The Alkaline Diet: Is There Evidence That an Alkaline pH Diet Benefits Health?

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This review looks at the role of an alkaline diet in health. Pubmed was searched looking for articles on pH, potential renal acid loads, bone health, muscle, growth hormone, back pain, vitamin D and chemotherapy. Many books written in the lay literature on the alkaline diet were also reviewed and evaluated in light of the published medical literature. There may be some value in considering an alkaline diet in reducing morbidity and mortality from chronic diseases and further studies are warranted in this area of medicine.

1. Background

Life on earth depends on appropriate pH levels in and around living organisms and cells. Human life requires a tightly controlled pH level in the serum of about 7.4 (a slightly alkaline range of 7.35 to 7.45) to survive [1].

As a comparison, in the past 100 years with increasing industrialization, the pH of the ocean has dropped from 8.2 to 8.1 because of increasing CO₂ deposition. This has a negative impact on life in the ocean [1, 2] and may lead to the collapse of the coral reefs [3]. Even the pH of the soil in which plants are grown can have considerable influence on the mineral content of the food we eat (as minerals are used as buffers to maintain pH). The ideal pH of soil for the best overall availability of essential nutrients is between 6 and 7. Acidic soils below pH of 6 may have reduced calcium and magnesium, and soil above pH 7 may result in chemically unavailable iron, manganese, copper and zinc. Adding dolomite and manure are ways of raising pH in an acid soil environment when the pH is below 6 [4].

When it comes to the pH and net acid load in the human diet, there has been considerable change from the hunter gather civilization to the present [5]. With the agricultural revolution (last 10,000 years) and even more recently with industrialization (last 200 years), there has been an decrease in potassium (K) compared to sodium (Na) and an increase in chloride compared to bicarbonate found in the diet [6]. The ratio of potassium to sodium has reversed, K/Na previously was 10 to 1 whereas the modern diet has a ratio of 1 to 3 [7]. It is generally accepted that agricultural humans today have a diet poor in magnesium and potassium as well as fiber and rich in saturated fat, simple sugars, sodium, and chloride as compared to the pre-agricultural period [6]. This results in a diet that may induce metabolic acidosis which is mismatched to the genetically determined nutritional requirements [8]. With aging, there is a gradual loss of renal acid-base regulatory function and a resultant increase in diet-induced metabolic acidosis while on the modern diet [9]. A low-carbohydrate high-protein diet with its increased acid load results in very little change in blood chemistry, and pH, but results in many changes in urinary chemistry. Urinary magnesium levels, urinary citrate and pH are decreased, urinary calcium, undissociated uric acid, and phosphate are increased. All of these result in an increased risk for kidney stones [10].

Much has been written in the lay literature as well as many online sites expounding on the benefits of the alkaline diet. This paper is an attempt to balance the evidence that is found in the scientific literature.
2. The Role of pH in Various Cells, Organs, and Membranes

The pH in our body may vary considerably from one area to another with the highest acidity in the stomach (pH of 1.35 to 3.5) to aid in digestion and protect against opportunistic microbial organisms. But even in the stomach, the layer just outside the epithelium is quite basic to prevent mucosal injury. It has been suggested that decreased gastric lining secretion of bicarbonates and a decrease in the alkaline/acid secretion in duodenal ulcer patients may play a significant role in duodenal ulcers [11]. The skin is quite acidic (pH 4–6.5) to provide an acid mantle as a protective barrier to the systemic circulation to bring about pH homeostasis [7].

The urine may have a variable pH from acid to alkaline depending on the need for balancing the internal environment. Acid excretion in the urine can be estimated by a formula described by Remer (sulfate + chloride + 1.8x phosphate + organic acids) minus (sodium + potassium + 2x calcium + 2x magnesium) mEq [14]. Foods can be categorized by the potential renal acid loads (PRALs) see Table 2. Fruits, vegetables, fruit juices, potatoes, and alkali-rich and low phosphorus beverages (red and white wine, mineral soda waters) having a negative acid load. Whereas, grain products, meats, dairy products, fish, and alkali poor and low phosphorus beverages (e.g., pale beers, cocoa) have relatively high acid loads [15]. Measurement of pH of the urine (reviewed in a recent study with two morning specimens done over a five-year span) did not predict bone fractures or loss of bone mineral density [16]. However, this may not be reflective of being on an alkaline or acid diet throughout this time. For more details, see Table 1.

3. Chronic Acidosis and Bone Disease

Calcium in the form of phosphates and carbonates represents a large reservoir of base in our body. In response to an acid load such as the modern diet these salts are released into the systemic circulation to bring about pH homeostasis [7]. It has been estimated that the quantity of calcium lost in the urine with the modern diet over time could be as high as almost 480 gm over 20 years or almost half the skeletal mass of calcium [21]. However, urinary losses of cal-cium are not a direct measure of osteoporosis. There are many regulatory factors that may compensate for the urinary calcium loss. When the arterial pH is in the normal range, a mild reduction of plasma bicarbonate results in a negative calcium balance which could benefit from supplementing bicarbonate in the form of potassium bicarbonate [22]. It has been found that bicarbonate, which increases the alkali content of a diet, but not potassium may attenuate bone loss in healthy older adults [23]. The bone minerals that are wasted in the urine may not have complete compensation through intestinal absorption, which is thought to result in osteoporosis. However, adequate vitamin D with a 25(OH)D level of >80 nmol/L may allow for appropriate intestinal absorption of calcium and magnesium and phosphate when needed [24]. Sadly, most populations are generally deficient in vitamin D especially in northern climates [25]. In chronic renal failure, correction of metabolic acidosis with bicarbonate significantly improves parathyroid levels and levels of the active form of vitamin D 1,25(OH)2D3 [26]. Recently, a study has shown the importance of phosphate in Remer’s PRAL formula. According to the formula it would be expected that an increase in phosphate should result in an increase in urinary calcium loss and a negative calcium balance in bone [27]. It should be noted that supplementation with phosphate in patients with bed rest reduced urinary calcium excretion but did not prevent bone loss [28]. The most recent systematic review and meta-analysis has shown that calcium balance is maintained and improved with phosphate which is quite contrary to the acid-ash hypothesis [29]. As well a recent study looking at soda intake (which has a significant amount of phosphate) and osteoporosis in postmenopausal American first nations women did not find a correlation [30]. It is quite possible that the high acid content according to Remer’s classification needs to be looked at again in light of compensatory phosphate intake. There is online information promoting an alkaline diet for bone health as well as a number of books. However, a recent systematic review of the literature looking for evidence supporting the alkaline diet for bone health found no protective role of dietary acid load in osteoporosis [31].

### Table 1: Ph of selected fluids, organs, and membranes.

<table>
<thead>
<tr>
<th>Organ, fluid or membrane</th>
<th>pH</th>
<th>Function of pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Skin</td>
<td>Natural pH is between 4 and 6.5 [17]</td>
<td>Barrier protection from microbes</td>
</tr>
<tr>
<td>(2) Urine</td>
<td>4.6 to 8.0 [18]</td>
<td>Limit overgrowth of microbes</td>
</tr>
<tr>
<td>(3) Gastric</td>
<td>1.35 to 3.5</td>
<td>Break down protein</td>
</tr>
<tr>
<td>(4) Bile</td>
<td>7.6 to 8.8</td>
<td>Neutralize stomach acid, aid in digestion</td>
</tr>
<tr>
<td>(5) Pancreatic fluid</td>
<td>8.8</td>
<td>Neutralize stomach acid, aid in digestion</td>
</tr>
<tr>
<td>(6) Vaginal fluid</td>
<td>&lt;4.7 [13]</td>
<td>Limit overgrowth of opportunistic microbes</td>
</tr>
<tr>
<td>(7) Cerebrospinal fluid</td>
<td>7.3</td>
<td>Bathes the exterior of the brain</td>
</tr>
<tr>
<td>(8) Intracellular fluid</td>
<td>6.0–7.2 [19]</td>
<td>Due to acid production in cells</td>
</tr>
<tr>
<td>(9) Serum venous</td>
<td>7.35</td>
<td>Tightly regulated</td>
</tr>
<tr>
<td>(10) Serum arterial</td>
<td>7.4</td>
<td>Tightly regulated</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Food or food group</th>
<th>PRAL mEq of: Cl + P0₄ + SO₄⁻ - Na - K - Ca - Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dairy</strong></td>
<td></td>
</tr>
<tr>
<td>Parmesan cheese</td>
<td>34.2</td>
</tr>
<tr>
<td>Processed cheese plain</td>
<td>28.7</td>
</tr>
<tr>
<td>Cheddar reduced fat</td>
<td>26.4</td>
</tr>
<tr>
<td>Hard cheese (average)</td>
<td>19.2</td>
</tr>
<tr>
<td>Fresh cheese (quark)</td>
<td>11.3</td>
</tr>
<tr>
<td>Cottage cheese plain</td>
<td>8.7</td>
</tr>
<tr>
<td>Yogurt whole milk</td>
<td>1.5</td>
</tr>
<tr>
<td>Ice Cream</td>
<td>0.8</td>
</tr>
<tr>
<td>Whole milk</td>
<td>0.7</td>
</tr>
<tr>
<td>Buttermilk</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Eggs</strong></td>
<td></td>
</tr>
<tr>
<td>Eggs yolk</td>
<td>23.4</td>
</tr>
<tr>
<td>Eggs white</td>
<td>1.1</td>
</tr>
<tr>
<td>Eggs chicken whole</td>
<td>8.2</td>
</tr>
<tr>
<td><strong>Meats</strong></td>
<td></td>
</tr>
<tr>
<td>Corned beef</td>
<td>13.2</td>
</tr>
<tr>
<td>Luncheon meat canned</td>
<td>10.2</td>
</tr>
<tr>
<td>Turkey</td>
<td>9.9</td>
</tr>
<tr>
<td>Veal</td>
<td>9.0</td>
</tr>
<tr>
<td>Lean beef</td>
<td>7.8</td>
</tr>
<tr>
<td>Frankfurters</td>
<td>6.7</td>
</tr>
<tr>
<td><strong>Sugars</strong></td>
<td></td>
</tr>
<tr>
<td>Sugar white</td>
<td>−0.1</td>
</tr>
<tr>
<td>Honey</td>
<td>−0.3</td>
</tr>
<tr>
<td><strong>Vegetables</strong></td>
<td></td>
</tr>
<tr>
<td>Cucumber</td>
<td>−0.8</td>
</tr>
<tr>
<td>Broccoli</td>
<td>−1.2</td>
</tr>
<tr>
<td>Tomato</td>
<td>−3.1</td>
</tr>
<tr>
<td>Eggplant</td>
<td>−3.4</td>
</tr>
<tr>
<td>Celery</td>
<td>−5.2</td>
</tr>
<tr>
<td>Spinach</td>
<td>−14.0</td>
</tr>
<tr>
<td><strong>Fats and Oils</strong></td>
<td></td>
</tr>
<tr>
<td>Butter</td>
<td>0.6</td>
</tr>
<tr>
<td>Margarine</td>
<td>−0.5</td>
</tr>
<tr>
<td>Olive oil</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Fruits and nuts and fruit juices</strong></td>
<td></td>
</tr>
<tr>
<td>Peanuts</td>
<td>8.3</td>
</tr>
<tr>
<td>Walnuts</td>
<td>6.8</td>
</tr>
<tr>
<td>Grape juice unsweetened</td>
<td>−1.0</td>
</tr>
<tr>
<td>Orange juice unsweetened</td>
<td>−2.9</td>
</tr>
<tr>
<td>Apples or apple juice unsweetened</td>
<td>−2.2</td>
</tr>
<tr>
<td>Apricots</td>
<td>−4.8</td>
</tr>
<tr>
<td>Banana</td>
<td>−5.5</td>
</tr>
<tr>
<td>Black currents</td>
<td>−6.5</td>
</tr>
<tr>
<td>Raisins</td>
<td>−21.0</td>
</tr>
<tr>
<td><strong>Grains and grain products</strong></td>
<td></td>
</tr>
<tr>
<td>Brown Rice</td>
<td>12.5</td>
</tr>
<tr>
<td>Rolled Oats</td>
<td>10.7</td>
</tr>
<tr>
<td>Spaghetti whole meal</td>
<td>7.3</td>
</tr>
<tr>
<td>Spaghetti white</td>
<td>6.5</td>
</tr>
</tbody>
</table>
Table 2: Continued.

<table>
<thead>
<tr>
<th>Food or food group</th>
<th>PRAL mEq of: Cl + P0&lt;sub&gt;4&lt;/sub&gt; + SO&lt;sub&gt;4&lt;/sub&gt;−</th>
<th>Na − K − Ca − Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornflakes</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>Rice white</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Bread rye flower</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>Bread whole wheat</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Legumes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lentils green and brown</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>Green beans</td>
<td>−3.1</td>
<td></td>
</tr>
<tr>
<td>Fish</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trout brown</td>
<td>10.8</td>
<td></td>
</tr>
<tr>
<td>Cod fillets</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>Beverages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beer pale</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Coca-Cola</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Beer draft</td>
<td>−0.2</td>
<td></td>
</tr>
<tr>
<td>Wine white</td>
<td>−1.2</td>
<td></td>
</tr>
<tr>
<td>Coffee infusion</td>
<td>−1.4</td>
<td></td>
</tr>
<tr>
<td>Wine red</td>
<td>−2.4</td>
<td></td>
</tr>
</tbody>
</table>

Another element of the modern diet is the excess of sodium in the diet. There is evidence that in healthy humans the increased sodium in the diet can predict the degree of hyperchloremic metabolic acidosis when consuming a net acid producing diet [32]. As well, there is evidence that there are adverse effects of sodium chloride in the aging population. A high sodium diet will exacerbate disuse-induced bone and muscle loss during immobilization by increasing bone resorption and protein wasting [33]. Excess dietary sodium has been shown to result in hypertension and osteoporosis in women [34, 35]. As well, dietary potassium which is lacking in the modern diet would modulate pressor and hypercalciuric effects of excess of sodium chloride [36].

Excess dietary protein with high acid renal load may decrease bone density if not buffered by ingestion of supplements or foods that are alkali rich [37]. However, adequate protein is necessary for prevention of osteoporosis and sarcopenia; therefore, increasing the amount of fruit and vegetables may be necessary rather than reducing protein [38].

4. Alkaline Diets and Muscle

As we age, there is a loss of muscle mass, which may predispose to falls and fractures. A three-year study looking at a diet rich in potassium, such as fruits and vegetables, as well as a reduced acid load, resulted in preservation of muscle mass in older men and women [39]. Conditions such as chronic renal failure that result in chronic metabolic acidosis result in accelerated breakdown in skeletal muscle [40]. Correction of acidosis may preserve muscle mass in conditions where muscle wasting is common such as diabetic ketosis, trauma, sepsis, chronic obstructive lung disease, and renal failure [41]. In situations that result in acute acidosis, supplementing younger patients with sodium bicarbonate prior to exhaustive exercise resulted in significantly less acidosis in the blood than those that were not supplemented with sodium bicarbonate [42].

5. Alkaline Supplementation and Growth Hormone

It has long been known that severe forms of metabolic acidosis in children, such as renal tubular acidosis, are associated with low levels of growth hormone with resultant short stature. Correction of the acidosis with bicarbonate [7] or potassium citrate [43] increases growth hormone significantly and improved growth. The use of enough potassium bicarbonate in the diet to neutralize the daily net acid load in postmenopausal women resulted in a significant increase in growth hormone and resultant osteocalcin [44]. Improving growth hormone levels may improve quality of life, reduce cardiovascular risk factors, improve body composition, and even improve memory and cognition [45]. As well this results in a reduction of urinary calcium loss equivalent to 5% of bone calcium content over a period of 3 years [46].

6. Alkaline Diet and Back Pain

There is some evidence that chronic low back pain improves with the supplementation of alkaline minerals [47]. With supplementation there was a slight but significant increase in blood pH and intracellular magnesium. Ensuring that there is enough intracellular magnesium allows for the proper function of enzyme systems and also allows for activation of vitamin D [48]. This in turn has been shown to improve back pain [49].
7. Alkalinity and Chemotherapy

The effectiveness of chemotherapeutic agents is markedly influenced by pH. Numerous agents such as epirubicin and Adriamycin require an alkaline media to be more effective. Others, such as cisplatin, mitomycin C, and thiopeta, are more cytotoxic in an acid media [50]. Cell death correlates with acidosis and intracellular pH shifts higher (more alkaline) after chemotherapy may reflect response to chemotherapy [51]. It has been suggested that inducing metabolic alkalosis may be useful in enhancing some treatment regimes by using sodium bicarbonate, carbicab, and furosemide [52]. Extracellular alkalization by using bicarbonate may result in improvements in therapeutic effectiveness [53]. There is no scientific literature establishing the benefit of an alkaline diet for the prevention of cancer at this time.

