to heart disease (cholesterol, high density lipoprotein, low density lipoprotein and triglycerides). Cranberry juice also showed no effect on 8-OHdG levels in urine or H_2O_2-induced oxidative DNA damage in lymphocytes (33). A German study, however, reported different results from their study. Healthy male subjects consumed either 700 mL of a red mixed berry juice (anthocyanin/polyphenolic-rich) or a polyphenol-depleted juice for 4 weeks. During intervention with the fruit juice, a decrease in oxidative DNA damage was observed that returned to pre-juice levels after conclusion of the study. Intervention with the control juice did not result in reduction of oxidative DNA damage (34). Stoner *et al*. conducted a study with patients diagnosed with colon cancer who had received no therapy. Patients consumed 60 g/day of oral administration of LBR powder for 2 – 4 weeks prior to surgery. Biopsies of normal-appearing and tumor tissues were taken before and after berry treatment and assayed for proliferation, apoptosis and angiogenesis. The results showed a significant reduction in proliferation rates and angiogenesis biomarkers in colon tumors but not in the normal-appearing cells whereas apoptosis increased in the colon tumors but not in the normal-appearing cells (25). Stoner's laboratory also conducted a 6 month chemopreventive pilot study administering 32 or 45 g (female, male respectively) of lyophilized black raspberry powder (LBR) to patients with Barrett's esophagus (BE). BE is a premalignant esophageal condition that confers a 30- to 40-fold

increased risk for the development of esophageal adenocarcinoma. Biopsies of Barrett's lesions were taken before and after treatment for biomarker analysis. Although berry treatment did not result in a reduction in segment length of Barrett's lesions at the end of the study in ten of the patients tested, a reduction in oxidative DNA damage was observed. Urine was collected at study baseline, week 12 and week 26 and evaluated for the oxidative damage biomarkers 8-OHdG and 8-epi-prostaglandin F2α (8-Iso-PGF2). There was a significant reduction in the levels of urinary 8-Iso-PGF2 but no significant change in mean levels of 8-OHdG (10).

DNA Repair

To investigate the anticancer effects of berry bioactives in reducing and repairing oxidative DNA damage, Maurya *et al.* performed studies in mice treated with gamma radiation and ferulic acid, a phenolic acid found in berries. In one instance, mice were administered ferulic acid (50 – 100 mg/kg body weight) intraperitoneally (IP) one hour prior to whole-body gamma radiation (4 Gy) to examine DNA repair activity. Peripheral blood leukocytes and bone marrow cells were examined and a dose-dependent decrease in DNA strand breaks was observed. In a second experiment, mice were given 50 mg/kg body weight of ferulic acid after whole-body irradiation and DNA strand breaks disappeared at a faster rate compared to the irradiated control mice, suggesting enhanced DNA repair in ferulic acid treated animals. Maurya reported similar results in troxerutin-treated mice, a flavonoid derivative of rutin, as well as in gamma-irradiated (2 Gy) human peripheral blood leukocytes. Maurya concluded the berry compounds ferulic acid and troxerutin are effective in enhancing DNA repair as demonstrated by a more rapid

rejoining of irradiation-induced DNA strand breaks in berry-treated cells (35, 36). Niture *et al*. tested the effectiveness of resveratrol and ellagic acid on the repair of O^6-alkylguanine adducts in the DNA of human lymphocytes and in glioblastoma and colon cancer cells. They found that resveratrol had only a modest effect and ellagic acid had no effect on the stimulation of the DNA repair protein O^6-methylguanine-DNA-methyltransferase, suggesting these berry compounds may have little or no effect on repair of O^6-alkylguanine adducts in DNA (37). In contrast to Niture's findings, Aiyer's laboratory showed that mice fed diets of raspberry, strawberry, blueberry and ellagic acid had reduced levels of estrogen-induced DNA adducts and upregulation of DNA repair genes. CD-1 mice were fed either a control diet or diet supplemented with ellagic acid (400 ppm) and dehydrated berries (5% w/w) with varying ellagic acid contents - blueberry (low), strawberry (medium) and red raspberry (high) for three weeks. Mice were treated with 17β-estradiol (E_2), a DNA mutagen causing oxidative damage and DNA adduct formation. In addition to the benchmark oxidative lesion 8-oxo-2′-deoxyguonosine (8-oxodG), four subgroups of novel, unidentified polar DNA adducts were detected following treatment. The results showed that mice on blueberry and strawberry diets had a 25% reduction in endogenous DNA adducts while red raspberry and ellagic acid diets showed significant reductions of 59 and 48% respectively. Both the red raspberry and ellagic acid diets resulted in a 3-8 fold over-expression of DNA repair genes such as XPA (xeroderma pigmentosum group A complementing protein), ERCC5 (DNA excision repair protein) and DNL3 (DNA ligase III). In addition, the red raspberry diet down-regulated genes such as MAPK14 (Mitogen activated protein kinase 14) and

MAPKK (MAP kinase kinase) that are involved in cell-signaling pathways by 5 – 15 fold. This study supports the role of berry polyphenols in maintaining genomic stability. Although the blueberry and strawberry diets were less effective in reducing oxidative DNA damage, red raspberries and pure ellagic acid were highly effective in reducing endogenous oxidative DNA damage, possibly by mechanisms that may involve an increase in DNA repair (38).

Anti-invasive Effect of Polyphenols

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that digest proteins of the extracellular matrix (ECM). They play important roles in regulating normal tissue development, tissue remodeling, wound healing, inflammation, and angiogenesis. However, unregulated MMP activity can contribute to various disease states such as arthritis, cardiovascular disease and cancer expansion. For instance, clinical studies in patients with coronary artery disease (CAD) have shown elevated levels of plasma MMP-9 (39). Experimental and cross-sectional studies have associated elevated MMP-9 levels to CAD risk factors (e.g. smoking, alcohol, and hypertension) before onset of disease, making MMP-9 a potential biomarker for future cardiovascular risk (39). Many studies have also shown that the MMPs, particularly MMP-2 and MMP-9, play prominent roles in tumor invasion and metastasis. It has been demonstrated that increased levels of these proteolytic enzymes correlate with the invasive potential of tumor cells (40), therefore much research has been conducted to find inhibitors of the MMPs to prevent cancer spread through metastasis. MMP inhibition by plant phytochemicals has been investigated by a number of laboratories with encouraging

results. Shin's laboratory tested the anti-invasive properties of an anthocyanin-rich extract isolated from *Vitis coignetiae* Pulliat (meoru in Korea), a fruit belonging to the grape family, on human hepatoma Hep3B cells. The Matrigel™ invasion assay was performed on the Hep3B cells in the presence or absence of anthocyanin extract (100 – 400 µg/mL) and resulted in inhibition of cell invasion in a dose-dependent manner. Anthocyanin treatment at 400 µg/mL inhibited cell invasion by 75% compared with controls. To further explore anthocyanin activity, Shin's lab also measured MMP-2 and MMP-9 protein levels and tumor necrosis-α (TNF-α)-induced-NF-κB activation. NF-κB is a transcription factor that plays a significant role in regulating MMP-2 and MMP-9 gene expression. Shin *et al*. found that the anthocyanin extract markedly suppressed the expression of MMP-2 and MMP-9 genes in Hep3B cells in a dose-dependent manner. In addition, the NF-κB activity induced by TNF-α was inhibited as well as its translocation into the nucleus of the cell. Their results indicate that the anti-invasive properties of the anthocyanin extract from meoru were due in part to their inhibition of NF-κB activation (41). Chen *et al*. also investigated the anti-metastasis potential of anthocyanins derived from black rice (*Oryza sativa* L. indica) on SKHep-1 cells (human hepatocellular carcinoma). Although black rice anthocyanins showed no significant toxic effects on cells treated with 0-200 µg/mL extract, it did inhibit cell invasion in a concentration-dependent manner as determined by the Matrigel™ invasion assay. The black rice anthocyanins were also examined for their effect on the secretion of MMPs and urokinase plasminogen activator (u-PA), a protease that degrades the ECM as part of the invasive process. They found that SKHep-1 cells pretreated with the anthocyanin extract showed

a significant reduction in MMP-9 and u-PA secretion. In contrast to Shin's findings, Chen determined the inhibitory activity of the black rice anthocyanins was effective against the endogenous MMP-9 inhibitor, TIMP-2 (tissue inhibitors of metalloproteinase-2). Treated cells showed a significant increase in TIMP-2 expression, which counteracts the action of MMP-9. Black rice anthocyanins were not effective against the u-PA inhibitor, PAI-1 (plasminogen activator inhibitor-1). In order to determine whether the inhibitory effects of the black rice anthocyanins on MMP-9 and u-PA were linked to the transcription factors NF-κB and AP-1 (activator protein-1) activity, nuclear extracts from SKHep-1 cells pretreated with the anthocyanin extract were analyzed for NF-κB and AP-1 binding activity. Although pre-treatment suppressed AP-1 binding activity, there was no effect upon NF-κB activity. According to Chen *et al.*, suppression of AP-1 activity by black rice anthocyanins may play a role in the inhibition of synthesis of MMP-9 or u-PA and present a potential in blocking tumor initiation, promotion and metastasis (42). Finally, MMP-2 and MMP-9 activity assays were performed by this laboratory by Tate *et al.* to examine the effect of raspberry, blackberry and muscadine grape extracts (1-2%) on MMP inhibition. All of the extracts exhibited significant inhibition of both MMP-2 and MMP-9 as determined by metalloproteinase activity assays. Suppression of MMP-2 gelatinase activity by the raspberry extract was approximately the same as measured by the MMP activity assay whereas the muscadine extract suppressed MMP-2 activity by 75%, less than observed in the MMP activity assay. Tate concluded the high concentration of polyphenols found in raspberries, blackberries and muscadine grapes

contribute to their anti-MMP activity and this inhibition could play a role in the suppression of carcinogenesis by diets high in fruit content (43).

Bioavailability of Polyphenols

Although there have been innumerable reports of the successes of polyphenolic compounds as chemopreventive agents, much of the work has been demonstrated *in vitro* on human and animal tumor cell lines and *in vivo* in animal model systems. Investigators suggest using caution in interpreting the results of the chemopreventive effects of antioxidants on normal and tumor cells in *in vitro* studies and applying these observations to *in vivo* activity. Seeram *et al*. stated "…there are several factors that must be considered when using cell culture studies to rank the chemopreventive activities of berry extracts. These include the cell line being used, the artificially high concentrations of extract, stability of extract components in different media, length of treatment time, differential uptake of phenolics and generation of artifacts such as H_2O_2 that is known to induce apoptosis" (19).

An additional concern according to Stoner *et al*. is extrapolating *in vitro* data to the *in vivo* situation in regard to dose extrapolation. Most, if not all, berry bioactives have demonstrated chemopreventive effects in cell cultures when used at micromolar concentrations (~10 to 150 µM). However, pharmacokinetic studies in animals and humans indicate that berry bioactives, such as the anthocyanins and ellagitannins, reach only nanomolar concentrations (~1 – 20 nM) in blood and tissues when administered in the diet. These levels are far below the levels required to exhibit anticarcinogenic effects *in vitro* (10). For instance, rats were administered 400 mg/kg body weight (BW) bilberry

anthocyanins and the plasma concentrations reached peak levels of 2 – 3 µg/mL after 15 minutes and then rapidly declined within 2 hours (44). A second study reported peak plasma levels of anthocyanins at 3.8 µM/L (1.8 µg/mL) after rats were fed 320 mg/kg BW red fruit anthocyanin via stomach intubation (44). Similar results have been reported in human studies as well. Human subjects ingesting a single dose of blackcurrant concentrate (3.57 mg/kg BW) resulted in plasma levels of anthocyanins of 0.120 nM. Frank and colleagues, however, measured anthocyanin concentrations in the plasma of subjects ingesting 400 mL red grape juice or red wine at much higher levels. Plasma levels peaked at 100 ng/mL for grape juice and 43 ng/mL for red wine, demonstrating variability in the bioavailability and absorption of anthocyanins among fruits (45).

Although phytochemicals differ greatly in their bioavailability, it is widely accepted that berry phenolics are "poorly bioavailable" due to their low levels in human circulation. For example, Stoner *et al*. investigated the absorption and metabolism of black raspberry anthocyanins in humans consuming 2.69 +/- 0.085 g/day and found that plasma levels peaked within 2 hours of berry consumption. The anthocyanins were excreted in the urine both as intact anthocyanins and as methylated derivatives within 4 – 8 hours of ingestion. When administered at this dose, Stoner reported that less than 1% of the berry anthocyanins was absorbed and excreted in the urine (25). A similar study was performed on healthy subjects who ingested 300 g of raspberries, however no detectable quantities were present in the plasma of either the native raspberry polyphenolics or their metabolites. Raspberry anthocyanins were excreted in the urine 0 – 7 hours after ingestion in quantities corresponding to <0.1% of berry intake (46).

Ellagitannin (ET) bioavailability was examined to investigate the metabolism of different dietary ellagitannins and ellagic acid (EA) derivatives in humans. Forty healthy volunteers consumed an ellagitannin-containing foodstuff (250 g strawberries, 225 g red raspberries, 35 g walnuts or 300 mL oak-aged red wine) and five urine samples were collected following intake. Neither ETs nor EA were detected in any of the urine samples; however, the microbial metabolite urolithin B glucoronide (an ellagic acid derivative) was found at significant levels in the urine of all the subjects, regardless of the foodstuff consumed. The mean percentage of metabolite excretion ranged from 2.8% (strawberries) to 16.6% (walnuts) of the ingested ETs, emphasizing the role of the colonic microflora in ET metabolism and bioavailability (47). Seeram proposes an explanation for the poor bioavailability of berry phenolics in human circulation. According to his studies, berry phenolics are extensively metabolized *in vivo* and further converted by colonic microflora into related compounds. These compounds may persist and accumulate in target tissues and contribute significantly to the biological effects that have been observed for berry fruits. He further points out that due to the current limitations in laboratory extraction procedures, the levels of berry phenolics *in vivo* may be underestimated because these compounds may bind to proteins, etc., causing their extraction for chemical analysis to be difficult (8).

Antimicrobial Effects of Polyphenols

Berry phytochemicals have also shown potent antimicrobial properties against a number of human pathogens. As early as 1985, Kaul *et al*. performed *in vitro* studies to determine the antiviral effect of several dietary flavonoids on the infectivity and

replication of herpes simplex virus type 1 (HSV-1), polio virus type 1, parainfluenza virus type 3 (Pf-3) and respiratory syncytial virus (RSV). Cell culture monolayers were infected with each virus and subsequently cultured in medium containing various concentrations of the flavonoid to be tested. Following incubation, plates were examined for viral plaque reduction. The flavonoid quercetin caused a reduction in both the infectivity of each virus as well as the intracellular replication of each virus in a concentration-dependent fashion. Hesperetin had no effect on infectivity but did reduce intracellular replication while naringin had no effect on either infectivity or replication of any of the viruses studied. According to Kaul, these results suggest that naturally occurring flavonoids possess a variable range of antiviral activity against certain RNA and DNA viruses to inhibit infectivity and replication (48). Wu *et al*. investigated the role of select flavonoids against HIV-1 (human immunodeficiency virus-1) replication in acutely infected H9 lymphocytes *in vitro*. Flavonoids were isolated from four species of plants of the genus *Desmos* and used to treat HIV-1–infected H9 T lymphocytes. While the majority of flavonoids tested showed only weak or no anti-HIV activity, one (Lawinal) showed potent anti-HIV activity while a second flavonoid (a chalcone), showed significant HIV inhibition (49).

In addition to antiviral properties, many berry fruits show antimicrobial activity against a wide range of bacteria. Because each berry fruit has its own unique complement of phytochemicals that influence its microbicidal activity, a wide range of inhibition between berry types is not uncommon. Berry bioactives have been shown to inhibit bacterial growth by a number of mechanisms, including destabilization or

permeabilization of the plasma membrane, inhibition of extracellular enzyme activity, direct actions on microbial metabolism and deprivation of bacterial substrates required for growth. In addition, berry compounds are able to prevent adhesion of bacteria to epithelial cells, a prerequisite for colonization and infection of many pathogens (50). A study by Cavanagh *et al.* tested commercial raspberry, blackcurrant, cranberry and blackberry cordials (100% berry juice) and fresh berries on 12 species of pathogenic bacteria (*Staphylococcus aureus,* methicillin-resistant *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella california, Salmonella enteritidis, Salmonella typhimurium, Shigella sonnei, Alcaligenes faecalis, Clostridium perfringens, Enterococcus faecalis* and *Mycobacterium phlei*). Dilutions (1:5, 1:10 and 1:100) of the cordial and fresh berry filtrate were prepared and mixed with nutrient agar; control plates contained nutrient agar alone. Plates were inoculated with bacteria from fresh 24 hour broth cultures and incubated 48 hours. Six different unidentified raspberry varieties were evaluated showing variable antibacterial activity, possibly due to differences in levels of ellagic acid and other phenolics in the raspberries. One variety showed complete inhibition of all bacteria tested while a second variety inhibited 10 of the 12 at both 1:5 and 1:10 dilutions. The remaining raspberry varieties were also effective to varying degrees but only at the 1:5 concentrations. Although the fresh raspberry extract inhibited the growth of six of the bacteria tested (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium* and *Clostridium perfringens* by 75% *and Shigella sonnei* by 100% at the 1:10 dilution), it was not 100% inhibitory to all the species. Of the other three berries tested, only the

blackcurrant at the 1:5 dilution completely inhibited all the bacteria while cranberry and blackberry showed variable results. None of the berries had antibacterial activity at dilutions of 1:100 (51).

Similar experiments were conducted by Nohynek *et al*. to test the effectiveness of berry phenolics against severe human pathogens of the gastrointestinal tract (GI). Phenolic berry extracts were prepared from a variety of fresh berries, including raspberries, cranberries, strawberries, black currant and cloudberries. The lyophilized berry extracts were suspended in 10 mL of growth media to a final concentration of 1 mg mL^{-1}. The bacterial strains used for the antimicrobial assays were grown in liquid cultures to late logarithmic or stationary phase and 1% of liquid microbial inoculums were added to the berry-treated medium. Cultures with no berry material served as controls. Liquid cultures were incubated 1 – 6 days depending on the growth rate of the studied microbe. Microbial growth was monitored by plate count of samples taken 4 – 6 times during the growth period of each organism. Test organisms included *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus cereus, Campylobacter jejuni, Escherichia coli, Helicobacter pylori, Salmonella typhimurium, Clostridium perfringens, and Lactobacillus rhamnosus.* Results of the study showed variability in the antimicrobial effects between berries as well as bacterial strains showing different sensitivities to the extracts. Cloudberry had the strongest antimicrobial activity followed by raspberry and strawberry. *Helicobacter pylori,* a causative agent of gastritis, peptic ulcers and gastric cancer, was the most sensitive to the extracts and was completely inhibited by these three berries. *Campylobacter jejuni,* a food-borne pathogen causing

gastroenteritis, was strongly inhibited by cloudberry and raspberry extract but unaffected by the other berry types. Of all the bacterial pathogens tested, cloudberry, raspberry and strawberry were clear inhibitors of microbial growth of all cultures while the other berry extracts showed no inhibition or weak bacteriostatic activity. Of the berries used in this study, cloudberry, raspberry and strawberry were the richest in ellagitannins, the main compound presumed to be responsible for their strong antimicrobial activity (52).

Immune Effects of Polyphenols

Although there is a large body of evidence supporting the role of phytochemicals as antioxidant and anticarcinogenic agents, much of the work has been demonstrated *in vitro* in human and animal tumor cell lines or *in vivo* in experimental animal systems. Human studies are limited and often focus on the chemopreventive and chemotherapeutic effects of phytochemicals in health and disease. For example, studies have been performed to determine the absorption, metabolism and excretion rates of phytochemicals or their metabolites in blood and urine as a measure of *in vivo* antioxidant activity. Other studies have evaluated phytochemicals as anti-inflammatory and anti-proliferative chemotherapeutic agents for the treatment of chronic illnesses such as cardiovascular disease and cancer. Very few *in vitro* or *in vivo* studies exist, however, that have examined the effect of phytonutrients on immune function. One area of focus has been upon the role certain dietary bioactive components have on the γδ T lymphocyte, a T cell type that has characteristics of both the innate and adaptive immune systems. While the majority of T cells express the αβ T cell receptor (TCR), a smaller population (1 - 10%) expresses the γδ TCR (53). In contrast to the αβ TCR that is characterized by its high

degree of antigen specificity, the γδ TCR appears to recognize classes of antigens that are present on groups of microbial pathogens, referred to as pathogen-associated molecular patterns (PAMPs) (54). Because the majority of γδ T cells reside in the epithelial linings of the gut, the lungs, the reproductive tract and skin, they are often considered the first line of defense against a vast number of foreign invaders that these cells are exposed to daily. The mucosal γδ T cells maintain the epithelial barrier by modulating inflammatory immune responses and promoting tissue repair in damaged epithelium (4). A second difference between the αβ- and γδ- T cells is that γδ T cells do not require the major histocompatibility complex (MHC) for antigen processing and presentation. The combination of rapid reactivity to frequently encountered pathogens without antigen processing or MHC presentation supports speculation that γδ T cells may function as an arm of the innate immune response (55). Gamma delta T cells also display the characteristics of adaptive immunity of expansion and memory. In addition to their residence in mucosal epithelial tissue, a second subset of γδ T cells is found in human blood where they continually expand and take on a memory phenotype during childhood from repeated exposure to foreign agents. These memory cells will recognize non-peptide PAMPs found in a variety of pathogens, undergo clonal expansion and release chemokines and cytokines that may play a regulatory role in recruiting αβ T cells, natural killer (NK) and NKT cells to the site of invasion (53). In addition to these immune activities, it has also been reported that subsets of γδ T cells recognize and lyse dying or metabolically-stressed or transformed host cells, possess anti-tumor activities and induce apoptosis in infected and malignant cells. They can also function as antigen presenting

cells and suppressor cells, where they kill activated macrophage at the end of an immune response (56). Because of the dual role γδ T cells have in innate and adaptive immunity, various phytochemicals capable of enhancing their activities has been explored. One *in vivo* study examined the effect of grape juice consumption on several immune parameters, including γδ T cell activity. Gamma delta T cells appear to have a unique response to certain phytochemicals, including the proanthocyanidins and anthocyanins found in grape juice. Apple polyphenols, tannins, and compounds found in green tea have also been shown to modify γδ T cell function (54). Recent research is beginning to show that some bioactive compounds such as those listed here may act like mild PAMP, priming cells to react quicker and stronger when confronted with a foreign invader (4). To examine this further, 78 healthy older volunteers consumed 355 mL of Concord grape juice daily for 10 weeks while a control group consumed a placebo. No other red, blue, or purple fruits or vegetables were consumed during the study. The results showed that the placebo group had a reduction in antioxidant activity in their serum (ORAC analysis), a reduction in γδ T cell proliferation *in vitro* and an increase in damage to lymphocyte DNA by oxidative stress, demonstrating the importance of red, blue, and purple fruits and vegetables in the diet. The participants ingesting the grape juice maintained these 3 functions and had an increased number of circulating γδ T cells in their blood compared to the control group. The investigator suggests that the phytochemicals found in grapes and grape products are recognized by γδ T cells because the chemical structures of the polyphenols resemble PAMP. This interaction serves to prime and support γδ T cell function and thus improves immune function (54). Nantz and colleagues performed a

double-blind, randomized, placebo-controlled study of 59 healthy law students who consumed either an encapsulated fruit and vegetable juice powder concentrate (FVJC) or placebo capsules for 77 days. Blood was collected on day 1, 35 and 77 to examine cytokine production, lymphocyte DNA damage, antioxidant status, carotenoid and vitamin C levels and the number of circulating αβ- and γδ-T cells. A log of illnesses and symptoms was also kept. In addition, Epstein-Barr virus (EBV) antibody titers were measured as an indicator of stress. According to Glaser, "Stress is known to have detrimental effects on immunity and may increase susceptibility to infectious agents, influence the severity of infections, reduce the response to vaccines, activate latent Epstein-Barr viruses and reduce the rate of wound healing" (56). In addition, the stress response is similar to the pro-oxidative state and can result in free radical damage to a variety of cells and tissues. At the conclusion of the study, the number of reported illnesses between the FVJP group and the placebo group did not differ, however the FVJP group reported fewer symptoms than the placebo group. EBV antibody titers did not change in either group over the course of the study although the participants reported they felt stressed. Cytokine levels, which can be altered under stress conditions, also did not change in either group. However, the level of INF-γ (interferon γ) produced by PMA (phorbol 12-myristate 13-acetate)-stimulated peripheral blood mononuclear cells was significantly lower (70%) in the FVJC group while the INF-γ levels in the placebo group did not change significantly over the course of the study. INF-γ plays a crucial role in the regulation of immune responses, and the reduction in INF-γ levels in the FVJC group suggests amelioration or reorganization of immunity to a non-inflamed state, according to

Nantz. The concentrations of the plasma carotenoids lutein, lycopene and β-carotene were significantly greater in the treatment group as well as plasma vitamin C levels. Plasma antioxidant ORAC values also increased 50% in the FVJC group over time and in the placebo group by the end of the study, but not to the degree observed in the treatment group. At baseline, lymphocyte DNA damage in the placebo group was significantly less than in the FVJC group, however, by day 35 DNA damage was reduced by 40% in the FVJC group to the same level as the placebo group with no changes occurring thereafter. Lastly, the FVJC group had a 30% increase in the number of circulating γδ T cells compared with their baseline or with the placebo group. The number of αβ T cells in the peripheral blood did not differ in either group. The investigators concluded the combination of fruit and vegetable juice phytochemicals consumed by study participants resulted in increased plasma nutrients and antioxidant capacity, a reduction in DNA strand breaks, and an increase in circulating γδ T cells (57). Holderness *et al*. focused on the response of γδ T cells to a plant-derived tannin from non-ripe apple peel (APP). The primary compounds in APP that are known to activate and prime γδ T cells are the procyanidins (also called condensed tannins), which are able to bind specific proteins with high affinity and induce immune responses (58). Experiments were designed to measure the *in vitro* proliferative response of human PBMC immune cell subsets to APP with IL-15 (interleukin-15) (1-20 µg/mL and 1 ng/mL respectively). Following a 48 hour treatment period, the majority of γδ T cells showed relatively little proliferation but did show an increase in the surface expression of IL-2Rα (interleukin-2 receptor α), an activation-related surface marker that indicates cells in a semi- activated, primed state. A

primed state allows for a more rapid and robust response to secondary stimuli, including antigen, cytokines or other types of agonists (58). The primed γδ T cells were then treated with IL-2 or IL-15 (secondary stimuli) and there was a 2 – 4 fold increase in proliferation versus the non-procyanidin-treated cultures. The small percentage of proliferating γδ T cells from the original culture increased 28% (mucosal γδ T cells) and 18% (peripheral γδ T cells) while the αβ T cells increased 18% following APP/IL-15 treatment. Although the B cell subset did not proliferate, there was an increase in expression of the activation marker CD69. Natural killer (NK) cells were the most responsive to procyanidin treatment with 55% of the total NK cell population proliferating. The monocyte/macrophage subset was depleted from the cultures and was not examined. According to Holderness, procyanidins such as those found in non-ripe apple peel with activating and proliferating effects upon γδ T cells may enhance host innate resistance to infection and modulate downstream adaptive immune responses. While Holderness reported a significant APP-induced proliferation of NK cells, a recent study in this laboratory by God focused on the effect of red raspberry consumption on NK cell cytotoxicity. Natural killer cells are part of the innate immune system and comprise 5 – 10% of lymphocytes in the peripheral blood. These cells display cytotoxic activity against a wide range of tumor cells and virally-infected host cells (55). To evaluate red raspberry polyphenols on NK cell cytotoxic activity, blood samples were acquired from 15 healthy volunteers before and after raspberry consumption. The monocytes/macrophages were depleted from the blood samples, leaving only the peripheral blood lymphocytes (PBLs) for the NK cytotoxicity assays. Chronic

myelogenous leukemia K-562 cells, the standard cell line for evaluating NK cytotoxicity, were treated with three concentrations of PBLs and allowed to incubate 4 hours. Cell viability measurements yielded variable results among volunteers. Five of the fifteen volunteers showed a significant increase in NK cytotoxicity levels following consumption of red raspberries while the remaining donors showed no effect or a negative effect. Preliminary assays were also done to test for the cytotoxic effect of plasma on MCF-7 breast cancer cells before and after raspberry consumption. MCF-7 cells were cultured in growth medium containing five concentrations of plasma (3, 5, 8, 10 or 12%) for 48 hours and cell viability measured. Each of the three participants showed a significant increase in cytotoxicity in the post-berry treated cells compared to the pre-berry samples. These results suggest that consumption of red raspberries can enhance NK cell cytotoxic activity in some individuals and lead to production of blood plasma components which are toxic to cancer cells *in vitro* (unpublished data, 18).

The current study was undertaken to test the feasibility of examining the effects of berry consumption on the immune response in humans in a new way which, if successful, should yield more rapid and trustworthy results than the traditional methods. The proposed approach would not require the long-term, large scale data collection used in epidemiological studies. It is expected that the results obtained with this approach will give a better approximation of the effects of berry consumption or other treatments than other currently used methods using either *in vitro* or animal models.

While *in vitro* studies of the effects of berry extracts on cell lines can give insight into whether a particular extract might contain biologically active compounds, there are

several problems with trying to extrapolate from these studies what the effects of these extracts will be *in vivo*. These include: 1) the cells are cultured in an artificial medium supplemented with fetal bovine serum (FBS) or some similar substitute. This is a very poor simulation of the actual environment of cells in the human organism. 2) The cells used are from lines which have been maintained in culture – usually for several passages – and their responses probably do not represent the responses of actual healthy or tumor cells in the organism. 3) The extracts tested contain only the compounds present in the berry extract. The effects of any changes in these compounds or any new compounds produced by *in vivo* metabolism cannot be evaluated by this approach.

Tests of the extracts in animal models also have defects. The changes induced in the test extracts by the metabolic system of the animal model are not likely to be the same as those induced in humans. Also, the immune responses in the animal model system are not exactly the same as in humans and perturbations induced by interaction with the test extracts and their metabolic products could be very different from those which take place in humans. There are numerous examples of this in the failures of drugs tested and giving promising results in animals, but failing to be effective or causing unacceptable side effects in humans.

In the proposed approach, human subjects are asked to consume the berries or berry extracts for several days and then donate blood samples for testing. The blood plasma is used to supplement culture medium for immune cells from the same donor. This avoids changes induced in the cells' responses by exposure to the alien components present in serum from fetal calves or other animals. The complement of hormones,

growth factors and other biological response modifiers present in the animal sera is completely different from that of human subjects and the responses of this subject's cells in this medium are probably completely different from what would take place *in vivo*. Clear evidence for this has been obtained from experiments in which DNA repair in human cells was found to be very different when cells collected from the same subject at the same time were cultured both in medium supplemented with FBS and in medium supplemented with their autologous plasma (59, 60). There are also even great differences in the levels of these growth regulatory components from one batch of animal serum to another. This is evidence of the fact that examination of different preparations of FBS differ by as much as 1000-fold in thyroid stimulating hormone (Hyclone Corporation newsletter). These variations could explain the differences in berry effectiveness observed by different investigators.

In addition to the fact that in this assay the cells are cultured in their own plasma, this plasma now contains not only components of the berry or extract, but also the compounds resulting from conversions carried out by the subject's own metabolic system. Thus, the experimental system in the proposed approach involves assaying the effects of the test compounds on a subject's cells in their autologous plasma containing metabolic products produced by processing of his own metabolic system.

Finally, an additional advantage to this over epidemiologic studies is that subjects can be studied individually. Each individual has his own genetic makeup resulting in his own peculiar metabolic system. This could result in different effects of berry consumption among different individuals. This is evident in the different ways people

can respond to the same drug. Since in our approach a subject's cells are tested in their own plasma before and after berry consumption, the results obtained are specific for that individual and the effects of berry consumption on that subject can be assessed as opposed to assuming that his responses will be those obtained by averaging over a large number of individuals who might have completely different genetic backgrounds. Using this novel experimental approach, a pilot study will be performed with healthy subjects to examine the effects of raspberry consumption on *in vivo* and *in vitro* immune parameters, including immune cell proliferation, plasma MMP-9 concentrations, and cytotoxicity toward human tumor cell lines.