8. Discussion

The human body has an amazing ability to maintain a steady pH in the blood with the main compensatory mechanisms being renal and respiratory. Many of the membranes in our body require an acid pH to protect us and to help us digest food. It has been suggested that an alkaline diet may prevent a number of diseases and result in significant health benefits. Looking at the above discussion on bone health alone, certain aspects have doubtful benefit. There does not seem to be enough evidence that milk or cheese may be as detrimental as Remer’s formula suggests since phosphate does benefit bone health and result in a positive calcium balance. However, another mechanism for the alkaline diet to benefit bone health may be the increase in growth hormone and resultant increase in osteocalcin. There is some evidence that the K/Na ratio does matter and that the significant amount of salt in our diet is detrimental. Even some governments are demanding that the food industry reduce the salt load in our diet. High-protein diets may also affect bone health but some protein is also needed for good bone health. Muscle wasting however seems to be reduced with an alkaline diet and back pain may benefit from this as well. An alkaline environment may improve the efficacy of some chemotherapy agents but not others.

9. Conclusion

Alkaline diets result in a more alkaline urine pH and may result in reduced calcium in the urine, however, as seen in some recent reports, this may not reflect total calcium balance because of other buffers such as phosphate. There is no substantial evidence that this improves bone health or protects from osteoporosis. However, alkaline diets may result in a number of health benefits as outlined below

1. Increased fruits and vegetables in an alkaline diet would improve the K/Na ratio and may benefit bone health, reduce muscle wasting, as well as mitigate other chronic diseases such as hypertension and strokes.

2. The resultant increase in growth hormone with an alkaline diet may improve many outcomes from cardiovascular health to memory and cognition.

3. An increase in intracellular magnesium, which is required for the function of many enzyme systems, is another added benefit of the alkaline diet. Available magnesium, which is required to activate vitamin D, would result in numerous added benefits in the vitamin D apocrine/exocrine systems.

4. Alkalinity may result in added benefit for some chemotherapeutic agents that require a higher pH.

From the evidence outlined above, it would be prudent to consider an alkaline diet to reduce morbidity and mortality of chronic disease that are plaguing our aging population. One of the first considerations in an alkaline diet, which includes more fruits and vegetables, is to know what type of soil they were grown in since this may significantly influence the mineral content. At this time, there are limited scientific studies in this area, and many more studies are indicated in regards to muscle effects, growth hormone, and interaction with vitamin D.

References


Biological significance of essential fatty acids.

Das UN¹.

Author information

Abstract

Essential fatty acids (EFAs)--linoleic acid (LA) and alpha-linolenic acid (ALA) are critical for human survival. EFAs are readily available in the diet. But, to derive their full benefit, EFAs need to be metabolized to their respective long-chain metabolites. EFAs not only form precursors to respective prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs), but also give rise to lipoxins (LXs), resolvins, isoprostanes, and hydroxy- and hydroperoxyeicosatetraenoates. Certain PGs, TXs, and LTs have pro-inflammatory actions whereas LXs and resolvins are anti-inflammatory in nature. Furthermore, EFAs and their long-chain metabolites modulate the activities of angiotensin converting and HMG-CoA reductase enzymes, enhance acetylcholine levels in the brain, increase the synthesis of endothelial nitric oxide, augment diuresis, and enhance insulin action. Thus, EFAs and their metabolites may function as endogenous ACE and HMG-CoA reductase inhibitors, nitric oxide enhancers, beta-blockers, diuretics, anti-hypertensive, and anti-atherosclerotic molecules. In addition, EFAs and their long-chain metabolites react with nitric oxide (NO) to yield respective nitroalkene derivatives that exert cell-signaling actions via ligation and activation of peroxisome proliferator-activated receptors (PPARs). Thus, EFAs and their derivatives have varied biological actions that may have relevance to their involvement in several physiological and pathological processes.

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[Indexed for MEDLINE]
Essential Fatty Acids

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Summary

› Linoleic acid (LA), an omega-6 fatty acid, and α-linolenic acid (ALA), an omega-3 fatty acid, are considered essential fatty acids (EFA) because they cannot be synthesized by humans. (More information)

› The long-chain omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), can be synthesized from ALA, but due to low conversion efficiency, it is recommended to obtain EPA and DHA from additional sources. (More information)

› LA, arachidonic acid (AA), and DHA are the most common polyunsaturated fatty acids (PUFA) accumulating in tissues. (More information)

› Both omega-6 and omega-3 fatty acids are important structural components of cell membranes, serve as precursors to bioactive lipid mediators, and provide a source of energy. Long-chain omega-3 PUFA in particular exert anti-inflammatory effects and it is recommended to increase their presence in the diet. (More information)

› Both dietary intake and endogenous metabolism influence whole-body status of EFA. Genetic
Disease Treatment
- Coronary heart disease
- Diabetes mellitus
- Inflammatory diseases
- Neuropsychiatric disorders
- Alzheimer's disease and dementia

Sources
- Food
- Biosynthesis of EPA and DHA
- Supplements
- Infant formula

Safety
- Adverse effects
- Infant formula
- Pregnancy and lactation
- Contaminants in fish
- Contaminants in supplements
- Drug interactions
- Nutrient interactions

Intake Recommendations
- US Institute of Medicine
- International recommendations
- AHA recommendation
- LPI Recommendation

Authors and Reviewers

References

- Polymorphisms in fatty acid synthesizing enzymes can have a significant impact on fatty acid levels in the body. (More information)
- DHA is important for visual and neurological development. Feeding infants formula enriched with DHA and AA appears to have no significant effect on cognitive development and a very modest effect on visual acuity. (More information)
- Replacing saturated fat in the diet with a mixture of PUFA (both omega-6 and omega-3) is associated with beneficial effects on the cardiovascular system. (More information)
- A large body of scientific research suggests that higher dietary omega-3 fatty acid intakes are associated with reductions in cardiovascular disease risk. Thus, the American Heart Association recommends that all adults eat fish, particularly oily fish, at least twice weekly. (More information)
- The results of prospective cohort studies and randomized controlled trials indicate that fish and fish oil consumption decreases the risk of coronary heart disease (CHD) mortality, including fatal myocardial infarction (heart attack) and sudden cardiac death. (More information)
- Low DHA status may be a risk factor for Alzheimer's disease and other types of dementia, but it is not yet known whether DHA supplementation can help prevent or treat such cognitive disorders. (More information)
- Increasing EPA and DHA intake may be beneficial in individuals with type 2 diabetes, especially those with elevated serum triglycerides. (More information)
- Randomized controlled trials have found that fish oil supplementation reduces the requirement for anti-inflammatory medication in patients with rheumatoid arthritis. (More information)
Although some data suggest that omega-3 fatty acid supplementation may be a beneficial adjunct in the therapy of depression, bipolar disorder, and schizophrenia, great heterogeneity between trials prevents conclusive determination of therapeutic efficacy. (More information)

**Introduction**

Omega-6 and omega-3 fatty acids are polyunsaturated fatty acids (PUFA), meaning they contain more than one cis double bond (1). In all omega-6 fatty acids, the first double bond is located between the sixth and seventh carbon atom from the methyl end of the fatty acid (n-6). Similarly, in all omega-3 fatty acids, the first double bond is located between the third and fourth carbon atom counting from the methyl end of the fatty acid (n-3). Scientific abbreviations for fatty acids tell the reader something about their chemical structure. One scientific abbreviation for α-linolenic acid (ALA) is 18:3n-3. The first part (18:3) tells the reader that ALA is an 18-carbon fatty acid with three double bonds, while the second part (n-3) tells the reader that the first double bond is in the n-3 position, which defines it as an omega-3 fatty acid (Figures 1a and 1b). Double bonds introduce kinks in the hydrocarbon chain that influence the structure and physical properties of the fatty acid molecule (Figure 1c).

**Figure 1a. Structures of Fatty Acids**

\[
\text{CH}_3 \quad \text{CHCH}_2(n) \quad \text{COOH} \\
\text{methyl or hydrocarbon carboxyl end chain} \\
\text{omega (ω) end}
\]

The general structure of a fatty acid (20).

**Figure 1b. Structures of Fatty Acids**

The chemical structure of α-linolenic acid (ALA), 18:3n-3. ALA has 18 carbon atoms (C) and 3 double bonds, the first of which is located 3 carbon atoms from the terminal methyl group (omega [ω] end).
Although humans and other mammals can synthesize saturated fatty acids and some monounsaturated fatty acids from carbon groups in carbohydrates and proteins, they lack the enzymes necessary to insert a cis double bond at the n-6 or the n-3 position of a fatty acid (1). Consequently, omega-6 and omega-3 fatty acids are essential nutrients. The parent fatty acid of the omega-6 series is linoleic acid (LA; 18:2n-6), and the parent fatty acid of the omega-3 series is ALA (Table 1 and Figure 2). Humans can synthesize long-chain (20 carbons or more) omega-6 fatty acids, such as dihomo-γ-linolenic acid (DGLA; 20:3n-6) and arachidonic acid (AA; 20:4n-6), from LA and long-chain omega-3 fatty acids, such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), from ALA (see Metabolism and Bioavailability).

Table 1. Names and Abbreviations of the Omega-6 and Omega-3 Fatty Acids
<table>
<thead>
<tr>
<th>Omega-6 Fatty Acids</th>
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<th>Omega-3 Fatty Acids</th>
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<tbody>
<tr>
<td>Linoleic acid</td>
<td>LA 18:2n-6</td>
<td>α-Linolenic acid ALA 18:3n-3</td>
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<tr>
<td>γ-Linolenic acid</td>
<td>GLA 18:3n-6</td>
<td>Stearadonic acid SDA 18:4n-3</td>
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<tr>
<td>Dihomo-γ-linolenic acid</td>
<td>DGLA 20:3n-6</td>
<td>Eicosatetraienoic acid ETA 20:4n-3</td>
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<tr>
<td>Arachidonic acid</td>
<td>AA 20:4n-6</td>
<td>Eicosapentaenoic acid EPA 20:5n-3</td>
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<td>Adrenic acid</td>
<td>22:4n-6</td>
<td>Docosapentaenoic acid DPA (n-3) 22:5n-3</td>
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<tr>
<td>Tetracosatetraenoic acid</td>
<td>24:4n-6</td>
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<td>Docosapentaenoic acid</td>
<td>DPA (n-6) 22:5n-6</td>
<td>Docosahexaenoic acid DHA 22:6n-3</td>
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Prior to absorption in the small intestine, fatty acids must be hydrolyzed from dietary fats (triglycerides and phospholipids) by pancreatic enzymes (2). Bile salts must also be present in the small intestine to allow for the incorporation of fatty acids and other fat digestion products into mixed micelles. Fat absorption from mixed micelles occurs throughout the small intestine and is 85-95% efficient under normal conditions.

Blood concentrations of fatty acids reflect both dietary intake and biological processes (3). Humans can synthesize longer omega-6 and omega-3 fatty acids from the essential fatty acids LA and ALA, respectively, through a series of desaturation (addition of a double bond) and elongation (addition of two carbon atoms) reactions (Figure 3) (4, 5). LA and ALA compete for the same elongase and desaturase enzymes in the synthesis of longer polyunsaturated fatty acids, such as AA and EPA.
The capacity to generate DHA from ALA is higher in women than men. Studies of ALA metabolism in healthy young men indicate that approximately 8% of dietary ALA is converted to EPA and 0-4% is converted to DHA (6). In healthy young women, approximately 21% of dietary ALA is converted to EPA and 9% is converted to DHA (7). The better conversion efficiency of young women compared to men appears to be related to the effects of estrogen (8, 9). Although ALA is considered the essential omega-3 fatty acid because it cannot be synthesized by humans, evidence that human conversion of EPA and, particularly, DHA is relatively inefficient suggests that EPA and DHA may be considered conditionally essential nutrients.

In addition to gender differences, genetic variability in enzymes involved in fatty acid metabolism influences one's ability to generate long-chain polyunsaturated fatty acids (LC-PUFA). Two key enzymes in fatty acid metabolism are delta-6 desaturase (FADS2) and delta-5 desaturase (FADS1) (see Figure 3 above) (10). Two common haplotypes (a cluster of polymorphisms) in the FADS genes

Humans can synthesize longer omega-6 and omega-3 fatty acids from the essential fatty acids LA and ALA through a series of desaturation (addition of a double bond) and elongation (addition of two carbon atoms) reactions. Delta-6 desaturase (FADS2) is considered the rate-limiting enzyme in this metabolic pathway. Retroconversion of DHA to EPA in peroxisomes occurs at low basal rates and following DHA supplementation (4, 5). FADS2, delta-6 desaturase; FADS1, delta-5 desaturase; Elvol2, Elvol5, elongases.
differ dramatically in their ability to generate LC-PUFA: haplotype D is associated with increased FADS activity (both FADS1 and FADS2) and is more efficient in converting fatty acid precursors (LA and ALA) to LC-PUFA (EPA, GLA, DHA, and AA) (11). These FADS polymorphisms are relatively common in the population and may explain up to 30% of the variability in blood levels of omega-3 and omega-6 fatty acids among individuals (3).

Finally, DHA is retroconverted to EPA at a low basal rate and following supplementation (see Figure 3 above) (12). After supplementing omnivores (n=8) and vegetarians (n=12) for six weeks with an EPA-free preparation of DHA (1.62 g/day), both EPA and DHA levels increased in serum and platelet phospholipids (13). Based on the measured changes, the estimated percent retroconversion of DHA to EPA was 7.4-11.4% (based on serum phospholipid data) and 12.3-13.8% (based on the platelet phospholipid data), with no significant difference between omnivores and vegetarians. Due to this nontrivial retroconversion efficiency, DHA supplementation represents an alternative to fish oil to increase blood and tissue levels of EPA, DPA, and DHA (5) (see Supplements).

**Biological Activities**

**Membrane structure and function**

Omega-6 and omega-3 PUFA are important structural components of cell membranes. When incorporated into phospholipids, they affect cell membrane properties, such as fluidity, flexibility, permeability, and the activity of membrane-bound enzymes (14). In addition to endogenous metabolism, dietary consumption of fatty acids can modify the composition and molecular structure of cellular membranes. Thus, increasing omega-3 fatty acid intake increases the omega-3 content of red blood cells, immune cells (15), atherosclerotic plaques (16), cardiac tissue (17), and other cell types throughout the body.

DHA is selectively incorporated into retinal cell membranes and postsynaptic neuronal cell membranes, suggesting it plays important roles in vision and nervous system function.

**Vision**

DHA is found at very high concentrations in the cell membranes of the retina; the retina conserves and recycles DHA even when omega-3 fatty acid intake is low (18). Animal studies indicate that DHA is required for the normal development and function of the retina. Moreover, these studies suggest that there is a critical period during retinal development when inadequate DHA will result in permanent abnormalities in retinal function. Research indicates that DHA plays an important role in the regeneration of the visual pigment rhodopsin, which plays a critical role in the visual transduction system that converts light hitting the retina to visual images in the brain (19).

**Nervous system**

The phospholipids of the brain’s gray matter contain high proportions of DHA and AA, suggesting they are important to central nervous system function (20). Brain DHA content may be particularly important, since animal studies have shown that depletion of DHA in the brain can result in
learning deficits. It is not clear how DHA affects brain function, but changes in DHA content of neuronal cell membranes could alter the function of ion channels or membrane-associated receptors, as well as the availability of neurotransmitters (21).

**Synthesis of lipid mediators**

**Eicosanoids**

Eicosanoids are potent chemical messengers that play critical roles in immune and inflammatory responses. The term 'eicosanoid' encompasses numerous bioactive lipid mediators that are derived from 20-carbon LC-PUFA. Following stimulation by hormones, cytokines, and other stimuli, DGLA, AA, and EPA are released from cell membranes and become substrates for eicosanoid production (Figure 4). Eicosanoid synthesis relies primarily on three families of enzymes: cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome p450 mono-oxygenases (P450s) (22). From 20-carbon lipid precursors, COX enzymes produce prostaglandins, prostacyclins, and thromboxanes (collectively known as prostanoids); LOX produces leukotrienes and hydroxy fatty acids; and P450s produce hydroxyeicosatetraenoic acids ("HETEs") and epoxides (Figure 5).
The cell membrane serves as a pool of PUFA available for further metabolism to various bioactive lipids. Various environmental signals induce the enzyme phospholipase A2 to cleave fatty acids from the sn2 position of membrane phospholipids. Liberated fatty acids serve as substrates for the production of various bioactive lipid mediators (22). DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; SPMs, specialized pro-resolving mediators.
Physiological responses to AA-derived eicosanoids differ from responses to EPA-derived eicosanoids. In general, eicosanoids derived from EPA are less potent inducers of inflammation, blood vessel constriction, and coagulation than eicosanoids derived from AA (23). Nonetheless, it is an oversimplification to label all AA-derived eicosanoids as pro-inflammatory. AA-derived prostaglandins induce inflammation but also inhibit pro-inflammatory leukotrienes and cytokines and induce anti-inflammatory lipoxins, thereby modulating the intensity and duration of the inflammatory response via negative feedback (see Figure 5 above) (16).

**Pro-resolving mediators**

A separate class of PUFA-derived bioactive lipids, specialized pro-resolving mediators (SPMs), has been recently identified (reviewed in 24). These molecules function as local mediators of the resolution phase of inflammation, actively turning off the inflammatory response. SPMs are derived from both omega-6 and omega-3 PUFA (see Figure 5 above) (25). The S-series of SPMs results from the LOX-mediated oxygenation of EPA and DHA, giving rise to S-resolvins, S-protectins, and S-maresins. A second class of SPMs, the R-series, is generated from the aspirin-dependent
acetylation of COX-2 and subsequent generation of aspirin-triggered SPMs from AA, EPA, and DHA. It appears that these mediators may explain many of the anti-inflammatory actions of omega-3 fatty acids that have been described (15, 26).

**Isoprostanes**

Isoprostanes are prostaglandin-like compounds that are formed by non-enzymatic, free radical-induced oxidation of any PUFA with three or more double bonds (see Figure 5 above) (22). Because they are produced upon exposure to free radicals, isoprostanes are often used as markers for oxidative stress. In contrast to prostanoids, isoprostanes are synthesized from esterified PUFA precursors and remain bound to the membrane phospholipid until cleaved by PLA2 and released into circulation. In addition to being used as markers of oxidative stress, isoprostanes may also function as inflammatory mediators, exerting both pro- and anti-inflammatory effects (22).

**Regulation of gene expression**

The results of cell culture and animal studies indicate that omega-6 and omega-3 fatty acids can modulate the expression of a number of genes, including those involved with fatty acid metabolism and inflammation (27, 28). Omega-6 and omega-3 fatty acids regulate gene expression by interacting with specific transcription factors, such as peroxisome proliferator-activated receptors (PPARs) (29). In many cases, PUFA act like hydrophobic hormones (e.g., steroid hormones) to control gene expression and bind directly to receptors like PPARs. These ligand-activated receptors then bind to the promoters of genes and function to increase/decrease transcription.

In other cases, PUFA regulate the abundance of transcription factors inside the cell's nucleus (30). Two examples include NFκB and SREBP-1. NFκB is a transcription factor involved in regulating the expression of multiple genes involved in inflammation. Omega-3 PUFA suppress NFκB nuclear content, thus inhibiting the production of inflammatory eicosanoids and cytokines. SREBP-1 is a major transcription factor controlling fatty acid synthesis, both de novo lipogenesis and PUFA synthesis. Dietary PUFA can suppress SREBP-1, which decreases the expression of enzymes involved in fatty acid synthesis and PUFA synthesis. In this way, dietary PUFA function as feedback inhibitors of all fatty acid synthesis.

**Deficiency**

**Essential fatty acid deficiency**

Clinical signs of essential fatty acid deficiency include a dry scaly rash, decreased growth in infants and children, increased susceptibility to infection, and poor wound healing (31). Omega-3, omega-6, and omega-9 fatty acids compete for the same desaturase enzymes. The desaturase enzymes show preference for the different series of fatty acids in the following order: omega-3 > omega-6 > omega-9. Consequently, synthesis of the omega-9 fatty acid eicosatrienoic acid (20:3n-9, mead acid, or 5,8,11-eicosatrienoic acid) increases only when dietary intakes of omega-3 and omega-6 fatty acids are very low; therefore, mead acid is one marker of essential fatty acid deficiency (32). A
plasma eicosatrienoic acid:arachidonic acid (triene:tetraene) ratio greater than 0.2 is generally considered indicative of essential fatty acid deficiency (31, 33). In patients who were given total parenteral nutrition containing fat-free, glucose-amino acid mixtures, biochemical signs of essential fatty acid deficiency developed in as little as 7 to 10 days (34). In these cases, the continuous glucose infusion resulted in high circulating insulin levels, which inhibited the release of essential fatty acids stored in adipose tissue. When glucose-free amino acid solutions were used, parenteral nutrition up to 14 days did not result in biochemical signs of essential fatty acid deficiency. Essential fatty acid deficiency has also been found to occur in patients with chronic fat malabsorption (35) and in patients with cystic fibrosis (36). Recently, it has been proposed that essential fatty acid deficiency may play a role in the pathology of protein-energy malnutrition (32).

**Omega-3 fatty acid deficiency**

At least one case of isolated omega-3 fatty acid deficiency has been reported. A young girl who received intravenous lipid emulsions with very little ALA developed visual problems and sensory neuropathy; these conditions were resolved when she was administered an emulsion containing more ALA (37). Isolated omega-3 fatty acid deficiency does not result in increased plasma triene:tetraene ratios, and skin atrophy and dermatitis are absent (1). Plasma DHA concentrations decrease when omega-3 fatty acid intake is insufficient, but no accepted plasma omega-3 fatty acid or eicosanoid concentrations indicative of impaired health status have been defined (1). Studies in rodents have revealed significant impairment of n-3 PUFA deficiency on learning and memory (38, 39) prompting research in humans to assess the impact of omega-3 PUFA on cognitive development and cognitive decline (see Visual and neurological development and Alzheimer's disease).

**Omega-3 index**

The omega-3 index is defined as the amount of EPA plus DHA in red blood cell (RBC) membranes expressed as the percent of total RBC membrane fatty acids (40). The EPA + DHA content of RBCs correlates with that of cardiac muscle cells (41, 42), and several observational studies indicate that a lower omega-3 index is associated with an increased risk of coronary heart disease (CHD) mortality (43). It is therefore proposed that the omega-3 index be used as a biomarker for cardiovascular disease risk, with proposed zones being high risk, <4%; intermediate risk, 4-8%; and low risk, >8% (44).

Supplementation with EPA + DHA from fish oil capsules for approximately five months dose-dependently increased the omega-3 index in 115 healthy, young adults (20-45 years of age), validating the use of the omega-3 index as a biomarker of EPA + DHA intake (45). Before the omega-3 index can be used in routine clinical evaluation, however, clinical reference values in the population must be established (46). Additionally, fatty acid metabolism may be altered in certain disease states, potentially making the omega-3 index less relevant for some cardiovascular conditions (5).
Disease Prevention

Visual and neurological development

The last trimester of pregnancy and first six months of postnatal life are critical periods for the accumulation of DHA in the brain and retina (47). Human milk contains a mixture of saturated fatty acids (~46%), monounsaturated fatty acids (~41%), omega-6 PUFA (~12%), and omega-3 PUFA (~1.3%) (48). Although human milk contains DHA in addition to ALA and EPA, ALA was the only omega-3 fatty acid present in conventional infant formulas until the year 2001.

Infant formulas

Although infants can synthesize DHA from ALA, they generally cannot synthesize enough to prevent declines in plasma and cellular DHA concentrations without additional dietary intake. Therefore, it was proposed that infant formulas be supplemented with enough DHA to bring plasma and cellular DHA levels of formula-fed infants up to those of breast-fed infants (49). Although formulas enriched with DHA raise plasma and red blood cell DHA concentrations in preterm and term infants, the results of randomized controlled trials (RCTs) examining measures of visual acuity and neurological development in infants fed formulas with or without added DHA have been mixed (50, 51). A 2012 meta-analysis of RCTs (12 trials, 1,902 infants) testing LC-PUFA supplemented versus unsupplemented formula, started within one month of birth, found no effect of LC-PUFA supplementation on infant cognition assessed at approximately one year of age (52). A lack of effect was observed regardless of the dose of LC-PUFA or the prematurity status of the infant. With respect to visual acuity, a 2013 meta-analysis of RCTs (19 trials, 1,949 infants) found a beneficial effect of LC-PUFA-supplemented formula, started within one month of birth, on infant visual acuity up to 12 months of age (53). Notably, two different types of visual acuity assessment were evaluated in the meta-analysis. Visual acuity assessed by using the visually evoked potential (VEP) (10 trials, 852 infants) showed a significant positive effect of LC-PUFA supplemented formula at 2, 4, and 12 months of age. When assessed by the behavioral method (BM) (12 trials, 1,095 infants), a significant benefit of LC-PUFA-supplemented formula on visual acuity was found only at the age of two months. No moderating effects of dose or prematurity status were observed.

Maternal supplementation (placental transfer and breast milk)

The effect of maternal omega-3 LC-PUFA supplementation on early childhood cognitive and visual development was evaluated in a 2013 systematic review and meta-analysis (54). Included in this assessment were 11 RCTs (a total of 5,272 participants) that supplemented maternal diet with omega-3 LC-PUFA during pregnancy or during pregnancy and lactation. Visual outcomes (eight trials) could not be evaluated in the meta-analysis due to variability in assessments; overall, four of six trials had null findings and the remaining two trials had very high rates of attrition. Cognitive outcomes (nine trials) included the Developmental Standard Score (DSS; in infants, toddlers, and preschoolers) or Intelligence Quotient (IQ; in children) and other aspects of neurodevelopment, such as language, behavior, and motor function. No differences were found between DHA and control groups for cognition measured with standardized psychometric scales in infants (<12
months), toddlers (12-24 months), and school aged children (5-12 years); preschool children (2-5 years) in the DHA treatment group had a 3.92 point increase in DSS compared to controls. The authors note that many of the trials of LC-PUFA supplementation in pregnancy had methodological weaknesses (e.g., high rates of attrition, small sample sizes, high risk of bias, multiple comparisons) limiting the confidence and interpretation of the pooled results.

Although epidemiological investigations have demonstrated that higher intakes of omega-3 LC-PUFA from fish and seafood during pregnancy are associated with improved developmental outcomes in offspring (54), trial evidence does not conclusively support or refute this relationship. At present, the potential benefits associated with obtaining long-chain omega-3 fatty acids through moderate consumption of fish (e.g., 1-2 servings weekly) during pregnancy and lactation outweigh any risks of contaminant exposure, though fish with high levels of methylmercury should be avoided (55). For information about contaminants in fish and guidelines for fish consumption by women of childbearing age, see Contaminants in fish.

**Gestation and pregnancy**

The results of randomized controlled trials (RCTs) during pregnancy suggest that omega-3 fatty acid supplementation does not decrease the incidence of gestational diabetes, pregnancy-induced hypertension, or preeclampsia (56-58) but may result in modest increases in length of gestation, especially in women with low omega-3 fatty acid consumption. A 2006 meta-analysis of six randomized controlled trials in women with low-risk pregnancies found that omega-3 PUFA supplementation during pregnancy resulted in an increased length of pregnancy by 1.6 days (59). A 2007 meta-analysis of randomized controlled trials in women with high-risk pregnancies found that supplementation with long-chain PUFA did not affect pregnancy duration or the incidence of premature births but decreased the incidence of early premature births (<34 weeks of gestation; 2 trials, N=291; Relative Risk [RR]: 0.39 (95% CI: 0.18-0.84) (60).

Because maternal dietary intake of LC-PUFA determines the DHA status of the newborn, several expert panels in the US recommend that pregnant and lactating women consume at least 200 mg DHA per day, close to the amount recommended for adults in general (250 mg/day) (47, 61). The European Food and Safety Authority (EFSA) recommends that pregnant and lactating women consume an additional 100-200 mg of preformed DHA on top of the 250 mg/day EPA plus DHA recommended for healthy adults (62).

**Cardiovascular disease**

**Omega-6 fatty acids: linoleic acid**

LA is the most abundant dietary PUFA and accounts for approximately 90% of dietary omega-6 PUFA intake (63). Taking into consideration the results from RCTs and observational cohort studies, a 2009 American Heart Association scientific advisory concluded that obtaining at least 5-10% of total caloric intake from omega-6 PUFA is associated with a reduced risk of coronary heart disease (CHD) relative to lower intakes (64, 65). A pooled analysis of 11 cohort studies, encompassing
344,696 individuals followed for 4 to 10 years, found that replacing 5% of energy from saturated fatty acids (SFAs) with PUFA was associated with a 13% lower risk of coronary events (95% CI: 0.77, 0.97) and a 26% lower risk of coronary deaths (95% CI: 0.61, 0.89) (66). A 2012 meta-analysis of seven RCTs corroborated this beneficial effect, with an estimated 10% reduction in CHD risk (RR: 0.90, 95% CI: 0.83-0.97) for each 5% energy increase in PUFA consumption (67).

In controlled feeding trials, replacing dietary SFA with PUFA consistently lowers serum total and LDL cholesterol concentrations (68, 69). In fact, LA has been shown to be the most potent fatty acid for lowering serum total and LDL cholesterol when substituted for dietary SFA (70). The mechanisms by which linoleic acid lowers blood cholesterol include (1) the upregulation of LDL receptor and redistribution of LDL-C from plasma to tissue, (2) increased bile acid production and cholesterol catabolism, and (3) decreased conversion of VLDL to LDL (71).

Although dietary LA lowers blood cholesterol levels, supplementation with concentrated sources of LA may have adverse cardiovascular effects in individuals with preexisting CHD (see Disease Treatment).

**Omega-3 fatty acids: α-linolenic acid**

Several prospective cohort studies have examined the relationship between dietary ALA intake and cardiovascular disease (CVD). A 2012 meta-analysis of observational studies evaluated the risk of incident CVD related to dietary consumption or biomarkers of ALA (72). The analysis included 27 studies, 251,049 individuals and 15,327 CVD events (fatal coronary heart disease [CHD], nonfatal CHD, total CHD, and stroke). Overall, the pooled analysis found a moderately lower risk of CVD with higher ALA exposure (Relative Risk [RR]: 0.86; 95% CI: 0.77, 0.97).

Unlike LA, the cardioprotective effects of higher ALA intakes do not appear to be related to changes in serum lipid profiles. A meta-analysis of 14 randomized controlled trials concluded that ALA supplementation had no effect on total cholesterol, LDL cholesterol, or triglyceride levels (73). However, several controlled clinical trials have found that increasing ALA intake decreased serum concentrations of C-reactive protein (CRP), a marker of inflammation that is strongly associated with the risk of cardiovascular events, such as MI and stroke (74-76).

**Long-chain omega-3 fatty acids: eicosapentaenoic acid and docosahexaenoic acid**

Evidence is accumulating that increasing intakes of long-chain omega-3 fatty acids (EPA and DHA) can decrease the risk of cardiovascular disease by (1) preventing arrhythmias that can lead to sudden cardiac death, (2) decreasing the risk of thrombosis (a clot) that can lead to myocardial infarction (MI) or stroke, (3) decreasing serum triglyceride levels, (4) slowing the growth of atherosclerotic plaque, (5) improving vascular endothelial function, (6) lowering blood pressure slightly, and (7) decreasing inflammation (77).
In spite of these possible biological effects, clinical trials have not shown a significant effect of long-chain omega-3 supplementation on major cardiovascular events. A 2006 systematic review and meta-analysis of randomized controlled trials and prospective cohort studies concluded that long-chain omega-3 fatty acids do not significantly reduce the risk of total mortality or cardiovascular events (78). Likewise, a 2012 meta-analysis of secondary prevention trials (20 RCTs, including 68,680 patients) found no significant effect of omega-3 supplements (~1.5 g/day of EPA + DHA for a median of 2 years) on all-cause mortality, cardiac death, sudden death, myocardial infarction, or stroke (79). The same lack of effect was observed in a 2012 systematic review and meta-analysis of RCTs investigating the impact of omega-3 supplementation on inflammatory biomarkers in both healthy and ill individuals (80).