2. MATERIALS AND METHODS

Effect of Red Raspberry Extract on Cancer Cells *in vitro*

Red raspberry extract was prepared from lyophilized red raspberries (Meeker variety, VanDrunen Farms, Momence, IL) to examine the cytotoxic or cytocidal effects of the extract upon five human cell lines. All cell lines were acquired from ATCC and cultured according to their recommended guidelines. Continuous cultures of cell lines were maintained in the appropriate medias supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (complete growth medium, CM). ATCC's standard protocol for subculturing by trypsinization was followed in all experiments. Cell lines tested were AGS (gastric adenocarcinoma), LoVo (colorectal adenocarcinoma), LNCaP (prostate carcinoma), HCC 1500 (ductal carcinoma from breast tissue), and CRL-2120 (normal skin fibroblast). Further information on each cell line is listed in Table 1.

Table 1. Cell Line Descriptions

ATCC® Number	Designation	Characteristics	Source
CRL-1739™	AGS	infected with Parainfluenza type 5; doubling time 20 hrs.	stomach
CCL-229™	LoVo	Dukes' type C, grade IV	colon
CRL-1740™	LNCaP	estrogen receptor, androgen receptor, doubling time 34 hrs.	prostate
CRL-2329™	HCC 1500	estrogen receptor, progesterone receptor, her2/neu$^-$, p53$^+$, TNM stage IIB, grade 2, doubling time 80 hrs.	mammary gland; breast
CRL-2120™	CCD-1094Sk	normal skin fibroblast isolated from breast with metastatic mammary carcinoma	normal skin
CCL-243™	K-562	lymphoblast, suspension culture, target for assessing natural killer cell activity	bone marrow

Red raspberry extract was prepared by blending 5 g of lyophilized berries with 35 mL distilled water and centrifuging for 10 minutes at 3000 rpm. The extract supernatant was removed and placed into a 50 mL conical tube and centrifuged for 10 minutes at 3000 rpm. The extract was removed and the process repeated again. The pH of the clarified extract was 2.94. The extract was sterilized through a 0.2 µm syringe filter (Nalgene) and mixed with complete growth medium appropriate for each cell line to give final concentrations of 10%, 15% and 20% raspberry extract.

Cell lines were trypsinized and counted with a hemocytometer using the trypan blue dye exclusion assay to determine viability. Cells were suspended in 250 µL complete growth medium at a density of 1.25×10^5 cells/sample. AGS and LoVo were suspended in F12-K CM, LNCaP and HCC 1500 in RPMI 1640 CM and CRL-2120 in EMEM CM. Two hundred fifty (250) µL of 10%, 15% and 20% raspberry extract were added to the samples to give final extract concentrations of 5%, 7.5% and 10%. A blank was prepared for each cell line containing complete medium only. Samples were vortexed immediately before they were aliquoted in quintuplicate into a 96 well plate, yielding a final cell density of 2.5×10^4 cells per 100 µL per well. The plate was incubated for 48 hours at 37°C in 5% CO_2.

Following incubation, the plate was centrifuged at 125 x g for 3 minutes and the supernatant decanted. Residual medium was removed by aspiration. To measure cell viability, the CellTiter 96®Aq_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) was performed according to the manufacturer's protocol. Briefly, this is a colorimetric assay in which MTS, a tetrazolium compound, is converted into formazan, a

product soluble in tissue culture medium, by dehydrogenase enzymes that are found in metabolically active cells. The absorbance of the formazan at 490 nm is directly proportional to the number of living cells in the sample.

A working solution was prepared by mixing MTS solution and culture medium at a ratio of 1 part MTS:5 parts culture medium. The MTS/culture medium solution was pipetted into each well; blank wells were prepared containing MTS/ culture medium solution only. The plate was incubated for 4 hours at 37°C in 5% CO_2 and the absorbance measured at 490 nm using the Tecan Infinite 200 microplate reader.

Effect of Ascorbic Acid on Cancer Cells *in vitro*

Due to the acidity of the raspberry extract (pH 2.9), the role of pH in cell killing was evaluated. Cell lines were treated with solutions of ascorbic acid adjusted to the same pH as the berry extract. In addition, the antioxidant property of ascorbic acid in cell killing was assessed.

A 19 mM solution of ascorbic acid was prepared (pH 2.91) in distilled water and sterilized through a 0.2 µm syringe filter. The sterile solution was mixed with complete growth medium appropriate for each cell line to give final concentrations of 10%, 15% and 20% ascorbic acid solutions. Cell lines were trypsinized and counted as above and suspended in 250 µL complete growth medium at a density of 1.25×10^5 cells/sample. Two hundred fifty (250) µL of 10%, 15% and 20% ascorbic acid stock solution was added to the samples to give final concentrations of 5% (0.95 mM), 7.5% (1.4 mM), and 10% (1.9 mM) ascorbic acid. A blank was prepared containing complete medium only for each cell line. Samples were vortexed immediately before they were aliquoted in

quintuplicate into a 96 well plate at a density of 2.5×10^4 cells per 100 µL per well. The plate was incubated for 48 hours at 37°C in 5% CO_2.

Following incubation, the plate was centrifuged at 125 x g for 3 minutes and the supernatant decanted. Residual medium was removed by aspiration. To measure cell viability, the MTS assay was performed as described above. The plate was incubated for 4 hours at 37°C in 5% CO_2 and the absorbance measured at 490 nm using the Tecan Infinite 200 microplate reader.

In Vivo **Red Raspberry Study**

Volunteers were asked to complete a questionnaire (Appendix A) to determine their eligibility to participate in the study. Sixteen healthy volunteers were selected as research subjects and asked to refrain from eating berries, fruits, or fruit-related foods or juices for two days. Volunteers were supplied with the Meeker variety of lyophilized red raspberries from the same lot as used in the *in vitro* assays described above. Volunteers consumed two servings of red raspberries daily for three days and one serving on day 4, for a total of seven servings (one serving = 22 grams). The freeze-dried raspberries were supplied to each volunteer in individual, pre-packaged servings. Participants were asked to refrain from eating any other berries, fruits, or fruit-related foods or juices during this time, but no other restrictions were placed on their diets. Each volunteer read and signed an informed consent form and completed the questionnaire in the appendix. The demographic data for the participants is listed in Table 2. Two blood samples were collected from each donor by venipuncture at Redfern Health Center by certified medical technologists according to standard clinical guidelines. The first blood sample was

Table 2. Demographic Profiles of Study Participants

Donor	Sex	Age	Exercise (per week)	Alcohol (per week)	Fruits/Juices (per week)	Medications
1	M	55	2	1-2	9	
2	M	62	4	2	21	
3	F	68	1	0	21	Synthroid, Fish Oil, Centrum, vitamin C, Caltrate, Osteobiflex
4	M	24	1	2-5	2	
5	F	51	5	1	14	Pravastin, Microgestin, Zomig Zmt, Alendronate,
6	F	55	0	0	7	Calcium,Magnesium
7	F	65	2	0	9	
8	F	31	2	0-1	14	Levothyroxine, Multivitamin
9	M	30	2	1	8	Multivitamin
10	M	52	7	4	7-12	Fish Oil, Niacin, Aspirin, Multivitamin
11	F	59	0	0	4	Calcium, Fem HRT
12	F	25	3	10	10	Birth control
13	F	20	2	0-1	10	Doryx, Birth control, Tri-Sprintec
14	F	21	2	0	5	Endial, Pantecta, Advair, Nasonex, Multivitamin
15	F	21	2	1-2	10	
16	F	21	3	0-1	12	

collected immediately before donors ate the first serving of berries, (and after having fasted from fruits and berries for two days), and the second 1-2 hours after eating the berries on day 4. Each time, 40 mL of donor blood was collected in 10 mL Vacutainer® tubes containing the anticoagulant sodium heparin. An additional 5 mL of blood was collected in a 5 mL Vacutainer® tube containing EDTA to determine the complete blood count (CBC) of the donor. The CBC was performed by Redfern Health Center medical technologists on a Beckman Coulter A^{C}•T^{TM} 5 diff Cap Pierce Hematology Analyzer.

Mononuclear Cell Isolation

To isolate the peripheral blood mononuclear cells (PBMCs), 5 mL of heparinized blood was layered onto 5 mL room temperature polysucrose sodium diatriazoate (Histopaque 1077; Sigma-Aldrich, St. Louis, MO) in 15 mL conical tubes. The tubes were centrifuged at 400 x g in a swinging bucket rotor for 30 minutes. The plasma was removed and reserved for medium supplementation and MMP-9 analysis. Plasma assayed for MMP-9 content was centrifuged an additional 10 minutes at 10,000 rpm to remove any remaining platelets. The MMP-9 plasma was immediately stored at -20° C until assayed. The buffy coat containing the mononuclear cells was removed and placed into two 15 mL conical tubes, each containing 10 mL phosphate buffered saline (PBS). Cells were vortexed and then centrifuged for 10 minutes at 250 x g. The PBS was decanted and the wash step repeated. Pelleted PBMCs were suspended in 3 mL RPMI 1640 complete medium (RPMI CM) containing 1% Penicillin/Streptomycin and 15% autologous plasma or 3 mL F-12K complete medium (F-12K CM) containing 1%

Penicillin/Streptomycin and 15% autologous plasma. The cells were counted in a hemocytometer using the Trypan Blue Dye Exclusion assay to determine viability.

PHA Assay

Three concentrations of phytohemagglutinin (PHA), a lymphocyte mitogen, (Sigma-Aldrich, St. Louis, MO), were prepared in RPMI CM at 2.5 µg/mL, 5 µg/mL and 7.5 µg/mL PHA. Three x 10^6 PBMCs in RPMI CM were aliquoted into each of three microcentrifuge tubes and centrifuged 3 minutes at 2000 rpm. The supernatant was removed and the cells resuspended in 300 µL PHA solution at each concentration. A control tube was prepared containing 3 x 10^6 PBMCs in RPMI CM only. Each sample was vortexed immediately before plating in a 96 well plate. All samples were prepared in triplicate, with 100 µL PHA solution containing 1 x 10^6 cells placed in each well. Cells were placed in a 37°C humidified incubator with 5% CO_2 for 72 hours. Following incubation, the plate was centrifuged at 125 x g for 3 minutes and the supernatant decanted; residual medium was removed by aspiration. To measure the extent of proliferation, the MTS Assay was performed as described above.

MMP-9 Immunoassay

To determine the effect of raspberry consumption on circulating levels of MMP-9, a matrix metalloproteinase enzyme, the Quantikine® MMP-9 Immunoassay (R&D Systems, Minneapolis, MN) was performed. This is a chromogenic sandwich ELISA assay that uses a monoclonal antibody specific for MMP-9 to measure the amount of total MMP-9 (pro and/or active) in a sample. A microplate reader set to 450 nm is used to

measure the optical density (OD) of the sample and the concentration is determined by comparing the absorbance to that of known MMP-9 standards supplied with the kit.

Plasma collected during mononuclear cell isolation was centrifuged one additional time at 10,000 rpm for 10 minutes to remove any remaining platelets in the plasma. MMP-9 is released upon platelet activation; therefore it is necessary to use platelet-free plasma in the assay. Samples were stored immediately at -20°C until assayed. To perform the assay, plasma samples were thawed and the assay completed according to the manufacturer's protocol. The OD values of the plasma samples and standards were measured at 450 nm on the Tecan microplate reader. A standard curve was constructed from the OD values of the known standards and is shown in Figure 1. These data were found to fit a polynomial with the following equation and R^2 value:

$$y = -0.0033x^2 + 0.1732x + 0.0074$$

$$R^2 = 0.9995$$

The amount of MMP-9 per donor sample was determined mathematically from the equation of the standard curve.

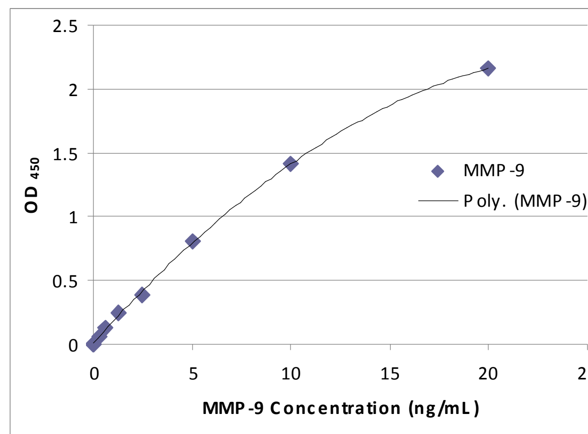


Figure 1. MMP-9 Standard Curve

Plasma Assay

Donor plasma collected before and after eating lyophilized red raspberries was assayed for its effects on the growth of five tumor cell lines. In addition to AGS (gastric), LoVo (colorectal), LNCaP (prostate) and HCC 1500 (breast), the chronic myelogenous leukemia cell line K-562, acquired from ATCC, was also tested (Table 1). The latter was added after samples from several donors had already been processed.

Adherent cell lines were trypsinized according to ATCC's subculturing protocol and suspended in media only: AGS and LoVo in F-12K and LNCaP and HCC 1500 in RPMI 1640. K-562 cells were centrifuged and resuspended in Iscove's Modified Dulbecco's Medium. For each cell line, the cells were counted with a hemocytometer and viability was assessed with the trypan blue dye exclusion assay.

Tumor cell lines were tested under four conditions to determine the effect of donor plasma on cell proliferation. Each cell line was assayed in quintuplicate at 1.25×10^4 cells/well and suspended in medium only, medium supplemented with 15% FBS, medium supplemented with 15% autologous plasma, or 100% autologous plasma only. Not all conditions or all cell lines were used for every donor. Cell suspensions were vortexed immediately before they were dispensed into wells. Cells were placed in a 37°C humidified incubator with 5% CO_2 for 72 hours. Following incubation, the plate was centrifuged at 125 x g for 3 minutes and the supernatant decanted; residual medium was removed by aspiration. The MTS Assay was performed to measure cell viability as described above.

PBMC Cytotoxicity Assay

The cytotoxicity assay was performed on blood samples collected before and after berry consumption to measure the effect of raspberries on PBMC activity against AGS, LoVo, LNCaP, HCC 1500 and K-562 tumor cell lines. The PBMCs served as effector cells (E) and the tumor cell lines were the target cells (T) in this assay.

Target cells were aliquoted into microcentrifuge tubes using 7.5×10^4 cells/sample. The PBMC effector cells were added to the target cells in the following ratios (effector:target): 5:1, 10:1 and 20:1. The mixed cell population was suspended in a total volume of 300 µL of the appropriate medium containing 15% autologous plasma. The cell suspensions were vortexed and dispensed in triplicate in a 96 well plate, resulting in 2.5×10^4 target cells/well. For some donor samples, the culture medium was omitted and replaced by 100% autologous plasma. The following wells were prepared in each assay: target only (T), effector only (E), and effector + target (ET). The plate was incubated 72 hours at 37°C in 5% CO_2. Four-hour incubations were also performed on some donor samples. Following incubation, the plate was centrifuged at 125 x g for 3 minutes and the supernatant decanted; residual medium was removed by aspiration. Cell viability was measured using the MTS assay as previously described. The calculation for cytotoxicity was performed as follows:

$$[((T) - (ET - E)) / T] \times 100 = \% \text{ cell death}$$

The calculation for determining the surviving fraction of cells after treatments was performed as follows:

$$[(ET - E) / T] \times 100 = \% \text{ surviving fraction}$$

Statistical Analysis

All absorbance values reported were based on the means of three or five replicate samples for each assay performed. The statistical analysis was done using the Student's *t* two-tailed matched pairs test to analyze the significance of the differences between baseline and post-raspberry ingestion samples. Analysis of variance was applied to test for significant differences between donor group means by ANOVA. Differences between assays and between donor groups were considered significant at $p \leq 0.05$. Pearson correlations, denoted by *r,* were used to assess the relationships between assays and between donor groups. Correlation coefficients between -1.0 to -0.5 and 0.5 to 1.0 were considered high.

3. RESULTS

Effects of Red Raspberry Extract and Ascorbic Acid on Cancer Cell Proliferation *in vitro*

Preliminary *in vitro* assays were performed to detect the effect of red raspberry extract on the proliferation of five human cell lines. The cell lines tested were: AGS (gastric), LoVo (colon), LNCaP (prostate), HCC 1500 (breast), and CRL-2120 (normal skin fibroblast). The cells were cultured for 48 hours in the presence and absence of extract and their viabilities measured. Due to the acidity of the berry extract (pH 2.94) it was necessary to consider the role of pH in cell killing. Each cell line was treated with a solution of ascorbic acid adjusted to have the same pH as the berry extract. In addition to its pH effect, ascorbic acid also served as a control for the antioxidant effect in cell killing.

The raspberry extract had a significant effect upon the proliferation of three of the five cell lines in a concentration-dependent manner, and the degrees of inhibition varied between the cell lines (Figure 2 and Table 3). AGS, LoVo, and LNCaP showed the greatest inhibition, with AGS being the most sensitive to the raspberry extract treatment. The AGS cells were inhibited to a higher degree than the other cell lines, with 90% cytotoxicity observed in the AGS samples treated with 10% raspberry extract while LNCaP and LoVo showed 52% and 71% cytotoxicity at this same dosage. Raspberry extract produced a slight stimulatory effect on HCC 1500 cells at the lowest treatment dosage but the two higher dosages were inhibitory at 24% and 56%. CRL 2120 normal skin fibroblasts followed a similar trend with 5% raspberry extract having no effect while concentrations of 7.5% and 10% extract were cytotoxic at 47% and 75% respectively.

Each cell line was also treated with solutions of ascorbic acid that served as controls for both the pH and antioxidant effects in cell killing. The cells were cultured for 48 hours in 5% (0.95 mM; 17 µg), 7.5% (1.43 mM; 25.5 µg), or 10% (1.90 mM; 34 µg) ascorbic acid and their viabilities measured. In contrast to the raspberry extract treatment, the same inhibition of cell proliferation was not observed in cells treated with ascorbic acid. The ascorbic acid produced a stimulatory effect in all five cell lines in a concentration-dependent manner, with LoVo, LNCaP, HCC 1500, and CRL 2120 cells increasing in proliferation between 18% – 80%. AGS cells were the least sensitive and increased only 13% at the highest ascorbic acid concentration.

Effects of Red Raspberries on Immune Parameters *in vivo*

The primary aim of the present study was to determine the effects of red raspberry consumption on: 1) *in vivo* and *in vitro* immune cell proliferation, 2) plasma matrix metalloproteinase 9 (MMP-9) levels, 3) blood plasma tumor cell cytotoxicity, and 4) PBMC tumor cell cytotoxicity.

Complete Blood Counts

Pre-berry and post-berry complete blood counts (CBCs) are listed for the study participants in Appendix B. For 12 of the 15 donors, increases were noted in their total white blood cell (WBC) counts, leukocyte subsets, and/or the WBC differential counts after eating raspberries for 3.5 days.

WBC counts are expressed as the absolute number of each cell type x 10^3/µL of blood. The pre-berry cell counts and their percent changes are listed in Table 4 for the subpopulations of WBCs where an increase was observed.

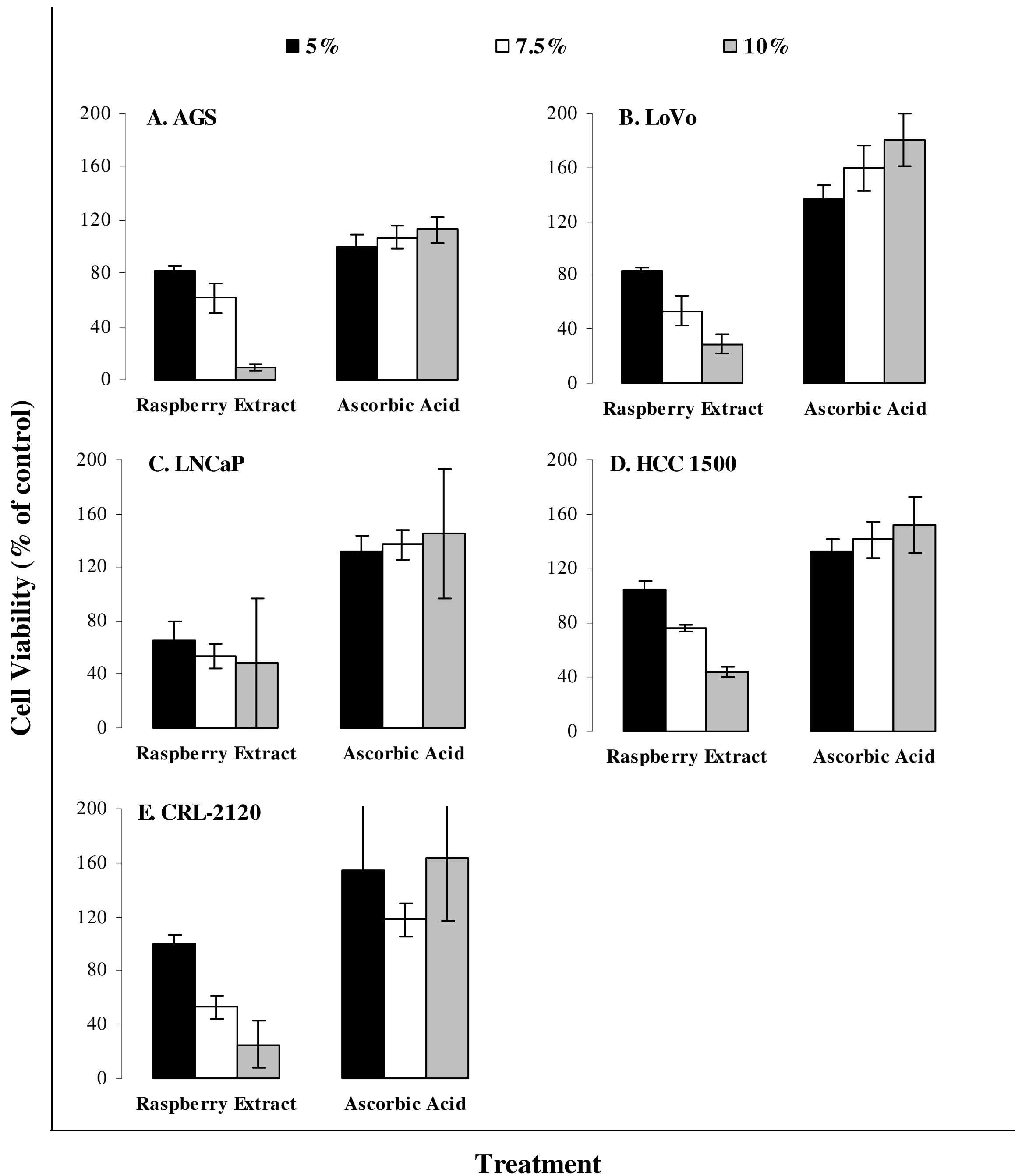


Figure 2. Dose effectiveness of red raspberry extract and ascorbic acid on cell line viability. A) AGS, B) LoVo, C) LNCaP, D) HCC 1500, and E) CRL-2120 cells were treated with three concentrations of raspberry extract or ascorbic acid for 48 hours and cell viabilities measured using the MTS assay. Results are presented as means of three replicate experiments ± standard error.

Table 3. Effect of Raspberry Extract and Ascorbic Acid on Cell Proliferation *in vitro*

	Cell Viability (% of control)					
	Raspberry Extract			Ascorbic Acid		
Cell Line	5%	7.5%	10%	5%	7.5%	10%
AGS	81±4.5	61±11.4	10±2.5	100±9.0	107±9.0	113±10.0
p-value	0.03	0.04	0.00	0.49	0.25	0.16
LoVo	83±2.5	54±11.3	29±7.2	136±10.8	159±16.8	180±19.5
p-value	0.01	0.03	0.00	0.04	0.04	0.03
LNCaP	65±14.5	53±9.5	48±8.1	132±11.9	137±11.6	145±17.3
p-value	0.07	0.02	0.01	0.06	0.04	0.06
HCC 1500	104±6.1	76±2.4	44±3.7	133±9.4	142±13.9	152±20.3
p-value	0.28	0.01	0.00	0.04	0.05	0.06
CRL 2120	100±6.4	53±8.2	25±17.3	155±58.0	118±12.0	164±46.5
p-value	0.49	0.01	0.02	0.22	0.14	0.15

Effects of three concentrations of red raspberry extract and ascorbic acid on the proliferation of five human cell lines. Results are presented as means ± standard error based on three replicate experiments. Significantly different from control: $p \leq 0.05$.

The percent increase was determined as follows:

$$\% \text{ difference} = \frac{\text{Post-berry absolute count - Pre-berry absolute count}}{\text{Pre-berry absolute count}} \times 100$$

At the completion of the 3.5 day study, 40% (6/15) of the donors showed an increase in the total number of circulating WBCs. Of these, 100% showed an increase in lymphocytes; 83% (5/6) increased in monocytes, and 67% (4/6) increased in neutrophils, eosinophils and basophils. Although the absolute numbers of these cell types increased following berry intake, they remained within the normal range of values for each cell type.

Additional changes were also noted in the relative WBC differential counts of three donors (Table 5). The WBC differential count is the proportion of each cell type expressed as a percent of the total WBC count. The normal relative differential ranges are 20.0 / 40.0% for lymphocytes, 45.0 / 70.0% for neutrophils, and 1.0 / 5.0 % for eosinophils. Before raspberry consumption, the relative WBC differential counts of donors 13 and 14 were outside the normal range of values for the lymphocytes and neutrophils, indicating lymphocytosis (elevated lymphocyte count) and neutropenia (decreased neutrophil count). In addition, donor 13 also had eosinophilia (elevated eosinophil count). Although donor 13's post-berry CBC still indicated these conditions, there was improvement toward the normal range in all three cell types. For donor 13, the lymphocytes and eosinophils decreased (2.1% and 1.9% respectively) and the neutrophils increased (4.5%) after berry consumption. For donor 14, the lymphocytes dropped 1.3% while the neutrophils increased 1.1% after berry consumption. The post-berry results for donor 15 showed a change in the relative WBC differential count as well. The pre-berry CBC showed a high relative neutrophil level that decreased 20.9% following raspberry ingestion.

Effect of Raspberry Intake on Mitogen Responsiveness

A mitogen stimulation assay was performed on freshly isolated peripheral blood mononuclear cells (PBMCs) collected before and after red raspberry consumption to assess the effect of raspberry phytochemicals on lymphocyte proliferation, which is related to immunocompetence. The stimulation index (SI) was determined for the pre- and post-berry samples from the absorbance values of the PHA-treated samples

Table 4. Proliferative Effect of Red Raspberries on Subpopulations of White Blood Cells *in vivo*

Donor		WBC (4.0/11.0) 10^3/µL	NE (2.0/7.5) 10^3/µL	LY (1.5/4.0) 10^3/µL	MO (0.2/0.8) 10^3/µL	EO (0.04/0.40) 10^3/µL	BA (0.02/0.10) 10^3/µL
1	Pre-berry				0.4		
	Post-berry ↑				32.5%		
3	Pre-berry					0.1	
	Post-berry ↑					10%	
4	Pre-berry	5.2	3.14	1.66	0.26	0.16	0.03
	Post-berry ↑	32.7%	42.4%	14.5%	26.9%	12.5%	66.7%
6	Pre-berry			1.2		0.09	0.02
	Post-berry ↑			15%		33.3%	50%
7	Pre-berry				0.44	0.12	
	Post-berry ↑				11.4%	25%	
8	Pre-berry		2.48				
	Post-berry ↑		1.6%				
9	Pre-berry	6.9		2.74	0.43	0.33	0.03
	Post-berry ↑	5.8%		8%	14%	18.2%	33.3%
11	Pre-berry	6.6	4.09	1.81	0.41	0.21	
	Post-berry ↑	10.6%	6.6%	21.6%	2.4%	42.9%	
13	Pre-berry		2.09				
	Post-berry ↑		5.74%				
14	Pre-berry	4.8	1.91	2.51	0.28		
	Post-berry ↑	6.3%	7.9%	2%	17.9%		
15	Pre-berry	8		1.76	0.27	0.14	0.04
	Post-berry ↑	3.8%		87.5%	77.9%	50%	25%
16	Pre-berry	5.7	2.76	2.26			0.03
	Post-berry ↑	14%	13.4%	20.8%			66.7%

Pre-berry total cell counts in WBC subpopulations and the percent increase after berry consumption. Key: WBC, white blood cell; NE, neutrophil; LY, lymphocyte; MO, monocyte; EO, eosinophil; BA, basophil. The reference range for each leukocyte count x 10^3/µL blood is listed in parenthesis.

Table 5. Effect of Red Raspberries on the Relative WBC Differential Counts *in vivo*

Donor		NE % 45.0 / 70.0	LY % 20.0 / 40.0	EO % 1.0 / 5.0	Summary
13	Pre-berry	30.8	50.1	13.5	Neutropenia, lymphocytosis, eosinophilia
	Post-berry	35.3	48.0	11.6	
14	Pre-berry	39.6	51.9		Neutropenia, lymphocytosis
	Post-berry	40.7	50.6		
15	Pre-berry	72.5			Neutrophilia
	Post-berry	51.6			

Pre-berry and post-berry relative WBC differential counts expressed as percents.
Key: WBC, white blood cell; NE, neutrophil; LY, lymphocyte; EO, eosinophil. The reference range for each leukocyte is expressed as a percent of the total WBC count and is listed in parenthesis.

normalized to the untreated, control PBMCs. A comparison was then made between the post-berry and pre-berry stimulation indices to determine whether berry consumption might affect cell-mediated immunity. The ratio of the post berry SI to the pre-berry SI was calculated. Ratios >1 indicate greater post-berry lymphocyte stimulation whereas ratios <1 signify greater pre-berry lymphocyte stimulation. In addition, resting MTS absorbance ratios were determined between untreated post-berry and pre-berry control PBMCs to examine the *in vivo* effect of berry phytochemicals upon the control PBMCs. Donors were divided into three groups based on the amount of change in their SIs. Results are shown in Figures 3 - 5 and Table 6. The SIs given in Table 6 represent the maximum of the three SIs measured for a given condition.

A significant difference was observed between the donor groups in lymphocyte responses to mitogen stimulation after berry consumption ($p < 0.0001$). Group 1 donors exhibited greater PHA-induced lymphocyte proliferation following raspberry consumption than prior to consumption ($p = 0.04$). For donors in Group 2 there was little change and for those in Group 3 there was a significant decrease in PHA response

following berry consumption (p = 0.002). Furthermore, the control PBMC SIs also showed a significant difference between groups (p = 0.007). Group 1 control PBMCs decreased in absorbances (p = 0.009) following berry intake while Group 3 control PBMCs increased (p = 0.09) in absorbance measurements. No significant changes were noted in the PBMC absorbances of Group 2 donors.

An inverse correlation was observed between the degree of mitogen-induced lymphocyte proliferation and the post-berry : pre-berry control PBMC ratios for the 16 donors (r = -0.6; p = 0.02). In Group 1, mitogen stimulation was greater after berry consumption, but the post : pre-berry control PBMC ratios were <1, ranging from 0.45 – 0.90 (r = -0.9). In contrast, for Group 3 donors mitogen responsiveness was less after berry consumption but the post : pre-berry control PBMC ratios were >1, ranging from 1.89 – 3.72 (r = 0.6), with the exception of donor 10 who was on a daily low-dose aspirin regimen. There was minimal or no differences in pre- and post-berry SIs for the Group 2 donors, and the post : pre-PBMC ratios were close to 1 except for donor 13, who was menstruating at the time.

Effect of Raspberry Intake on Plasma Levels of MMP-9

The effect of raspberry consumption on MMP-9 plasma levels was measured in pre- and post-berry plasma samples for fifteen of the study participants. The enzyme concentration was determined in each sample and a ratio of the post-berry to pre-berry MMP-9 concentration was calculated for each donor. Ratios <1 indicate greater pre-berry MMP-9 concentrations whereas ratios >1 reflect greater post-berry MMP-9 concentrations. Ratios differing from 1.0 by 10% or less are not considered meaningful

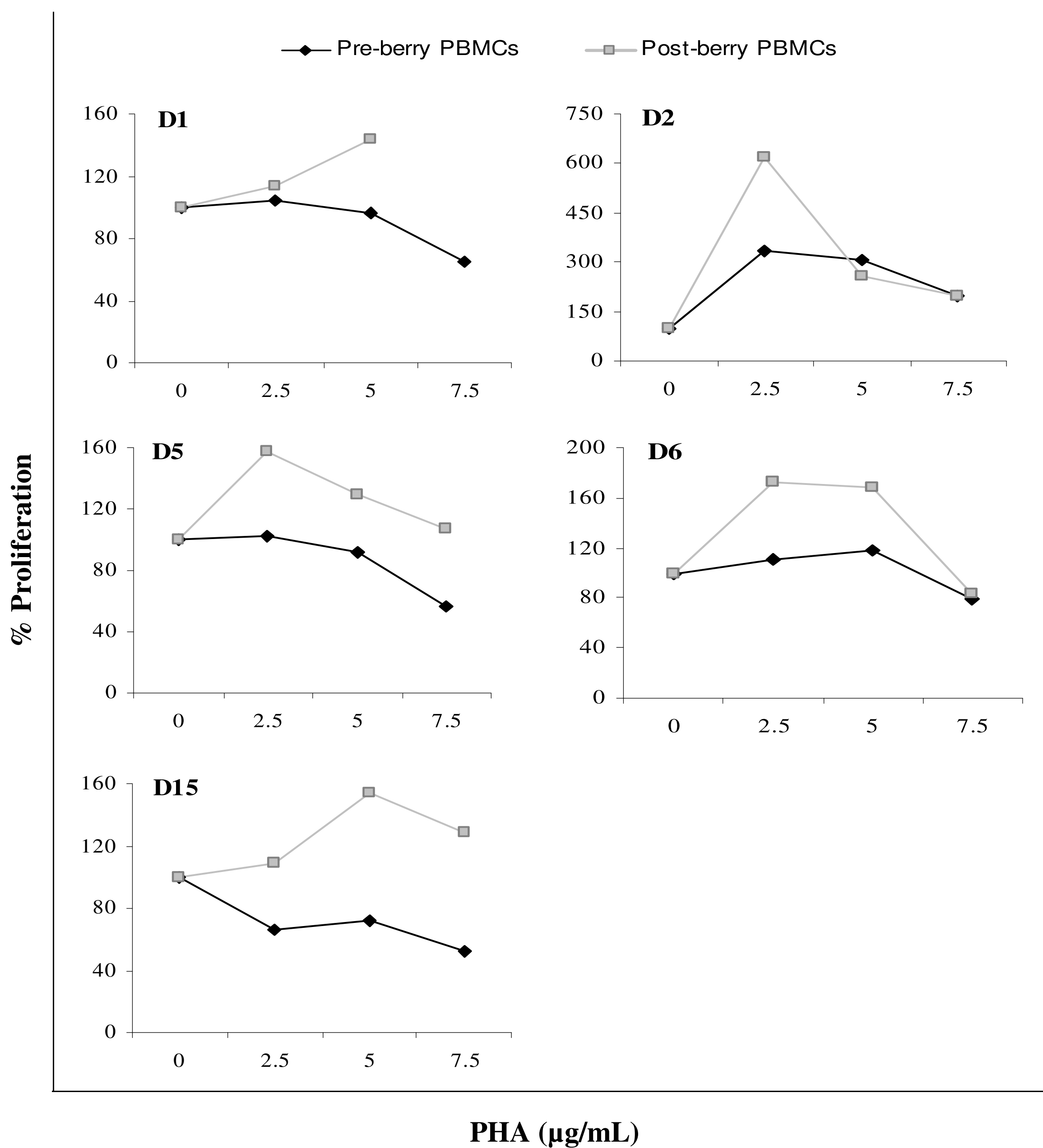


Figure 3. Group 1 mitogen-induced stimulation of T lymphocytes pre- and post-raspberry consumption. Peripheral blood mononuclear cells were treated with three concentrations of PHA in RPMI medium supplemented with 15% autologous plasma. PBMCs were incubated for 72 hours and cell viabilities measured using the MTS assay. Results are presented as the means of PBMCs prepared in triplicate.

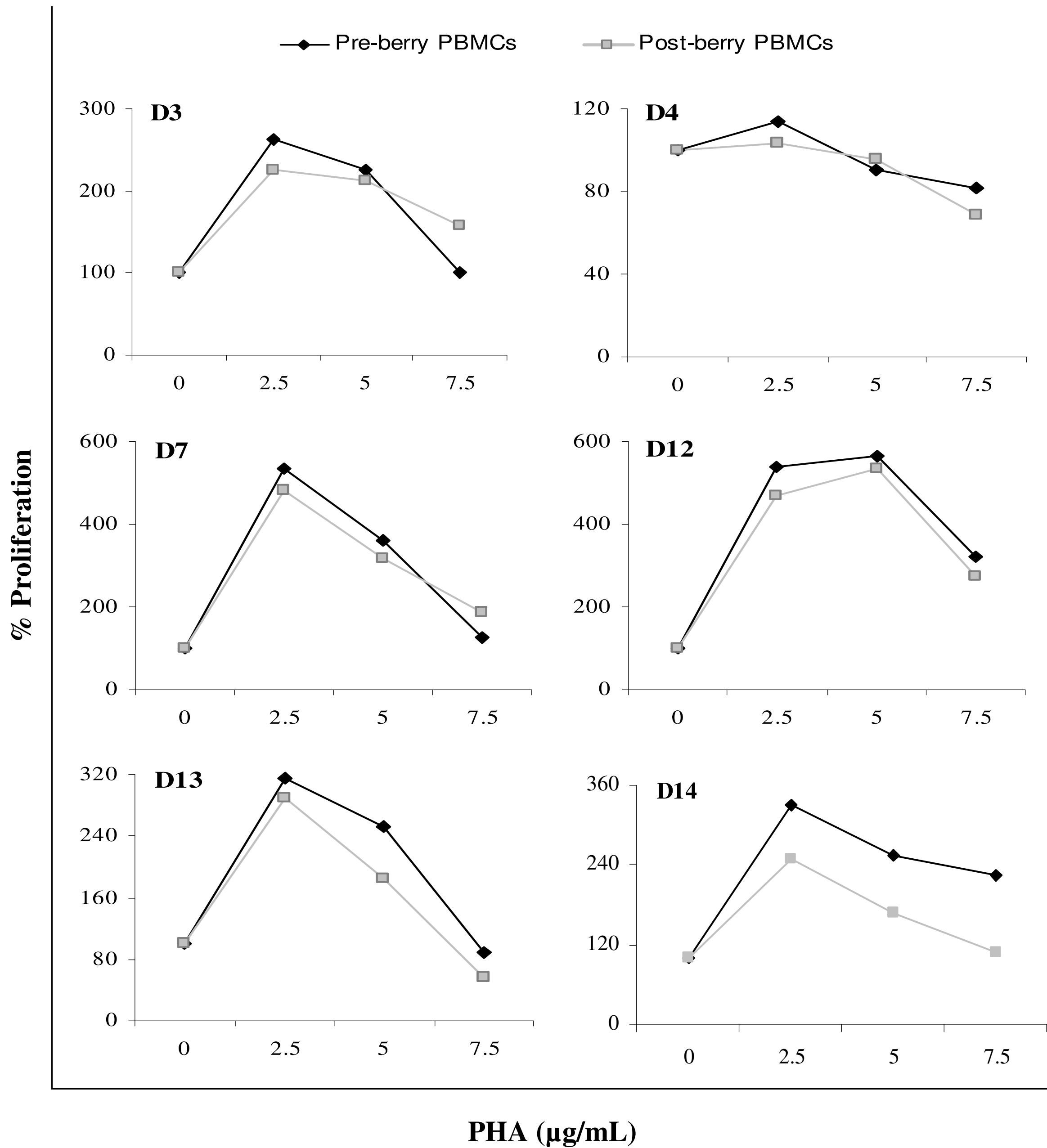


Figure 4. Group 2 mitogen-induced stimulation of T lymphocytes pre- and post- raspberry consumption. Peripheral blood mononuclear cells were treated with three concentrations of PHA in RPMI medium supplemented with 15% autologous plasma. PBMCs were incubated for 72 hours and cell viabilities measured using the MTS assay. Results are presented as the means of PBMCs prepared in triplicate.

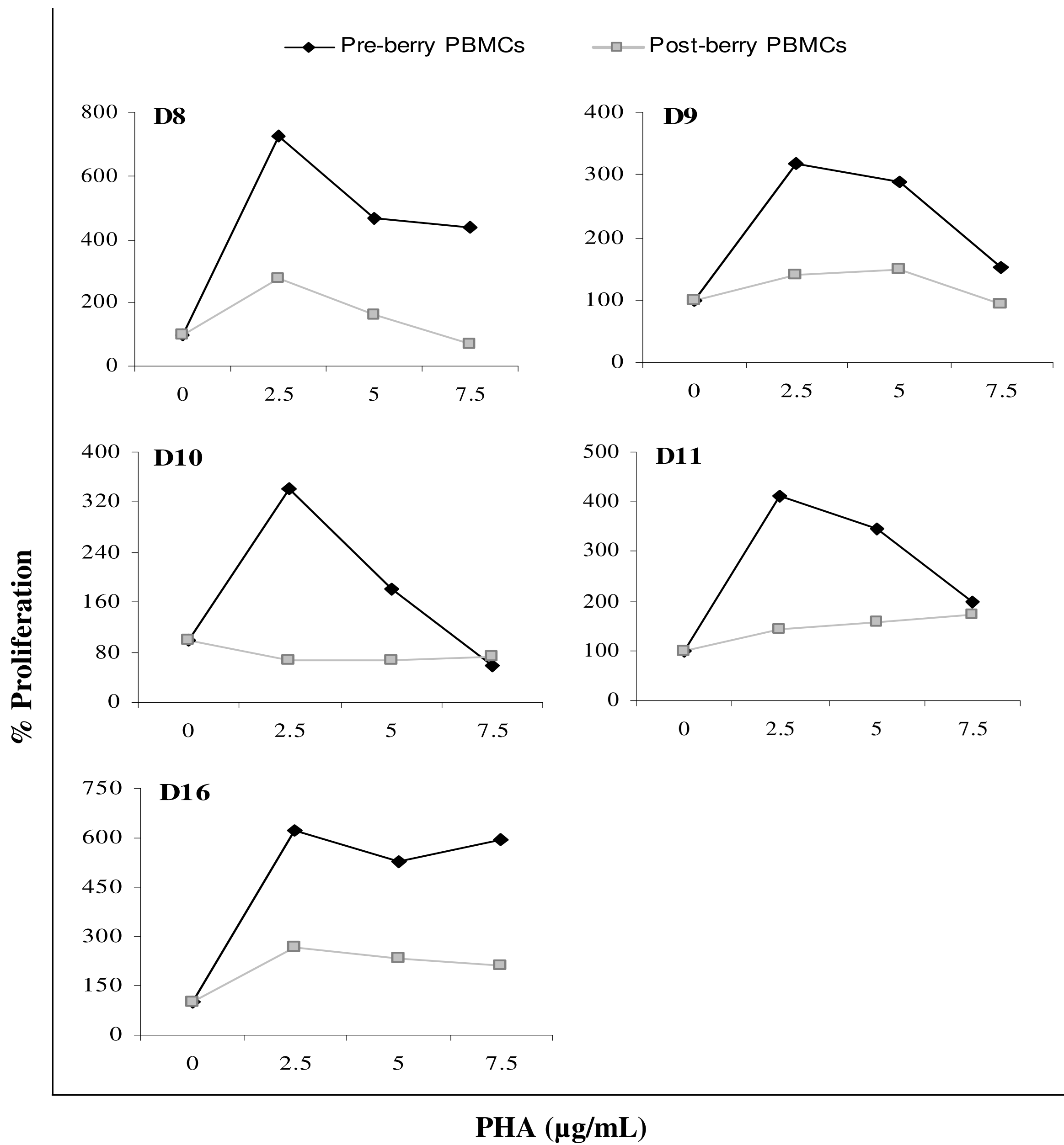


Figure 5. Group 3 mitogen-induced stimulation of T lymphocytes pre- and post-raspberry consumption. Peripheral blood mononuclear cells were treated with three concentrations of PHA in RPMI medium supplemented with 15% autologous plasma. PBMCs were incubated for 72 hours and cell viabilities measured using the MTS assay. Results are presented as the means of PBMCs prepared in triplicate.

Table 6. Effect of Red Raspberries on Mitogen Responsiveness

Donors		Cell Proliferation (% of control)			Post:Pre	Post:Pre
		2.5%	5%	7.5%	Berry	PBMC
Group 1		PHA	PHA	PHA	SI	Control SI
1	Pre-berry	104.4	96.8	65.4		
	Post-berry	113.7	143.8		1.4	0.9
2	Pre-berry	333.0	309.3	198.0		
	Post-berry	620.7	256.4	197.3	1.9	0.5
5	Pre-berry	102.0	91.9	56.7		
	Post-berry	157.9	129.7	107.3	1.6	0.9
6	Pre-berry	110.7	117.3	79.4		
	Post-berry	173.0	169.0	83.9	1.7	0.9
15	Pre-berry	66.6	71.5	52.7		
	Post-berry	109.0	154.7	128.2	2.2	0.5
Group 2						
3	Pre-berry	262.3	225.4	101.0		
	Post-berry	225.9	212.0	157.5	0.9	0.9
4	Pre-berry	114.1	90.2	81.3		
	Post-berry	103.7	95.9	68.5	0.9	1.0
7	Pre-berry	533.5	360.1	125.8		
	Post-berry	484.2	317.9	185.2	0.9	0.9
12	Pre-berry	538.7	563.1	323.7		
	Post-berry	468.5	534.1	274.9	1.0	0.9
13	Pre-berry	316.0	252.8	89.6		
	Post-berry	289.6	184.4	57.1	0.9	0.6
14	Pre-berry	330.4	254.6	226.0		
	Post-berry	248.4	167.8	107.6	0.8	1.1
Group 3						
8	Pre-berry	726.0	468.8	438.3		
	Post-berry	277.3	159.0	66.5	0.4	3.6
9	Pre-berry	319.3	290.3	152.7		
	Post-berry	140.5	148.9	94.4	0.5	1.9
10	Pre-berry	341.7	181.6	59.5		
	Post-berry	67.1	66.1	73.2	0.2	0.5
11	Pre-berry	411.5	346.0	197.9		
	Post-berry	144.7	157.4	171.1	0.4	3.7
16	Pre-berry	619.9	529.1	592.0		
	Post-berry	268.3	235.6	209.5	0.4	2.2

relative to a change in MMP-9 concentration. The results for the three donor groups are presented in Table 7.

Although variability was observed among donor groups in plasma MMP-9 levels after berry consumption, the differences were not significant ($p = 0.16$). Overall, 3 of the 15 participant's post-berry plasma decreased in MMP-9 between 18% – 39% while 6 of the 15 participants increased between 23% – 68%, as shown in Figure 6. Results of one of the two MMP-9 concentrations from donors 7 and 11 measured outside the dynamic range of the MMP-9 assay and cannot be accurately quantified; therefore they are excluded from the evaluation. However, an increase or decrease in MMP-9 concentration for these donors can be noted. The results for donor 7 show a post-berry decrease in MMP-9 while donor 11's MMP-9 concentration increased.

The three donors whose post-berry plasma decreased in MMP-9 were in Group 2. While two of the donors increased in neutrophils or monocytes, none had an increase in lymphocytes. All 3 donors decreased in their resting post : pre-berry PBMC control ratios (0.6 – 0.9)., and a strong correlation ($r = 0.7$) was noted between decreases in MMP-9 levels and the control PBMC SIs.

Of the six donors with increased post-berry plasma MMP-9 concentrations, four were in Group 3. All six of the donors (100%) had an increase in one or more WBC types as determined by their CBCs. Among the donors, five increased in absolute lymphocyte numbers ranging from 2 – 22%, four increased in neutrophils and five increased in monocytes, eosinophils, and/or basophils. In addition, among these six donors, five also increased in post : pre-berry control PBMC SIs after 72 hours in culture.

This is demonstrated in Figure 7 and Table 8. For Group 3 donors, a strong positive correlation was noted between increases in MMP-9 levels and neutrophil and lymphocyte counts ($r = 0.6$ and 0.8 respectively) as well as PBMC control SIs ($r = 0.7$).

Effect of Raspberry Phytochemicals on Plasma Cytotoxicity *in vitro*

A plasma assay was performed to examine the effect of raspberry intake on the cytotoxic activity of donor plasma against AGS, LNCaP, HCC 1500, LoVo, and K-562 tumor cell lines. The cell lines were cultured for 72 hours in complete growth medium containing either fetal bovine serum (FBS) or pre- or post-berry plasma and assayed for cell survival. The results from the plasma-treated samples were normalized to the control samples cultured in FBS. It is possible that changes in the characteristics of the cultured cells might vary between the pre- and post-assays, so to compensate for this, an FBS standard was used. For both the pre- and post-assays, the absorbance of the cells in donor plasma was divided by the absorbance of the same number of cells in FBS. The normalized donor survival indices were then compared. Ratios were determined between the post-berry and pre-berry plasma results. Ratios <1 indicate greater post-berry plasma cytotoxicity whereas ratios >1 reflect greater pre-berry plasma cytotoxicity. The results for the three donor groups are presented in Figures 8 - 9 and Tables 9 – 10. In addition, a summary of the total percentages of cytotoxicity for the donors as well as the average change in post-berry plasma cytotoxicity for each group and cell line are given in Tables 11 - 12.

Table 7. Effect of Red Raspberries on Total MMP-9 Plasma Levels

Donor	Pre-Plasma MMP-9	Post-Plasma MMP-9	% Change	Post/Pre MMP-9 SI
Group 1	(range 13.2 – 105 ng/mL)			
1	60.6	52.1	-14.0	0.9
2	35.3	38.3	8.6	1.1
5	24.1	26.0	7.7	1.1
6	43.3	55.2	27.7	1.3
15	61.6	58.2	-5.6	0.9
Group 2				
4	39.7	43.2	8.6	1.1
7	146.9*	72.7	-50.5	0.5
12	20.6	16.9	-18.0	0.8
13	44.3	26.9	-39.2	0.6
14	22.3	37.5	68.2	1.7
Group 3				
8	30.0	36.8	22.6	1.2
9	37.7	48.2	27.7	1.3
10	35.9	33.5	-6.7	0.9
11	76.2	178.4*	134.2	2.3
16	27.2	41.4	52.4	1.5

Total MMP-9 in donor plasma pre- and post-raspberry consumption as measured by ELISA. Post/pre-berry ratios <1 indicate greater pre-berry MMP-9 concentrations while ratios >1 reflect greater post-berry MMP-9 concentrations. Ratios differing from 1.0 by 10% or less are not considered meaningful relative to a change in MMP-9 concentration.
*Value falls outside the dynamic range of the assay.

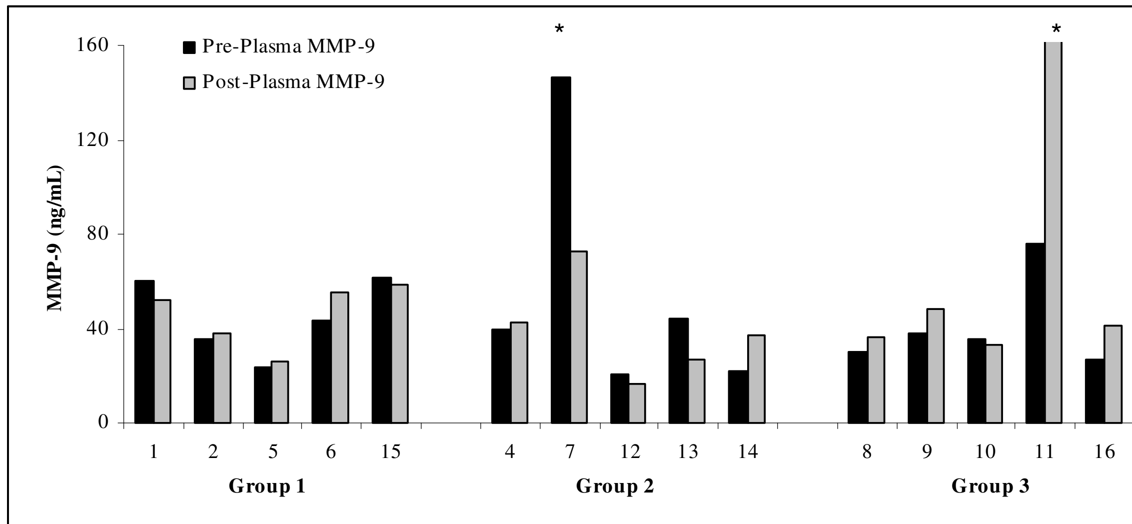


Figure 6. Total MMP-9 levels in donor plasmas pre- and post-raspberry consumption.
*Denotes value that falls outside the dynamic range of the MMP-9 ELISA assay.

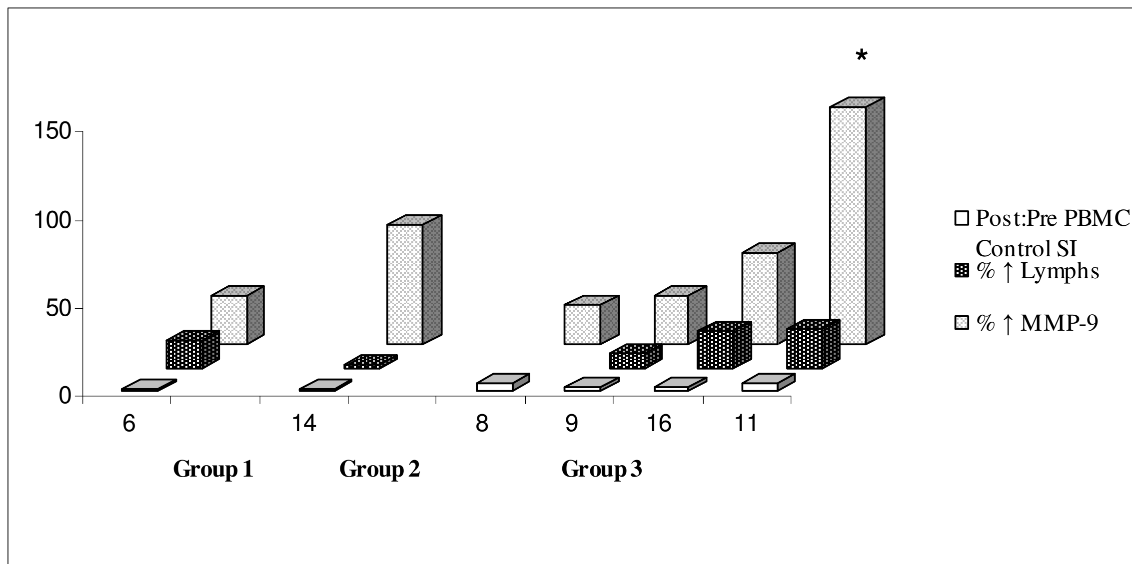


Figure 7. Donors with increased MMP-9 levels, absolute lymphocyte counts, and resting PBMC activities post-raspberry consumption. Donor 8 did not increase in lymphocyte levels after berry consumption.
*Denotes value that falls outside the dynamic range of the MMP-9 ELISA assay.

Table 8. Donors with Increased MMP-9 SIs, PBMC Control SIs, and PBMC Counts Post-berry Consumption

Donor	Post:Pre MMP-9 SI	Post:Pre PBMC Control SI	% Increase in PBMCs		
			NE	LY	MO
6	1.3	0.9		15	
8	1.2	3.6	1.6		
9	1.3	1.9		8	14
11	2.3	3.7	6.6	21.6	2.4
14	1.7	1.1	7.9	2	17.9
16	1.5	2.2	13.4	20.8	

Key: NE, neutrophil; LY, lymphocyte; MO, monocyte

Variability was observed in plasma cytotoxicity among donors and cell lines following raspberry consumption. However, when comparing responses of all donors and cell lines, 57% - 69% of the participants' plasmas showed increased cytotoxicity after consuming raspberries compared to their pre-berry plasma samples. A significant increase in plasma cytotoxicity was noted in the HCC 1500 breast cell line ($p = 0.05$), with an average change of 28.3±6.7% cytotoxicity among donors after berry consumption.

Although variability in plasma cytotoxicity was observed among donors and cell lines, no major differences were noted between donor groups. Group 3 donors showed a significant increase in post-berry plasma cytotoxicity in the AGS cell line ($p = 0.005$). A strong positive correlation was noted between the AGS and HCC 1500 cell lines in post-

berry plasma cytotoxicity in both Group 2 and Group 3 donors (r = 0.6 and 0.9 respectively) while an inverse relationship was observed in Group 1 (r = -0.8).

Effect of Raspberry Intake on PBMC Cytotoxicity *in vitro*

Results of the PBMC cytotoxicity assay varied widely among donors and cell lines. However, trends were noted among donors within cell lines. As expected, there was an inverse relationship between the E:T ratio (5:1, 10:1, 20:1) and cell survival in the AGS gastric cell line Following 72-hours incubation, the percentage of surviving AGS cells decreased as the number of PBMCs per sample increased. Although both the pre-berry and post-berry PBMCs exhibited tumoricidal activity, 57% (4/7) of the donors (6, 4, 7, and 9) showed increased post-berry killing in at least two of the effector concentrations. The difference in post-berry killing ranged from 8 – 44% above the pre-berry killing between the three effector concentrations.

The tumoricidal activity of donor PBMCs against AGS cells was also examined following a 4-hour treatment period and a similar inverse relationship was observed. However, only donor 15 showed increased post-berry killing. At the 20:1 E:T concentrations the absorbance measurements increased, suggesting PBMC activation or cellular replication (p = 0.08). When AGS cells were incubated with donor PBMCs and 100% plasma concentrations, post-berry killing improved in 80% (4/5) of the donors (donors 15, 13, 14, and 16). Cell killing increased between 8.26 – 123.92% at the 20:1 effector concentration compared to the original samples in 15% plasma.

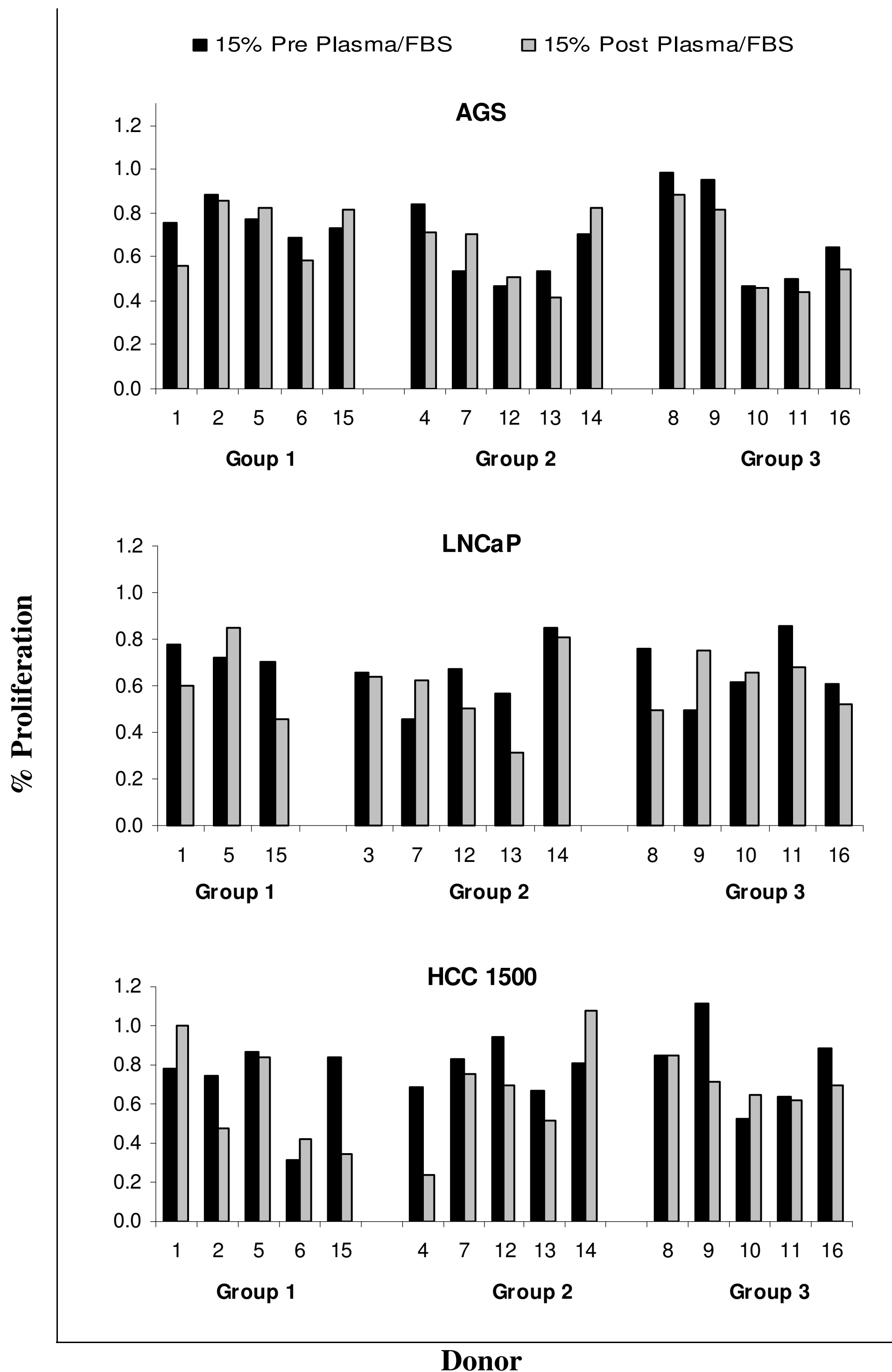


Figure 8. Cytotoxic effect of pre- and post-raspberry plasma on AGS, LNCaP, and HCC 1500 tumor cell growth at 72-hours. Tumor cells (2.5 x 10^4 cell/well) were plated in appropriate medium containing 15% autologous plasma or FBS for 72 hours. The MTS assay was performed to determine the number of viable cells after treatment.

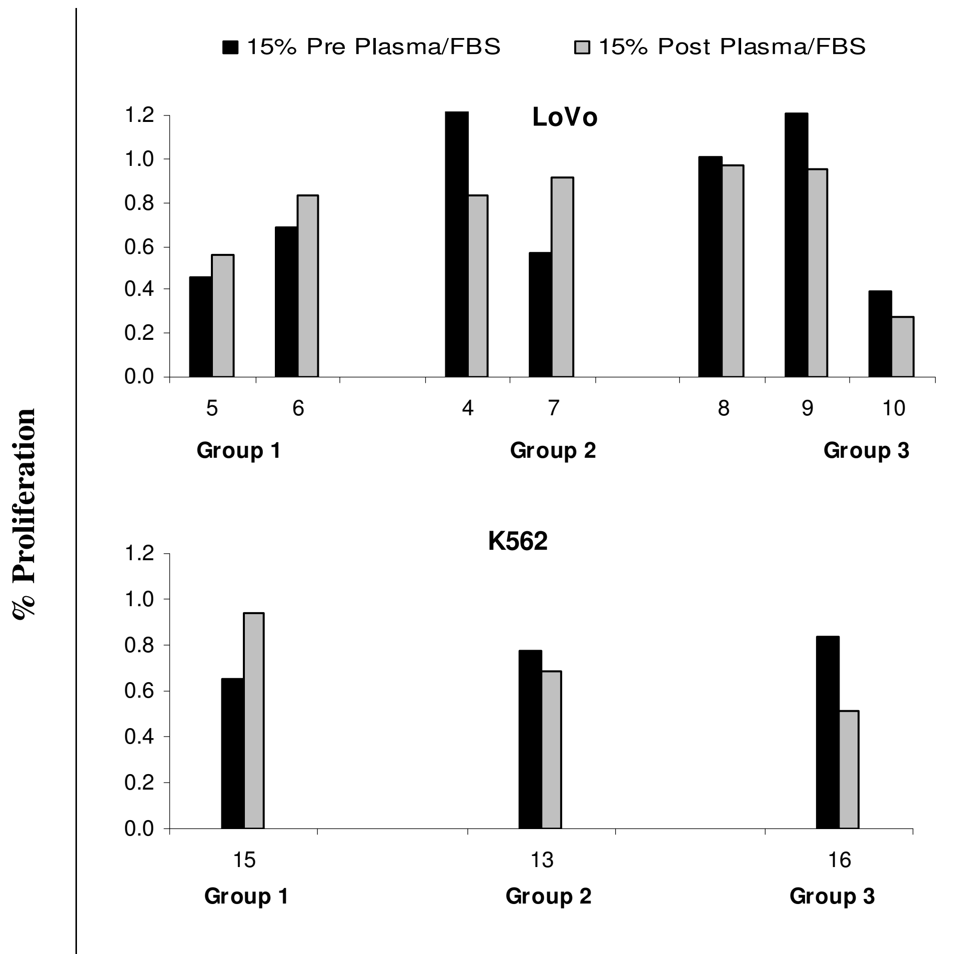


Figure 9. Cytotoxic effect of pre- and post-raspberry plasma on LoVo and K-562 tumor cell growth at 72-hours. Tumor cells (2.5×10^4 cell/well) were plated in appropriate medium containing 15% autologous plasma or FBS for 72 hours. The MTS assay was performed to determine the number of viable cells after treatment.