Although supplementation trials have not demonstrated a clear clinical benefit of omega-3 supplements, a recent multicenter, prospective, observational cohort study found a strong relationship between plasma phospholipid omega-3 PUFA levels (a biomarker of omega-3 status) and cardiovascular mortality (81). The Cardiovascular Health Study (CHS) related circulating levels of total and individual LC-PUFA (EPA, DPA, and DHA) to risk of total and CVD-specific mortality in 2,692 older (≥65 years) US adults. Higher levels of individual and combined total omega-3 PUFA in plasma phospholipids were associated with lower total mortality (HR for total omega-3: 0.73, 95% CI: 0.61-0.86). Looking more closely at cause-specific mortality, the observed reduction in risk was attributed mainly to fewer arrhythmic cardiac deaths (HR: 0.52, 95% CI: 0.31-0.86) and specifically with higher circulating DHA content (45% lower risk). Only EPA was associated with nonfatal MI (28% lower risk), while DPA was most strongly associated with stroke death (47% lower risk).

**Coronary heart disease:** A 2012 meta-analysis of 17 cohort studies with 315,812 participants and an average follow-up of 15.9 years calculated the pooled effect of fish consumption on coronary heart disease (CHD) mortality (82). Low (1 serving/week) or moderate fish consumption (2-4 servings/week) had a significant beneficial effect on the prevention of CHD mortality. Specifically, compared with the lowest fish consumption (<1 serving/month or 1-3 servings/month), consumption of 1 serving of fish per week and 2-4 servings/week was associated with a 16% (RR: 0.84, 95% CI: 0.75, 0.95) and 21% (RR: 0.79, 95% CI: 0.67,0.92) lower risk of fatal CHD, respectively.

Overall, among the various CVD outcomes (Figure 6), findings from prospective cohort studies and RCTs consistently indicate that consumption of fish or fish oil significantly reduces CHD mortality, including fatal myocardial infarction and sudden cardiac death (77, 83, 84). There is little evidence that these effects differ by sex, age, or race/ethnicity (77).
Cardiovascular disease (CVD) is an umbrella term that encompasses all diseases of the heart and blood vessels. There is a general consensus that adequate intakes of fish, fish oil, or omega-3 PUFA supplements have favorable effects on cardiovascular outcomes. However, the primary cardioprotective effect of long-chain omega-3 PUFA seems to be on the last step of CHD, arrhythmia. For this reason, sudden cardiac death (SCD) appears to be the most consistently affected clinical outcome (77, 81).

**Sudden cardiac death:** Sudden cardiac death (SCD) is the result of a fatal ventricular arrhythmia, which usually occurs in people with CHD. Studies in cell culture indicate that long-chain omega-3 fatty acids decrease the excitability of cardiac muscle cells (myocytes) by modulating ion channel conductance (85). The results of epidemiological studies suggest that regular fish consumption is inversely associated with the risk of SCD. A 2011 systematic review and meta-analysis of eight prospective cohort studies evaluated the impact of consuming <250 mg versus ≥250 mg omega-3 PUFA per day on various CHD outcomes (86). Consumption of ≥250 mg omega-3 PUFA per day was associated with a significant, 35% reduction in the risk of SCD (RR: 0.65; 95% CI: 0.54, 0.79).

A meta-analysis of nine randomized controlled trials found no significant effect of omega-3 supplements on SCD or ventricular arrhythmias in patients with previous MI compared to those taking placebo (87). Notably, although the pooled analysis reported no significant effect, the included trials reported either a protective effect (six trials) or null effect (three trials), with no harmful outcomes reported.
**Stroke:** Ischemic strokes are the result of insufficient blood flow to an area of the brain, which may occur when an artery supplying the brain becomes occluded by a clot. Hemorrhagic strokes occur when a blood vessel ruptures and bleeds into the brain. In the United States, 87% of strokes are ischemic strokes (88). A 2012 meta-analysis of 16 prospective studies, encompassing 402,127 individuals for a mean of 12.8 years, found that increased fish intake was associated with a decreased risk of ischemic stroke, but not hemorrhagic stroke (89). According to this analysis, consuming fish even once per week may significantly reduce the risk of ischemic stroke. In a separate dose-response meta-analysis of these prospective studies, a 3-servings/week increase in fish consumption was associated with a 6% decreased risk of total stroke (95% CI: 0.89-0.99) (90). Again, the association remained significant only for ischemic stroke (RR: 0.90, 95% CI: 0.84-0.97).

Although the protective effect of fish intake could be attributed to many things (e.g., the displacement of red meat, a marker of an overall healthier lifestyle and dietary pattern, nutrient interactions (91, 92)), its high content of omega-3 PUFA may be a major contributing factor. A meta-analysis of eight prospective studies that assessed the association between omega-3 PUFA intake on stroke risk found evidence of a nonlinear relationship between LC-PUFA intake and stroke risk, with only moderate intakes of 200-400 mg/day omega-3 PUFA associated with significantly reduced risk of total stroke (93). Additionally, when analyzed by stroke type, the risk for ischemic stroke was lower in the highest versus lowest category of long-chain omega-3 PUFA intake (RR: 0.82, 95% CI: 0.71-0.94).

Another 2012 systematic review and meta-analysis assessed both prospective cohort studies and RCTs that investigated fish consumption or omega-3 supplementation on cerebrovascular disease (any fatal or non-fatal ischemic stroke, hemorrhagic stroke, cerebrovascular accident, or transient ischemic attack) (91). From 26 prospective cohort studies, the pooled relative risk (RR) for cerebrovascular disease for 2-4 versus ≤1 serving of fish per week was 0.94 (95% CI: 0.90-0.98); for >5 servings versus ≤1 serving per week, the RR was 0.88 (95% CI: 0.81-0.96). No significant association was found between long-chain omega-3 biomarkers and risk of cerebrovascular disease. From the 12 RCTs analyzed, 10 of which recruited subjects with preexisting cardiovascular disease at baseline, no significant effect of omega-3 supplementation (mean dose of 1.8 g/day for a mean duration of 3 years) on cerebrovascular disease outcomes was observed. The same lack of effect of omega-3 supplementation on total stroke risk was observed in a second meta-analysis of RCTs (nine trials) (79).

**Serum triglycerides:** A meta-analysis of 17 prospective studies found hypertriglyceridemia (serum triglycerides >200 mg/dL) to be an independent risk factor for cardiovascular disease (94). Numerous controlled clinical trials have demonstrated that increasing intakes of EPA and DHA significantly lower serum triglyceride concentrations (95). The triglyceride-lowering effects of EPA and DHA increase with dose (96), but clinically meaningful reductions in serum triglyceride concentrations have been demonstrated at doses of 2 g/day of EPA + DHA (97). In its
recommendations regarding omega-3 fatty acids and cardiovascular disease (see Intake Recommendations), the American Heart Association indicates that an EPA + DHA supplement may be useful in patients with hypertriglyceridemia (23).

A 2011 meta-analysis of RCTs compared the effect of EPA alone (10 trials), DHA alone (17 trials), or EPA versus DHA (6 trials) on serum lipids (98). Although both EPA and DHA reduce triglyceride levels, they have different effects on LDL and HDL levels. DHA raises LDL and HDL, whereas EPA has no significant effect. Importantly, DHA may increase LDL via increased conversion of VLDL to LDL and by producing larger, more buoyant LDL particles (3).

**Summary: omega-3 and omega-6 PUFA and cardiovascular disease prevention**

The results of observational studies and randomized controlled trials suggest that replacing dietary SFA with omega-6 and omega-3 PUFA (from both plant and marine sources) lowers LDL cholesterol and decreases cardiovascular disease risk. Additionally, the results of epidemiological studies provide consistent evidence that increasing dietary omega-3 fatty intake is associated with significant reductions in cardiovascular disease risk through mechanisms other than lowering LDL cholesterol. In particular, increasing fish consumption to at least two servings of oily fish per week has been associated with significant reductions in fatal myocardial infarction and sudden cardiac death (77). This amount would provide about 400-500 mg/day of EPA + DHA (23).

**Alzheimer's disease**

Alzheimer's disease is the most common cause of dementia in older adults. Alzheimer's disease is characterized by the formation of amyloid plaque in the brain and nerve cell degeneration. Disease symptoms, including memory loss and confusion, worsen over time (99). Some epidemiological studies have associated high intake of fish with lower risks of impaired cognitive function (100), dementia (101), and Alzheimer's disease (101, 102). Proposed mechanisms for a protective effect of long-chain omega-3 fatty acids in the brain and vascular system include (1) the mitigation of inflammation, (2) improved cerebral blood flow, and (3) reduced amyloid aggregation (103).

A 2009 systematic review reported on the association between eating fish (as a source of long-chain omega-3 fatty acids) or taking an omega-3 supplement and the risk of cognitive decline or Alzheimer's disease (103). Out of 11 observational studies, three reported a significant benefit of omega-3 fatty acids on cognitive decline; four of eight observational studies reported positive findings on incident Alzheimer's disease or dementia. The four small clinical trials reviewed showed no evidence for prevention or treatment of any form of dementia (103).

DHA, the major omega-3 fatty acid in the brain, appears to be protective against Alzheimer's disease (104). Observational studies have found that lower DHA status is associated with increased risk of Alzheimer's disease (105-107), as well as other types of dementia (106). The relationship between DHA status and cognitive decline may be dependent on apolipoprotein E (APOE) genotype. Of three common APOE alleles (epsilon 2 [ε2], ε3, and ε4), the presence of the APOE ε4 (E4) allele is associated with increased risk and earlier onset of Alzheimer's disease (108).
protective effect of consumption of fatty fish on the risk for dementia and AD may not apply to carriers of the E4 allele (109, 110). EPA + DHA supplementation did not increase plasma levels of these omega-3 PUFA to the same extent in E4 carriers compared to non-carriers (111), and [13C]DHA tracer studies indicate that DHA metabolism differs in E4 carriers, with greater oxidation and lower plasma levels in E4 positive versus negative individuals (112).

Overall, the data favor a role for diets rich in long-chain omega-3 fatty acids in slowing cognitive decline but not for supplementation in the prevention or treatment of any type of dementia. The efficacy of omega-3 supplementation may depend on the underlying pathology of AD (i.e., the involvement of a vascular issue) (103) or the presence of the APOE4 allele (110, 111). Additionally, consistency in outcome measures and diagnostic criteria, and longer duration trials may be necessary to see a consistent effect.

Disease Treatment

Coronary heart disease

_Dietary intervention trials_

**Omega-6 fatty acids** (linoleic acid): In a reanalysis of the Sydney Heart Health Study (SHHS), a single-blind, RCT in 458 men (ages 30-59 years) with a recent coronary event, the replacement of dietary saturated fat with omega-6 linoleic acid led to higher rates of death from all-causes, cardiovascular disease, and coronary heart disease (CHD) compared to controls (113). Furthermore, a meta-analysis that included the SHHS and two other secondary prevention trials revealed an increased risk of mortality when saturated fat is replaced with concentrated sources of linoleic acid. There are some important limitations and considerations with the SHHS to keep in mind: (1) LA intake went from 6% to 15% of total energy for the study participants; in US adults, the average intake of LA is approximately 7% of total energy (114); (2) there may have been displacement of monounsaturated fatty acids and other PUFA in addition to saturated fats in the experimental intervention; and (3) the experimental formulation of safflower oil margarine may have provided _trans_ fat. Regardless of these issues, substituting dietary saturated fat with mixed PUFA (both omega-6 and omega-3) rather than linoleic acid alone reduces CVD risk (113) and is recommended for both the primary and secondary prevention of CVD (65, 67).

**Omega-3 fatty acids**: In the Diet and Reinfarction Trial (DART), total mortality and fatal MI decreased by 29% in male MI survivors advised to increase their weekly intake of oily fish to 200-400 g (7-14 oz)—an amount estimated to provide an additional 500-800 mg/day of long-chain omega-3 fatty acids (EPA + DHA) (115). The Diet and Reinfarction Trial 2 (DART-2) administered similar dietary advice but to a different cohort of high-risk individuals: those with stable angina (116). In this case, advice to eat oily fish or fish oil did not affect all-cause mortality but was associated with an increased risk of sudden cardiac death. This increased risk was confined to the use of fish oil capsules rather than dietary fish intake. Though the results of the DART trials seem to contradict each other, there are important differences that offer explanations, namely the timing of the
intervention (shortly after first MI in the first trial) and the stage of CHD (early versus stable) in the study population. These trials suggest that fish oil may reduce mortality during recovery from MI but perhaps not during later stages of the disease.

The Alpha Omega Trial tested if low doses of EPA + DHA (400 mg per day), ALA (2 g per day), or both in margarines reduced the risk of cardiovascular (CV) events among 4,837 patients (78% male; mean age, 69 years) who had a MI in the previous 10 years (117). After 40 months, none of the omega-3 PUFA treatments significantly affected the rate of major CV events compared to placebo. Notably, medication use in the study population was high: antithrombotic agents (97.5%), antihypertensive drugs (89.7%), and statins (85%).

**Supplementation trials**

In the largest randomized controlled trial of supplemental omega-3 fatty acids to date, the GISSI-Prevenzione Trial, CHD patients who received supplements providing 850 mg/day of EPA + DHA for 3.5 years had a risk of sudden death that was 45% lower than those who did not take supplements; supplement users also experienced a 20% lower risk of death from all causes compared to non-supplement users (118). Interestingly, it took only three months of supplementation to demonstrate a significant decrease in total mortality and four months to demonstrate a significant decrease in sudden death (119).

The results of a meta-analysis that pooled the findings of 29 randomized controlled trials of dietary or supplementary omega-3 fatty acids indicated that omega-3 fatty acids were not associated with a statistically significant reduction in all-cause mortality or risk of restenosis in high-risk cardiovascular patients (120). Heterogeneity in trial size and follow-up time limited the analysis, and the authors note that the probability of benefit from omega-3 fatty acids still remains high for both endpoints.

**Summary**

The results of randomized controlled trials in individuals with documented CHD suggest a beneficial effect of dietary and supplemental omega-3 fatty acids. Based on the results of these trials, the American Heart Association recommends that individuals with documented CHD consume approximately 1 g/day of EPA + DHA, preferably by consumption of oily fish (see Intake Recommendations) (121).

**Diabetes mellitus**

Cardiovascular disease are the leading causes of death in individuals with diabetes mellitus (DM). The dyslipidemia typically associated with diabetes is characterized by a combination of hypertriglyceridemia (serum triglycerides >200 mg/dL), low HDL-C, and abnormal LDL composition (122).
A 2009 meta-analysis of 23 randomized controlled trials (RCTs), including 1,075 individuals with type 2 diabetes, found that omega-3 fatty acid supplementation (mean dose, 3.5 g/day) lowered serum triglyceride levels by 0.45 mmol/L, lowered VLDL-C by 0.07 mmol/L, but raised LDL-C by 0.11 mmol/L (123). No significant changes in total cholesterol, HDL-C, HbA1c, fasting glucose, fasting insulin, or body weight were observed.

Since 2009, the results of two additional trials of omega-3 supplementation in diabetic patients have been published. The Alpha Omega Trial evaluated the effect of low-dose supplementation with omega-3 fatty acids on ventricular arrhythmias and fatal CHD in stable, post-MI patients (117). While the main analysis found no effect of supplementation, a secondary analysis of 1,014 diabetic participants found that low-dose supplementation of combined omega-3 fatty acids (~400 mg EPA + DHA and 2 g ALA per day in an experimental margarine spread for 40 months) resulted in fewer ventricular arrhythmia-related events (HR 0.16, 95% CI 0.04-0.69) compared to placebo margarine (124). In the ORIGIN trial, 1 g/day of omega-3 fatty acids (465 mg EPA + 375 mg DHA per day for 6 years) had no effect on rates of major vascular events, all-cause mortality, cardiovascular mortality, or death from arrhythmia in 12,536 dysglycemic patients at high risk for cardiovascular events compared to placebo (125). Triglyceride levels were reduced by 14.5 mg/dL (0.16 mmol/L), but no other blood lipids were affected by supplementation. In both of these trials, a high proportion of study participants used cardiovascular medications.

**Summary**

Increasing EPA and DHA intakes may be beneficial to diabetic individuals, especially those with elevated serum triglycerides or with a history of MI (126). There is no compelling evidence that daily EPA + DHA intakes of less than 3 g/day adversely affect long-term glycemic control in diabetics (127-129). The American Diabetes Association recommends that diabetic individuals increase omega-3 fatty acid consumption by consuming two to three 3-oz servings of fish weekly (121).