Table 9. Cytotoxic Effect of Pre- and Post-Raspberry Plasma on AGS and LNCaP Tumor Cell Growth at 72 Hours

Donor	AGS				LNCaP			
Group 1	Pre-Berry	Post-Berry	Post/Pre Ratio	Post/Pre % Killing	Pre-Berry	Post-Berry	Post/Pre Ratio	Post/Pre % Killing
1	0.8	0.6	0.7	26.2	0.8	0.6	0.8	22.6
2	0.9	0.9	1.0	2.6				
5	0.8	0.8	1.1	-6.9	0.7	0.9	1.2	-17.7
6	0.7	0.6	0.9	14.2				
15	0.7	0.8	1.1	-11.4	0.7	0.5	0.7	35.3
Group 2								
3					0.66	0.64	0.97	3.01
4	0.8	0.7	0.9	15.0				
7	0.5	0.7	1.3	-32.3	0.5	0.6	1.4	-37.9
12	0.5	0.5	1.1	-9.6	0.7	0.5	0.7	25.7
13	0.5	0.4	0.8	21.9	0.6	0.3	0.6	44.7
14	0.7	0.8	1.2	-16.4	0.9	0.8	1.0	5.0
Group 3								
8	1.0	0.9	0.9	10.8	0.8	0.5	0.7	35.5
9	1.0	0.8	0.9	14.8	0.5	0.8	1.5	-50.6
10	0.5	0.5	1.0	1.6	0.6	0.7	1.1	-6.7
11	0.5	0.5	0.9	11.2	0.9	0.7	0.8	20.1
16	0.6	0.5	0.8	15.9	0.6	0.5	0.9	13.9

Cytotoxic effect of pre-berry and post-berry plasma on AGS and LNCaP tumor cell growth following 72 hour treatment in medium supplemented with 15% autologous plasma. Post:pre-raspberry ratios <1 indicate greater post-berry plasma cytotoxicity whereas ratios >1 indicate greater pre-berry cytotoxicity. The percent plasma killings with negative values represent donor samples showing greater pre-berry plasma killing.

Table 10. Cytotoxic Effect of Pre- and Post-Raspberry Plasma on HCC 1500, LoVo, and K-562 Tumor Cell Growth at 72 Hours

Donor	HCC 1500				LoVo			
Group 1	Pre-Berry	Post-Berry	Post/Pre Ratio	Post/Pre % Killing	Pre-Berry	Post-Berry	Post/Pre Ratio	Post/Pre % Killing
1	0.8	1.0	1.3	-27.3				
2	0.7	0.5	0.7	35.4				
5	0.9	0.8	1.0	3.8	0.5	0.6	1.2	-21.1
6	0.3	0.4	1.3	-34.0	0.7	0.8	1.2	-21.5
15	0.8	0.4	0.4	58.4				
Group 2								
4	0.7	0.2	0.3	65.9	1.3	0.8	0.7	34.4
7	0.8	0.8	0.9	9.4	0.6	0.9	1.6	-62.8
12	0.9	0.7	0.7	26.0				
13	0.7	0.5	0.8	22.2				
14	0.8	1.1	1.3	-33.5				
Group 3								
8	0.8	0.9	1.0	-0.8	1.0	1.0	1.0	3.47
9	1.1	0.7	0.6	36.4	1.2	1.0	0.8	21.2
10	0.5	0.6	1.2	-23.5	0.4	0.3	0.7	30.3
11	0.6	0.6	1.0	4.0				
16	0.9	0.7	0.8	21.1				
	K-562							
Group 1								
15	0.7	0.9	1.4	-43.5				
Group 2								
13	0.8	0.7	0.9	11.1				
Group 3								
16	0.8	0.5	0.6	38.7				

Cytotoxic effect of pre-berry and post-berry plasma on HCC 1500, LoVo, and K-562 tumor cell growth following 72 hour treatment in medium supplemented with 15% autologous plasma. Post:pre-raspberry ratios <1 indicate greater post-berry plasma cytotoxicity whereas ratios >1 indicate greater pre-berry cytotoxicity. The percent plasma killings with negative values represent donor samples showing greater pre-berry plasma killing.

Table 11. Donors with Increased Post-Raspberry Plasma Cytotoxicity

Cell Line	Group 1 % Donors	Group 2 % Donors	Group 3 % Donors	Males % Donors	Females % Donors
AGS	60	40	100	100	50
LNCaP	67	80	60	33	80
HCC 1500	60	80	60	60	70
LoVo	0	50	100	100	25
K-562	0	100*	100*	**	67

* indicates Group containing 1 donor only.
**No male plasma was tested on K-562 cells.

Table 12. Average Change in Post-Raspberry Plasma Cytotoxicity in Donor Groups

Cell Line	Total Average % Cytotoxicity	Group 1 Average % Cytotoxicity	Group 2 Average % Cytotoxicity	Group 3 Average % Cytotoxicity
AGS	13.4±2.5	14.3±6.8	18.4±3.5	10.9±2.5
LNCaP	22.9±4.7	29.0±6.4	19.6±9.8	23.2±6.4
HCC 1500	28.3±6.7	32.6±15.8	30.8±12.2	20.5±9.4
LoVo	22.3±6.9	0	*	18.3±7.9
K562	24.9±13.8	0	*	*

Average post-berry plasma cytotoxicity ± SEM in donor groups.
* indicates Group containing 1 donor only.

A linear trend was observed in the LoVo colon cells between the E:T concentration and cell survival following a 72-hour treatment period. Both the pre- and post-berry samples from six donors (5, 6, 4, 7, 8, and 9) showed an increase in cell survival as the PBMC concentration increased in at least two of the concentrations. Sixty-seven percent (4/6) of the donors (6, 7, 8, and 9), however, demonstrated increased

post-berry killing in at least one of the effector concentrations ranging from 7.6 – 45.4% compared to the pre-berry samples.

Of the four donor samples used to treat LNCaP prostate cells, proliferation occurred in two donor samples while cytotoxicity resulted in the two remaining donor samples. Post-berry cytotoxicity was enhanced 2.6% in only one of these donors. When incubation time was reduced to 4 hours, LNCaP cells continued to show proliferation when treated with pre- and post-berry PBMCs from six donors ($p = 0.08$). However, by increasing the plasma concentration, 22.7% - 66.4% cytotoxicity was observed. The cell killing occurred in both the pre- and post-berry samples from four of the five donors.

PBMC-killing of the HCC 1500 breast cells also followed a trend. This cell line showed greater sensitivity to cell killing at the 5:1 and 10:1 E:T concentrations after a 72-hour treatment period. Of the pre- and post-berry samples from five donors (5, 4, 7, 8, and 9), all of the samples, with the exception of one pre-berry sample from donor 7, demonstrated the greatest tumoricidal activity at the two lower effector concentrations. Cell-killing occurred between 25.1% – 95.3% in these samples compared to the 20:1 E:T samples that ranged between 9% – 75.8% killing. At the 20:1 E:T concentrations the absorbance measurements increased significantly, suggesting PBMC activation or cellular replication ($p = 0.05$). When the treatment time was reduced to 4 hours, the HCC 1500 cells proliferated in both the pre- and post-berry samples in at least two effector concentrations in 80% (4/5) of the donors (15, 10, 12, and 13). When plasma concentrations were increased, the HCC 1500 cells continued to proliferate in samples from donors 15, 12, and 14.

K-562 lymphocytic leukemia cells also exhibited proliferation when treated 4 hours with pre- and post-berry PBMCs and 15% plasma. Only one of four donor samples demonstrated cytotoxicity after berry consumption. At the 20:1 E:T concentrations the absorbance measurements increased significantly, suggesting PBMC activation or cellular replication ($p = 0.04$).

The enhanced post-berry proliferative effect observed in the 72 hour and 4 hour treatments of the HCC 1500, AGS, LNCaP, and K-562 tumor cell lines could be explained as an activation of the donor PBMCs from the berry phytochemicals. While the tumor cells and/or PBMCs could proliferate during a 72 hour incubation, it is unlikely they would proliferate above the control samples during the brief 4 hour incubation period. Post-berry proliferation or activation at 4 hours was significant for the HCC 1500 and K-562 cell lines ($p = 0.05$ and 0.04 respectively) and moderately significant for the AGS and LNCaP cell lines (0.08).

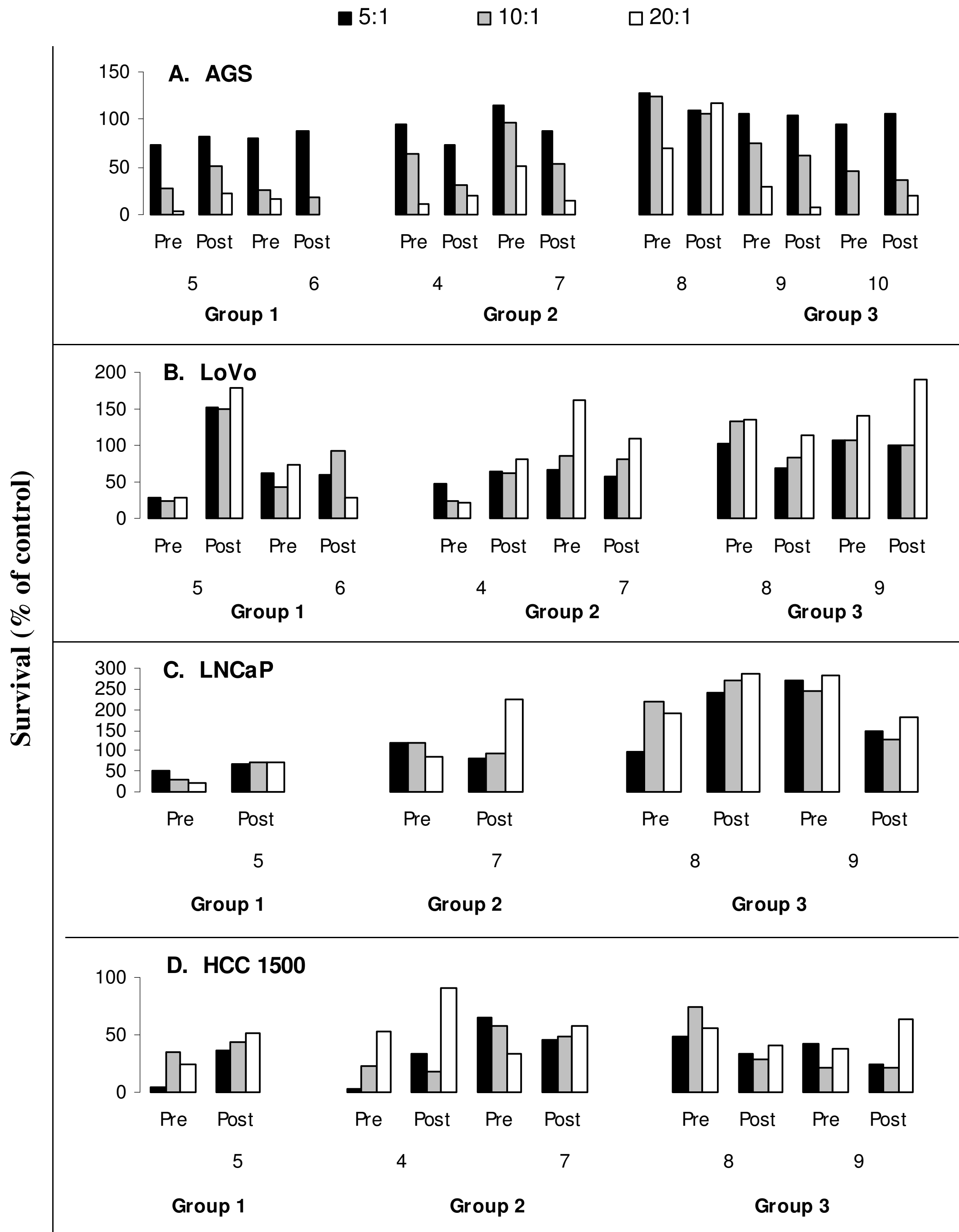


Figure 10. PBMC treatment of AGS, LoVo, LNCaP, and HCC 1500 tumor cells at 72 hours. Tumor cells (2.5 x 10^4 cells/well) were incubated with three concentrations of PBMCs in medium containing 15% autologous plasma pre- and post-raspberry ingestion and cell viabilities measured.

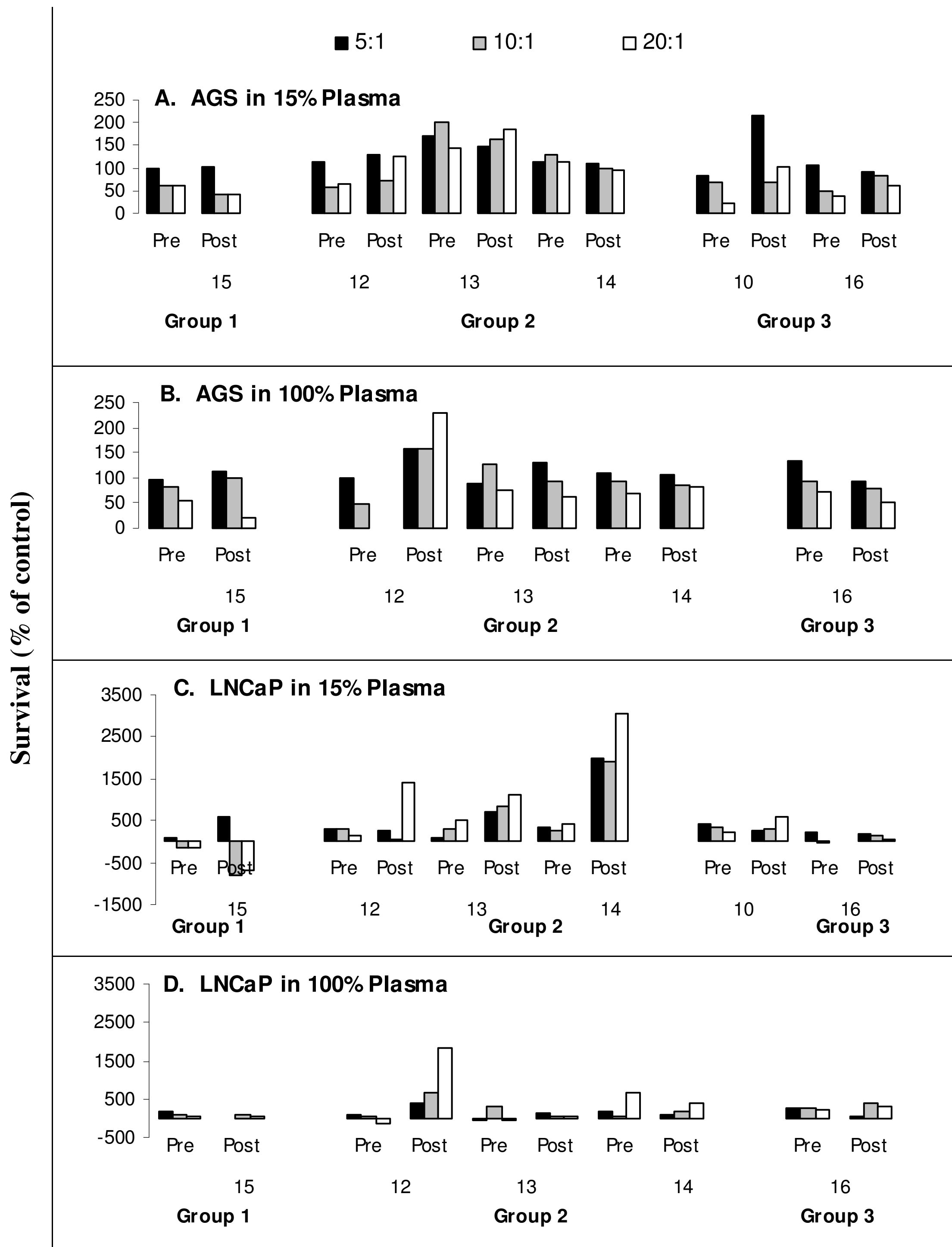


Figure 11. PBMC treatment of AGS and LNCaP tumor cells at 4 hours. Tumor cells (2.5 x 10^4 cells/well) were incubated with three concentrations of PBMCs in medium containing 15% or 100% autologous plasma pre- and post-raspberry ingestion and cell viabilities measured.

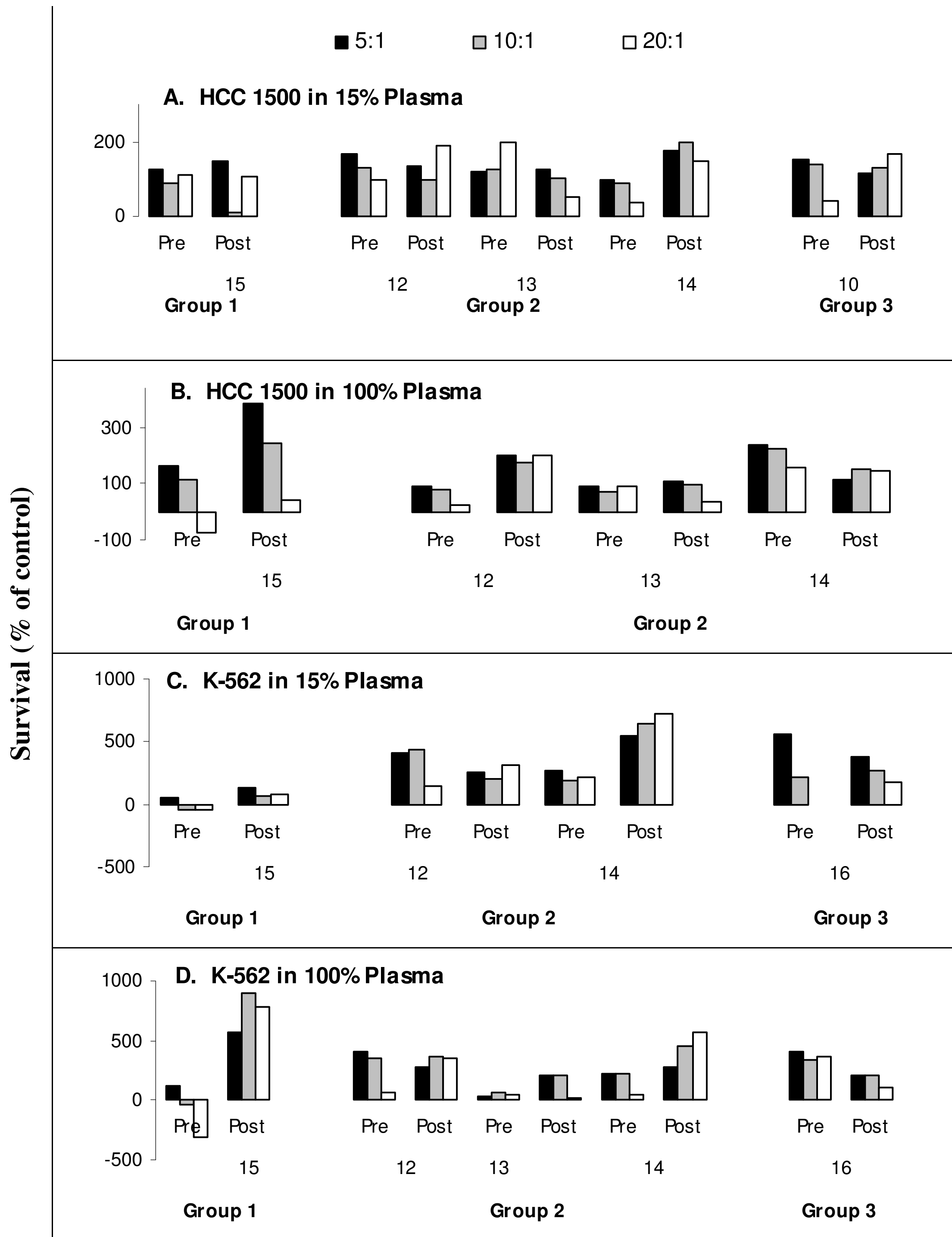


Figure 12. PBMC treatment of HCC 1500 and K-562 tumor cells at 4 hours. Tumor cells (2.5 x 10^4 cells/well) were incubated with three concentrations of PBMCs in medium containing 15% or 100% autologous plasma pre- and post-raspberry ingestion and cell viabilities measured.

Table 13. PBMC Treatment of AGS, LoVo, LNCaP, and HCC 1500 Tumor Cells at 72 Hours

Donor		AGS			LoVo		
Group 1		5:1	10:1	20:1	5:1	10:1	20:1
5	Pre	72.9	27.7	3.0	28.2	23.8	27.6
	Post	82.5	52.0	21.9	151.8	149.2	179.6
6	Pre	79.7	26.3	15.7	62.8	41.7	74.9
	Post	87.3	18.2	0.00	60.0	92.3	29.5
Group 2							
4	Pre	94.4	64.1	10.2	48.1	23.0	22.4
	Post	74.1	30.2	19.6	64.9	60.8	80.1
7	Pre	115.6	97.1	50.4	65.6	86.1	162.4
	Post	87.3	53.1	14.9	56.6	80.9	110.0
Group 3							
8	Pre	127.7	124.4	69.9	103.0	133.1	135.1
	Post	109.4	105.6	117.5	70.2	84.0	113.4
9	Pre	105.8	75.3	30.0	106.5	107.8	139.7
	Post	104.5	61.5	6.6	98.9	99.4	191.2
10	Pre	94.8	45.2	0.00			
	Post	105.7	37.3	20.7			

Group 1		LNCaP			HCC 1500		
5	Pre	50.5	29.7	19.1	4.7	35.1	24.2
	Post	68.9	70.4	72.2	36.0	43.6	51.9
Group 2							
4	Pre				3.3	22.2	52.6
	Post				33.1	18.8	91.0
7	Pre	118.4	118.8	83.6	64.7	57.5	33.1
	Post	80.9	92.5	222.1	45.1	49.0	57.3
Group 3							
8	Pre	98.8	219.4	189.8	48.5	74.9	56.0
	Post	239.6	272.2	288.7	33.4	29.3	40.2
9	Pre	270.9	245.2	282.2	42.7	20.6	38.2
	Post	147.7	124.9	181.7	23.8	20.9	63.8

Percent surviving fraction of AGS, LoVo, LNCaP, and HCC 1500 tumor cells following 72 hours incubation in complete medium containing 15% autologous plasma and PBMCs at a 5:1, 10:1, or 20:1 effector:target concentration.

Table 14. PBMC Treatment of AGS and LNCaP Tumor Cells at 4 Hours

Donor		AGS in 15% Plasma			AGS in 100% Plasma		
Group 1		5:1	10:1	20:1	5:1	10:1	20:1
15	Pre	99.6	58.9	59.9	95.8	82.1	54.6
	Post	101.5	40.3	43.1	113.7	98.2	21.9
Group 2							
12	Pre	114.1	55.7	65.0	97.9	49.2	
	Post	127.3	71.6	123.4	158.6	159.1	229.9
13	Pre	169.6	199.6	143.9	90.6	126.3	74.7
	Post	149.2	164.0	186.6	131.7	91.1	62.7
14	Pre	113.5	128.8	114.8	110.3	92.3	70.0
	Post	108.9	99.2	93.1	105.2	86.5	81.8
Group 3							
10	Pre	85.0	66.4	21.7			
	Post	214.1	68.7	101.0			
16	Pre	107.2	49.5	36.5	132.4	92.1	72.3
	Post	92.0	82.4	61.2	92.5	78.5	53.0

Group 1		LNCaP in 15% Plasma			LNCaP in 100% Plasma		
15	Pre	113.6	-142.9	-162.6	170.9	100.5	50.6
	Post	598.8	-819.9	-676.7	4.4	80.7	58.2
Group 2							
12	Pre	294.6	293.4	121.4	62.5	45.2	-159.7
	Post	264.3	71.5	1408.3	395.0	673.2	1855.8
13	Pre	104.8	317.0	507.4	-59.8	287.6	-63.6
	Post	728.7	833.9	1105.4	142.1	47.8	33.6
14	Pre	355.1	247.7	433.6	180.1	45.	688.0
	Post	1999.4	1917.5	3049.8	77.3	177.0	400.2
Group 3							
10	Pre	405.7	343.5	231.8			
	Post	246.5	286.7	582.1			
16	Pre	203.4	-39.4	21.5	270.1	256.0	216.4
	Post	166.1	129.2	63.9	55.5	400.3	330.6

Percent surviving fraction of AGS and LNCaP tumor cells following a 4-hour incubation in complete medium containing 15% or 100% autologous plasma and PBMCs at a 5:1, 10:1, or 20:1 effector:target concentration.

Table 15. PBMC Treatment of HCC 1500 and K-562 Tumor Cells at 4 Hours

Donor		HCC 1500 in 15% Plasma			HCC 1500 in 100% Plasma		
Group 1		5:1	10:1	20:1	5:1	10:1	20:1
15	Pre	125.7	85.5	113.0	162.3	114.9	-77.8
	Post	148.4	10.9	107.4	386.1	241.4	40.9
Group 2							
12	Pre	165.4	128.6	98.9	93.2	78.9	25.0
	Post	134.9	99.2	188.2	202.6	178.9	203.0
13	Pre	119.7	125.0	196.5	90.3	72.3	90.5
	Post	123.5	103.1	48.8	107.2	97.0	37.8
14	Pre	94.7	89.1	35.7	237.4	227.9	155.5
	Post	173.2	200.8	145.5	116.5	153.0	142.8
Group 3							
10	Pre	150.2	138.0	43.3			
	Post	114.2	128.8	167.6			

Group 1		K-562 in 15% Plasma			K-562 in 100% Plasma		
15	Pre	50.1	-43.5	-46.9	116.5	-42.0	-313.1
	Post	128.1	57.7	83.5	567.6	904.1	781.7
Group 2							
12	Pre	411.0	431.6	143.2	409.4	354.8	58.3
	Post	257.8	202.7	308.5	283.0	371.6	345.0
13	Pre				37.8	57.4	45.0
	Post				206.3	208.7	19.7
14	Pre	265.9	193.9	220.6	225.3	216.5	47.8
	Post	551.1	643.3	727.9	272.1	453.5	568.3
Group 3							
16	Pre	563.5	216.2	0.1	413.4	333.6	367.2
	Post	375.2	266.0	175.4	202.1	203.5	106.3

Percent surviving fraction of HCC 1500 and K-562 tumor cells following a 4-hour incubation in complete medium containing 15% or 100% autologous plasma and PBMCs at a 5:1, 10:1, or 20:1 effector:target concentration.

4. DISCUSSION

Many health benefits have been attributed to the consumption of fruits and berries, including raspberries. There is a large body of scientific data that supports the health benefits of eating raspberries. According to an executive summary published in 2007 by the Washington Red Raspberry Commission, raspberries have been shown to: protect against free radical-induced cell damage and reduce oxidative stress, prevent the growth of cancer cells *in vitro* and in animal models, reduce the risk of cardiovascular disease, protect against infectious bacterial and viral diseases, provide protection against food-induced allergic reactions, help regulate blood glucose and reduce the risk of diabetes, and lower the risk of developing age-related neurodegenerative diseases (61). Almost all of these effects have been demonstrated in cell culture or animal models. However, in humans any benefits of berry consumption relative to tumorigenesis or any anticancer properties in patients need to be demonstrated. The benefits derived from raspberry consumption have been attributed to their content of polyphenols, flavonoids, anthocyanins, ellagitannins, and vitamin C. It is the phytochemicals that are responsible for many of the biological activities of raspberries, including antioxidant, anti-inflammatory and anticancer properties (8). Raspberries are strong antioxidants and have a high free radical scavenging capacity, due primarily to their ellagitannins. Among fruits and berries, raspberries contain some of the highest levels of ellagitannins, which are abundant in the pulp and seeds. The Meeker red raspberry, as used in this study, contains the most ellagitannins of the raspberry cultivars, and when in lyophilized, or freeze-dried form, the concentration of the ellagitannins is increased 9-10 fold (62).

Numerous studies have demonstrated the anticancer properties of raspberries on tumor cell proliferation *in vitro* (15, 16, 17), and studies from this laboratory support these findings (18, 43). Preliminary *in vitro* assays were performed to detect the effect of three concentrations of lyophilized red raspberry extract on the proliferation of four tumor cell lines. The cell lines used in this study represent some of the most commonly reported cancers and have a high rate of incidence globally. They include prostate, breast, colon, and stomach cancers (63). Our results show that the extracts had a potent cytotoxic effect on all the cell lines tested. The inhibition of the proliferation was concentration dependent, and the degrees of inhibition varied for the different cell types. Of the tumor cell lines, AGS cells were the most sensitive to the cytotoxic effects of raspberry extract treatment, with significant killing measured at all extract dosages ($p = 0.04 – 0.00$). Efficient killing also occurred in the LoVo, LNCaP, and HCC 1500 cells. Both the LNCaP prostate cells and HCC 1500 breast cells are estrogen receptor positive, and plant flavonoids and anthocyanins have been shown to bind to estrogen receptors and exert an anti-estrogenic, or anti-proliferative effect upon the cells (12, 3). This could be one mechanism contributing to the >50% reduction in proliferation in these two cell lines.

A normal cell line was also included in the study to compare the anti-proliferative effect of raspberry extract on normal vs. cancer cells. CRL-2120 normal skin fibroblasts were treated with the same concentrations of raspberry extract as used on the tumor cell lines. Although fibroblast proliferation was unaffected at the lowest extract dosage of 5%, the higher concentrations were cytotoxic to a similar degree as measured in the tumor cells. Han *et al* reported a similar finding when human oral cavity cells were

treated with black raspberry extract. They determined it was the ellagic acid in the berries that exerted the cytotoxic effect on the cells (64). It is possible the concentrations of ellagic acid in the 7.5% and 10% lyophilized raspberry extracts used in this assay were sufficiently high to explain the cytotoxic effect on the CRL 2120 normal skin fibroblasts. Because the concentrations of ellagic acid are unknown in these treatments, it is uncertain if these concentrations can be attained *in vivo*. Therefore, caution must be used when extrapolating the *in vitro* results obtained in this study to *in vivo* situations. The cytotoxicity observed in the CRL 2120 normal skin fibroblasts might not accurately represent the effects that would be seen on healthy cells *in vivo*. However, results from this assay indicate that the concentrations of phytochemicals in the raspberry extracts used in this study were sufficient to kill tumor cells of four commonly occurring cancers *in vitro*.

Each of the cell lines was also treated with three concentrations of ascorbic acid that served as controls for both the pH and antioxidant effects in cell killing. Numerous studies of fruits and berries containing ascorbic acid (vitamin C) show inhibition of cancer cell proliferation *in vitro*. In contrast to the raspberry extract treatment, the ascorbic acid produced a stimulatory effect in the same five cell lines in a concentration-dependent manner. While proliferation was not significant in the AGS and CRL 2120 cell lines, LoVo, LNCaP, and HCC 1500 cells increased up to 80% ($p = 0.03$). A study by Olsson *et al* also noted a slight stimulatory effect upon tumor cells treated with ascorbic acid. They compared the antiproliferative effect of 10 fruits and berries containing vitamin C to treatment with ascorbic acid alone on HT29 and MCF-7 cells.

While inhibition of proliferation was observed in the tumor cells treated with the fruit and berry extracts, the same inhibition of proliferation was not seen when the cancer cells were treated with ascorbic acid only. These investigators proposed that the inhibition of cancer cell proliferation by the fruit and berry extracts containing vitamin C could be due to a synergism between vitamin C and other phytochemicals in the fruit, whereas vitamin C, or ascorbic acid alone was not effective (16). In the current study, the concentrations of ascorbic acid were not sufficient to exert a cytotoxic effect on the cell lines tested. The antioxidant activity of the ascorbic acid may have enhanced proliferation of the metabolically active tumor cells by scavenging reactive oxygen species that cause cell damage and trigger apoptosis.

Although numerous studies have been published supporting the beneficial antitumor effects of fruit and berry phytochemicals *in vitro* and in animal models (5, 7, 8, 10), human studies are limited. This pilot study was undertaken to test the feasibility of examining the effects of berry consumption on the immune response in humans in a novel way that will give a better approximation of the effects of berry consumption. Cells shown to be killed by berry extracts *in vitro* were tested for their sensitivity to killing by blood plasma and immune cells taken from donors consuming berries from the same source. Volunteers were asked to abstain from eating fruits or berries other than the lyophilized raspberries during the study; otherwise no changes were made in the participants' diets or lifestyles. Blood samples were obtained before and after berry consumption and *in vitro* assays were performed on the plasmas and PBMCs of the donors.

This experimental study design offers a number of benefits compared to *in vitro* assays examining berry extract treatments of tumor cells. While *in vitro* studies of the effects of berry extracts on cell lines can give insight into whether a particular extract might contain biologically active compounds, the extracts tested contain only the compounds present in the extract. The effects of any changes in these compounds or any new compounds produced by *in vivo* metabolism cannot be evaluated by the traditional *in vitro* method. In the current approach, raspberries are digested *in vivo* where they undergo extensive modification by gut microflora as well as the individual's own metabolic system. The subject's plasma will contain berry components as well as the metabolic by-products formed after digestion. In addition, the rate of absorption and excretion and the bioavailability of berry metabolites vary among individuals. Using our approach, these factors are built into the model and a more accurate assessment of the influence of berry bioactives on immune cell function can be assessed individually. Furthermore, the full complement of biological mediators (hormones, cytokines, growth factors, blood proteins, etc.), many of which play an essential role in immune cell activity, are present in the plasma of the individual. This plasma is used to supplement the culture medium for immune cells from the same donor compared to the standard method of culturing cells in fetal bovine serum (FBS). Since the PBMCs of the individual are tested in his autologous plasma *in vitro,* this approach more closely mimics the host's *in vivo* conditions and avoids changes induced in the immune cells' responses by exposure to the alien components present in serum from fetal calves or other animals. It is expected that the results obtained with this experimental approach will yield more

rapid and trustworthy results and give a better approximation of the effects of berry consumption than other currently used methods.

The pilot study was performed on 16 participants ranging from 20 – 68 years of age. After 3.5 days of raspberry consumption the results show that there were measurable differences in immune parameters, and the degrees of response seemed dependent on the characteristics of the individual. Of the female participants, five were post-menopausal and three were menstruating at the time of the study. For the females on birth control medication, hormone replacement therapy, or menstruating at the time of the study, hormonal effects cannot be excluded when interpreting the study results. In addition, five participants were on a daily multivitamin and/or vitamin C regimen and any contributions to the antioxidant effect from these supplements must be considered in the analysis. Although variability was noted in immune responses to berry phytochemicals, trends emerged among participants in lymphocyte mitogen stimulation, circulating MMP-9 concentrations, and plasma and PBMC cytotoxicity on tumor cells.

Complete blood counts were collected from 15 donors before and after berry consumption to determine an *in vivo* effect of raspberry phytochemicals on PBMC proliferation. The CBC provides valuable information about the hematopoietic system and an individual's health status. White blood cell counts vary daily simply due to intra-individual biological variation. Mean WBC counts are also affected by age and race and can increase as a result of exercise, stress or smoking. According to Brigden and Heathcoate, a change in the WBC count of >36% would be considered a critical or true difference in 95% of. cases (65). In this study 80% of the participants had an increase in

the absolute numbers of one or more white blood cell types ranging from 2 – 88% after eating raspberries. Fifty eight percent of the donors increased in lymphocytes, monocytes, and eosinophils, and 50% increased in neutrophils, and a strong positive correlation was noted between increases in lymphocyte and monocyte numbers after berry consumption ($r = 0.7$). Although the differential changes in WBC counts remained within the normal range of values, the increased numbers of phagocytes and lymphocytes could augment the response of both the innate and adaptive arms of the immune system and provide improved protection against foreign invaders and aberrant cells arising in the host. A study by Nantz *et al.* also reported an increase in PBMCs in healthy study participants following ingestion of a fruit and vegetable juice powder (FVJP) concentrate. They determined the percentage of $\gamma\delta$-$CD3^+$ T cells had increased 30% in the FVJP group relative to their baselines or the placebo group. The percentage of $\alpha\beta$-$CD3^+$ T cells in the peripheral blood of this group was not affected (57). Percival reported a similar increase in $\gamma\delta$-$CD3^+$ T cells after healthy study participants consumed Concord grape juice daily for 10 weeks (54). Studies show that the chemical structures of polyphenols in fruits and berries resemble pathogen-associated molecular patterns (PAMPs) that are recognized by $\gamma\delta$ T cells, and this interaction could serve to prime the cells or stimulate them to proliferate (57). Although lymphocyte identification was not performed in this study, this could be one mechanism contributing to the increase in lymphocyte numbers observed in a large number of donors after berry consumption.

Results from this study suggest raspberry phytonutrients may also play a regulatory role, directly or indirectly, in maintaining WBC homeostasis. Increases and

decreases in WBCs are associated with infections and conditions such as leukemias, or in other instances the adverse effects of medications. The relative WBC differential counts of three female participants before berry intake indicated neutropenia, lymphocytosis, eosinophilia, and neutrophilia. Two of the donors were menstruating at the time, and for one, the neutropenia and eosinophilia could be attributed to a daily medication used to treat acne. However, in all three donors the relative WBC differential counts improved toward the normal range after raspberry consumption.

The effect of berry phytochemicals on adaptive immunity was also examined. The T lymphocyte mitogen proliferation assay, an *in vitro* assay used to assess host immunocompetence, was performed on donor PBMCs before and after raspberry consumption. Donors were divided into three groups based on their lymphocyte responses to mitogen stimulation after berry consumption. Variability was observed among donors, with Group 1 donors exhibiting the greatest mitogen-induced T lymphocyte proliferation following berry intake. For donors in Group 2 there was little change and for those in Group 3 there was a significant decrease in mitogen response following berry ingestion.

Group 1 donors, or 31% of the study participants, had a significant increase in mitogen-activated T lymphocyte proliferation up to two fold above baseline after berry intake ($p = 0.04$). Research from several laboratories supports these findings. Their results show that numerous polyphenolic compounds can induce a priming, or semi-activation of lymphocyte populations, including $\gamma\delta$ T cells and NK cells. These cells become primed for expansion and, when exposed to secondary stimuli (e.g. mitogen

stimulation), the primed cells undergo clonal expansion (4, 58, 66). Increases in these lymphocyte populations could result in greater immune protection against pathogens and aberrant cells *in vivo*. A large correlation was observed in Group 1 donors between mitogen stimulation and lymphocyte numbers *in vivo* ($r = 0.96$). Two of the Group 1 donors (donors 6 and 15) had an increase in lymphocytes *in vivo* as reported in the CBC results. Their absolute lymphocyte numbers measured at the low end of the lymphocyte range before berry consumption but increased 15% and 88% respectively after eating berries. This suggests in some individuals that berry phytochemicals may serve to prime and initiate lymphocyte proliferation, possibly leading to a more rapid immune response to an antigen.

The lymphocytes of donors in Group 2 were unresponsive to mitogen stimulation following berry intake. This is denoted by the stimulation indices (SIs) of the donors. Stimulation indices >1 indicate greater post-berry lymphocyte stimulation whereas SIs <1 signify greater pre-berry lymphocyte stimulation. The SIs of donors in Group 2 ranged from 0.8 – 1, indicating little or no change between the pre- and post-berry responses to mitogen stimulation. This suggests the berry phytochemicals had little or no priming effect on the lymphocytes of these donors, or that the concentration of berry phytochemicals in their plasmas was insufficient to stimulate the lymphocytes *in vivo*.

In comparison to Group 1 and 2 donors, the SIs were substantially lower in Group 3 and ranged from 0.2 – 0.5 ($p = 0.002$), which suggests a possible immunosuppressive effect of berry phytochemicals on lymphocyte proliferation. However, some of the participants increased in their absolute lymphocyte numbers *in vivo* after berry

consumption (donors 9, 11, 16). One possible explanation is that the lymphocytes of these donors had undergone proliferation *in vivo* following berry consumption, and additional mitogen stimulation *in vitro* failed to induce further proliferation. The remaining Group 3 donors whose lymphocytes failed to mitogen-stimulate (donors 8, 10) also decreased in lymphocytes *in vivo*.

The untreated control PBMCs were also monitored for changes in their resting metabolic activity (MTS absorbance without PHA stimulation) in the mitogen stimulation assay before and after berry intake. A significant difference was noted between donor groups in the control PBMC SIs ($p = 0.007$). Although minimal changes were noted in the PBMC SIs of Group 2 donors, .Group 1 control PBMCs SIs decreased ($p = 0.009$) while Group 3 control PBMC SIs increased ($p = 0.09$). An inverse relationship was observed between the degree of mitogen-induced lymphocyte proliferation and the control PBMC SIs ($r = -0.6$; $p = 0.02$). Although mitogen stimulation was greater for Group 1 donors after berry consumption, their control PBMC SIs were <1 and ranged from 0.5 – 0.9. In contrast, Group 3 donors decreased in lymphocyte mitogen-responsiveness after berry consumption but their control PBMC SIs were >1, and ranged from 1.9 – 3.7, with the exception of donor 10 who was on a daily low-dose aspirin regimen. The increased activity of the control PBMCs from Group 3 donors could be a measurement of post-berry monocyte proliferation, which could result in an enhanced innate immune response *in vivo*. Alternatively, a second explanation for the observed effect could be that the control PBMCs were in a primed, or activated state following berry consumption, but not toward proliferation. While the mitogen stimulation assay is

a measure of T lymphocyte proliferation, the basis of the MTS assay relies on the activity of dehydrogenase enzymes that are found in metabolically active cells. Dehydrogenase enzymes function to reduce MTS into formazan, and it is generally accepted that the concentration of formazan is proportional to the number of cells in a sample. However, in metabolically active cells there are numerous types of dehydrogenase enzymes, many of which function in glycolysis and the TCA cycle (tricarboxylic acid cycle) for energy production. Increased energy availability is essential to maintain the cellular activity of activated lymphocytes and monocytes, which could lead to enhanced immunity in the host. Therefore, for donors who did not mitogen stimulate but increased in control PBMC activity, we cannot exclude the possibility that we are measuring a form of PBMC activation following berry intake. Lastly, for Group 2 donors there was little or no difference between the mitogen response and control PBMC stimulation indices. With the exception of donor 13, both SIs were close to 1, suggesting that in some individuals berry phytochemicals appear to have little or no effect on lymphocyte proliferation or immune cell activation as measured by this assay. Donor 13 was menstruating at the time of the study and we cannot exclude possible hormonal effects on her PBMC responses to berry phytochemicals.

This study also examined the effect of raspberry ingestion on plasma matrix metalloproteinase-9 (MMP-9). MMPs play a critical role in tumor invasion and metastasis; therefore the inhibition or reduction of MMP-9 by berry phytochemicals could help to decrease or prevent the spread of cancer. However, results from this study show that plasma levels of MMP-9 did not change significantly following berry intake.

Forty percent of the donors' post-berry plasma increased in MMP-9 (23% – 68%) while 20% decreased (18% – 39%), and the remaining donors' post-berry MMP-9 levels were relatively unchanged (≤ 10%).

Of the six donors with increased post-berry plasma MMP-9 concentrations, four were in Group 3. All six of the donors had an increase in one or more WBC types as determined by their CBCs. Among the donors, five increased in absolute lymphocyte numbers, four increased in neutrophils and five increased in monocytes, eosinophils, and/or basophils. The increase in neutrophils and monocytes could be one factor contributing to the increase in MMP-9. Pro-MMP-9, a precursor of MMP-9, is secreted by monocytes, macrophages, and neutrophils. Five of the donors increased in one or both of these cell types. The same five donors also increased in their untreated control PBMC SIs (1.1 – 3.7), suggesting an upregulation, or activation of the PBMCs following berry intake. For Group 3 donors, a strong positive correlation was noted between increases in MMP-9 levels and neutrophil and lymphocyte counts ($r = 0.6$ and 0.8 respectively) as well as PBMC control SIs ($r = 0.7$). One explanation for this could be due to the priming effect of polyphenols on γδ T cells. A number of cytokines, including GM-CSF (granulocyte-macrophage colony stimulating factor) and IL-8 (interleukin 8) are released from the primed lymphocytes and serve as activators of neutrophils and monocytes (58). This activation results in the production of pro-inflammatory cytokines (IL-1, TNF-α) and reactive oxygen species (ROS) that have been shown to upregulate the production of MMP-9 (39, 67, 68). While the increase in WBCs and MMP-9 levels in these donors

could aid in enhanced innate immunity, tissue repair, and wound healing, it could also affect the potential for cancer metastasis.

The three female donors whose post-berry plasma decreased in MMP-9 were in Group 2. Two of them were menstruating at the time of the study, and the reduction in MMP-9 could have been influenced by hormonal changes. While two of the donors increased in neutrophils or monocytes, none increased in lymphocytes. All 3 donor's lymphocytes failed to mitogen-stimulate, and they also decreased in their resting post : pre-berry PBMC control ratios (0.6 – 0.9). A strong correlation (r = 0.7) was noted between decreases in MMP-9 levels and the control PBMC SIs.

Similarly, of the six donors whose MMP-9 levels remained relatively constant, five had lower levels of control PBMC SIs (0.4 – 0.9), and the last donor's SI w as unchanged. For the donors whose MMP-9 levels decreased or remained relatively constant, their results suggest little or no *in vivo* γδ T cell priming occurred after berry intake and therefore no activation of the neutrophils and monocytes. An alternate explanation is that the antioxidant activity of the berry phytochemicals *in vivo* could have decreased the levels of ROS in the plasma, which could have resulted in lower concentrations or steady-state levels of MMP-9 in these donors.

Additional studies were performed on donor plasma to assess changes in plasma tumor cell cytotoxicity following raspberry intake. The results observed need not have been caused by the direct effects of berry phytochemicals, but could also have resulted from changes induced in donor metabolism or physiology. Any changes induced by berry phytochemicals are dependent on sufficient bioavailability. Studies from numerous

laboratories have reported that the serum bioavailability of many berry polyphenols is low, making it unclear whether the *in vivo* concentrations are sufficient to provide anti-carcinogenic protection (10, 25, 46). Although most berry bioactives have demonstrated chemopreventive effects *in vitro* when used at micromolar concentrations, pharmacokinetic studies in humans indicate that berry bioactives, such as the anthocyanins and ellagitannins, reach only nanomolar concentrations in blood and tissues when administered in the diet. These levels are far below the levels required to exhibit anticarcinogenic effects *in vitro* (10). However, results from this study clearly demonstrate that the levels of berry bioactives present in the plasmas of the study participants following berry intake were sufficient to induce or produce cytotoxic effects on tumor cells *in vitro*. Consumption of the lyophilized, or freeze-dried form of the raspberries may have contributed to the plasma levels attained in this study. Freeze-drying increases the concentration of polyphenols 9-10 fold and likely increases the bioavailability of the phytonutrients in the serum. Although our results show variability among individuals as well as donor groups, 100% of the donors' post-berry plasma increased in tumor cell cytotoxicity against one and up to four of the cell lines tested. While changes were modest in the AGS and LNCaP tumor cell lines ($p = 0.1$), the greatest increase in plasma cytotoxicity among donors was noted in the HCC 1500 breast cell line, with an average change of 28.3±6.7% cytotoxicity after berry consumption ($p = 0.05$). As mentioned previously, both the LNCaP prostate cells and HCC 1500 breast cells are estrogen receptor positive, and the anti-estrogenic properties of the berry

constituents present in the donor plasmas could be one mechanism contributing to the increased cytotoxicity measured in these cells.

Group 1 and 2 donors exhibited increased post-berry plasma cytotoxicity in the AGS, LNCaP, and HCC 1500 cell lines, and to a similar degree. Their results indicate *in vivo* changes were induced in the plasmas of these donors to increase tumor cell cytotoxicity *in vitro*. While the two groups differed in their responses to lymphocyte mitogen-stimulation after berry intake, there were similarities in their control PBMC SIs and MMP-9 levels. For both groups, control PBMC SIs and MMP-9 levels either decreased or remained constant ($r = 0.7$). In addition, of the ten donors with CBC data, 50% or more had decreased levels of lymphocytes, neutrophils, and monocytes after berry consumption (Appendix C). Although there was a measurable difference in the plasma cytotoxicity of these individuals, it cannot be ascertained from this assay if the berry phytochemicals exerted an immunosuppressive effect on donor PBMCs *in vivo*. Overall there was a decrease in WBC expansion *in vivo*, which could have resulted in the decrease in control PBMC activation that was observed *in vitro* in these donors. This lack of apparent activation could have influenced the decrease or maintenance of the MMP-9 levels. Regardless, the bioavailability of the berry phytochemicals in the plasmas of these donors could have directly or indirectly exerted a cytotoxic effect on the cell lines tested.

The largest fraction of donors with increased post-berry plasma cytotoxicity against all five cell lines tested occurred in Group 3. These donors showed a significant increase in post-berry plasma cytotoxicity in the AGS cell line ($p = 0.005$) and a modest

increase in the HCC 1500 cell line (p = 0.1). Although this groups' T lymphocytes did not mitogen-stimulate *in vitro*, they all had increased activity in their control PBMC SIs. In addition, the majority of the donors had increased levels of lymphocytes (r = 0.5) and MMP-9 (r = 0.7) *in vivo*. These findings suggest the bioavailability and plasma levels of the berry phytochemicals is greatest in these individuals and exert an activating effect on their immune cells *in vivo*.

The HCC 1500 breast and LNCaP prostate cell lines were susceptible to cell killing in both the male and female donor plasmas, possibly due to the anti-estrogenic effect from the berry metabolites present in the donor plasmas.

The donors' plasma cytotoxicity after berry intake did not correlate with an increase in PBMC cytotoxicity in all donors. Results of the cytotoxicity assay varied widely among donors and cell lines. While berry phytochemicals enhanced tumor cell killing in some individuals, it decreased PBMC cytotoxicity in others as measured by this assay. The greatest increase in post-berry PBMC tumoricidal activity was measured in the AGS gastric, LoVo colon, and HCC 1500 breast cell lines after a 72 hour treatment period. Although donor plasma contributed to cell death, PBMC tumoricidal activity increased in 50% or more of the donors tested following berry intake.

Possible proliferation of tumor cells or immune cells was observed with the AGS, HCC 1500, LNCaP, and K-562 cell lines. While tumor cell killing also occurred in the LNCaP and K-562 cell lines at different PBMC concentrations after berry intake, activation or proliferation was more prevalent. To examine this further, the incubation time was reduced to four hours to minimize cell proliferation. However, at the 20:1 E:T

concentrations the absorbance measurements increased in the AGS, LNCaP and K-562 cell lines, suggesting PBMC activation or cellular replication. These findings differ from a similar study from this laboratory by God who examined the effect of raspberry consumption on NK cell cytotoxicity toward K-562 cells. He reported a significant increase in NK cell cytotoxicity toward these cells in 33% of his study participants following berry intake (18). However, in God's study the monocytes were depleted from donor PBMCs before treatment with K-562 cells while the current study sought to investigate the effect of berry phytochemicals on the monocytes as well. The inclusion of monocytes in this study could explain the difference between the enhanced NK cell cytotoxicity in God's results and those reported here. Natural killer cells are cytotoxic against a wide range of tumor cells, and once they are activated, NK cells produce interferon gamma (IFN γ), a potent immunoregulatory cytokine and powerful mediator of macrophage activation. As monocytes undergo differentiation and activation, numerous physical and metabolic changes occur. The cells enlarge 5-10 fold and increase in phagocytic ability. They also increase in the number and complexity of intracellular organelles and produce higher levels of hydrolytic enzymes, including reactive oxygen and nitrogen species (ROS, RNS) and inducible nitric oxide synthase (iNOS). In addition, they secrete a variety of soluble factors and inflammatory cytokines, such as IL1, IL6, and TNF-α (55). Activated macrophages are therefore more effective in eliminating pathogens, killing tumor cells, and activating the adaptive arm of the immune system. However, results from the LNCaP and K-562 samples do not reflect enhanced cytocidal activity. Minimal or no cytocidal activity was noted for the PBMCs from 10

donors before and after berry consumption. Tumor cell killing could have occurred by the lymphocytic cells (NK cells, γδ T cells, and cytotoxic T cells) but the upregulated metabolic activity of the macrophages could have obscured detection of tumor cell death. The apparent absence or decrease in cytotoxicity could be due in part to the strong antioxidant and anti-inflammatory effects of the raspberry polyphenols. The plasma assay performed in this study show that there were sufficient changes in donor plasma to increase plasma tumor cell cytotoxicity following berry intake. Elevated levels of raspberry antioxidants could therefore quench the ROS, RNS, and lytic enzymes produced by the activated macrophages and minimize or eliminate tumor cell killing. Schauss *et al* showed that extremely low doses of antioxidants in freeze-dried acai were able to cross the cell membrane of freshly isolated human neutrophils and quench the free radicals produced within the neutrophils (69). It is possible the levels of raspberry antioxidants or induced antioxidants reached in the donor plasmas were sufficient to negate the tumoricidal activity of the activated macrophages. Alternatively, based upon the assay results we cannot discount PBMC and/or tumor cell proliferation in some donor samples. Holderness *et al* demonstrated γδT cells and NK cells primed by apple-derived tannins responded rapidly to secondary stimuli and proliferated up to four-fold. Donor PBMCs that were primed *in vivo* following berry intake and subsequently exposed to tumor cells *in vitro* could then rapidly expand and account for the results reported here.

The enhanced post-berry proliferative effect observed in the 72 hour and 4 hour treatments of the HCC 1500, AGS, LNCaP, and K-562 tumor cell lines suggests an activation of the donor PBMCs from the berry phytochemicals. While the tumor cells

and/or PBMCs could proliferate during a 72 hour treatment period, it is unlikely they would proliferate above the control samples during the brief 4 hour incubation period. Post-berry proliferation was significant for the HCC 1500 and K-562 cell lines ($p = 0.05$ and 0.04 respectively) and moderately significant for the AGS and LNCaP cell lines (0.08). However, based upon the limitations of this assay, it cannot be determined conclusively if PBMC activation or proliferation was occurring in these samples.

In conclusion, preliminary *in vitro* results show that lyophilized red raspberry extract exhibited a potent cytotoxic effect on four commonly occurring cancers, including gastric, prostate, colon, and breast. By comparison to ascorbic acid that served as a control, it could be determined that the cytotoxicity of the raspberry extract was not solely attributable to pH or antioxidant effects in cell killing.

The results of the *in vivo* study show that consumption of red raspberries may have the capacity to enhance immune activity in some individuals. Although we did not have adequate control over factors known to influence immune function, such as diet, sleep habits, medications, or stress, there were measurable differences in immune parameters in some donors following raspberry consumption. More than half of the donors had increased levels of one or more subsets of leukocytes after eating berries. This increase in both innate and adaptive immune cell populations could lead to better protection against microbial pathogens as well as cancer cells arising in the host. In addition, the beneficial effect of berry phytochemicals on the adaptive immune response was noted in some donors by their increased T lymphocyte responses to mitogen stimulation. Other donors showed an increase in the resting levels of PBMCs, and it is

unclear if this was due to an increase in metabolic activity or proliferation. These findings suggest that berry phytochemicals could possibly enhance immunocompetence and augment immune cell activity in some individuals, leading to greater protection in the host. While MMP-9 levels varied among donors, those who increased in MMP-9 also increased in one or more subsets of leukocytes, with the majority increasing in lymphocytes. In contrast, the majority of donors whose MMP-9 levels decreased or remained relatively constant had decreased levels of lymphocytes. Although a correlation between PBMC numbers and MMP-9 plasma levels is reported here, the significance of this is not known and warrants further investigation. Despite numerous studies reporting the low bioavailability of polyphenols *in vivo*, our results clearly show that consumption of lyophilized red raspberries provided sufficient phytochemical-induced changes in the plasmas of all the study participants to kill tumor cells *in vitro*. These results demonstrate the antiproliferative and antitumor properties of plasma phytonutrients alone without the need for direct immune cell participation. However, an increase in PBMC cytotoxicity was also observed in a large number of donors following raspberry intake. While donor plasma contributed to the cell death observed in several cases, PBMC tumoricidal activity increased as well. Because of the fact that the surviving fraction of cells is negative in some cases, the quantity (E+T) is larger than (ET), where E represents the absorbance of the effector PBMCs, T represents the absorbance of the target tumor cells, and ET represents the absorbance of the mixture. This implies that the effectors are probably being activated or proliferating. This effect is observed in the AGS, HCC 1500, LNCaP, and K-562 cell lines. We don't know the

significance of the activation or if it relates to better immune responses or immune protection. Taken together, these results demonstrate that in some individuals raspberry phytochemicals appear to have an immunostimulatory effect on both the innate and adaptive arms of the immune system.

Findings from the current study warrant further investigation to determine whether raspberry consumption has an activating effect on PBMCs *in vivo*, and if this activation results in enhanced immune function in the host. Based on the experimental approach used in this study, *in vivo* studies could also be conducted to clarify the overall effect of berry consumption on patients with premalignant conditions such as colonic polyps or Barrett's esophagus.

APPENDICES

A. *In Vivo* Red Raspberry Study Questionnaire

1. Do you have an adverse reaction to having blood drawn? (i.e. fainting, dizziness, excessive bleeding, etc.)
   YES NO

2. Do you have an adverse reaction to consuming berries or using berry-related products?
   YES NO

3. Do you have an infectious or autoimmune disease (such as multiple sclerosis or arthritis)?
   YES NO

4. Do you have or have you ever had cancer?
   YES NO

5. Have you had a cold, the flu, other infections or allergy problems during the past week?
   YES NO

6. Are you taking any medications?
   YES NO

7. If you answered yes to question 6, what type(s) of medication do you take?

8. How often do you exercise?

9. When was the last time you exercised?

10. Are fruits and berries a normal part of your diet?
    YES NO

11. If you answered yes to question 10, how many times a week do you eat fruits and/or berries?

12. What types of fruits or berries do you eat?

13. Have you eaten fruit or berries in the last two days?
    YES NO

14. If you answered yes to question 13, what type of fruit did you eat and how much was consumed?

15. Do you drink fruit juice or consume other fruit-related products (e.g. Fruit roll-ups)?
    YES NO

16. If you answered yes to question 15, how many times a week do you consume fruit-related products?

17. Have you had any fruit juice or fruit-related product in the past two days?
    YES NO

18. If you answered yes to question 17, what were those products and how much did you consume?

19. Are you a diabetic?
    YES NO

20. Do you consume alcoholic beverages?
    YES NO

21. If the answer to question 20 is yes, approximately how many drinks per week do you have?

22. What is your age:

23. Are you:
    MALE FEMALE

24. If you have been exposed to tanning radiation, X-rays, or a CT scan in the past week, please describe (for tanning, how long were you exposed?).

25. For women, since the levels of various hormones can affect the immune system, we would like to know the following:
    a. Are you on any form of hormone replacement therapy or birth control?
       YES NO
    b. What is the date of you last menstruation?

If you choose not to answer question 25, please feel free to omit.