**Inflammatory diseases**

**Rheumatoid arthritis**

A 2012 meta-analysis of 10 randomized controlled trials, involving 270 rheumatoid arthritis (RA) patients, assessed the efficacy of high-dose omega-3 PUFA supplementation on clinical outcomes of RA (130). Omega-3 consumption of ≥2.7 g/day for a minimum of three months reduced nonsteroidal anti-inflammatory drug (NSAID) use but had no significant effect on tender joint count, swollen joint count, morning stiffness, or physical function compared to placebo. On the other hand, a 2012 systematic review, which included 23 trials investigating omega-3 supplementation (mainly as fish oil) in RA patients, concluded that there appears to be a consistent, though modest, benefit of marine-derived omega-3 PUFA (average intake ~3g/day) on some clinical symptoms of RA (131). Thus, high-dose supplementation of long-chain omega-3 PUFA spares the need for anti-inflammatory medications and may reduce joint pain and swelling in some RA patients.
**Inflammatory bowel disease**

**Crohn's disease:** A 2013 systematic review evaluated the efficacy of omega-3 supplementation in Crohn's disease (CD) patients, considering the evidence base from both short-term (9 to 24 weeks) and long-term (1 year) trials (132). Among five trials that evaluated the efficacy of omega-3 supplementation on CD relapse rates, conflicting outcomes were reported. Most trials were limited by small sample sizes and short duration; up to three years may be necessary to see an effect on CD relapse rates given the natural relapsing-remitting course of the disease. The two largest and most recent trials (EPIC-1 and EPIC-2) showed no significant effect of omega-3 supplementation on symptoms of CD remission compared to placebo (133). Two additional systematic reviews and a meta-analysis reached similar conclusions (134, 135). Three short-term trials showed positive effects of omega-3 supplementation on plasma biochemical parameters (e.g., reduced inflammatory cytokine expression, increased plasma EPA and DHA levels) compared to controls (132). In spite of its impact on biochemical changes in the short-term, however, the ability of omega-3 supplementation to maintain remission or effect clinically meaningful changes in CD is not supported by the current evidence.

**Ulcerative colitis:** Seven randomized controlled trials of fish oil supplementation in active ulcerative colitis (UC) patients reported significant improvement in at least one outcome measure, such as decreased corticosteroid use, improved disease activity scores, or improved histology scores (135). In inactive UC patients, omega-3 supplementation had no effect on relapse rates compared to placebo in four separate trials (134, 135).

While no serious side effects were reported in any trials of fish oil supplementation for the maintenance or remission of inflammatory bowel disease, diarrhea and upper gastrointestinal symptoms occurred more frequently with omega-3 treatment (134, 135).

**Asthma**

Inflammatory eicosanoids (leukotrienes) derived from arachidonic acid (AA; 20:4n-6) are thought to play an important role in the pathology of asthma (28). Since increasing omega-3 fatty acid intake has been found to decrease the formation of AA-derived leukotrienes, a number of clinical trials have examined the effects of long-chain omega-3 fatty acid supplementation on asthma. Although there is some evidence that omega-3 fatty acid supplementation can decrease the production of inflammatory mediators in asthmatic patients (136, 137), evidence that omega-3 fatty acid supplementation decreases the clinical severity of asthma in controlled trials has been inconsistent (138). Three systematic reviews of randomized controlled trials of long-chain omega-3 fatty acid supplementation in asthmatic adults and children found no consistent effects on clinical outcome measures, including pulmonary function tests, asthmatic symptoms, medication use, or bronchial hyperreactivity (139-141).

**Immunoglobulin A nephropathy**
Immunoglobulin A (IgA) nephropathy is a kidney disorder that results from the deposition of IgA in the glomeruli of the kidney. The cause of IgA nephropathy is not clear, but progressive renal failure may eventually develop in 15-40% of patients (142). Since glomerular IgA deposition results in increased production of inflammatory mediators, omega-3 fatty acid supplementation could potentially modulate the inflammatory response and preserve renal function.

A 2012 meta-analysis assessed the efficacy of omega-3 fatty acid treatment on adult IgA nephropathy (143). Five randomized controlled trials were included in an analysis involving 239 patients (mean age range, 37 to 41 years) who received placebo or supplemental EPA + DHA at doses of 1.4 to 5.1 g/day for 6 to 24 months. Compared with control groups, omega-3 treatment had no significant effect on urine protein excretion or glomerular filtration rate. Only two trials measured changes in serum creatinine, a marker of renal function, and end-stage renal disease; in both trials, omega-3 treatment had a beneficial effect on these two parameters. No adverse events associated with omega-3 treatment were reported in any of the trials.

**Neuropsychiatric disorders**

**Major depression and bipolar disorder**

Data from ecologic studies across different countries suggest an inverse association between seafood consumption and national rates of major depression (144) and bipolar disorder (145). Several small studies have found omega-3 fatty acid concentrations to be lower in the plasma (146-148) and adipose tissue (fat) (149) of individuals suffering from depression compared to controls. Although it is not known how omega-3 fatty acid intake affects the incidence of depression, modulation of neuronal signaling pathways and eicosanoid production have been proposed as possible mechanisms (150).

There may be some benefit of omega-3 PUFA supplementation on depressive disorders, but it is difficult to compare studies and draw conclusions due to great heterogeneity among the trials (151, 152). Small sample sizes, lack of standardization of therapeutic doses, type of omega-3 PUFA administered, co-treatment with pharmacological agents, and diagnostic criteria vary among the trials.

A 2012 systematic review of all published RCTs investigated the effect of omega-3 PUFA supplementation on the prevention and treatment of several types of depression and other neuropsychiatric disorders (151). With respect to major depression, a majority of studies reported a positive effect for omega-3 supplements on depressive symptoms, though efficacy is still considered inconclusive given the great variability among trials. A few themes emerged from this review: more trials reported positive effect for omega-3 PUFA supplements as an adjunct to pharmacological treatment; in monotherapy trials, EPA alone was more effective than DHA alone; and in combination trials, positive effects were more likely if an EPA:DHA ratio of >1.5–2.0 was administered.
Unipolar depression and bipolar disorder are considered distinct psychiatric conditions, although major depression occurs in both. As with major depression, the 2012 review of RCTs indicated that omega-3 supplementation may have a positive effect as an adjunct to therapy in patients with bipolar disorder (151).

While there is some promising evidence for the use of omega-3 fatty acids for major depression and bipolar disorder, additional trials that account for dietary omega-3 intake, changes in red blood cell PUFA levels, the ratio of EPA:DHA provided, co-treatment with medications, and of sufficient duration are necessary.

**Schizophrenia**

A 2013 meta-analysis of 18 studies compared the PUFA composition of red blood cell (RBC) membranes in schizophrenic patients to those of normal controls (153). The majority of studies investigated medicated patients, though the authors separated the analysis into three groups of patients at time of measurement in order to account for possible confounding from pharmacologic agents: antipsychotic-medicated, antipsychotic-naïve, and antipsychotic-free. Overall, decreased levels of DPA, DHA, and AA in RBC membranes were associated with the schizophrenic state. Several mechanisms may account for the altered PUFA levels in schizophrenia, such as altered lipid metabolism, increased oxidative stress, or changes in diet consequent to disease-related behavior.

The use of long-chain omega-3 fatty acid supplements to alleviate symptoms of schizophrenia or to mitigate adverse effects of antipsychotic medications has been investigated in a number of clinical trials (154). Overall, there was no effect of fish oil or LC-PUFA supplements on symptoms of schizophrenia. However, given the high safety profile of fish oil supplements and some evidence of a positive effect of EPA supplementation in a subset of trials, some clinicians may consider EPA a useful adjunct to antipsychotic therapy in schizophrenic patients.

**Alzheimer's disease and dementia**

Some epidemiological studies have associated decreased DHA status with Alzheimer's disease and other types of dementia (see above). Although results of studies in animal models have been promising (reviewed in 155), the existing clinical trial data do not support a recommendation for DHA supplementation in the treatment of Alzheimer's disease in humans. A randomized controlled trial (RCT) was conducted to determine if supplementation with DHA slows cognitive and functional decline in individuals with Alzheimer disease (156). Two hundred ninety-five individuals with mild to moderate Alzheimer's disease (Mini-Mental State Examination [MMSE] scores, 14-26) received algal DHA supplement (1 g twice daily) or placebo (corn or soy oil) for 18 months. Although DHA supplementation significantly increased levels of DHA in plasma phospholipids and cerebrospinal fluid, it had no effect on the rate of change on the cognitive subscale of the Alzheimer Disease Assessment Scale (ADAS-cog), the Clinical Dementia Rating (CDR) sum of boxes, or brain atrophy compared to placebo. No adverse effects were associated with DHA treatment in this trial.
Sources

Food sources

**Omega-6 fatty acids**

Linoleic acid: Food sources of LA include vegetable oils, such as soybean, saflower, and corn oil; nuts; seeds; and some vegetables. Dietary surveys in the US indicate that the average adult intake of LA ranges from 17-20 g/day for men and 12-13 g/day for women (63). Some foods that are rich in LA are listed in Table 2.

<table>
<thead>
<tr>
<th>Food</th>
<th>Serving</th>
<th>Linoleic Acid (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Safflower oil</td>
<td>1 tablespoon</td>
<td>10.1</td>
</tr>
<tr>
<td>Sunflower seeds, oil roasted</td>
<td>1 oz</td>
<td>9.7</td>
</tr>
<tr>
<td>Pine nuts</td>
<td>1 oz</td>
<td>9.4</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>1 tablespoon</td>
<td>8.9</td>
</tr>
<tr>
<td>Corn oil</td>
<td>1 tablespoon</td>
<td>7.3</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>1 tablespoon</td>
<td>6.9</td>
</tr>
<tr>
<td>Pecans, oil roasted</td>
<td>1 oz</td>
<td>6.4</td>
</tr>
<tr>
<td>Brazil nuts</td>
<td>1 oz</td>
<td>5.8</td>
</tr>
<tr>
<td>Sesame oil</td>
<td>1 tablespoon</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Arachidonic acid: Animals, but not plants, can convert LA to AA. Therefore, AA is present in small amounts in meat, poultry, and eggs.

**Omega-3 fatty acids**

α-Linolenic acid (ALA): Flaxseeds, walnuts, and their oils are among the richest dietary sources of ALA. Canola oil is also an excellent source of ALA. Dietary surveys in the US indicate that average adult intakes for ALA range from 1.8-2.0 g/day for men and from 1.4-1.5 g/day for women (63). Some foods that are rich in ALA are listed in Table 3.

<table>
<thead>
<tr>
<th>Food</th>
<th>Serving</th>
<th>α-Linolenic acid (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flaxseed oil</td>
<td>1 tablespoon</td>
<td>7.3</td>
</tr>
</tbody>
</table>
Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA): Oily fish are the major dietary source of EPA and DHA. Dietary surveys in the US indicate that average adult intakes of EPA range from 0.03-0.06 g/day, and average adult intakes of DHA range from 0.05-0.10 g/day (63). Omega-3 fatty acid-enriched eggs are also available in the US. Some foods that are rich in EPA and DHA are listed in Table 4.

Table 4. Food Sources of EPA (20:5n-3) and DHA (22:6n-3) (97)

<table>
<thead>
<tr>
<th>Food</th>
<th>Serving</th>
<th>EPA (g)</th>
<th>DHA (g)</th>
<th>Amount Providing 1 g of EPA + DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herring, Pacific</td>
<td>3 oz*</td>
<td>1.06</td>
<td>0.75</td>
<td>1.5 oz</td>
</tr>
<tr>
<td>Salmon, chinook</td>
<td>3 oz</td>
<td>0.86</td>
<td>0.62</td>
<td>2 oz</td>
</tr>
<tr>
<td>Sardines, Pacific</td>
<td>3 oz</td>
<td>0.45</td>
<td>0.74</td>
<td>2.5 oz</td>
</tr>
<tr>
<td>Salmon, Atlantic</td>
<td>3 oz</td>
<td>0.28</td>
<td>0.95</td>
<td>2.5 oz</td>
</tr>
<tr>
<td>Oysters, Pacific</td>
<td>3 oz</td>
<td>0.75</td>
<td>0.43</td>
<td>2.5 oz</td>
</tr>
<tr>
<td>Salmon, sockeye</td>
<td>3 oz</td>
<td>0.45</td>
<td>0.60</td>
<td>3 oz</td>
</tr>
<tr>
<td>Trout, rainbow</td>
<td>3 oz</td>
<td>0.40</td>
<td>0.44</td>
<td>3.5 oz</td>
</tr>
<tr>
<td>Tuna, canned, white</td>
<td>3 oz</td>
<td>0.20</td>
<td>0.54</td>
<td>4 oz</td>
</tr>
<tr>
<td>Crab, Dungeness</td>
<td>3 oz</td>
<td>0.24</td>
<td>0.10</td>
<td>9 oz</td>
</tr>
<tr>
<td>Tuna, canned, light</td>
<td>3 oz</td>
<td>0.04</td>
<td>0.19</td>
<td>12 oz</td>
</tr>
</tbody>
</table>

*A three-ounce serving of fish is about the size of a deck of cards.
Mango Supplementation Prevents Gut Microbial Dysbiosis and Modulates Short Chain Fatty Acid Production Independent of Body Weight Reduction in C57BL/6 Mice Fed a High Fat Diet

Babajide Ojo1, Guadalupe El-Rassi2, Penelope Perkins-Veazie3, Stephen Clarke1, Brenda J Smith1 and Edralin A Lucas1

Abstract

Fermentable non-digestible carbohydrates and fiber from plant food sources are suggested to prevent high fat (HF) diet-induced gut microbial dysbiosis and other obesity-related outcomes. Various parts of mango have been studied for their anti-obesogenic, immunomodulatory and gastro-protective properties. This study investigated the effects of 12-week freeze-dried mango pulp supplementation on the gut microbiota and their fermentation products, and its impact on body composition, glucose homeostasis and gut inflammatory markers in C57BL/6 mice fed a HF diet. Six-week old male C57BL/6 mice were randomly assigned to four dietary treatment groups: Control (AIN-93M, 10% kcal from fat), HF (60% kcal from fat), and HF+1% or 10% mango. Cecal content analyses using 16S rDNA sequencing showed that HF feeding reduced Bifidobacteria and Akkermansia while mango supplementation prevented the loss of these populations in a dose-dependent manner. In contrast to previous studies, mango supplementation did not reduce body weight or fasting blood glucose. Interestingly, both mango doses lowered HF-induced hypertriglyceridemia. The HF+10% mango significantly lowered plasma non-esterified fatty acids, but increased plasma total cholesterol. In comparison to the HF group, a dose-dependent increase in microbial fermentation was observed with mango supplementation, as evident in increased fecal and cecal acetate and butyric acid, but not propionic acid. Furthermore, mango supplementation modulated gut inflammation, as observed with an increase in ileal and colonic interleukin (IL)-10 gene expression compared to the HF group. These findings demonstrated that mango supplementation in high fat feeding modulated some of the adverse effects that accompanies high fat diet-induced obesity.

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Footnotes

This abstract is from the Experimental Biology 2016 Meeting. There is no full text article associated with this abstract published in The FASEB Journal.
Mango Supplementation Prevents Gut Microbial Dysbiosis and Modulates Short Chain Fatty Acid Production Independent of Body Weight Reduction in C57B…
In vitro and in vivo evaluation of the prebiotic activity of water-soluble blueberry extracts

World Journal of Microbiology and Biotechnology


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Abstract

The prebiotic effects of water extracts of two blueberry (BBE) cultivars (‘Centurion’ and ‘Maru’) were studied using pure and mixed cultures of human faecal bacteria. The results demonstrated for the first time that addition of BBE from both cultivars to broth media containing pure cultures of Lactobacillus rhamnosus and Bifidobacterium breve resulted in a significant increase (P < 0.05–0.0001) in the population size of these strains. Batch fermentation system was used to monitor the effect of BBE addition on the mixed faecal bacterial populations (obtained from healthy human donors). Addition of BBE from both cultivars to batch cultures inoculated with mixed human faecal cultures resulted in a significant increase in the number of lactobacilli (P < 0.01–0.0001) and bifidobacteria (P < 0.05–0.0001). Furthermore, a significant influence on the population size of
lactobacilli and bifidobacteria was observed after administration of extracts from both
cultivars to rats daily for 6 days in comparison with the control group. In rats gavaged
orally with 4 ml kg⁻¹ day⁻¹ of BBE for 6 days, the population size of lactobacilli (P < 0.05)
and bifidobacteria (P < 0.05–0.01) was increased significantly. We hypothesize that BBE
could modify the bacterial profile by increasing the numbers of beneficial bacteria and
thereby improving gut health.

Keywords

Blueberry extract Prebiotic activity Lactobacilli Bifidobacteria

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metabolism%20of%20organic%20compounds&amp;author=MR.%20Alberto&amp;author=MC.%20Manca%20de%20Nadra&amp;journal=J%20Agric%20Food%20Chem
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Culture-independent microbial community analysis reveals that inulin in the diet
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on%20the%20mouse%20cecum&amp;author=JH.%20Apajalahti&amp;author=H.%20Kettunen&amp;author=WE.%20Holben&amp;author=PH.%20Nurminen&amp;author=N.%20Rautonen&amp;author=
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transgalactooligosaccharides increases faecal bifidobacteria and modifies colonic


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(Google Scholar)


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Berry and Citrus Phenolic Compounds Inhibit Dipeptidyl Peptidase IV: Implications in Diabetes Management

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Beneficial health effects of fruits and vegetables in the diet have been attributed to their high flavonoid content. Dipeptidyl peptidase IV (DPP-IV) is a serine aminopeptidase that is a novel target for type 2 diabetes therapy due to its incretin hormone regulatory effects. In this study, well-characterized anthocyanins (ANC) isolated from berry wine blends and twenty-seven other phenolic compounds commonly present in citrus, berry, grape, and soybean, were individually investigated for their inhibitory effects on DPP-IV by using a luminescence assay and computational modeling. ANC from blueberry-blackberry wine blends strongly inhibited DPP-IV activity (IC$_{50}$, 0.07 ± 0.02 to >300 μM). Of the twenty-seven phenolics tested, the most potent DPP-IV inhibitors were resveratrol (IC$_{50}$, 0.6 ± 0.4 nM), luteolin (0.12 ± 0.01 μM), apigenin (0.14 ± 0.02 μM), and flavone (0.17 ± 0.01 μM), with IC$_{50}$ values lower than diprotin A (4.21 ± 2.01 μM), a reference standard inhibitory compound. Analyses of computational modeling showed that resveratrol and flavone were competitive inhibitors which could dock directly into all three active sites of DPP-IV, while luteolin and apigenin docked in a noncompetitive manner. Hydrogen bonding was the main binding mode of all tested phenolic compounds with DPP-IV. These results indicate that flavonoids, particularly luteolin, apigenin, and flavone, and the stilbenoid resveratrol can act as naturally occurring DPP-IV inhibitors.

1. Introduction

Type 2 diabetes is characterized by excessive blood glucose and insulin resistance due to an improper insulin response of the body to manage glucose from the diet [1]. Dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5), a serine peptidase, is one of the newest pharmaceutical targets for type 2 diabetes treatment [1]. On the other hand, incretin-based therapy has several potential sites of action for the treatment of type 2 diabetes ranging from increasing insulin secretion, reducing glucagon secretion, and regulating glucose control [2]. It is well known that glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are major human incretin hormones that stimulate insulin release in a glucose-dependent manner in healthy individuals [3, 4]. However, DPP-IV rapidly transforms these two gut incretin hormones after secretion by cleavage of the penultimate proline or alanine at N-terminus, and thus forms their inactive metabolites [5–7]. Both hormones have very short half-lives (approximately 2 min) due to the rapid degradation by DPP-IV [8]. Among the several peptide substrates of DPP-IV, GLP-1 is one of the well-characterized physiological and pharmacological substrates of the enzyme. GLP-1, which is secreted in a nutrient-dependent manner, stimulates glucose-dependent insulin secretion and regulates glycemia. However, the actions of GLP-1 do not last long due to degradation by DPP-IV. For this reason, DPP-IV inhibition is expected to result in elevated plasma insulin levels by inhibiting the degradation of active GLP-1 after oral glucose intake. This in turn leads to the suppression of blood glucose elevation. Therefore, development of DPP-IV inhibitors is being actively conducted worldwide, and control of blood glucose levels...
by enhancement of GLP-1 action is a new option for the treatment of diabetes.

In recent years, protein-ligand docking has become a powerful tool for drug development, and is also a method to be able to identify binding modes with high accuracy. For DPP-IV, computational docking analyses have been commonly used for designing inhibitors [9], screening of potential inhibitors [10], and explaining the differences in activity of drugs with different structures [11]. However, most of the previously investigated inhibitors of DPP-IV have been synthetically derived. As for naturally occurring flavonoids, the binding modes with DPP-IV are still not yet established.

Phenolic compounds, such as flavonoids, widely abundant in fruits and vegetables, have been suggested as important compounds for diabetes reduction [9, 10]. However, so far only a few phenolic compounds have been investigated to inhibit DPP-IV activity. These include procyanidin from grape seeds [12] and naringin from orange peel [13]. Therefore, it is necessary to further elucidate the modulating effect on DPP-IV activity of phenolic compounds from other natural sources.

In epidemiological studies, berries were the most important contributors to lowering risk for type 2 diabetes [14]. Additionally, an inverse relationship between intake of flavonoids, specifically those from berries, and risk of type 2 diabetes was found [15]. However, there is lack of evidence for the role of specific phenolics in clinical trials, and there is not yet sufficient data to confirm that anthocyanins have a protective effect against the risk of type 2 diabetes [16]. Additionally, anthocyanins found in berries have been found to have a beneficial effect on glucose metabolism; however, stronger scientific evidence is needed.

Anthocyanins (ANC) from blueberry-blackberry wine blends have been evaluated for DPP-IV and carbohydrate-utilizing enzymes inhibitor studies in our laboratory, and they have exhibited potent DPP-IV and α-glucosidase inhibitory activities [17]. Thus, the aim of the present study was to further characterize the ANC-rich fractions from blueberry-blackberry wine blends by HPLC and analyze their DPP-IV inhibitory effect in vitro. Furthermore, a variety of other phenolic compounds commonly present in berries, citrus, and other plant foods were studied for their DPP-IV inhibitory activity. We hypothesized that berry and citrus phenolics could bind to the active sites of DPP-IV, thus inhibiting DPP-IV enzyme activity. For the most potent compounds, kinetic and computational docking analyses were used to elucidate the binding modes with the DPP-IV enzyme.

2. Materials and Methods

2.1. Materials. Wines were produced from highbush blueberry (Vaccinium corymbosum) cultivars Blue Chip, Bluecrop, Blue Haven, Blue Jay, Blueray, Bluetsa, Collins, Coville, Darrow, Earlblue, Elliot, Jersey, Late Blue, and Spartan and blackberry (Rubus fruticosus) cultivars A-1937, A-2215, A-2241 Natchez, A-2315, APF 27, APF 40, APF 41, and Prime Jan, collected from Dixon Springs Agricultural Center in Simpson, IL, USA during the ripening season of 2010. Blueberry wine and blackberry wine were separately fermented using Saccharomyces bayanus as previously described [17]. After the fermentation, blends ranging from 100% blueberry to 100% blackberry were made using room temperature fermented wines. Blends were prepared with different ratios of blueberry : % blackberry. The ratios were 100:0, 75:25, 25:75, and 0:100 of blueberry: blackberry wine blends, respectively.

All solvents used for phenolic extraction were HPLC-grade and were purchased from Fisher Scientific (Pittsburg, PA). Amberlite XAD-7 was purchased from Sigma-Aldrich (St. Louis, MO). Sephadex LH-20 was purchased from GE Life Sciences (Buckinghamshire, UK). Porcine kidney DPP-IV enzyme (88% sequence homology with human; both are homodimers with a subunit molecular mass of ~30 kDa) and diprotin A were purchased from Sigma-Aldrich. DPP-IV Glo™ Protease Assay kits were purchased from Promega (Madison, WI). Flavonoids with high purities that were purchased from Sigma-Aldrich included luteolin (>98%), apigenin (>95%), quercetin (>98%), kaempferol (>97%), rutin hydrate (>94%), naringenin (>95%), neo-hesperidin (>90%), flavone (>97%), naringin (>90%), hesperidin (>80%), cyanidin-3-glucoside (>95%), cyanidin (>95%), malvidin (>95%), resveratrol (>99%), protocatechuic acid (>97%), catechin (>98%), epicatechin (>90%), epigallocatechin gallate (EGCG, >95%), gallic acid (>97.5%), caffeic acid (>98%), and chlorogenic acid (>95%). Hesperetin (>95%) was purchased from Sigma-Aldrich (Wicklow, Ireland) and limonin (>90%) from MP BioMedicals (Solon, OH). Narirutin (>93.9%) and eriocitrin (>97.4%) were purchased from Chromadex (Irvine, CA). Genistein (>90%) and genistin (>90%) were kindly donated by Dr. Mark Berhow, USDA. All other reagents were of analytical grade.

2.2. Phenolic Extraction and Preparation of ANC Fractions. Phenolic extraction and preparation of ANC fractions were conducted as previously described [17]. Briefly, each wine was firstly acidified, dealkoholized, and then mixed with amberlite XAD-7 resin to remove sugars and phenolic acids. After nonpolar compounds were further removed from the crude polyphenolics, the polar eluate was loaded onto a Sephadex LH-20 column to generate ANC-enriched fractions. With an isocratic elution using water: methanol (80:20, containing 0.1% TFA) and then 50% methanol, five anthocyanin-rich fractions (ANC 1–5) were obtained. ANC 2–5 from each blend of blueberry and blackberry were analyzed by HPLC to determine their ANC composition.

2.3. Anthocyanin Analysis. ANC analyses were conducted as previously published [17] using a 1200 HPLC (Agilent Technologies, Santa Clara, CA) with a Supelcosil LC-18 RP column (250 × 4.6 mm, 5 μM) (Supelco, Bellefonte, PA). ANC were detected at 520 nm using a diode array detector (DAD). Specific anthocyanins were identified based on comparison to our previously published data [18, 19]. A previously well-characterized blueberry extract [19] was included with each sample run to verify compound separation and identification. Using the peak areas as measured by HPLC at 520 nm, total ANC were quantified from a standard curve generated from
0.125, 0.25, 0.5, and 1.0 mg/mL of cyanidin-3-glucoside (C3G) and ANC amounts are presented as C3G equivalents.

2.4. DPP-IV Inhibition. Measurement of the activity and potential inhibition of DPP-IV, a type II membrane glycoprotein, was done using the DPP-IV Glo™ Protease Assay following the manufacturer’s protocol (Promega, Madison, WI). Briefly, 50 μL of DPP-IV Glo™ reagent was added to a white-walled 96-well plate containing 50 μL of blank, positive control, or treatment. The blank contained the vehicle only while positive control contained the vehicle and purified DPP-IV enzyme (at a final concentration of 1 ng/mL). Treatments used were enriched ANC fractions (0.5, 5, 20, and 40 μg/mL), phenolic compounds (0.5, 5, 20 and 40 μg/mL) or known inhibitor, diprotin A (1, 2, 12, 24, 125, and 250 μM), and the purified DPP-IV enzyme at a final concentration of 1 ng/mL. The content of the wells was gently mixed using an Ultra Microplate Reader (Biotek Instruments, Winooski, VT) at medium intensity for 4 s. DPP-IV cleavage of the provided Gly-Pro-amino methyl coumarin (AMC) substrate generated a luminescent signal by luciferase reaction, with the amount of DPP-IV enzyme available to bind Gly-Pro-AMC proportional to relative light units (RLU) produced. This signal in RLU was measured after 30 min in the Ultra Microplate Reader and then compared to the blank. Diprotin A linear standard curve (y = 41.936x + 27.294, R² = 0.91), where y was the % inhibitory activity of diprotin A and x was the log₁₀ of the concentration (μM) of known inhibitor diprotin A, was used to calculate IC₅₀ value: the concentration needed to decrease the activity of the enzyme by 50% of its original activity. IC₅₀ values were calculated based on the molecular mass of each compound or C3G as the equivalent for ANC-enriched fractions.

2.5. Inhibitory Kinetics Study. Porcine kidney DPP-IV activity was measured at various concentrations of three flavonoids (5 and 10 mg/mL for luteolin, apigenin, and flavone; and 0.25 and 0.5 mg/mL for resveratrol). Each concentration was evaluated in the presence of various concentrations of Gly-Pro-AMC (0–60 μM). DPP-IV activity was measured using the DPP-IV Glo Protease Assay as mentioned above. The inhibition pattern was evaluated utilizing the Lineweaver-Burk plot. Enzyme-inhibition constant Kᵢ was determined by plotting the reciprocal of the initial luminescence versus the reciprocal of the initial substrate concentration.

2.6. Molecular Modeling and Computational Docking Study. The DPP-IV enzyme exists as a dimer in the crystal form, and each monomer consists of 726 amino acids [20]. The docking studies were conducted with the monomeric unit of the enzyme, as the active site of the enzyme resides deep within each monomer of the receptor protein and not on the enzyme surface [21]. The molecular docking analysis of flavonoids was carried out using AUTODOCK 4.2 (CCDC, UK; http://www.ccdc.cam.ac.uk/products/csd/) [22]. The crystal structure of the DPP-IV enzyme (Protein Data Bank (PDB) ID: 2I03) was obtained from the protein data bank (http://www.rcsb.org/pdb), and the protein structure was prepared using Accelrys Discovery Studio 3.5 program (Accelrys Software Inc., San Diego, CA). For the computational docking study, the energies of diprotin A and flavonoids were minimized by applying a CHARMM22 force field, using the Accelrys Discovery Studio 3.5 program. After removing water molecules and adding all the hydrogen atoms, Gasteiger-Hücke charges were assigned to the enzyme. The ligand conformers were treated as flexible and protein structures were treated as rigid during the docking process. The docking was carried for 100 genetic algorithm runs, which was optimum to validate the crystal structure of the ligand. Most of the other genetic algorithm parameters such as the population size were maintained at their default values. The best docking results were considered to be the conformation having the lowest binding energy (∆G) using:

\[
\Delta G = \Delta G \text{ (intermolecular)} + \Delta G \text{ (internal)} + \Delta G \text{ (tor)} - \Delta G \text{ (unbound extended)},
\]

where ∆G (intermolecular) denotes the sum (kcal/mol) of Van der Waals energy, hydrogen bond energy, electrostatic energy, and desolvation energy; ∆G (internal) is the final total internal energy (kcal/mol); ∆G (tor) denotes torsional free energy (kcal/mol); and ∆G (unbound extended) is the unbound system’s energy (kcal/mol).

In the context of Autodocking, inhibition constant (Ki) is directly related to the binding energy:

\[
K_i = e^{\Delta G/(RT)};
\]

where e is the base number of natural logarithm (approximately equals 2.72), R is the gas constant (kcal/mol), and T is the absolute temperature. Smaller Kᵢ and more negative ∆G mean tighter binding.

2.7. Statistical Analyses. Data were expressed as means of independent duplicates with at least three replicates. The dose-response analysis of each compound on DPP-IV activity was performed using nonlinear or linear regression (curve fit) using EXCEL Microsoft (e.g., see Supplementary Information available online at http://dx.doi.org/10.1155/2013/479505). Statistical analysis was conducted using the proc GLM procedures of SAS version 9.3 (SAS Inst. Inc., Cary, NC, 2009). Group mean comparisons were conducted using Duncan means and were considered to be significant at P < 0.05 based on the least significant differences (LSD) from one-way analysis of variance (ANOVA) with alpha = 0.05. Correlations were made using Pearson’s correlation values with P < 0.05.

3. Results

3.1. Blackberry Wine Presented High Concentrations of Delphinidin-3-arabinoside. Anthocyanin relative distributions in the extracts of blueberry-blackberry wine blends are shown in Table 1. Chromatographic analyses revealed up to seventeen ANC present in blueberry-blackberry wine blends. Malvidin-3-galactoside and cyanidin-3-glucoside were the main ANC present in the blueberry wine, while delphinidin-3-arabinoside was the predominant ANC present in the
Table 1: Anthocyanin (ANC) identification and quantification by HPLC at maximum absorption of 520 nm.