B: Complete Blood Counts of Study Participants

Donor 1

PRE-Berry
Donor 01
10/26/09

REDFERN HEALTH CENTER
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4054

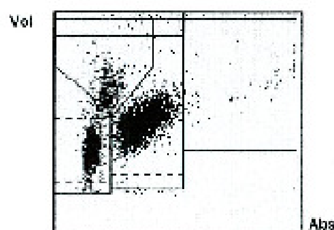
864-656-3566

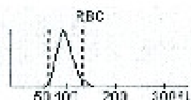
Sample ID: 0110262009 Patient Name: 01
Run Date/Time: 10/26/2009 11:29:03 AM Patient ID: 0110262009 Collect Date/Time:
Seq#: 390 Gender: Unknown DOB: Physician:
OPR: BCI Age: Location:
Flagging Set: New Standard Ranges
Comments:

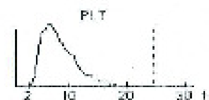
			Range	
WBC	5.5	10^3/µL	4.0	/ 11.0
RBC	5.44	10^6/µL	3.80	/ 6.50
HGB	17.0	g/dL	12.0	/ 18.0
HCT	49.0	%	32.0	/ 54.0
MCV	90	fL	75	/ 95
MCH	31.3	pg	27.0	/ 32.0
MCHC	34.7	g/dL	31.0	/ 35.0
RDW	12.4	%	11.0	/ 13.0
PLT	274	10^3/µL	150	/ 400
MPV	8.4	fL	6.0	/ 13.0

				Range	
NE	64.7		%	45.0	/ 70.0
LY	26.0		%	20.0	/ 40.0
MO	7.3		%	3.0	/ 10.0
EO	1.4		%	1.0	/ 5.0
BA	0.6	H	%	0.0	/ 0.5
NE#	3.53		10^3/µL	2.00	/ 7.50
LY#	1.42	L	10^3/µL	1.50	/ 4.00
MO#	0.40		10^3/µL	0.20	/ 0.80
EO#	0.08		10^3/µL	0.04	/ 0.40
BA#	0.03		10^3/µL	0.02	/ 0.10

Flags and Messages







Microscopic Examination

Neutrophils		Metamyelocytes		Anisocytosis		Retics	
Bands		Myelocytes		Hypochromia		Sed. Rate	
Lymphocytes		Promyelocytes		Polychromasia			
Monocytes		Blast		Poikilocytosis			
Eosinophils		Atyp. Lymph		Microcytosis			
Basophils		NRBC s		Macrocytosis			

Comment :

Requested by :
Reviewed by : 10-26-09 @ 1130

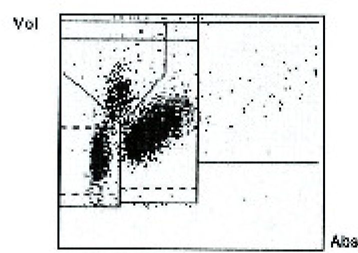
Out of Action Range Out of Patient Range XXX

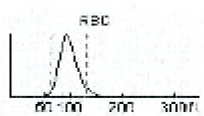
post-Berry

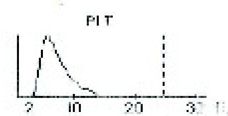
REDFERN HEALTH CENTER
CLEMSON UNIVERSITY
BOX 344054
CLEMSON SC 29634-4064
864-656-3666

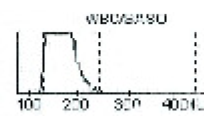
Sample ID: 0110292009 Patient Name: 0110292009
Run Date/Time: 10/29/2009 09:47:39 AM Patient ID:
Collect Date/Time:
Seq#: 505 Gender: Unknown DOB:
Physician:
OPR: BCI Age:
Location:
Flagging Set: New Standard Ranges
Comments:

			Range					Range	Flags and Messages
WBC	5.3	10^3/µL	4.0 / 11.0	NE	64.6		%	45.0 / 70.0	
				LY	23.5		%	20.0 / 40.0	
RBC	5.33	10^6/µL	3.80 / 6.50	MO	10.0		%	3.0 / 10.0	
HGB	16.1	g/dL	12.0 / 18.0	EO	1.4		%	1.0 / 5.0	
HCT	47.9	%	35.0 / 54.0	BA	0.5		%	0.0 / 0.5	
MCV	90	fL	76 / 96						
MCH	30.2	pg	27.0 / 32.0						
MCHC	33.6	g/dL	31.0 / 35.0	NE#	3.45		10^3/µL	2.00 / 7.50	
RDW	12.6	%	11.0 / 16.0	LY#	1.25	L	10^3/µL	1.50 / 4.00	
				MO#	0.53		10^3/µL	0.20 / 0.80	
PLT	297	10^3/µL	150 / 400	EO#	0.07		10^3/µL	0.04 / 0.40	
MPV	7.4	fL	6.0 / 10.0	BA#	0.03		10^3/µL	0.02 / 0.10	









Microscopic Examination

Neutrophils	______	Metamyelocytes	______	Anisocytosis	______	Retics	______
Bands	______	Myelocytes	______	Hypochromia	______	Sed. Rate	______
Lymphocytes	______	Promyelocytes	______	Polychromasia	______		
Monocytes	______	Blast	______	Poikilocytosis	______		
Eosinophils	______	Atyp. Lymph	______	Microcytosis	______		
Basophils	______	NRBC s	______	Macrocytosis	______		

Comment : ______________________

Requested by :

Reviewed by : *GHW 950 10-29-09*

Out of Action Range [shaded] Out of Patient Range XXX

Printed: 10/29/2009 09:47:44 AM

Donor 03
Arc - ferragut 864-656-3556

REDFERN HEALTH CENTER
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4054

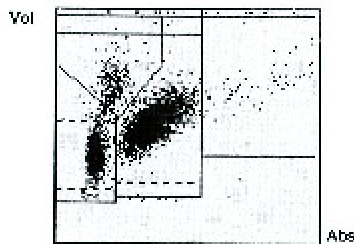
Sample ID: 0311092009 Patient Name: 0311092009
Run Date/Time: 11/09/2009 01:53:57 PM Patient ID:
Seq#: 619 Gender: Unknown DOB:
OPR: BCI Age:
Flagging Set: New Standard Ranges
Comments:

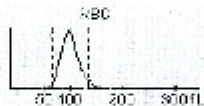
Collect Date/Time:
Physician:
Location:

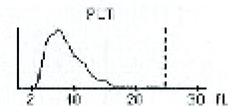
			Range
WBC	6.1	10^3/µL	4.0 / 11.0
RBC	4.92	10^6/µL	3.80 / 5.90
HGB	15.4	g/dL	12.0 / 18.0
HCT	46.0	%	36.0 / 54.0
MCV	93	fL	76 / 96
MCH	31.3	pg	27.0 / 32.0
MCHC	33.5	g/dL	31.0 / 35.0
RDW	12.0	%	11.0 / 16.0
PLT	181	10^3/µL	150 / 400
MPV	9.2	fl	6.0 / 10.0

				Range
NE	64.5		%	45.0 / 70.0
LY	26.3		%	20.0 / 40.0
MO	6.9		%	3.0 / 10.0
EO	1.7		%	1.0 / 5.0
BA	0.6	H	%	0.0 / 0.5
NE#	3.93		10^3/µL	2.00 / 7.50
LY#	1.60		10^3/µL	1.50 / 4.00
MO#	0.42		10^3/µL	0.20 / 0.80
EO#	0.10		10^3/µL	0.04 / 0.40
BA#	0.04		10^3/µL	0.02 / 0.10

Flags and Messages







Microscopic Examination

Neutrophils		Metamyelocytes		Anisocytosis		Retics	
Bands		Myelocytes		Hypochromia		Sed. Rate	
Lymphocytes		Promyelocytes		Polychromasia			
Monocytes		Blast		Poikilocytosis			
Eosinophils		Atyp. Lymph		Microcytosis			
Basophils		NRBC s		Macrocytosis			

Comment :

Requested by :
Reviewed by : GHW 55 P 11-9-09

Out of Action Range XXX Out of Patient Range XXX

Printed 11/09/2009 01:54:32 PM

Donor 03
Post-bleeding

REDFERN HEALTH CENTER
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4064

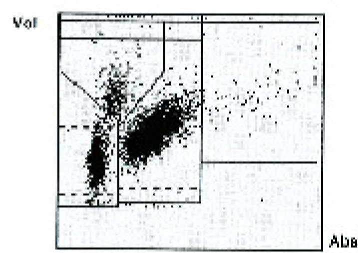
864-656-3666

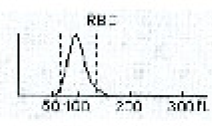
Sample ID: 0311122009 Patient Name:
Run Date/Time: 11/12/2009 08:19:22 AM Patient ID: 0311122009 Collect Date/Time: 11/12/2009 08:16:00 AM
Seq#: 929 Gender: Unknown DOB: Physician:
OPR: BCI Age: Location:
Flagging Set: New Standard Ranges
Comments:

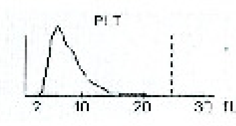
Test	Result	Flag	Units	Range
WBC	4.9		10^3/µL	4.0 / 11.0
RBC	4.80		10^6/µL	3.80 / 6.50
HGB	15.4		g/dL	12.0 / 18.0
HCT	44.9		%	36.0 / 54.0
MCV	94		fL	78 / 98
MCH	32.0	H	pg	27.0 / 32.0
MCHC	34.2		g/dL	31.0 / 35.0
RDW	12.2		%	11.0 / 16.0
PLT	164		10^3/µL	150 / 400
MPV	9.1		fL	6.0 / 10.0

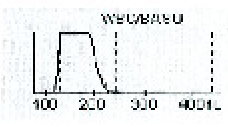
Test	Result	Flag	Units	Range
NE	60.9		%	45.0 / 70.0
LY	28.8		%	20.0 / 40.0
MO	7.5		%	3.0 / 10.0
EO	2.2		%	1.0 / 5.0
BA	0.6	H	%	0.0 / 0.5
NE#	2.99		10^3/µL	2.00 / 7.50
LY#	1.41	L	10^3/µL	1.50 / 4.00
MO#	0.37		10^3/µL	0.20 / 0.80
EO#	0.11		10^3/µL	0.04 / 0.40
BA#	0.03		10^3/µL	0.02 / 0.10

Flags and Messages









Microscopic Examination

Neutrophils	______	Metamyelocytes	______	Anisocytosis	______	Retics	______
Bands	______	Myelocytes	______	Hypochromia	______	Sed. Rate	______
Lymphocytes	______	Promyelocytes	______	Polychromasia	______		
Monocytes	______	Blast	______	Poikilocytosis	______		
Eosinophils	______	Atyp. Lymph	______	Microcytosis	______		
Basophils	______	NRBC s	______	Macrocytosis	______		

Comment : ______________________

Requested by :

Reviewed by : PBS 11-12-09 0820

Out of Action Range XXXX Out of Patient Range XXX

Printed: 11/12/2009 08:19:29 AM

Donor 04
PRE-DIFF
12/4/09

REDFERN HEALTH CENTER
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4054

864-656-3566

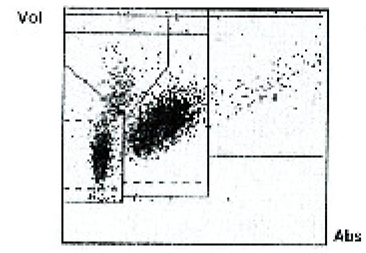
Sample ID: 1204200904 **Patient Name: 1204200904**

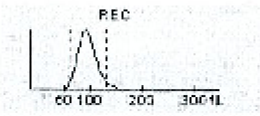
Run Date/Time:	12/04/2009 09:33:03 AM	Patient ID:	1204200904		Collect Date/Time: 12/04/2009 09:25:00 AM
Seq#:	431	Gender:	Unknown	DOB:	Physician:
OPR:	DCI			Age:	Location:
Flagging Set:	New Standard Ranges				
Comments:					

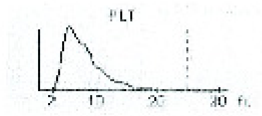
			Range	
WBC	5.2	10^3/µL	4.0 / 11.0	
RBC	5.41	10^6/µL	3.80 / 6.50	
HGB	16.1	g/dL	12.0 / 18.0	
HCT	48.2	%	35.0 / 54.0	
MCV	89	fL	76 / 96	
MCH	29.8	pg	27.0 / 32.0	
MCHC	33.4	g/dL	31.0 / 35.0	
RDW	13.2	%	11.0 / 16.0	
PLT	205	10^3/µL	150 / 400	
MPV	8.3	fL	5.0 / 10.0	

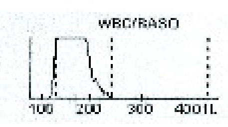
				Range
NE	59.9		%	45.0 / 70.0
LY	31.6		%	20.0 / 40.0
MO	4.8		%	3.0 / 10.0
EO	3.0		%	1.0 / 5.0
BA	0.5	H	%	0.0 / 0.5
NE#	3.14		10^3/µL	2.00 / 7.50
LY#	1.06		10^3/µL	1.50 / 4.00
MO#	0.26		10^3/µL	0.20 / 0.80
EO#	0.16		10^3/µL	0.04 / 0.40
BA#	0.03		10^3/µL	0.02 / 0.10

Flags and Messages









Microscopic Examination

Neutrophils	______	Metamyelocytes	______	Anisocytosis	______	Retics	______
Bands	______	Myelocytes	______	Hypochromia	______	Sed. Rate	______
Lymphocytes	______	Promyelocytes	______	Polychromasia	______		
Monocytes	______	Blast	______	Poikilocytosis	______		
Eosinophils	______	Atyp. Lymph	______	Microcytosis	______		
Basophils	______	NRBC s	______	Macrocytosis	______		

Comment : ______________________

Requested by : ______________

Reviewed by : DBS 12-4-09 0930

Out of Action Range Out of Patient Range XXX

Donor 04
Post-berry
12-7-09

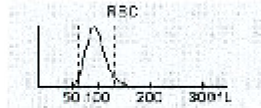
REDFERN HEALTH CENTER
CLEMSON UNIVERSITY

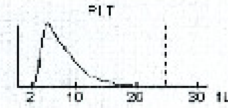
BOX 344054
CLEMSON SC 29634-4054

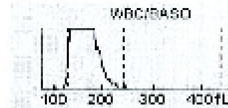
864-656-3566

Sample ID: 1207200904 Patient Name: 1207200904
Run Date/Time: 12/07/2009 03:23:25 PM Patient ID: 1207200904 Collect Date/Time: 12/07/2009 03:20:00 PM
Seq#: 468 Gender: Unknown DOB: Physician:
OPR: BCI Age: Location:
Flagging Set: New Standard Ranges
Comments:

			Range					Range	Flags and Messages
WBC	6.9	10^3/µL	4.0 / 11.0	NE	64.5		%	45.0 / 70.0	
				LY	27.4		%	20.0 / 40.0	
RBC	5.20	10^6/µL	3.60 / 6.50	MO	4.8		%	3.0 / 10.0	
HGB	15.7	g/dL	12.0 / 18.0	EO	2.6		%	1.0 / 5.0	
HCT	46.5	%	36.0 / 54.0	BA	0.7	H	%	0.0 / 0.5	
MCV	89	fL	76 / 96						
MCH	30.2	pg	27.0 / 32.0						
MCHC	33.7	g/dL	31.0 / 35.0	NE#	4.47		10^3/µL	2.00 / 7.50	
RDW	13.7	%	11.0 / 16.0	LY#	1.90		10^3/µL	1.50 / 4.00	
				MO#	0.33		10^3/µL	0.20 / 0.80	
PLT	241	10^3/µL	150 / 400	EO#	0.18		10^3/µL	0.04 / 0.40	
MPV	8.2	fL	6.0 / 10.0	BA#	0.05		10^3/µL	0.02 / 0.10	







Microscopic Examination

Neutrophils		Metamyelocytes		Anisocytosis		Retics	
Bands		Myelocytes		Hypochromia		Sed. Rate	
Lymphocytes		Promyelocytes		Polychromasia			
Monocytes		Blast		Poikilocytosis			
Eosinophils		Atyp. Lymph		Microcytosis			
Basophils		NRBC s		Macrocytosis			

Comment : ____________________

Requested by : ____________________

Reviewed by : DJS 12-7-09 3:25

Out of Action Range XXXX Out of Patient Range XX.X

Printed 12/07/2009 03:23 PM

Donor 05
Pre-ferry
12/08/09

REDFERN HEALTH CENTER
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4054

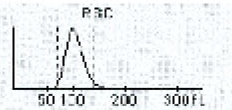
864-656-3566

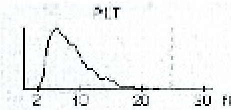
Sample ID:	0512082009	Patient Name:			
Run Date/Time:	12/08/2009 08:17:02 AM	Patient ID:		Collect Date/Time:	
Seq#:	486	Gender:	Unknown	DOB:	Physician:
OPR:	BCI			Age:	Location:
Flagging Set:	New Standard Ranges				
Comments:					

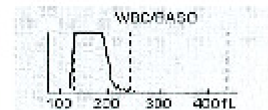
				Range
WBC	5.2		10^3/µL	4.0 / 11.0
RBC	3.96		10^6/µL	3.80 / 5.50
HGB	12.7		g/dL	12.0 / 18.0
HCT	37.6		%	35.0 / 54.0
MCV	95		fL	78 / 98
MCH	32.1	H	pg	27.0 / 32.0
MCHC	33.7		g/dL	31.0 / 35.0
RDW	11.7		%	11.0 / 16.0
PLT	191		10^3/µL	150 / 400
MPV	8.7		fL	6.0 / 10.0

			Range
NE	55.5	%	45.0 / 70.0
LY	36.7	%	20.0 / 40.0
MO	5.5	%	3.0 / 10.0
EO	1.9	%	1.0 / 5.0
BA	0.4	%	0.0 / 0.5
NE#	2.90	10^3/µL	2.00 / 7.50
LY#	1.92	10^3/µL	1.50 / 4.00
MO#	0.29	10^3/µL	0.20 / 0.80
EO#	0.10	10^3/µL	0.04 / 0.40
BA#	0.02	10^3/µL	0.02 / 0.10

Flags and Messages







Microscopic Examination

Neutrophils	______	Metamyelocytes	______	Anisocytosis	______	Retics	______
Bands	______	Myelocytes	______	Hypochromia	______	Sed. Rate	______
Lymphocytes	______	Promyelocytes	______	Polychromasia	______		
Monocytes	______	Blast	______	Poikilocytosis	______		
Eosinophils	______	Atyp. Lymph	______	Microcytosis	______		
Basophils	______	NRBC s	______	Macrocytosis	______		

Comment : ______________________

Requested by :

Reviewed by : GLW 0820 12-8-09

Crit of Action Range XX.X Out of Patient Range XX.X

Printed 12/08/2009 08:17 09 AM

Donor 05
Post-berry
12-11-09

REDFERN HEALTH CENTER
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4054

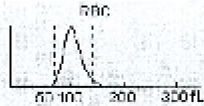
864-656-3586

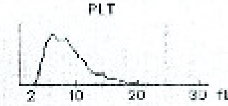
Sample ID:	0512112009	Patient Name:			
Run Date/Time:	12/11/2009 08:17:13 AM	Patient ID:			Collect Date/Time:
Seq#:	573	Gender:	Unknown	DOB:	Physician:
OPR:	BCI			Age:	Location:
Flagging Set:	New Standard Ranges				
Comments:					

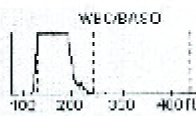
			Range	
WBC	4.6	10^3/µL	4.0	/ 11.0
RBC	4.04	10^6/µL	3.80	/ 5.50
HGB	12.8	g/dl	12.0	/ 18.0
HCT	38.4	%	35.0	/ 54.0
MCV	95	fL	75	/ 96
MCH	31.6	pg	27.0	/ 32.0
MCHC	33.3	g/dL	31.0	/ 35.0
RDW	11.7	%	11.0	/ 16.0
PLT	190	10^3/µL	150	/ 400
MPV	8.8	fL	6.0	/ 10.0

				Range	
NE	56.1		%	45.0	/ 70.0
LY	36.7		%	20.0	/ 40.0
MO	5.0		%	3.0	/ 10.0
EO	2.0		%	1.0	/ 6.0
BA	0.4		%	0.0	/ 0.5
NE#	2.59		10^3/µL	2.00	/ 7.50
LY#	1.55		10^3/µL	1.50	/ 4.00
MO#	0.27		10^3/µL	0.20	/ 0.80
EO#	0.09		10^3/µL	0.04	/ 0.40
BA#	0.02	L	10^3/µL	0.02	/ 0.10

Flags and Messages







Microscopic Examination

Neutrophils		Metamyelocytes		Anisocytosis		Retics	
Bands		Myelocytes		Hypochromia		Sed. Rate	
Lymphocytes		Promyelocytes		Polychromasia			
Monocytes		Blast		Poikilocytosis			
Eosinophils		Atyp. Lymph		Microcytosis			
Basophils		NRBC s		Macrocytosis			

Comment : ______________________

Requested by : ______________________

Reviewed by : [illegible signature] 12-11-09

Out of Action Range [illegible] Out of Patient Range XXX

Printed 12/11/2009 [illegible] AM

Donor 6b
Pre-berry
12-15-09

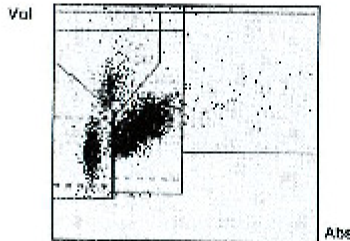
REDFERN HEALTH CENTER
CLEMSON UNIVERSITY

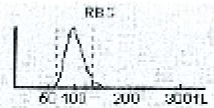
BOX 344054
CLEMSON SC 29634-4054

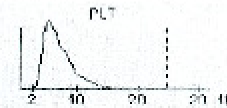
864-656-3566

Sample ID: 1215200906 Patient Name: 1215200906
Run Date/Time: 12/15/2009 08:36:32 AM Patient ID: 1215200906 Collect Date/Time: 12/15/2009 08:00:00 AM
Seq#: 809 Gender: Unknown DOB: Physician:
OPR: DCI Age: Location:
Flagging Set: New Standard Ranges
Comments:

			Range					Range	Flags and Messages
WBC	5.5		10^3/µL	4.0 / 11.0	NE	68.5	%	40.0 / 70.0	
					LY	21.9	%	20.0 / 40.0	
RBC	4.34		10^6/µL	3.80 / 6.50	MO	7.6	%	3.0 / 10.0	
HGB	13.6		g/dL	12.0 / 18.0	EO	1.6	%	1.0 / 5.0	
HCT	40.2		%	36.0 / 54.0	BA	0.4	%	0.0 / 0.5	
MCV	93		fL	76 / 96					
MCH	31.3		pg	27.0 / 32.0					
MCHC	33.8		g/dL	31.0 / 35.0					
RDW	12.3		%	11.0 / 16.0	NE#	3.77	10^3/µL	2.00 / 7.50	
					LY#	1.20 L	10^3/µL	1.50 / 4.00	
PLT	343		10^3/µL	150 / 400	MO#	0.42	10^3/µL	0.20 / 0.80	
MPV	7.4		fL	5.0 / 10.0	EO#	0.09	10^3/µL	0.04 / 0.40	
					BA#	0.02	10^3/µL	0.02 / 0.10	







Microscopic Examination

Neutrophils	____	Metamyelocytes	____	Anisocytosis	____	Retics	____
Bands	____	Myelocytes	____	Hypochromia	____	Sed. Rate	____
Lymphocytes	____	Promyelocytes	____	Polychromasia	____		
Monocytes	____	Blast	____	Poikilocytosis	____		
Eosinophils	____	Atyp. Lymph	____	Microcytosis	____		
Basophils	____	NRBC s	____	Macrocytosis	____		

Comment: ____________

Requested by : ____________

Reviewed by : JBS 12-15-09 0535

Out of Action Range ▒▒ Out of Patient Range XXX

Printed 12/15/2009 08:36:37 AM

Dinnau 06
Post-[illegible]
12-18-09

REDFERN HEALTH CENTER
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4054

864-656-3566

Sample ID:	1218200906	Patient Name: 1218200906			
Run Date/Time:	12/18/2009 08:49:35 AM	Patient ID:	1218200906	Collect Date/Time:	12/18/2009 08:45:00 AM
Seq#:	847	Gender:	Unknown	DOB:	Physician:
OPR:	BCI			Age:	Location:
Flagging Set:	New Standard Ranges				
Comments:					

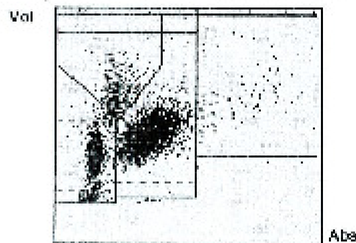
			Range		
WBC	4.8	10^3/µL	4.0	/	11.0
RBC	4.53	10^6/µL	3.80	/	6.50
HGB	13.9	g/dL	12.0	/	18.0
HCT	42.3	%	36.0	/	54.0
MCV	93	fL	76	/	98
MCH	30.6	pg	27.0	/	32.0
MCHC	32.8	g/dL	31.0	/	36.0
RDW	12.5	%	11.0	/	16.0
PLT	282	10^3/µL	150	/	400
MPV	7.9	fL	6.0	/	10.0

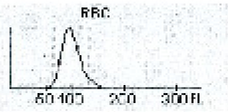
				Range		
NE	62.5	R	%	45.0	/	70.0
LY	28.5	R	%	20.0	/	40.0
MO	6.5	R	%	3.0	/	10.0
EO	2.4	R	%	1.0	/	5.0
BA	0.7	H	%	0.0	/	0.5
NE#	3.03	R	10^3/µL	2.00	/	7.50
LY#	1.36	RL	10^3/µL	1.50	/	4.00
MO#	0.29	R	10^3/µL	0.20	/	0.80
EO#	0.12	R	10^3/µL	0.04	/	0.40
BA#	0.03		10^3/µL	0.02	/	0.10

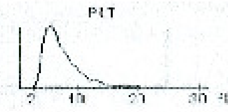
Flags and Messages

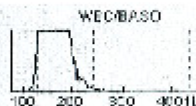
Diffplot and Histogram Flags
SL, SL1

Interpretive Messages
NRBC









Microscopic Examination

Neutrophils	______	Metamyelocytes	______	Anisocytosis	______	Retics	______
Bands	______	Myelocytes	______	Hypochromia	______	Sed. Rate	______
Lymphocytes	______	Promyelocytes	______	Polychromasia	______		
Monocytes	______	Blast	______	Poikilocytosis	______		
Eosinophils	______	Atyp. Lymph	______	Microcytosis	______		
Basophils	______	NRBC s	______	Macrocytosis	______		

Comment : ______________________

Requested by : ______________

Reviewed by : DAS 12-18-09 0850

Out of Active Range XXXX   Out of Patient Range XXX

Printed 12/18/2009 08:49:16 AM

Donor 07
Pre - [illegible]
1-8-10

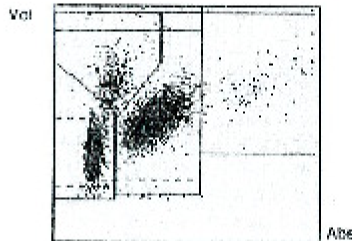
REDFERN HEALTH CENTER
CLEMSON UNIVERSITY

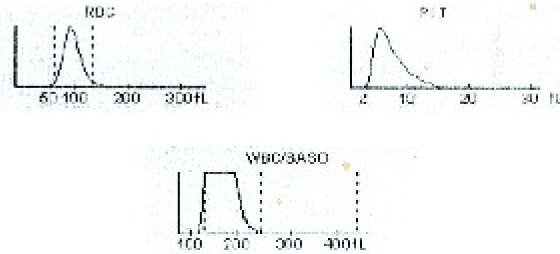
BOX 344054
CLEMSON SC 29634-4054

864-656-3566

Sample ID:	701082010	Patient Name:			
Run Date/Time:	01/09/2010 08:27:12 AM	Patient ID:		Collect Date/Time:	
Seq#:	812	Gender:	Unknown	DOB:	Physician:
OPR:	BCI			Age:	Location:
Flagging Set:	New Standard Ranges				
Comments:					

			Range					Range	Flags and Messages
WBC	6.2	10^3/µL	4.0 / 11.0	NE	54.5		%	45.0 / 70.0	
				LY	35.4		%	20.0 / 40.0	
RBC	4.57	10^6/µL	3.80 / 6.50	MO	7.1		%	3.0 / 10.0	
HGB	13.6	g/dL	12.0 / 18.0	EO	2.0		%	1.0 / 5.0	
HCT	40.9	%	36.0 / 54.0	BA	0.6	H	%	0.0 / 0.5	
MCV	89	fL	76 / 96						
MCH	29.8	pg	27.0 / 32.0						
MCHC	33.3	g/dL	31.0 / 35.0	NE#	3.39		10^3/µL	2.00 / 7.50	
RDW	12.2	%	11.0 / 16.0	LY#	2.19		10^3/µL	1.50 / 4.00	
				MO#	0.44		10^3/µL	0.20 / 0.80	
PLT	343	10^3/µL	150 / 400	EO#	0.12		10^3/µL	0.04 / 0.40	
MPV	7.6	fL	6.0 / 10.0	BA#	0.04		10^3/µL	0.02 / 0.10	





Microscopic Examination

Neutrophils		Metamyelocytes		Anisocytosis		Retics	
Bands		Myelocytes		Hypochromia		Sed. Rate	
Lymphocytes		Promyelocytes		Polychromasia			
Monocytes		Blast		Poikilocytosis			
Eosinophils		Atyp. Lymph		Microcytosis			
Basophils		NRBC s		Macrocytosis			

Comment : ____________________

Requested by : ____________________

Reviewed by : [illegible] 1-8-10

Donor 07
Post - [illegible]
1-11-10

REDFERN HEALTH CENTER
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4054

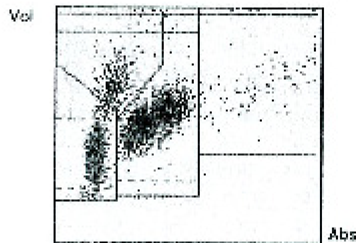
864-656-3566

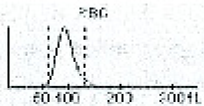
Sample ID:	0701112010	Patient Name:			
Run Date/Time:	01/11/2010 08:27:44 AM	Patient ID:		Collect Date/Time:	
Seq#:	940	Gender:	Unknown	DOB:	Physician:
OPR:	BCI			Age:	Location:
Flagging Set:	New Standard Ranges				
Comments:					

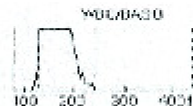
			Range
WBC	6.0	10^3/µL	4.0 / 11.0
RBC	4.56	10^6/µL	3.80 / 6.50
HGB	13.6	g/dL	12.0 / 18.0
HCT	40.8	%	35.0 / 54.0
MCV	89	fL	76 / 96
MCH	29.8	pg	27.0 / 32.0
MCHC	33.3	g/dL	31.0 / 35.0
RDW	12.4	%	11.0 / 16.0
PLT	345	10^3/µL	150 / 400
MPV	7.6	fL	5.0 / 10.0

			Range
NE	53.1	%	45.0 / 70.0
LY	35.8	%	20.0 / 40.0
MO	8.1	%	3.0 / 10.0
EO	2.5	%	1.0 / 5.0
BA	0.5	%	0.0 / 0.5
NE#	3.18	10^3/µL	2.00 / 7.50
LY#	2.15	10^3/µL	1.50 / 4.00
MO#	0.49	10^3/µL	0.20 / 0.80
EO#	0.15	10^3/µL	0.04 / 0.40
BA#	0.03	10^3/µL	0.02 / 0.10

Flags and Messages







Microscopic Examination

Neutrophils		Metamyelocytes		Anisocytosis		Retics	
Bands		Myelocytes		Hypochromia		Sed. Rate	
Lymphocytes		Promyelocytes		Polychromasia			
Monocytes		Blast		Poikilocytosis			
Eosinophils		Atyp. Lymph		Microcytosis			
Basophils		NRBC s		Macrocytosis			

Comment : ______________________

Requested by : ______________________

Reviewed by : EL B30 1-11-2010

Outside Action Range XX.X Outside Patient Range XX.X Printed On [illegible]

Donor 08
1/12/10
Pre-berry

REDFERN HEALTH CENTER
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4054

864-656-3566

Sample ID: 0112201008 Patient Name: 0112201008

Run Date/Time: 01/12/2010 08:47:36 AM Patient ID: 0112201008

Collect Date/Time: 01/12/2010 08:40:00 AM

Seq#: 891 Gender: Unknown DOB:

Physician:

OPR: BCI Age:

Location:

Flagging Set: New Standard Ranges

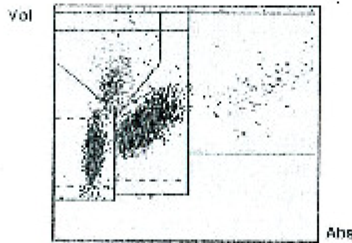
Comments:

				Range
WBC	5.0		10^3/µL	4.0 / 11.0
RBC	4.86		10^6/µL	3.60 / 6.50
HGB	12.2		g/dL	12.0 / 18.0
HCT	37.0		%	35.0 / 54.0
MCV	76		fL	78 / 98
MCH	25.1	L	pg	27.0 / 32.0
MCHC	33.0		g/dL	31.0 / 35.0
RDW	12.5		%	11.0 / 16.0
PLT	241		10^3/µL	150 / 400
MPV	7.8		fL	8.0 / 10.0

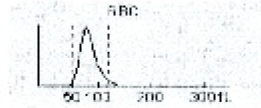
				Range
NE	50.0		%	45.0 / 70.0
LY	39.4		%	20.0 / 40.0
MO	7.4		%	3.0 / 10.0
EO	2.8		%	1.0 / 5.0
BA	0.4		%	0.0 / 0.5
NE#	2.49		10^3/µL	2.00 / 7.50
LY#	1.96		10^3/µL	1.50 / 4.00
MO#	0.37		10^3/µL	0.20 / 0.80
EO#	0.14		10^3/µL	0.04 / 0.40
BA#	0.02	L	10^3/µL	0.02 / 0.10

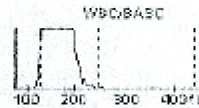
Flags and Messages

Vol



Abs





Microscopic Examination

Neutrophils	______	Metamyelocytes	______	Anisocytosis	______	Retics	______
Bands	______	Myelocytes	______	Hypochromia	______	Sed. Rate	______
Lymphocytes	______	Promyelocytes	______	Polychromasia	______		
Monocytes	______	Blast	______	Poikilocytosis	______		
Eosinophils	______	Atyp. Lymph	______	Microcytosis	______		
Basophils	______	NRBC s	______	Macrocytosis	______		

Comment : ______________________

Requested by : ______________

Reviewed by : TBS 1-12-10 0845

Out of Action Range: XXX Out of Patient Range: XXX Printed 01/12/2010 08:47 [illegible]

Donor 08
Post
1/15/10

REDFERN HEALTH CENTER
CLEMSON UNIVERSITY

BOX 344064
CLEMSON SC 29634-4064

864-656-3565

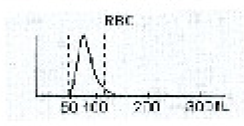
Sample ID: 001152010 Patient Name:
Run Date/Time: 01/15/2010 08:32:48 AM Patient ID:
Seq#: 73 Gender: Unknown DOB:
OPR: BCI Age:
Flagging Set: New Standard Ranges
Comments:

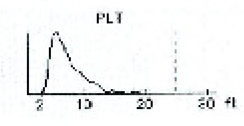
Collect Date/Time:
Physician:
Location:

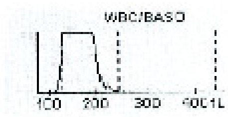
				Range
WBC	4.7		10^3/µL	4.0 / 11.0
RBC	4.78		10^6/µL	3.80 / 6.50
HGB	12.1		g/dL	12.0 / 18.0
HCT	36.5		%	35.0 / 54.0
MCV	76		fL	76 / 96
MCH	25.2	L	pg	27.0 / 32.0
MCHC	33.0		g/dL	31.0 / 35.0
RDW	12.6		%	11.0 / 16.0
PLT	221		10^3/µL	150 / 400
MPV	7.6		fL	6.0 / 10.0