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>ANC ID</th>
<th>100:0 ANC</th>
<th>75:25 ANC</th>
<th>25:75 ANC</th>
<th>0:100 ANC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ANC2</td>
<td>ANC3</td>
<td>ANC4</td>
<td>ANC5</td>
</tr>
<tr>
<td>24.44</td>
<td>Delphinidin-3-galactoside</td>
<td>6.3</td>
<td>10.8</td>
<td>10.0</td>
<td>nd</td>
</tr>
<tr>
<td>25.98</td>
<td>Delphinidin-3-glucoside</td>
<td>nd</td>
<td>26.8</td>
<td>45.5</td>
<td>nd</td>
</tr>
<tr>
<td>27.33</td>
<td>Cyanidin-3-galactoside</td>
<td>6.1</td>
<td>6.5</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>28.86</td>
<td>Delphinidin-3-arabinoside</td>
<td>nd</td>
<td>11.9</td>
<td>30.9</td>
<td>nd</td>
</tr>
<tr>
<td>29.69</td>
<td>Cyanidin-3-glucoside</td>
<td>6.4</td>
<td>23.4</td>
<td>nd</td>
<td>60.6</td>
</tr>
<tr>
<td>30.82</td>
<td>Cyanidin-3-arabinoside</td>
<td>nd</td>
<td>140.2</td>
<td>7.4</td>
<td>nd</td>
</tr>
<tr>
<td>31.62</td>
<td>Petunidin-3-glucoside</td>
<td>9.7</td>
<td>7.3</td>
<td>24.9</td>
<td>10.5</td>
</tr>
<tr>
<td>32.69</td>
<td>Peonidin-3-glucoside</td>
<td>6.0</td>
<td>39.8</td>
<td>nd</td>
<td>8.3</td>
</tr>
<tr>
<td>33.95</td>
<td>Petunidin-3-arabinoside</td>
<td>5.9</td>
<td>178.5</td>
<td>22.5</td>
<td>nd</td>
</tr>
<tr>
<td>34.39</td>
<td>Malvidin-3-galactoside</td>
<td>74.8</td>
<td>292.1</td>
<td>13.2</td>
<td>6.2</td>
</tr>
<tr>
<td>35.19</td>
<td>Malvidin-3-glucoside</td>
<td>63.1</td>
<td>11.1</td>
<td>19.0</td>
<td>6.1</td>
</tr>
<tr>
<td>37.14</td>
<td>Malvidin-3-arabinoside</td>
<td>5.9</td>
<td>278.9</td>
<td>19.7</td>
<td>8.3</td>
</tr>
<tr>
<td>39.03</td>
<td>Delphinidin-6-acetyl-3-glucoside</td>
<td>nd</td>
<td>73</td>
<td>17.9</td>
<td>6.7</td>
</tr>
<tr>
<td>40.55</td>
<td>Cyanidin-6-acetyl-3-glucoside</td>
<td>nd</td>
<td>7.8</td>
<td>6.7</td>
<td>nd</td>
</tr>
<tr>
<td>41.70</td>
<td>Malvidin-6-acetyl-galactoside</td>
<td>nd</td>
<td>26.7</td>
<td>7.1</td>
<td>nd</td>
</tr>
<tr>
<td>42.25</td>
<td>Petunidin-6-acetyl-3-glucoside</td>
<td>nd</td>
<td>12.9</td>
<td>6.0</td>
<td>nd</td>
</tr>
<tr>
<td>44.40</td>
<td>Malvidin-6-acetyl-arabinoside</td>
<td>10.8</td>
<td>10.5</td>
<td>11.5</td>
<td>19.4</td>
</tr>
<tr>
<td>Sub-total ANC</td>
<td>190.5</td>
<td>111.7</td>
<td>275.6</td>
<td>74.0</td>
<td>864.0</td>
</tr>
<tr>
<td>Total ANC</td>
<td>1653.8</td>
<td>2241.7</td>
<td>2907.5</td>
<td>3267.8</td>
<td></td>
</tr>
</tbody>
</table>

*Bold numbers indicate the dominant flavonoids in that particular ANC fraction. 
nd: peak not detected.
blackberry wine. Total ANC ranged from 1653.8 mg C3G equivalents/L for blueberry wine to 3267.8 mg C3G equivalents/L for blackberry wine. It was also observed that there was an obvious difference between ANC amounts of different fractions generated as ANC2–5.

3.2. Anthocyanins from Blackberry Wine Potently Inhibited DPP-IV. ANC-enriched fractions (ANC 1–5) isolated from blueberry-blackberry wine blends were analyzed for their DPP-IV inhibitory effect. Table 2 shows the IC50 values of ANC from blueberry-blackberry wine blends needed to inhibit DPP-IV enzyme. Compared to a standard curve of diprotin A (IC50, 4.21 ± 2.01 µM), a known DPP-IV inhibitor with an Ile-Pro-Ile sequence, ANC 2–5 tested at concentrations of 0.5, 5, 20, and 40 µM in C3G equivalents obtained from each blend had IC50 values ranging from 2.64 ± 1.40 µM in ANC 2 from blueberry wine to 0.07 ± 0.02 µM in ANC 3 from blackberry wine (Table 2). Table 2 also shows that ANC from blackberry wine were the most effective of the blends at reducing the activity of DPP-IV (with IC50 values of no more than 0.22 µM C3G).

3.3. Resveratrol, a Stilbenoid, Luteolin, Apigenin, and Flavone, Flavonoids Commonly Present in Fruits, Have Strong DPP-IV Inhibitory Activity. Twenty-seven phenolic compounds commonly present in citrus, berries, grape, soybeans, and other plants were tested for DPP-IV inhibitory effect (Table 3). Sixteen phenolic compounds demonstrated DPP-IV inhibitory activity with IC50 values ranging from 0.6 ± 0.4 nM (resveratrol) to 10.36 ± 0.09 µM (eriocitrin). Eleven compounds did not have DPP-IV inhibitory activity including rutin, narirutin, naringin, hesperidin, limonin, neohesperidin, genistin, catechin, epicatechin, chlorogenic acid, and protocatechuic acid (data not shown).

Of the sixteen effective phenolic compounds, three had IC50 values higher than diprotin A (4.21 ± 2.01 µM) including eriocitrin (IC50 value of 10.36 ± 0.09 µM), EGCG (10.21 ± 0.75 µM), and gallic acid (4.65 ± 0.1 µM). However, IC50 values of the other thirteen compounds were lower than that of diprotin A, indicating that less of these compounds was needed to inhibit DPP-IV. These thirteen phenolics could be divided into three categories according to the results of statistical differences on their DPP-IV inhibitory effect: less active with high IC50 values (1.31–3.37 µM), intermediate activity with IC50 values of 0.24–0.74 µM, and very high activity with low IC50 values (0.0006–0.17 µM). The phenolic compounds with high IC50 values were cyanidin, quercetin, and caffeic acid; the ones with intermediate activity were naringenin, hesperetin, cyanidin-3-glucoside, kaempferol, and malvidin. The four phenolics with very high activity included resveratrol, luteolin, apigenin, and flavone. IC50 value of resveratrol had the highest DPP-IV inhibitory activity among all of the compounds tested (P < 0.05).

3.4. Resveratrol and Flavone Inhibited DPP-IV Activity in a Competitive Manner, While Luteolin and Apigenin Inhibited Noncompetitively. To examine whether the most potent phenolic compounds, resveratrol, luteolin, apigenin and flavone, inhibited DPP-IV through interaction with the active site of the enzyme, we tested the enzyme kinetics. The inhibitory manner of the flavonoids was determined through generating a Lineweaver-Burk plot (Figure 1). As noted in Figures 1(a) and 1(d), both the slope and the x-intercept were changed by the addition of inhibitors, but there was no effect on the y-intercept. This is the definition of linear competitive inhibition. Therefore, resveratrol (Figure 1(a)) and flavone (Figure 1(d)) inhibited DPP-IV activity in a competitive manner. The KI values were calculated to be 0.2 ± 0.01 µM for resveratrol and 18.6 ± 0.3 µM for flavone. As for luteolin and apigenin (Figures 1(b) and 1(c)), both the slope and the y-intercept were changed by the added inhibitors, but there was no effect on the x-intercept. Therefore, luteolin and apigenin noncompetitively inhibited the enzyme, with KI values at 4.9 ± 0.2 µM and 7.9 ± 1.4 µM, respectively.

3.5. Diprotin A and Natural Phenolic Compounds Inhibit DPP-IV Activity by Binding Tightly into the Active Site of the Enzyme. Binding pose of diprotin A, resveratrol and flavone in the DPP-IV active site is indicated in Figures 2 and 3, showing that these three compounds interact closely with key residues of sites S1, S2 and S3 within the active pocket.
Table 3: DPP-IV inhibition by flavonoids (IC<sub>50</sub>), their number of hydroxyl groups (OH), binding energy, inhibition constant (K<sub>i</sub>)<sup>2</sup>, H bonds involved, and π interactions.

<table>
<thead>
<tr>
<th>Flavonoids</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>Number of OH groups</th>
<th>Binding energy (kcal/mol)</th>
<th>K&lt;sub&gt;i&lt;/sub&gt; (μM)</th>
<th>H Bonds&lt;sup&gt;3&lt;/sup&gt;</th>
<th>π interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>4.21 ± 2.01&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0</td>
<td>-7.31</td>
<td>4.42</td>
<td>TYR547:HH-UNK:O22</td>
<td>TYR666-UNK:N13</td>
</tr>
<tr>
<td>Diprotin A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Berry flavonoids</td>
<td>1.41 ± 0.25&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5</td>
<td>-5.95</td>
<td>43.43</td>
<td>TRP563:HN-UNK:O15</td>
<td>UNK-B:TRP629</td>
</tr>
<tr>
<td>Cyanidin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyanidin-3-glucoside</td>
<td>0.42 ± 0.09&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>8</td>
<td>-6.35</td>
<td>22.33</td>
<td>ARG356:HH11-UNK:O13</td>
<td>UNK-PHE357</td>
</tr>
<tr>
<td>Malvidin</td>
<td>1.41 ± 0.44&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>3</td>
<td>-6.36</td>
<td>21.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrus flavonoids</td>
<td>0.12 ± 0.01&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4</td>
<td>-6.26</td>
<td>25.83</td>
<td>ARG356:HH11-UNK:O13</td>
<td>UNK-PHE357</td>
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<td>Luteolin</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Apigenin</td>
<td>0.14 ± 0.02&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>3</td>
<td>-6.14</td>
<td>31.77</td>
<td>ARG356:HH11-UNK:O13</td>
<td>UNK-PHE669</td>
</tr>
<tr>
<td>Quercetin</td>
<td>2.92 ± 0.68&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5</td>
<td>-6.33</td>
<td>23.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kaempferol</td>
<td>0.49 ± 0.02&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>4</td>
<td>-6.62</td>
<td>13.99</td>
<td>SER209:HG-UNK:O21</td>
<td>UNK-ARG358:NH2</td>
</tr>
<tr>
<td>Flavone</td>
<td>0.17 ± 0.01&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0</td>
<td>-6.64</td>
<td>13.57</td>
<td>No hydrogen bonds</td>
<td>No π interactions</td>
</tr>
</tbody>
</table>

<sup>1</sup> IC<sub>50</sub> values for each flavonoid were determined using a competitive inhibition assay.

<sup>2</sup> Binding energy values were calculated using molecular docking software.

<sup>3</sup> H bond interactions were identified using molecular docking software and visualized using PyMOL.

<sup>4</sup> π interactions were identified using molecular docking software and visualized using PyMOL.
Table 3: Continued.

<table>
<thead>
<tr>
<th>Flavonoids</th>
<th>IC$_{50}$ (µM)</th>
<th>Number of OH groups</th>
<th>Binding energy (kcal/mol)</th>
<th>$K_i$ (µM)</th>
<th>H Bonds$^3$</th>
<th>π interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hesperetin</td>
<td>0.28 ± 0.07$^{ef}$</td>
<td>3</td>
<td>−6.85</td>
<td>9.57</td>
<td>ARG358:HH22-UNK:O2 UNK:CH2-GLU206:O1E UNK:H1-UNK:O2 UNK:H3-ARG358:O</td>
<td>π-cation UNK-ARG358:NH1</td>
</tr>
<tr>
<td>Soy isoflavone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grape stilbenoid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other flavonoids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^1$IC$_{50}$ values were determined from at least two independent duplicates done in triplicate for each of the concentrations tested. Concentrations (µM) were calculated based on the molecular mass of each pure compound. Values are means ± SEM. Means with different letters in each column are significantly different for DPP-IV (P < 0.05).

$^2$K$_i$ values were obtained from computational docking as indicated in Materials and Methods section.

$^3$UNK refers to phenolic compound or diprotin A.
Diprotin A is a potent DPP-IV inhibitor with Ile-Pro-Ile sequence commonly used as a reference compound. Figure 2 shows the binding mode of diprotin A with DPP-IV. The binding site of diprotin A is located at the S1, S2 and S3 sites (Figure 2(A)). In the S2 site (Figure 2(A)), the N-terminal amino group of diprotin A is hydrogen-bonded to the carboxyl oxygens of two Glu residues (Glu205 and Glu206). Furthermore, the N-terminal amino group forms a π interaction to the Tyr666. The carbonyl oxygen of Ile-1 of diprotin A forms an electrostatic interaction with Tyr662, Arg125, and Asn710 residues. Pro-2 of diprotin A is located in the S1 site and forms a hydrophobic interaction with the phenol rings of Tyr666, and Tyr547. The carbonyl oxygen of Ile-3 of diprotin A also forms double hydrogen bonds to Tyr547 and Tyr666. In the S3 site, Van der Waals interactions are also seen between diprotin A and Ser209 and Phe357 residues of DPP-IV. These observations agree with the reported results obtained from X-ray crystal structure complex of DPP-IV and diprotin A [20].

The overlay of binding poses of resveratrol (green) and flavone (yellow) in the DPP-IV active site is shown in Figure 3(A). As observed in Figure 3(A), resveratrol and flavone dock very well into all three active sites S1, S2, and S3 of DPP-IV. Resveratrol showed hydrogen bonding of 4'-OH-, 3'-OH-, and 5'-OH-group with hydroxyl of side chain of Ser630 (S1 pocket) and Ser209 (S3 pocket). Hydrogen bonds were also seen between 5'-OH of resveratrol, NH2-group of side chain of Arg669 residues, and C=O groups of side chains of Glu205 (S1 pocket) and Ser209 (S3 pocket). Hydrogen bonds were also seen between 5'-OH of resveratrol, NH2-group of side chain of Arg669 residues, and C=O groups of side chains of Glu205 (S1 pocket) and Ser209 (S3 pocket). At the same time, electrostatic interactions were also observed between resveratrol and S1 pocket (His740, Tyr631, Ser630, His125), S2 pocket (Glu205, Glu206), S3 pocket (Ser209), and Arg669 of DPP-IV.
Figure 2: Key interactions of diprotin A ($A_1$, $A_2$) with active sites of DPP-IV enzyme. Binding of diprotin A ($A_1$, grey) in the DPP-IV active site is indicated (surface view: blue), wherein it interacts closely with key residues of active sites S1, S2, and S3. Residues with pink circles indicate hydrogen bond, or ionic or polar interactions; residues with green circles indicate Van der Waals interactions. The arrows indicate hydrogen bonds to side chain residues in blue and backbone residues in green.

Figure 3: Key interactions of resveratrol ($A_1$, $A_2$), flavone ($A_1$, $A_3$), luteolin ($B_1$, $B_2$), apigenin ($B_1$, $B_3$), quercetin ($C_1$, $C_2$), and genistein ($C_1$, $C_3$) with active sites of DPP-IV enzyme. Binding pose of resveratrol ($A_1$, green) and flavone ($A_2$, yellow) in the DPP-IV active site is indicated (surface view: blue), wherein two compounds interact closely with key residues of active sites S1, S2, and S3. Binding pose of luteolin ($B_1$, green), apigenin ($B_2$, yellow), quercetin ($C_1$, green) and genistein ($C_3$, yellow) in the DPP-IV binding site is indicated, wherein these flavonoids interact closely with the key residues of sites S2, and S3. Residues with pink circles indicate hydrogen-bond, or ionic or polar interactions, residues with green circles indicate Van der Waals interactions. The arrows indicate hydrogen bonds to side chain residues in blue and backbone residues in green.
No hydrogen bonds were seen between flavone and amino acids in the pockets of DPP-IV (Figure 3(A)). However, electrostatic interactions between flavone and amino acid residues in S1 pocket (Tyr547, Ser630, Asn710, and His740), S2 pocket (Arg125, Glu205), and Van der Waals interactions between flavone and amino acid residues in S1 pocket (Tyr631, Val656, Tyr666, Val711), S2 pocket (Glu206) and S3 pocket (Phe357), allowed flavone to anchor in the active sites of DPP-IV.

The overlay of binding poses of luteolin (green) and apigenin (yellow) in the DPP-IV active site is also shown in Figure 3(B). As shown in Figure 3(B), luteolin and apigenin had almost identical binding modes with the active sites of DPP-IV with each having ring B and C docked into sites S2 and S3. Three common features of binding with DPP-IV exist between both flavonoids (Figures 3(B2) and 3(B3)). Firstly, hydrogen bonds and \( \pi \)-interactions played important roles in docking both the flavonoids into the active pockets S2 and S3 of DPP-IV enzyme. In the S2 pocket, the B ring \( 5' \)-hydroxyl of luteolin formed a hydrogen bond with hydroxyl group of side chain of Ser209 (S3 pocket), while the \( 4' \)-hydroxyl group on B ring of apigenin forms a similar hydrogen bond within the S3 pocket. Luteolin also showed H-bonding by B ring \( 4' \)-hydroxyl with C=O groups of side chains of Glu205 (S2 pocket). Secondly, in the S3 pocket, both compounds formed a hydrogen bond of the C ring \( 1' \)-oxygen with the NH of Arg358's guanidine side chain. H-bonding of the A ring \( 8' \)-hydroxyl with C=O groups of side chains of Glu361 favors strong binding of both flavonoids to the DPP-IV active site. A third common feature of both flavonoids was shown by \( \pi \)-cation interactions of ring B and the NH2 of Arg669. Additional features of each flavonoid added to their unique docking within the active site of DPP-IV. A hydrogen bond between the A ring \( 5' \)-hydroxyl of apigenin and the NH of Arg361's guanidine side chain also enhanced the docking of apigenin and DPP-IV. For luteolin, a \( \pi \)-sigma interaction was also seen between the side chain of Phe357 and C ring of luteolin (S3 pocket).

Quercetin and genistein had a comparable binding position to luteolin and apigenin (Figure 3(c)). Hydroxyl groups in A and B rings were also important for quercetin and genistein to bind into the S2 and S3 sites (Figures 3(C2) and 3(C3)). At the same time, \( \pi \)-interactions between these flavonoids and Arg358 and Arg669 also contributed to the tethering of the two flavonoids to the active sites. All hydrogen bonds formed between phenolic compounds and DPP-IV are indicated in Table 3.

The binding energies obtained by computational docking analyses were compared among the compounds tested (Table 3). Gallic acid had the highest binding energy (−3.96 kcal/mol), while diprotin A had the lowest binding energy (−7.31 kcal/mol). ICH50 values of the phenolic compounds that were found to inhibit DPP-IV activity correlated with their binding energies (\( r = 0.67, P < 0.05 \)). Both a lower ICH50 value and lower binding energy indicate stronger inhibitory potency. The inhibition constant (\( K_i \)) obtained by computational docking analyses is also shown in Table 3. The \( K_i \) values of these phenolic compounds varied from 1.25 \( \mu \)M for gallic acid to 604.91 \( \mu \)M for EGCG. A highly significant correlation existed between \( K_i \) values and ICH50 values (\( r = 0.82, P = 0.0002 \)). Significant correlations were also found between \( K_i \) values and binding energies (\( r = 0.56, P < 0.05 \)), and between \( K_i \) values and number of hydroxyl group (\( r = 0.56, P < 0.05 \)).

4. Discussion and Conclusions

This study showed that ANC from berry wine and a variety of other phenolic compounds commonly present in fruits and vegetables had strong DPP-IV inhibitory effect in vitro and in silico. Computational docking analyses also showed for the first time that these natural phenolics could inhibit DPP-IV activity by binding tightly into the active sites of the enzyme. The biological activities, stability, and bioavailability of anthocyanins depend on their chemical structures. Blends were created to generate a mixture of potentially bioactive compounds commonly present in both blueberries and blackberries after fermentation, which can be optimized based on the characterization and potential benefit.

Previous studies on wine compounds and biological activity indicated that it is not the presence of a single compound that is responsible for beneficial effects such as antioxidant capacity or ability to reduce inflammation, but rather involves several phenolic compounds. Major contributions are from compounds such as transresveratrol as well as minor contributions from cinnamic and hydroxycinnamic acids, cyanidin, and some phenolic acids [23]. The combination of these phenolic compounds within the blends produced from fermented blueberry and blackberry provided a unique potential for inhibition of DPP-IV. Therefore, while the inhibitory effects demonstrated by the anthocyanin-enriched blends are primarily due to the major anthocyanin components, the presence of other compounds also influenced the demonstrated potency.

In general, anthocyanins may protect beta-cells, increase the secretion of insulin, reduce the digestion of sugars in the small intestine, and thereby have multiple and simultaneous antidiabetic effects. Inhibitors of DPP-IV have been found to prevent pancreatic beta cell destruction in mice [24]. Extracts enriched in flavonoids have been seen to inhibit plasma DPP-IV [25].

The primary anthocyanin in the blackberry blends was delphinidin, which has previously shown potency to inhibit enzymatic activity of a glyoxalase I, which is being investigated as a target for prevention of cancer. Compared to other anthocyanins found in berries, (cyanidin and pelargonidin), delphinidin had the most potent DPP-IV inhibitory effect, suggesting the importance of interactions of the hydroxy groups on the B ring of anthocyanins. Further, binding modes indicated that the hydroxyl groups located at the R1 position greatly contribute to inhibitory potency and specificity to the binding site [26]. This previous study, along with the results from our research, indicates that the anthocyanin delphinidin can form several hydrogen bonds to several amino acids due to its hydroxyl groups at R1 position.

Our previous study also showed that the blueberry-blackberry wine contained high amounts of total anthocyanin [17]. However, correlation was not seen between...
DPP-IV inhibitory effect and anthocyanin concentration in ANC fractions ($P > 0.05$) from berry wines. For example, ANC3, ANC4, and ANC5 from blackberry wine were of similar IC$_{50}$ values to inhibit DPP-IV, while the anthocyanin concentration was almost 4 times higher in ANC3 than in ANC4 and ANC5. Additionally, ANC4 and ANC5 had the same IC$_{50}$ values and ANC concentration, but their anthocyanin compositions differed. These results indicate that delphinidin-3-arabinoside, as the major anthocyanin identified in blackberry blends, could contribute to the DPPIV inhibition, however, other ANC may also play an important role in DPP-IV inhibition. Therefore, DPP-IV inhibitory effect of ANC could depend on not only the concentration but the composition and structures of flavonoids present. More research should be conducted to clarify the relationship between DPP-IV inhibitory effect and anthocyanin structure of ANC from berries.

Phenolic compounds are widely recognized for their ability to improve diabetic conditions by decreasing blood glucose levels $[27]$. It is interesting that most of the ANC fractions showed potent DPP-IV inhibitory activity, with the lowest IC$_{50}$ value from blackberry wine. Grape seed-derived procyanidins (GSPE) were also able to inhibit recombinant human DPP-IV activity, achieving around 70% inhibition at 200 mg/L of GSPE $[12]$. In order to compare with the GSPE, the percentage inhibition was given in this study as IC$_{50}$ values. The concentrations of all ANC from blackberry wine for achieving the same inhibitory effect on DPP-IV were less than 200 mg/L. Especially for ANC3 from blackberry wine, the concentration of 41.9 mg/L could lead to around 70% inhibition of DPP-IV activity. These results suggest that ANC from blueberry and blackberry wine have strong DPP-IV inhibitory activity. The efficacy of the ANC to inhibit DPP-IV enzyme activity at a rate comparable to diprotin A and GSPE indicated that ANC may be able to act as naturally occurring DPP-IV inhibitors.

Many kinds of natural flavonoids exist in plants but only a few have been reported for DPP-IV inhibitory effect $[12, 13]$. In the present study of twenty-seven phenolic compounds commonly present in berries, citrus, soybeans, and other plant commodities, most flavonoids were determined to have DPP-IV inhibitory effect. It is interesting that most of the flavonoids tested in the present study showed lower IC$_{50}$ values and therefore were more potent than the reference inhibitor standard diprotin A. Resveratrol, luteolin, apigenin and flavone showed the most potent DPP-IV inhibitory activity due to their lowest IC$_{50}$ values. In particular, this study demonstrated that resveratrol was the most potent DPP-IV inhibitor with IC$_{50}$ value at 0.6 nM exhibiting even lower values than sitaglitrin (18 nM) and vildagliptin (3.5 nM) $[10]$, two current pharmacologic drug inhibitors of DPP-IV. A summary of current foods and food components in the prevention of diabetes by Thomas and Pfeiffer $[16]$ has indicated that the potential evidence for phenolic compounds is not conclusive; however, resveratrol was found to have a beneficial effect on protecting beta cells, which may be due to its ability to modulate the activity of DPP-IV.

DPP-IV has three binding pockets/active sites (S1, S2 and S3). The specificity pocket S1 is composed of the side chains of catalytic triad (Ser630, Asn710, and His740), which are involved in strong hydrophobic interactions $[10]$. The cavity near Glu205, Glu206 and Tyr662 residues is referred to as the S2 pocket. The S3 pocket of DPP-IV consists of Ser209, Arg358, and Phe357 $[21]$. The outside position of the S3 pocket in DPP-IV allows larger groups access to the site; on the other hand, the inside position of the S3 pocket favors smaller groups $[28]$. The four most potent compounds, resveratrol, luteolin, apigenin and flavone, had low $K_{i}$ values to inhibit DPP-IV, which indicated that they had high affinity to the active sites of DPP-IV. The kinetic analysis showed that resveratrol and flavone inhibited DPP-IV activity in a competitive manner, while luteolin and apigenin were in a noncompetitive manner. Further computational docking analyses are consistent with the tested inhibitory manner of the phenolic compounds. Docking analysis showed that resveratrol and flavone bound well into all the three sites S1, S2 and S3 of DPP-IV, while luteolin and apigenin could only bind into S2 and S3 pockets. Although luteolin and apigenin could dock into S2 and S3 pockets, the kinetic analysis showed that they inhibited DPP-IV in a noncompetitive manner. We presume that the binding of luteolin and apigenin into S2 and S3 may lead to DPP-IV conformational changes, or changes in the side chain of amino acid residues of DPP-IV, and the catalytic activity will be decreased when the substrate is also bound.

We found that apigenin had a similar effect as resveratrol to directly inhibit DPP-IV activity, and genistein also exhibited a potent DPP-IV inhibitory effect. In the present study, most of the glycosylated flavonoids with two sugar groups, including naringin, rutin, narirutin, hesperidin, and neohesperidin, had no DPP-IV inhibitory effect. One explanation is that conjugation of bulky sugar groups to the flavonoid core structure could sterically hinder binding to the active sites within DPP-IV, thus resulting in no inhibitory capacity of the tested flavonoids. The computational docking analyses further supported this phenomenon. However, cyanidin-3-glucoside, which has been identified as the major ANC in different blackberry species $[29]$, showed no statistical difference ($P > 0.05$) on DPP-IV inhibitory activity (IC$_{50}$, 0.42 ± 0.09 μM) than cyanidin (IC$_{50}$, 1.31 ± 0.34) and malvidin (IC$_{50}$, 0.74 ± 0.16). Considering ANC-enriched fractions from blueberry and blackberry wines contain a mixture of flavonoids with only one sugar group, flavonoids with monosugar groups may have better DPP-IV inhibitory effects than flavonoids with more sugar groups due to less steric hindrance.

Flavone, luteolin, and apigenin have the same flavone core structure. However, flavone could dock into all three active sites of DPP-IV, while luteolin and apigenin could dock into only two of them. Computational docking showed comparably strong binding of luteolin and apigenin due to hydrogen bonds of ring B hydroxyls with residue Ser209 in the S3 pocket, for ring C 1′-oxygen with the NH of guanidine side chain of Arg358 in the S3 pocket, and for ring A 8′-hydroxyls with C=O groups of side chains of Glu361. These features also exist in the binding of other citrus flavonoids (including kaempferol, quercetin, hesperetin, and naringenin) to DPP-IV, which have the same flavone core
structure. Even the binding of genistein, a soy isoflavone, to DPP-IV also had these features. Therefore, hydroxyls in these flavonoids are important to dock into active sites of DPP-IV with the same binding modes. Furthermore, the formation of $\pi$-interaction between A or B ring of citrus flavonoids and Arg669 or Arg358 also favors the binding of citrus flavonoids into S2 and S3 sites. Flavone has no hydroxyl residues capable of hydrogen bonding with residues in S2 and S3 pockets with the same binding modes as flavonoids like luteolin. Therefore, although it could dock into all the three pockets of DPP-IV, flavone had a higher $K_i$ value due to absence of hydroxyl groups.

Significant correlations were seen between IC$_{50}$ values of these flavonoids and their binding energies and $K_i$ values determined computationally in the present study. In the docking studies, if a compound shows lower binding energy compared to the standard, it proves that the compound has higher activity [30]. These results indicated that more negative binding energy and smaller $K_i$ result in tighter binding, and then more potent inhibitory effect. Meanwhile, a significant correlation also exists between the $K_i$ values determined in silico and the number of hydroxyl groups of flavonoids ($r = 0.56$, $P < 0.05$), which indicates that more hydroxyl groups of flavonoids can result in higher inhibition constant and therefore higher IC$_{50}$ value, indicating less affinity to bind the active site. This could explain why quercetin with five hydroxyls has a higher IC$_{50}$ value (less potent) than the other citrus compounds, despite sharing the same flavone core structure. IC$_{50}$ values of citrus compounds were also found to be significantly correlated with their numbers of hydroxyls.

We obtained $K_i$ values using the computational analyses as well as experimentally. $K_i$ values determined with the computational analyses were calculated from the binding energy. However, the binding energy is designed to score and rank conformations of ligand and protein and not designed to give accurate binding energy. Therefore, $K_i$ values generated from autodock correlated with free binding energies significantly ($r = 0.56$, $P < 0.05$) but differed from the experimental $K_i$ values.

In conclusion, our study demonstrated that ANC isolated from blueberry-blackberry wine blends and a variety of other phenolic compounds commonly present in citrus, berry, soy, and other plants could strongly inhibit DPP-IV activity. Resveratrol and flavone were competitive inhibitors which could dock into all the three active sites, while luteolin and apigenin bound to DPP-IV in a noncompetitive manner. Results obtained from this study further support the efficacy of flavonoids as naturally occurring DPP-IV inhibitors.

**Abbreviations**

ANC: Anthocyanins  
C3G: Cyanidin-3-glucoside  
DPP-IV: Dipeptidyl peptidase IV  
GLP-1: Glucagon-like peptide-1  
GIP: Glucose-dependent insulinotropic polypeptide  
IC$_{50}$: Concentration to inhibit 50% enzyme activity  
$K_i$: Enzyme inhibitor constant.

### Conflict of Interests

The authors have declared no conflict of interest.

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### References


Cytotoxic effects of ellagitannins isolated from walnuts in human cancer cells.

Le V\(^1\), Esposito D, Grace MH, Ha D, Pham A, Bortolazzo A, Bevens Z, Kim J, Okuda R, Komarnytsky S, Lila MA, White JB.

Author information

Abstract
Walnuts contain many bioactive components that may slow cancer growth. A previous report showed that a diet supplemented with walnuts decreased the tumor size formed by MDA-MB-231 human cancer cells injected into nude mice. However, the mechanism of action was never determined. We characterized the effects of a methanol extract prepared from walnuts on human MDA-MB-231, MCF7, and HeLa cells. The extract was cytotoxic to all cancer cells. We identified compounds from the methanol extract that induced this cytotoxicity. The predominant compounds were Tellimagrandin I and Tellimagrandin II, members of the ellagitannin family. We also show a walnut extract decreases the intracellular pH, depolarizes the mitochondrial membrane with release of cytochrome c and phosphatidylserine flipping. The antimitogenic effects of walnut extract were associated with a twofold reduction of mitochondria respiration. These results suggest impairment of mitochondrial function and apoptosis as relevant mechanism of anticancer effects of the walnut extract.

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