				Range
NE	53.9		%	45.0 / 70.0
LY	38.7		%	20.0 / 40.0
MO	5.1		%	3.0 / 10.0
EO	2.0		%	1.0 / 5.0
BA	0.3		%	0.0 / 0.5
NE#	2.52		10^3/µL	2.00 / 7.50
LY#	1.81		10^3/µL	1.50 / 4.00
MO#	0.24		10^3/µL	0.20 / 0.80
EO#	0.09		10^3/µL	0.04 / 0.40
BA#	0.01	L	10^3/µL	0.02 / 0.10

Flags and Messages







Microscopic Examination

Neutrophils		Metamyelocytes		Anisocytosis		Retics	
Bands		Myelocytes		Hypochromia		Sed. Rate	
Lymphocytes		Promyelocytes		Polychromasia			
Monocytes		Blast		Poikilocytosis			
Eosinophils		Atyp. Lymph		Microcytosis			
Basophils		NRBC s		Macrocytosis			

Comment :

Requested by :
Reviewed by : [signature] 1-15-2010

Out of Action Range: XXXX Critical Patient Range: XXX

Printed 01/15/2010 08:32 AM

Donor 09
Pre
1/19/10

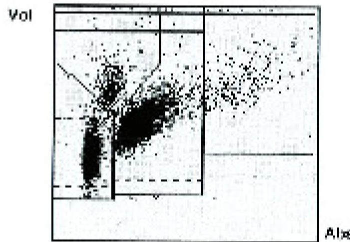
REDFERN HEALTH CENTER
CLEMSON UNIVERSITY

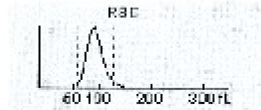
BOX 344054
CLEMSON SC 29634-4054

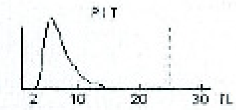
864-656-3566

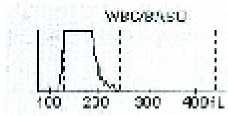
Sample ID:	09donor	Patient Name:	09donor		
Run Date/Time:	01/19/2010 08:34:50 AM	Patient ID:	09donor	Collect Date/Time:	01/19/2010 08:30:00 AM
Seq#:	105	Gender:	Unknown	DOB:	Physician:
OPR:	BCI			Age:	Location:
Flagging Set:	New Standard Ranges				
Comments:					

			Range				Range	Flags and Messages
WBC	6.9	10^3/µL	4.0 / 11.0	NE	49.1	%	45.0 / 70.0	
				LY	39.5	%	20.0 / 40.0	
RBC	4.99	10^6/µL	3.80 / 6.50	MO	6.2	%	3.0 / 10.0	
HGB	14.8	g/dL	12.0 / 18.0	EO	4.8	%	1.0 / 5.0	
HCT	43.4	%	36.0 / 54.0	BA	0.4	%	0.0 / 0.5	
MCV	87	fL	78 / 96					
MCH	29.7	pg	27.0 / 32.0					
MCHC	34.2	g/dL	31.0 / 35.0	NE#	3.40	10^3/µL	2.00 / 7.50	
RDW	12.0	%	11.0 / 16.0	LY#	2.74	10^3/µL	1.50 / 4.00	
				MO#	0.43	10^3/µL	0.20 / 0.80	
PLT	326	10^3/µL	150 / 400	EO#	0.33	10^3/µL	0.04 / 0.40	
MPV	7.2	fL	6.0 / 10.0	BA#	0.03	10^3/µL	0.02 / 0.10	









Microscopic Examination

Neutrophils	______	Metamyelocytes	______	Anisocytosis	______	Retics	______
Bands	______	Myelocytes	______	Hypochromia	______	Sed. Rate	______
Lymphocytes	______	Promyelocytes	______	Polychromasia	______		
Monocytes	______	Blast	______	Poikilocytosis	______		
Eosinophils	______	Atyp. Lymph	______	Microcytosis	______		
Basophils	______	NRBC s	______	Macrocytosis	______		

Comment : ______________________

Requested by :
Reviewed by : LBT 1-19-10 @ 8:35

Out of Action Range: Out of Patient Range: XX.X

Printed 01/19/2010 08:34:50 AM

Donor 09
Post
1/22/10

REDFERN HEALTH CENTER
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4054

864-656-3566

Sample ID: 0901222010 Patient Name:

Run Date/Time: 01/22/2010 09:16 12 AM Patient ID:

Collect Date/Time:

Seq#: 230 Gender: Unknown DOB:

Physician:

OPR: BCI Age:

Location:

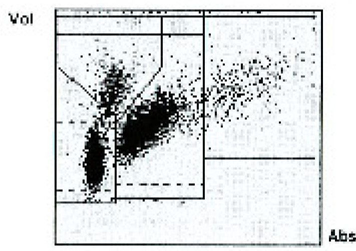
Flagging Set: New Standard Ranges

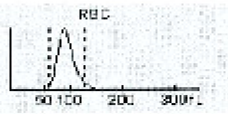
Comments:

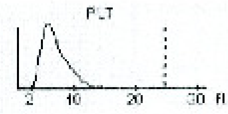
			Range
WBC	7.3	10^3/µL	4.0 / 11.0
RBC	4.85	10^6/µL	3.80 / 6.50
HGB	14.6	g/dL	12.0 / 18.0
HCT	42.2	%	35.0 / 54.0
MCV	87	fL	75 / 96
MCH	30.0	pg	27.0 / 32.0
MCHC	34.5	g/dL	31.0 / 35.0
RDW	11.5	%	11.0 / 16.0
PLT	297	10^3/µL	150 / 400
MPV	7.2	fL	6.0 / 10.0

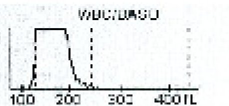
				Range
NE	46.4		%	45.0 / 70.0
LY	40.8	H	%	20.0 / 40.0
MO	5.9		%	3.0 / 10.0
EO	5.4	H	%	1.0 / 5.0
BA	0.6	H	%	0.0 / 0.5
NE#	3.37		10^3/µL	2.00 / 7.50
LY#	2.96		10^3/µL	1.50 / 4.00
MO#	0.49		10^3/µL	0.20 / 0.80
EO#	0.39		10^3/µL	0.04 / 0.40
BA#	0.04		10^3/µL	0.02 / 0.10

Flags and Messages









Microscopic Examination

Neutrophils		Metamyelocytes		Anisocytosis		Retics	
Bands		Myelocytes		Hypochromia		Sed. Rate	
Lymphocytes		Promyelocytes		Polychromasia			
Monocytes		Blast		Poikilocytosis			
Eosinophils		Atyp. Lymph		Microcytosis			
Basophils		NRBC s		Macrocytosis			

Comment :

Requested by :

Reviewed by : [signature] 1-22-10

Out of Action Range | Out of Patient Range XX.X

Printed 01/22/2010 09:17 AM

Donor 10
Pre-Burg
2-2-10

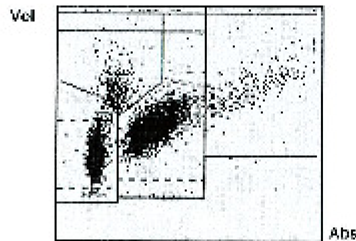
REDFERN HEALTH
CLEMSON UNIVERSITY

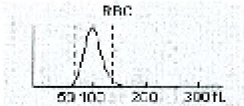
BOX 344054
CLEMSON SC 29634-4054

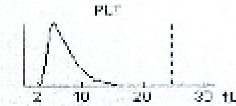
Sample ID: 0202201010 Patient Name: 0202201010
Run Date/Time: 02/02/2010 09:35:35 AM Patient ID: 0202201010
Collect Date/Time: 02/02/2010 09:35:00 AM
Seq#: 114 Gender: Unknown DOB:
Physician:
OPR: KC Age:
Location:
Flagging Set: Standard Range
Comments:

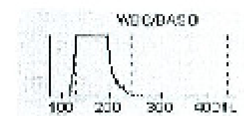
			Range					Range
WBC	6.4	10^3/µL	4.0 / 11.0	NE	49.9		%	45.0 / 70.0
				LY	38.7		%	20.0 / 40.0
RBC	4.87	10^6/µL	3.80 / 6.50	MO	5.9		%	3.0 / 10.0
HGB	15.2	g/dL	12.0 / 18.0	EO	4.8		%	1.0 / 5.0
HCT	45.2	%	36.0 / 54.0	BA	0.7	H	%	0.0 / 0.5
MCV	93	fL	75 / 96					
MCH	31.1	pg	27.0 / 32.0					
MCHC	33.6	g/dL	31.0 / 35.0	NE#	3.21		10^3/µL	2.00 / 7.50
RDW	12.1	%	11.0 / 16.0	LY#	2.49		10^3/µL	1.50 / 4.00
				MO#	0.38		10^3/µL	0.20 / 0.80
PLT	306	10^3/µL	150 / 400	EO#	0.31		10^3/µL	0.04 / 0.40
MPV	7.4	fL	5.0 / 10.0	BA#	0.05		10^3/µL	0.02 / 0.10

Flags and Messages









Microscopic Examination

Neutrophils		Metamyelocytes		Anisocytosis		Retics	
Bands		Myelocytes		Hypochromia		Sed. Rate	
Lymphocytes		Promyelocytes		Polychromasia			
Monocytes		Blast		Poikilocytosis			
Eosinophils		Atyp. Lymph		Microcytosis			
Basophils		NRBC s		Macrocytosis			

Comment :

Requested by :

Reviewed by : DBS 2-2-10 0935

Out of Action Range Out of Patient Range XXX

Printed 02/02/2010 09:35:45 AM

Donor 10
Post-
2-5-10

REDFERN HEALTH
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4054

Sample ID: 0205201010 **Patient Name: 0205201010**
Run Date/Time: 02/05/2010 08:43:04 AM Patient ID: 0205201010 Collect Date/Time: 02/05/2010 08:40:00 AM
Seq#: 236 Gender: Unknown DOB: Physician:
OPR: DCI Age: Location:
Flagging Set: Standard Range
Comments:

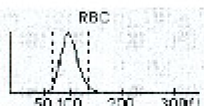
			Range
WBC	5.5	10^3/µL	4.0 / 11.0
RBC	4.90	10^6/µL	3.80 / 6.50
HGB	15.4	g/dL	12.0 / 18.0
HCT	45.7	%	35.0 / 54.0
MCV	93	fL	75 / 96
MCH	31.4	pg	27.0 / 32.0
MCHC	33.7	g/dL	31.0 / 36.0
RDW	12.0	%	11.0 / 16.0
PLT	300	10^3/µL	150 / 400
MPV	7.3	fL	6.0 / 10.0

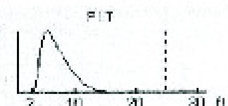
				Range
NE	50.6		%	45.0 / 70.0
LY	37.6		%	20.0 / 40.0
MO	6.0		%	3.0 / 10.0
EO	5.2	H	%	1.0 / 5.0
BA	0.6	H	%	0.0 / 0.5
NE#	2.83		10^3/µL	2.00 / 7.50
LY#	2.11		10^3/µL	1.50 / 4.00
MO#	0.34		10^3/µL	0.20 / 0.80
EO#	0.29		10^3/µL	0.04 / 0.40
BA#	0.03		10^3/µL	0.02 / 0.10

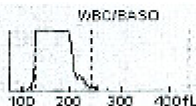
Flags and Messages

Vol

Abs







Microscopic Examination

Neutrophils	______	Metamyelocytes	______	Anisocytosis	______	Retics	______
Bands	______	Myelocytes	______	Hypochromia	______	Sed. Rate	______
Lymphocytes	______	Promyelocytes	______	Polychromasia	______		
Monocytes	______	Blast	______	Poikilocytosis	______		
Eosinophils	______	Atyp. Lymph	______	Microcytosis	______		
Basophils	______	NRBC s	______	Macrocytosis	______		

Comment: ______

Requested by: ______
Reviewed by: DSS 2-5-10 0845

Out of Action Range XXX Out of Patient Range XX.X

Printed 02/05/2010 08:43:12 AM

Donor 11
Pre
2-9-10

REDFERN HEALTH
CLEMSON UNIVERSITY

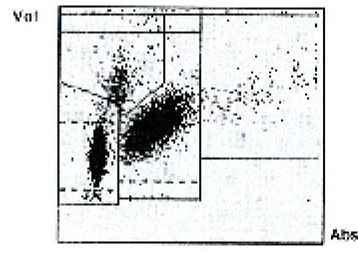
BOX 344054
CLEMSON SC 29634-4054

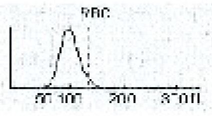
Sample ID: 0209201011 **Patient Name:**
Run Date/Time: 02/09/2010 08:21:56 AM Patient ID: Collect Date/Time:
Seq#: 329 Gender: Unknown DOB: Physician:
OPR: BCI Age: Location:
Flagging Set: Standard Range
Comments:

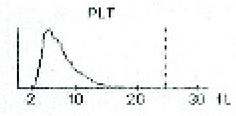
			Range
WBC	6.6	10^3/µL	4.0 / 11.0
RBC	4.25	10^6/µL	3.80 / 6.50
HGB	13.0	g/dl	12.0 / 18.0
HCT	38.8	%	36.0 / 54.0
MCV	91	fL	76 / 96
MCH	30.6	pg	27.0 / 32.0
MCHC	33.6	g/dL	31.0 / 35.0
RDW	13.5	%	11.0 / 16.0
PLT	213	10^3/µL	150 / 400
MPV	7.9	fL	6.0 / 10.0

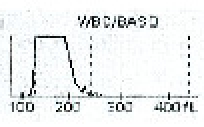
				Range
NE	62.4		%	45.0 / 70.0
LY	27.6		%	20.0 / 40.0
MO	6.2		%	3.0 / 10.0
EO	3.2		%	1.0 / 5.0
BA	0.6	H	%	0.0 / 0.5
NE#	4.09		10^3/µL	2.00 / 7.50
LY#	1.81		10^3/µL	1.50 / 4.00
MO#	0.41		10^3/µL	0.20 / 0.80
EO#	0.21		10^3/µL	0.04 / 0.40
BA#	0.04		10^3/µL	0.02 / 0.10

Flags and Messages









Microscopic Examination

Neutrophils		Metamyelocytes		Anisocytosis		Retics	
Bands		Myelocytes		Hypochromia		Sed. Rate	
Lymphocytes		Promyelocytes		Polychromasia			
Monocytes		Blast		Poikilocytosis			
Eosinophils		Atyp. Lymph		Microcytosis			
Basophils		NRBC s		Macrocytosis			

Comment :

Requested by :

Reviewed by : GW BZ9 2-9-10

Out of Action Range Out of Patient Range XX.X

Printed 02/09/2010

Donor 11
Post
2-12-10

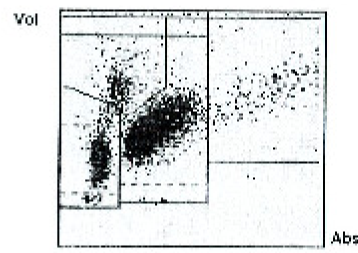
REDFERN HEALTH
CLEMSON UNIVERSITY

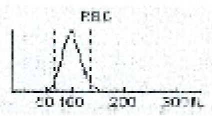
BOX 344054
CLEMSON SC 29634-4054

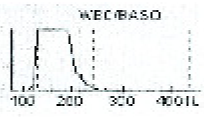
Sample ID: 0212201011 Patient Name:
Run Date/Time: 02/12/2010 08:26:49 AM Patient ID: Collect Date/Time:
Seq#: 455 Gender: Unknown DOB: Physician:
OPR: BCI Age: Location:
Flagging Set: Standard Range
Comments:

			Range					Range
WBC	7.3	10^3/µL	4.0 / 11.0	NE	59.5		%	45.0 / 70.0
				LY	30.0		%	20.0 / 40.0
RBC	4.28	10^6/µL	3.80 / 6.50	MO	5.8		%	3.0 / 10.0
HGB	13.3	g/dL	12.0 / 16.0	EO	4.1		%	1.0 / 5.0
HCT	39.6	%	35.0 / 54.0	BA	0.6	H	%	0.0 / 0.5
MCV	92	fL	76 / 99					
MCH	31.2	pg	27.0 / 32.0					
MCHC	33.8	g/dL	31.0 / 35.0	NE#	4.36		10^3/µL	2.00 / 7.50
RDW	13.2	%	11.0 / 16.0	LY#	2.20		10^3/µL	1.50 / 4.00
				MO#	0.42		10^3/µL	0.20 / 0.80
PLT	226	10^3/µL	150 / 400	EO#	0.30		10^3/µL	0.04 / 0.40
MPV	7.8	fL	6.0 / 10.0	BA#	0.04		10^3/µL	0.02 / 0.10

Flags and Messages







Microscopic Examination

Neutrophils	____	Metamyelocytes	____	Anisocytosis	____	Retics	____
Bands	____	Myelocytes	____	Hypochromia	____	Sed. Rate	____
Lymphocytes	____	Promyelocytes	____	Polychromasia	____		
Monocytes	____	Blast	____	Poikilocytosis	____		
Eosinophils	____	Atyp. Lymph	____	Microcytosis	____		
Basophils	____	NRBC s	____	Macrocytosis	____		

Comment : ____________________

Requested by : ____________________

Reviewed by : GLW B3g 2-12-10

Out of Action Range XXX Out of Patient Range XX.X Printed 02/12/2010 08:28:56 AM

Donor 12
Pre
2-9-10

REDFERN HEALTH
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4054

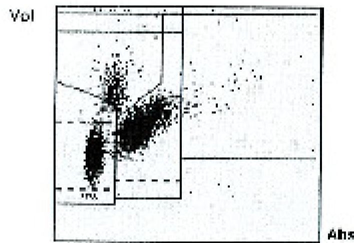
Sample ID: 0209201012 Patient Name: 0209201012
Run Date/Time: 02/09/2010 08.35.33 AM Patient ID: 0209201012 Collect Date/Time: 02/09/2010 08.35.00 AM
Seq#: 330 Gender: Unknown DOB: Physician:
OPR: BCI Age: Location:
Flagging Set: Standard Range
Comments:

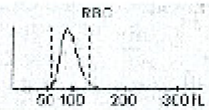
			Range	
WBC	4.5	10^3/µL	4.0	/ 11.0
RBC	4.22	10^6/µL	3.80	/ 6.50
HGB	12.3	g/dL	12.0	/ 18.0
HCT	37.[illegible]	%	36.0	/ 54.0
MCV	88	fL	76	/ 95
MCH	29.0	pg	27.0	/ 32.0
MCHC	33.1	g/dL	31.0	/ 35.0
RDW	12.6	%	11.0	/ 16.0
PLT	261	10^3/µL	150	/ 400
MPV	7.6	fL	6.0	/ 10.0

				Range	
NE	50.1		%	45.0	/ 70.0
LY	39.8		%	20.0	/ 40.0
MO	8.1		%	3.0	/ 10.0
EO	1.7		%	1.0	/ 5.0
BA	0.3		%	0.0	/ 0.5
NE#	2.24		10^3/µL	2.00	/ 7.50
LY#	1.78		10^3/µL	1.50	/ 4.00
MO#	0.36		10^3/µL	0.20	/ 0.90
EO#	0.08		10^3/µL	0.04	/ 0.40
BA#	0.01	L	10^3/µL	0.02	/ 0.10

Flags and Messages

✓





Microscopic Examination

Neutrophils	______	Metamyelocytes	______	Anisocytosis	______	Relics	______
Bands	______	Myelocytes	______	Hypochromia	______	Sed. Rate	______
Lymphocytes	______	Promyelocytes	______	Polychromasia	______		
Monocytes	______	Blast	______	Poikilocytosis	______		
Eosinophils	______	Atyp. Lymph	______	Microcytosis	______		
Basophils	______	NRBC s	______	Macrocytosis	______		

Comment: ______________________

Requested by: ______________

Reviewed by: FDS 2-9-10 0840

Out of Action Range XX.X Out of Patient Range XX.X

Printed 02/09/2010 [illegible]

Donor 12
Post
2-12-10

REDFERN HEALTH
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4054

Sample ID: 0212201012 **Patient Name:** 0212201012

Run Date/Time: 02/12/2010 10:55:13 AM Patient ID: 0212201012 Collect Date/Time: 02/12/2010 10:50:00 AM

Seq#: 456 Gender: Unknown DOB: Physician:

OPR: BCI Age: Location:

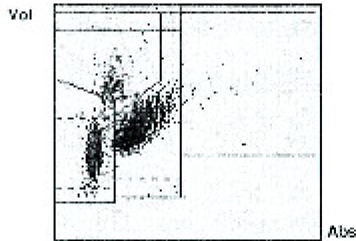
Flagging Set: Standard Range

Comments:

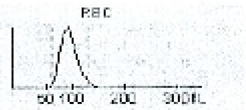
				Range						Range
WBC	3.5	L	10^3/µL	4.0 / 11.0	NE	49.2		%		45.0 / 70.0
					LY	42.7	H	%		20.0 / 40.0
RBC	4.45		10^6/µL	3.00 / 6.50	MO	6.8		%		3.0 / 10.0
HGB	13.1		g/dL	12.0 / 18.0	EO	1.0		%		1.0 / 5.0
HCT	38.8		%	36.0 / 54.0	BA	0.3		%		0.0 / 0.5
MCV	87		fL	76 / 95						
MCH	29.5		pg	27.0 / 32.0						
MCHC	33.9		g/dL	31.0 / 35.0	NE#	1.73	L	10^3/µL		2.00 / 7.50
RDW	12.5		%	11.0 / 16.0	LY#	1.50		10^3/µL		1.00 / 4.00
					MO#	0.24		10^3/µL		0.20 / 0.80
PLT	277		10^3/µL	150 / 400	EO#	0.04	L	10^3/µL		0.04 / 0.40
MPV	7.7		fL	6.0 / 10.0	BA#	0.01	L	10^3/µL		0.02 / 0.10

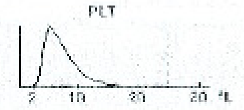
Flags and Messages

Vol



Abs





Microscopic Examination

Neutrophils	______	Metamyelocytes	______	Anisocytosis	______	Retics	______
Bands	______	Myelocytes	______	Hypochromia	______	Sed. Rate	______
Lymphocytes	______	Promyelocytes	______	Polychromasia	______		
Monocytes	______	Blast	______	Poikilocytosis	______		
Eosinophils	______	Atyp. Lymph	______	Microcytosis	______		
Basophils	______	NRBC s	______	Macrocytosis	______		

Comment: ______________________ Requested by: ______________

Reviewed by: JBS 2-12-10 1055

Out of Action Range XXXX Out of Patient Range XX.X

Donor 13
Pre
2/19/10

REDFERN HEALTH
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4054

Sample ID: 0219201014 **Patient Name: 0219201014**

Run Date/Time: 02/19/2010 09:06:13 AM Patient ID: 0219201014 Collect Date/Time: 02/19/2010 09:05:00 AM

Seq#: 666 Gender: Unknown DOB: Physician:

OPR: BC Age: Location:

Flagging Set: Standard Range

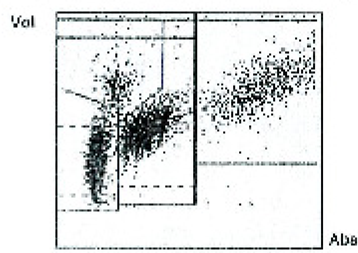
Comments:

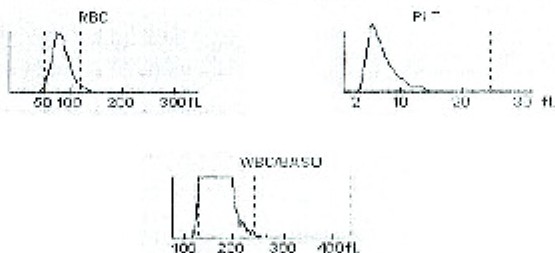
				Range
WBC	6.9		10^3/µL	4.0 / 11.0
RBC	4.50		10^6/µL	3.80 / 6.50
HGB	11.8	L	g/dL	12.0 / 18.0
HCT	35.2		%	35.0 / 54.0
MCV	79		fL	76 / 96
MCH	25.8	L	pg	27.0 / 32.0
MCHC	32.3		g/dL	31.0 / 35.0
RDW	14.0		%	11.0 / 16.0
PLT	312		10^3/µL	150 / 400
MPV	7.2		fL	6.0 / 10.0

				Range
NE	[illegible]	L	%	40.0 / 75.0
LY	[illegible]	H	%	20.0 / 40.0
MO	4.8		%	3.0 / 10.0
EO	[illegible]	H	%	1.0 / 5.0
BA	0.8	H	%	0.0 / 0.5
NE#	2.09		10^3/µL	2.00 / 7.50
LY#	3.40		10^3/µL	1.50 / 4.00
MO#	0.33		10^3/µL	0.20 / 0.80
EO#	0.32	H	10^3/µL	0.04 / 0.30
BA#	0.05		10^3/µL	0.02 / 0.10

Flags and Messages

Interpretive Messages
Lymphocytosis
Neutropenia
Eosinophilia





Microscopic Examination

Neutrophils	______	Metamyelocytes	______	Anisocytosis	______	Retics	______
Bands	______	Myelocytes	______	Hypochromia	______	Sed. Rate	______
Lymphocytes	______	Promyelocytes	______	Polychromasia	______		
Monocytes	______	Blast	______	Poikilocytosis	______		
Eosinophils	______	Atyp. Lymph	______	Microcytosis	______		
Basophils	______	NRBC s	______	Macrocytosis	______		

Comment : ______

Requested by : ______

Reviewed by : DBS 2-19-10 0910

Out of Action Range XX.X Out of Patient Range XX.X

Donor 13
Post
2-22-10

REDFERN HEALTH
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4054

Sample ID: 02222010~~14~~ 13 **Patient Name:** 0222201014

Run Date/Time: 02/22/2010 09:36:40 AM Patient ID: 0222201014 Collect Date/Time: 02/22/2010 09:35:00 AM

Seq#: 707 Gender: Unknown DOB: Physician:

OPR: BCI Age: Location:

Flagging Set: Standard Range

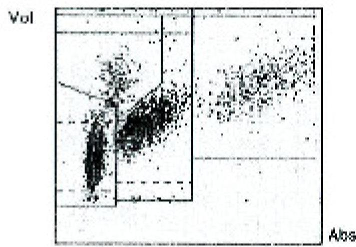
Comments:

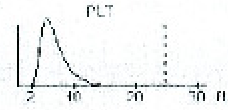
				Range
WBC	6.3		10^3/µL	4.0 / 11.0
RBC	4.79		10^6/µL	3.80 / 6.50
HGB	12.5		g/dL	12.0 / 18.0
HCT	38.1		%	36.0 / 54.0
MCV	80		fL	76 / 96
MCH	26.2	L	pg	27.0 / 32.0
MCHC	32.8		g/dL	31.0 / 35.0
RDW	10.8		%	11.0 / 16.0
PLT	231		10^3/µL	150 / 400
MPV	6.9		fL	6.0 / 10.0

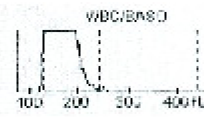
				Range
NE	[illegible]	[illegible]	%	[illegible]
LY	[illegible]	H	%	[illegible]
MO	4.5		%	3.0 / 10.0
EO	[illegible]	H	%	[illegible]
BA	0.8	H	%	0.0 / 0.5
NE#	2.21		10^3/µL	2.00 / 7.50
LY#	3.31		10^3/µL	1.50 / 4.00
MO#	0.28		10^3/µL	0.20 / 0.80
EO#	[illegible]	H	[illegible]	[illegible]
BA#	0.04		10^3/µL	0.02 / 0.10

Flags and Messages

<u>Interpretive Messages</u>
Lymphocytosis
Neutropenia
Eosinophilia







<u>Microscopic Examination</u>

Neutrophils		Metamyelocytes		Anisocytosis		Retics	
Bands		Myelocytes		Hypochromia		Sed. Rate	
Lymphocytes		Promyelocytes		Polychromasia			
Monocytes		Blast		Poikilocytosis			
Eosinophils		Atyp. Lymph		Microcytosis			
Basophils		NRBC s		Macrocytosis			

Comment : __________________

Requested by : ____________

Reviewed by : TBC 2-22-10 0935

Out of Action Range XXXX Out of Patient Range XXX Printed 02/22/2010 09:40:49 AM

Lorenn 14
Pre
3/9/10

REDFERN HEALTH
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4054

Sample ID: 14 Patient Name: 14
Run Date/Time: 03/09/2010 08:33:25 AM Patient ID: 14 Collect Date/Time: 03/09/2010 08:30:00 AM
Seq#: 1244 Gender: Unknown DOB: Physician:
OPR: BCI Age: Location:
Flagging Set: Standard Range
Comments:

			Range
WBC	4.5	10^3/µL	4.0 / 11.0
RBC	4.13	10^6/µL	3.80 / 5.50
HGB	12.6	g/dL	12.0 / 18.0
HCT	37.9	%	35.0 / 54.0
MCV	92	fL	76 / 96
MCH	30.5	pg	27.0 / 32.0
MCHC	33.2	g/dL	31.0 / 35.0
RDW	11.7	%	11.0 / 16.0
PLT	245	10^3/µL	150 / 400
MPV	7.9	fL	6.0 / 10.0

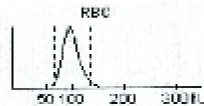
				Range
NE	39.6	L	%	45.0 / 70.0
LY	51.8	HH	%	20.0 / 40.0
MO	5.7		%	3.0 / 10.0
EO	2.0		%	1.0 / 5.0
BA	0.6	H	%	0.0 / 0.5
NE#	1.91	L	10^3/µL	2.00 / 7.50
LY#	2.61		10^3/µL	1.50 / 4.00
MO#	0.23		10^3/µL	0.20 / 0.90
EO#	0.10		10^3/µL	0.04 / 0.40
BA#	0.04		10^3/µL	0.02 / 0.10

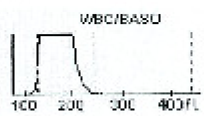
Flags and Messages

Interpretive Messages
Lymphocytosis
Neutropenia

Vol

Abs





Microscopic Examination

Neutrophils		Metamyelocytes		Anisocytosis		Retics	
Bands		Myelocytes		Hypochromia		Sed. Rate	
Lymphocytes		Promyelocytes		Polychromasia			
Monocytes		Blast		Poikilocytosis			
Eosinophils		Atyp. Lymph		Microcytosis			
Basophils		NRBC s		Macrocytosis			

Comment :

Requested by :

Reviewed by : 3-9-10 @ 835

Out of Action Range XX.X Out of Patient Range XX.X

Printed 03/09/2010 08:33:41 AM

Roman 14
Post
3-12-10

REDFERN HEALTH
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4054

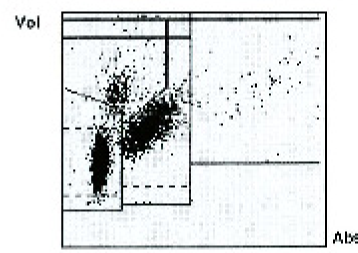
Sample ID: 0312201014 **Patient Name:** 0312201014
Run Date/Time: 03/12/2010 08:58:04 AM **Patient ID:** 0312201014 **Collect Date/Time:** 03/12/2010 08:55:00 AM
Seq#: 1361 **Gender:** Unknown **DOB:** **Physician:**
OPR: BC **Age:** **Location:**
Flagging Set: Standard Range
Comments:

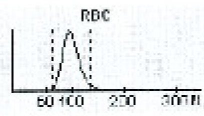
			Range
WBC	5.1	10^3/µL	4.0 / 11.0
RBC	4.25	10^6/µL	3.80 / 6.50
HGB	13.0	g/dL	12.0 / 18.0
HCT	38.7	%	36.0 / 54.0
MCV	91	fL	78 / 98
MCH	30.5	pg	27.0 / 32.0
MCHC	33.5	g/dL	31.0 / 35.0
RDW	11.6	%	11.0 / 16.0
PLT	258	10^3/µL	150 / 400
MPV	8.1	fL	6.0 / 10.0

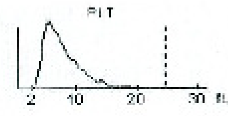
				Range
NE	40.7	L	%	45.0 / 70.0
LY	50.6	HH	%	20.0 / 40.0
MO	6.5		%	3.0 / 10.0
EO	1.7		%	1.0 / 5.0
BA	0.5		%	0.0 / 0.5
NE#	2.06		10^3/µL	2.00 / 7.50
LY#	2.58		10^3/µL	1.50 / 4.00
MO#	0.33		10^3/µL	0.20 / 0.80
EO#	0.09		10^3/µL	0.04 / 0.40
BA#	0.03		10^3/µL	0.02 / 0.10

Flags and Messages

Interpretive Messages
Lymphocytosis







Microscopic Examination

Neutrophils		Metamyelocytes		Anisocytosis		Retics	
Bands		Myelocytes		Hypochromia		Sed. Rate	
Lymphocytes		Promyelocytes		Polychromasia			
Monocytes		Blast		Poikilocytosis			
Eosinophils		Atyp. Lymph		Microcytosis			
Basophils		NRBC s		Macrocytosis			

Comment : ____________

Requested by : ____________

Reviewed by : DDS 3-12-10 9a

Out of Action Range XXX Out of Patient Range XXX

Printed 03/12/2010 08:59:41 AM

Dover 15
Pre-Berry
3/26/10

REDFERN HEALTH
CLEMSON UNIVERSITY

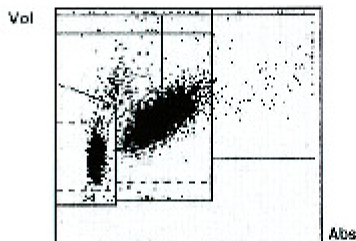
BOX 344054
CLEMSON SC 29634-4054

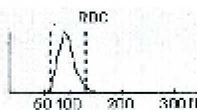
Sample ID: 0326201015   Patient Name:
Run Date/Time: 03/26/2010 12:32:14 PM   Patient ID: Collect Date/Time:
Seq#: 1624   Gender: Unknown   DOB: Physician:
OPR: BCI   Age: Location:
Flagging Set: Standard Range
Comments:

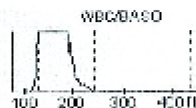
			Range
WBC	8.0	10^3/µL	4.0 / 11.0
RBC	4.39	10^6/µL	3.80 / 6.50
HGB	13.2	g/dL	12.0 / 18.0
HCT	39.5	%	36.0 / 54.0
MCV	90	fL	76 / 96
MCH	30.1	pg	27.0 / 32.0
MCHC	33.5	g/dL	31.0 / 35.0
RDW	12.0	%	11.0 / 16.0
PLT	306	10^3/µL	150 / 400
MPV	7.4	fL	5.0 / 10.0

				Range
NE	72.5	H	%	45.0 / 70.0
LY	21.9		%	20.0 / 40.0
MO	3.4		%	3.0 / 10.0
EO	1.7		%	1.0 / 5.0
BA	0.5		%	0.0 / 0.5
NE#	5.83		10^3/µL	2.00 / 7.50
LY#	1.76		10^3/µL	1.50 / 4.00
MO#	0.27		10^3/µL	0.20 / 0.80
EO#	0.14		10^3/µL	0.04 / 0.40
BA#	0.04		10^3/µL	0.02 / 0.10

Flags and Messages







Microscopic Examination

Neutrophils		Metamyelocytes		Anisocytosis		Retics	
Bands		Myelocytes		Hypochromia		Sed. Rate	
Lymphocytes		Promyelocytes		Polychromasia			
Monocytes		Blast		Poikilocytosis			
Eosinophils		Atyp. Lymph		Microcytosis			
Basophils		NRBC s		Macrocytosis			

Comment :

Requested by :
Reviewed by : GW-1235 3-26-10

Out of Action Range XXXXX   Out of Patient Range XXXX

Printed 03/26/2010 12:32:14 PM

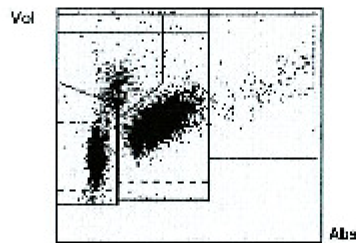
Donor 15
Post-berry
3/29/10
Research

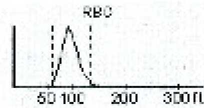
REDFERN HEALTH
CLEMSON UNIVERSITY

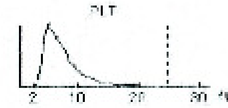
BOX 344054
CLEMSON SC 29634-4054

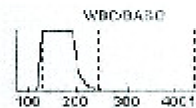
Sample ID: 0329201014 Patient Name: 0329201014
Run Date/Time: 03/29/2010 04:37:01 PM Patient ID: 0329201014 Collect Date/Time: 03/29/2010 04:30:00 PM
Seq#: 1667 Gender: Unknown DOB: Physician:
OPR: BCI Age: Location:
Flagging Set: Standard Range
Comments:

				Range						Range	Flags and Messages
WBC	8.3		10^3/µL	4.0 / 11.0	NE	51.6		%	45.0 / 70.0	
					LY	39.8		%	20.0 / 40.0	
RBC	4.02		10^6/µL	3.80 / 6.50	MO	5.7		%	3.0 / 10.0	
HGB	12.3		g/dL	12.0 / 18.0	EO	2.5		%	1.0 / 5.0	
HCT	35.8	L	%	36.0 / 54.0	BA	0.6	H	%	0.0 / 0.5	
MCV	89		fL	76 / 96						
MCH	30.6		pg	27.0 / 32.0						
MCHC	34.3		g/dL	31.0 / 35.0	NE#	4.30		10^3/µL	2.00 / 7.50	
RDW	12.0		%	11.0 / 16.0	LY#	3.30		10^3/µL	1.50 / 4.00	
					MO#	0.49		10^3/µL	0.20 / 0.80	
PLT	279		10^3/µL	150 / 400	EO#	0.21		10^3/µL	0.04 / 0.40	
MPV	7.7		fL	6.0 / 10.0	BA#	0.05		10^3/µL	0.02 / 0.10	









Microscopic Examination

Neutrophils	______	Metamyelocytes	______	Anisocytosis	______	Retics	______
Bands	______	Myelocytes	______	Hypochromia	______	Sed. Rate	______
Lymphocytes	______	Promyelocytes	______	Polychromasia	______		
Monocytes	______	Blast	______	Poikilocytosis	______		
Eosinophils	______	Atyp. Lymph	______	Microcytosis	______		
Basophils	______	NRBC s	______	Macrocytosis	______		

Comment : ______________________

Requested by : ______
Reviewed by : PSS 3-29-10 ·/BS

Out of Action Range XXXX Out of Patient Range XXX

Donor 16
Pre-berry
4-2-10

REDFERN HEALTH
CLEMSON UNIVERSITY

BOX 344054
CLEMSON SC 29634-4054

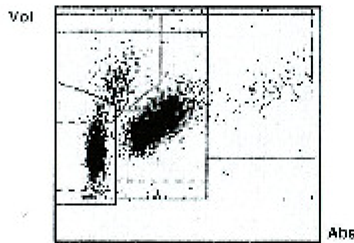
Sample ID: 0006000450 Patient Name: [redacted]
Run Date/Time: 04/02/2010 08:47:44 AM Patient ID:
Seq#: 1792 Gender: Unknown DOB:
OPR: BCI Age:
Flagging Set: Standard Range
Comments:

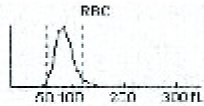
Collect Date/Time: 04/02/2010 08:40:30 AM
Physician:
Location:

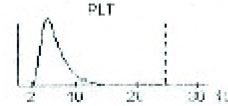
				Range
WBC	5.7		10^3/µL	4.0 / 11.0
RBC	4.71		10^6/µL	3.80 / 6.00
HGB	12.6		g/dL	12.0 / 18.0
HCT	38.6		%	36.0 / 54.0
MCV	82		fL	76 / 98
MCH	26.8	L	pg	27.0 / 32.0
MCHC	32.7		g/dL	31.0 / 35.0
RDW	13.7		%	11.0 / 16.0
PLT	329		10^3/µL	150 / 400
MPV	5.8		fL	6.0 / 10.0

			Range
NE	48.7	%	45.0 / 70.0
LY	39.9	%	20.0 / 40.0
MO	6.6	%	3.0 / 10.0
EO	4.3	%	1.0 / 5.0
BA	0.5	%	0.0 / 0.5
NE#	2.76	10^3/µL	2.00 / 7.50
LY#	2.26	10^3/µL	1.00 / 4.00
MO#	0.37	10^3/µL	0.20 / 0.80
EO#	0.24	10^3/µL	0.04 / 0.40
BA#	0.03	10^3/µL	0.02 / 0.10

Flags and Messages







Microscopic Examination

Neutrophils	____	Metamyelocytes	____	Anisocytosis	____	Retics	____
Bands	____	Myelocytes	____	Hypochromia	____	Sed. Rate	____
Lymphocytes	____	Promyelocytes	____	Polychromasia	____		
Monocytes	____	Blast	____	Poikilocytosis	____		
Eosinophils	____	Atyp. Lymph	____	Microcytosis	____		
Basophils	____	NRBC s	____	Macrocytosis	____		

Comment : ____________

Requested by : ____________
Reviewed by : ____________

Out of Action Range Out of Patient Range XX.X

Donor 16
Post- Berry
4-5-10

REDFERN HEALTH
CLEMSON UNIVERSITY

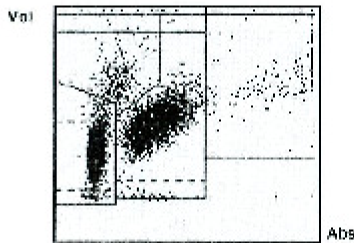
BOX 344054
CLEMSON SC 29634-4054

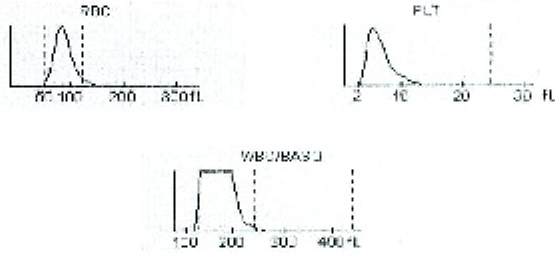
Sample ID: DONOR16 Patient Name:
Run Date/Time: 04/05/2010 02:05:43 PM Patient ID: DONOR16 Collect Date/Time: 04/05/2010 02:00:00 PM
Seq#: 1364 Gender: Unknown DOB: Physician:
OPR: BCI Age: Location:
Flagging Set: Standard Range
Comments:

			Range	
WBC	6.5	*	10^3/µL	4.0 / 11.0
RBC	4.69		10^6/µL	3.80 / 6.50
HGB	12.7		g/dL	12.0 / 18.0
HCT	38.3		%	36.0 / 54.0
MCV	82		fL	76 / 96
MCH	27.2		pg	27.0 / 32.0
MCHC	33.2		g/dL	31.0 / 35.0
RDW	13.5		%	11.0 / 16.0
PLT	335		10^3/µL	150 / 400
MPV	6.8		fL	6.0 / 10.0

				Range
NE	48.0	*	%	45.0 / 70.0
LY	41.9	*H	%	20.0 / 40.0
MO	5.6	*	%	3.0 / 10.0
EO	3.7	*	%	1.0 / 5.0
BA	0.8	*H	%	0.0 / 0.5
NE#	3.13	*	10^3/µL	2.00 / 7.50
LY#	2.73	*	10^3/µL	1.50 / 4.00
MO#	0.37	*	10^3/µL	0.20 / 0.80
EO#	0.24	*	10^3/µL	0.04 / 0.40
BA#	0.05	*	10^3/µL	0.02 / 0.10

Suspect and Messages
WBC
WBC/BA DIFF





Microscopic Examination

Neutrophils		Metamyelocytes		Anisocytosis		Retics	
Bands		Myelocytes		Hypochromia		Sed. Rate	
Lymphocytes		Promyelocytes		Polychromasia			
Monocytes		Blast		Poikilocytosis			
Eosinophils		Atyp. Lymph		Microcytosis			
Basophils		NRBC s		Macrocytosis			

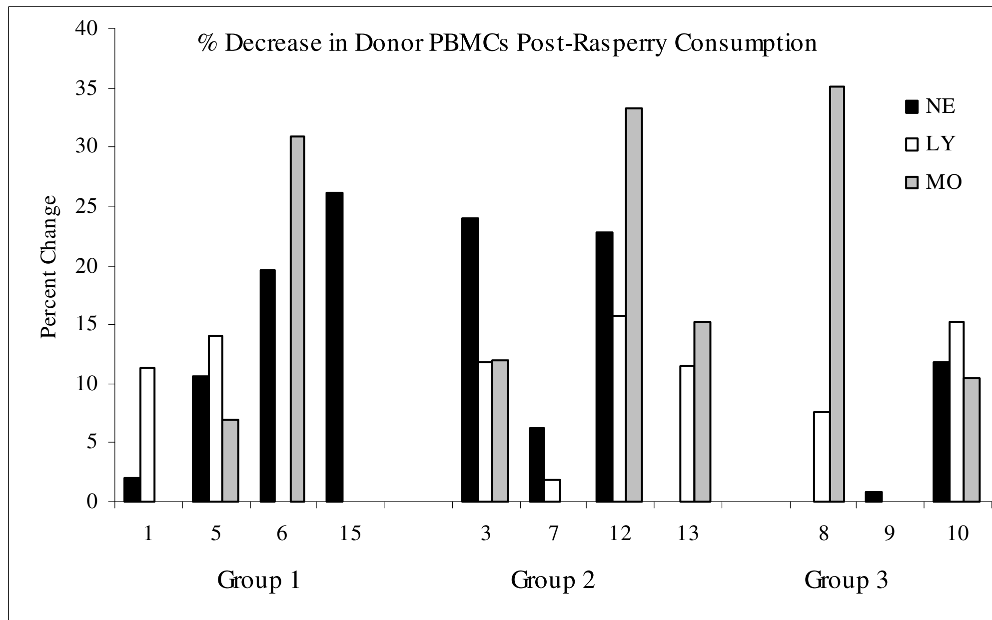
Comment :

Requested by
Reviewed by JST 4-5-10 @ 2:05

Out of Action Range XXXX Out of Patient Range XX.X

Printed 04/05/2010 02:04:17 PM

C. Percent Decrease in Donor PBMCs Post-Raspberry Consumption



Group 1, 2, and 3 donors who decreased in subpopulations of WBCs after raspberry intake.

LITERATURE CITED

1. World Health Organization (2003). Diet, Nutrition and the Prevention of Diseases. WHO Technical Report Series 916.

2. World Health Organization (2010). Global Strategy on Diet, Physical Activity and Health. http://www.who.int

3. Nichenametla SN, Taruscio TG (2006). A Review of the Effects and Mechanisms of Polyphenolics in Cancer. *Food Science and Nutrition*. **46**, 161 – 183.

4. Holderness J, Hedges J, Daughenbaugh K, Kimmel E, Graff J, Freedman B, Jutila M (2008). Response of γδ T Cells to Plant-derived Tannins. *Critical Review in Immunology*. **28(5)**, 377 – 402.

5. Magrone T, Candore G, Caruso C, Jirillo E, Covelli V (2008). Polyphenols from Red Wine Modulate Immune Responsiveness: Biological and Clinical Significance. *Current Pharmaceutical Design*. **14**, 2733 – 2748.

6. Tuñon MJ, Garcia-Mediavilla MV, Sánchez-Campos S, González-Gallego J (2009). Potential of Flavonoids as Anti-inflammatory Agents: Modulation of Pro-inflammatory Gene Expression and Signal Transduction Pathways. *Current Drug Metabolism*. **10**, 256 – 271.

7. Mullen W, McGinn J, Lean ME, MacLean MR, Gardner P, duthie GG, Yokota T, Crozier A (2002). Ellagitannins, Flavonoids, and Other Phenolics in Red Raspberries and Their Contribution to Antioxidant Capacity and Vasorelaxation Properties. *Journal of Agricultural and Food Chemistry*. **50**, 5191 – 5196.

8. Seeram NP (2008). Berry Fruits: Compositional Elements, Biochemical Activities, and the Impact of their Intake on Human Health, Performance, and Disease. *Journal of Agricultural and Food Chemistry*. **56**, 627 – 629.

9. Beekwilder J, Hall RD, Ric de Vos CH (2005). Identification and Dietary Relevance of Antioxidants from Raspberry. *BioFactors*. **23**, 197 – 205.

10. Stoner GD, Wang LS, Casto BC (2008). Laboratory and Clinical Studies of Cancer Chemoprevention by Antioxidants in Berries. *Carcinogenesis*. **29**, 1665 – 1674.

11. Nijveldt RJ, Van Nood E, Van Hoorn DE, Boelens PG, Van Norren K, Van Leeuwen PA (2001). Flavonoids: a Review of Probable Mechanisms of Action and Potential Applications. *American Journal of Clinical Nutrition*. **74(4)**, 418 – 425.

12. Hodek P, Trefil P, Stiborová (2002). Flavonoids-Potent and Versatile Biologically Active Compounds Interacting with Cytochromes P450. *Chemico-Biological Interactions*. **139**, 1 – 21.

13. Van Acker SA, Tromp MN, Haenen GR, Van der Vijgh WJ, Bast A (1995). Flavonoids as Scavengers of Nitric Oxide Radical. *Biochemical and Biophysical Research Communications*. **214**, 755 – 759.

14. García-Lafuente A, Guillamón E, Villares A, Rostagno M, Martínez J (2009). Flavonoids as Anti-inflammatory Agents: Implications in Cancer and Cardiovascular Disease. *Inflammatory Research*. **58**, 537 – 552.

15. Liu M, Li XQ, Weber C, Lee CY, Brown J, Liu RH (2002). Antioxidant and Antiproliferative Activities of Raspberries. *Journal of Agricultural and Food Chemistry*. **50**, 2926 – 2930.

16. Olsson ME, Gustavsson KE, Andersson S, Nilsson A, Duan RD (2004). Inhibition of Cancer Cell Proliferation *in vitro* by Fruit and Berry Extracts and Correlations with Antioxidant Levels. *Journal of Agricultural and Food Chemistry*.52, 7264 – 7271.

17. McDougall GJ, Ross HA, Ikejk M, Stewart D (2008). Berry Extracts Exert Different Antiproliferative Effects Against Cervical and Colon Cancer Cells Grown *in vitro*. *Journal of Agricultural and Food Chemistry*. **56**, 3016 – 3023.

18. God J. “Red Raspberries Possess Anticarcinogenic and Immunostimulatory Potential.” Diss. Clemson University, 2009.

19. Seeram NP, Adams LS, Zhang Y, Lee R, Sand D, Scheuller HS, Heber D (2006). Blackberry, Black Raspberry, Blueberry, Cranberry, Red Raspberry, and Strawberry Extracts Inhibit Growth and Stimulate Apoptosis of Human Cancer Cells *in vitro*. *Journal of Agricultural and Food Chemistry.* **54**, 9329 – 9339.

20. Yang JH, Hsia TC, Kuo HM, Chao PD, Chou CC, Wei YH, Chung JG (2006). Inhibition of Lung Cancer Cell Growth by Quercetin Gulcuronides via G_2/M Arrest and Induction of Apoptosis. *Drug Metabolism and Disposition*. **34**, 296 – 304.

21. Feng R, Ni HM, Wang SY, Tourkova IL, Shurin MR, Harada H, Yin XM (2007). Cyanidin-3-rutinoside, a Natural Polyphenol Antioxidant, Selectively Kills Leukemic Cells by Induction of Oxidative Stress. *Journal of Biological Chemistry*. **282(18)**, 13468 – 13476.

22. Santangelo C, Vari R, Scazzocchio B, Di Benedetto R, Filesi C, Masella R (2007). Polyphenols, Intracellular Signaling and Inflammation. *Ann lst Super Sanita*. **43**, 394 – 405.

23. Huang C, Huang Y, Li J, Hu W, Aziz R, Tang M, Sun N, Cassady J, Stoner GD (2002). Inhibition of Benzo(a)pyrene diol-epoxide-induced Transactivation of Activated Protein 1 and Nuclear Factor κB by Black Raspberry Extracts. *Cancer Research*. **62**, 6857 – 6863.

24. Rodrigo KA, Rawal Y, Renner RJ, Schwartz SJ, Tian Q, Larsen PE, Mallery SR (2006). Suppression of the Tumorigenic Phenotype in Human Oral Squamous Cell Carcinoma Cells by an Ethanol Extract Derived from Freeze-dried Black Raspberries. *Nutrition and Cancer*. **54(1)**, 58 – 68.

25. Wang LS, Stoner GD (2008). Anthocyanins and Their Role in Cancer Prevention. *Cancer Letters*. **269(2)**, 281 – 290.

26. Bagchi D, Sen CK, Bagchi M, Atalay M (2003). Anti-angiogenic, Antioxidant, and Anti-carcinogenic Properties of a Novel Anthocyanin-rich Berry Extract Formula. *Biochemistry*. **69(1)**, 95 – 102.

27. Kresty LA *et al*. (2001). Chemoprevention of Esophageal Tumorigenesis by Dietary Administration of Lyophilized Black Raspberries. *Cancer Research*. **61**, 6112 – 6119.

28. Casto BC et al. (2002). Chemoprevention of Oral Cancer by Black Raspberries. *Anticancer Research*. **22**, 4005 – 4016.

29. Carlton PS *et al.* (2000). Failure of Dietary Lyophilized Strawberries to Inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-1 butanone-and Benzo(*a*)pyrene-induced Lung Tumorigenesis in Strain A/J Mice. *Cancer Letters*. **159**, 113 – 117.

30. Prior RL, Wu X (2006). Anthocyanins: Structural Characteristics that Result in Unique Metabolic Patterns and Biological Activities. *Free Radical Research*. **40**, 1014 – 1028.

31. Harris GK, Gupta A, Nines RG, Kresty LA, Habib SG, Frankel WL, LaPerle K, Gallaher DD, Schwartz SJ, Stoner GD (2001). Effects of Lyophilized Black Raspberries on Azoxymethane-induced Colon Cancer and 8-hydroxy-2'deoxyguanosine Levels in the Fischer 344 Rat. *Nutrition and Cancer*. **40**, 125 – 133.

32. Afaq F, Syed DN, Malik A, Hadi N, Sarfaraz S, Kweon Mh, Khan N, Zaid MA, Mukhtar H (2007). Delphinidin, an Anthocyanidin in Pigmented Fruits and Vegetables, Protects Human HaCaT Keratinocytes and Mouse Skin Against

UVB-mediated Oxidative Stress and Apoptosis. *Journal of Investigative Dermatology*. **121(1)**, 222 – 232.

33. Duthie SJ, Jenkinson AM, Crozier A, Mullen W, Pirie L, Kyle J, Yap LS, Christen P, Duthie GG (2005). The Effects of Cranberry Juice Consumption on Antioxidant Status and Biomarkers Relating to Heart Disease and Cancer in Healthy Human Volunteers. *European Journal of Nutrition*. **45(2)**, 113 – 122.

34. Weisel T, Baum M, Eisenbrand G, Dietrich H, Will F, Stockin JP, Kulling S, Rufer C, Johannes C, Janzowski C (2006). An Anthocyanin/Polyphenolic-rich Fruit Juice Reduces Oxidative DNA Damage and Increases Glutathione Level in Healthy Probands. *Biotechnology Journal*. 1(4), 388 – 397.

35. Maurya DK, Balakrishnan S, Salvi VP, Nair CK (2005). Protection of Cellular DNA from γ-radiation-induced Damages and Enhancement in DNA Repair by Troxerutin. *Molecular and Cellular Biochemistry*. **280**, 57 – 68.

36. Maurya D, Salvi V, Nair C (2005). Radiation Protection of DNA by Ferulic Acid Under *in vitro* and *in vivo* Conditions. *Molecular and Cellular Biochemistry*.

37. Niture S, Velu C, Smith Q, Bhat G, Srivenugopal K (2007). Increased Expression of the MGMT Repair Protein Mediated by Cysteine Pro-drugs and Chemopreventative Natural Products in Human Lymphocytes and Tumor Cell Lines. *Carcinogenesis*. **28**, 378 – 389.

38. Aiyer H, Vadhanam M, Stoyanova R, Caprio G, Clapper M, Gupta R (2008). Dietary Berries and Ellagic Acid Prevent Oxidative DNA Damage and Modulate Expression of DNA Repair Genes. *International Journal of Molecular Sciences*. 9, 327 – 341.

39. Garvin P, Nilsson L, Carstensen J, Jonasson L, Kristenson M (2008). Circulating Matrix Metalloproteinase-9 Associated with Cardiovascular Risk Factors in a Middle-aged Normal Population. *PLoS One*. **3(3)**, 1 – 7.

40. Kessenbrock K, Plaks V, Werb Z (2010). Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. *Cell*. **141**, 52 – 67.

41. Shin DY, Lee WS, Kim SH, Kim MJ, Yun JW, Lu JN, Lee SJ, Tsoy I, Kim HJ, Ryu CH, Kim GY, Kang HS, Shin SC, Choi YH (2009). Anti-invasive Activity of Anthocyanins Isolated from *Vitis coignetiae* in Human Hepatocarcinoma Cells. *Journal of Medicinal Food*. **12(5)**, 967 – 972.

42. Chen PN, Kuo WH, Chiang CL, Chiou HL, Hsieh YS, Chu SC (2006). Black Rice Anthocyanins Inhibit Cancer Cells Invasion via Repressions of MMPs and u-PA Expression. *Chemico-Biological Interactions*. **163**, 218 – 229.

43. Tate P, God J, Bibb R, Lu Q, Larcom L (2004). Inhibition of Metalloproteinase Activity by Fruit Extracts. *Cancer Letters*. **212**, 153 – 158.

44. McGhie TK, Walton MC (2007). The Bioavailability and Absorption of Anthocyanins: Towards a Better Understanding. *Molecular Nutrition and Food Research*. **51**, 702 – 713.

45. Frank T, Netzel M, Strass G, Bitsch R, Bitsch I (2003). Bioavailability of Anthocyanins-3-glucosides Following Consumption of Red Wine and Red Grape Juice. *Canadian Journal of Physiology and Pharmacology*. **81**, 423 – 435.

46. González-Barrio R, Borges G, Mullen W, Crozier A (2010). Bioavailability of Anthocyanins and Ellagitannins Following Consumption of Raspberries by Healthy Humans and Subjects with an Ileostomy. *Journal of Agricultural Food Chemistry*. **58(7)**, 3933 – 3939.

47. Cerdá B, Tomás-Barberán F, Espín J (2005). Metabolism of Antioxidant and Chemopreventive Ellagitannins from Strawberries, Raspberries, Walnuts, and Oak-aged Wine in Humans: Identification of Biomarkers and Individual Variability. *Journal of Agricultural and Food Chemistry*. **53**, 227 – 235.

48. Kaul T, Middleton E, Ogra P (1985). Antiviral Effect of Flavonoids on Human Viruses. *Journal of Medical Virology*. **15(1)**, 71 – 79.

49. Wu J, Wang X, Yi Y, Lee K (2003). Anti-AIDS Agents 54. A Potent Anti-HIV Chalcone and Flavonoids from Genus *Desmos*. *Bioorganic and Medicinal Chemistry Letters*. **13**, 1813 – 1815.

50. Puupponen-Pimia R, Nohynek L, Alakomi H, Oksman-Caldentey KM (2005). Bioactive Berry Compounds – Novel Tools Against Human Pathogens. *Applied Microbiology and Biotechnology*. **67(1)**, 8 – 18.

51. Cavanagh H, Hipwell M, Wilkinson J (2003). Antibacterial Activity of Berry Fruits Used for Culinary Purposes. *Journal of Medicinal Food*. **6(1)**, 57 – 61.

52. Nohynek L, Alakomi H, Kahkonen M, Heinonen M, Helander I, Oksman-Caldentey K, Puupponen-Pimia R (2006). Berry Phenolics: Antimicrobial Properties and Mechanisms of Action Against Severe Human Pathogens. *Nutrition and Cancer*. **54(1)**, 18 – 32.

53. Girardi M (2006). Immunosurveillance and Immunoregulation by γδ T Cells. *Journal of Investigative Dermatology*. **126**, 25 – 31.

54. Percival S, (2009). Grape Consumption Supports Immunity in Animals and Humans. *Journal of Nutrition*. **139**, 1801S – 1805S.

55. Kindt T, Goldsby R, Osborne B. Kuby Immunology. New York: W.H. Freeman and Company, 2007.

56. Glaser R, Kiecolt-Glaser J (2005). Stress-induced Immune Dysfunction: Implications for Health. *Nature Reviews. Immunology*. **5**, 243 – 251.

57. Nantz M, Rowe C, Nieves C, Percival S (2006). Immunity and Antioxidant Capacity in Humans is Enhanced by Consumption of a Dried, Encapsulated Fruit and Vegetable Juice Concentrate. *Journal of Nutrition*. **136**, 2606 – 2610.

58. Holderness J, Jackiw L, Kimmel E, Kerns H, Radke M, Hedges J, Petrie C, McCurley P, Glee P, Palecanda A, Jutila M (2007). Select Plant Tannins Induce IL-2Rα Up-regulation and Augment Cell Division in γδ T Cells. *Journal of Immunology*. **179**, 6468 – 6478.

59. Skoner J, Sigmon J, Larcom L (1995). Suppressed DNA Repair Capacity of Peripheral Lymphocytes in Pregnant Women. *Molecular and Cellular Endocrinology*. **108,** 179 – 183.

60. Kumar G, Smith S, Larcom L (1997). Human Peripheral Mononuclear Cell Responses to UV Damage are Affected by Radiation-induced Changes in Plasma. *Photochemistry and Photobiology*. **65(3)**, 436 – 440.

61. Powers J (2007). Washington Red Raspberries Executive Summary. www.red-raspberries.org

62. .Stoner G, Wang L, Zikri N, chen T, Hecht S, Huang C, Sardo C, Lechner J (2007). Cancer Prevention with Freeze-Dried Berries and Berry Components. *Semin Cancer Biol*. **17(5)**, 403 – 410.

63. U.S. Cancer Statistics Working Group. *United States Cancer Statistics*: 1999 - 2007 Incidence and Mortality. http://apps.nccd.cdc.gov/uscs/toptencancers.aspx

64. Han C, Ding H, Casto B, Stoner G, D'Ambrosio S (2005). Inhibition of the Growth of Premalignant and Malignant Human Oral Cell Lines by Extracts and Components of Black Raspberries. *Nutrition and Cancer*. **51(2),** 207 – 217.

65. Brigden M, Heathcote J (2000). Problems in Interpreting Laboratory Tests. What do Unexpected Results Mean? *Postgraduate Medicine*. **107(7)**, 145 – 158.

66. Percival S, Bukowski J, Milner J (2008). Bioactive Food Components that Enhance γδ T Cell Function May Play a Role in Cancer Prevention. *Journal of Nutrition*. **138**, 1 – 4.

67. Nelson K, Melendez J (2004). Mitochondrial Redox Control of Matrix Metalloproteinases. *Free Radical Biology and Medicine*. **37**, 768 – 784.

68. Bub A, Watzl B, Blockhaus M, Briviba K, Liegibel U, Müller H, Zobel B, Rechkemmer G (2003). Fruit Juice Consumption Modulates Antioxidative Status, Immune Status and DNA Damage. *Journal of Nutritional Biochemistry*. **14(2)**, 90 – 98.

69. Schauss A, Wu X, Prior R, Ou B, Huang D, Owens J, Agarwal A, Jensen G, Hart A, Shanbrom E (2006). Antioxidant Capacity and Other Bioactivities of the Freeze-Dried Amazonian Palm Berry, *Euterpe oleraceae* Mart. (Acai). *Journal of Agricultural and Food Chemistry*. **54**, 8604 – 8610.