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Atherosclerosis and Antioxidants

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Abstract:

Cellular lipid oxidation is a known cause for the cascade leading to the formation of lipid laden foam cells, which can cause of atherosclerosis. While statins and antioxidants have recently come under question in the amelioration of atherosclerosis, Flavonoids have recently been touted as a powerful antioxidant and suppresser of atherosclerosis. This paper will attempt to show why statins and vitamin E have come under scrutiny, and how the desired effects of Flavonoids can be attributed to the role it plays in increased paraoxonase-1 activity (a known anti inflammatory associated with HDL), decreased C- Reactive protein activity, and increased nitric oxide (NO) in endothelial cells among other factors.

Introduction:

Coronary artery disease (CAD) is the leading cause of death in the United States every year. According to the Center for Disease Control and the National Center for Health Statistics, data for the year 2004 indicate that heart disease killed 652,486 people (U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES, 2008). This is nearly 100,000 more than the second leading cause of death in the United States, cancer. Atherosclerotic plaque build-up in the arteries is the cause of CAD, and has been the primary focus of scientists in search of a cure for CAD. Needless to say, much research has been done on finding the causes of heart disease and developing drugs that inhibit its development. Other then pharmacological products, there is a very large body of research into more natural remedies for atherosclerosis, including antioxidants such as vitamin E that have been touted as providing protection against lipid oxidation. Recently, however, there is a growing body of evidence that statins and antioxidant supplementation may not be beneficial as thought. In contrast, Flavonoids, by operating under mechanisms different then antioxidants and statins, offers new hope in the treatment of atherosclerosis.

Coronary Artery Disease:

While there are many factors that contribute to CAD, there are a number of factors specifically related to atherosclerosis that play a key role in its development. Atherosclerosis is a progressive disease of the arteries and is the result of plaque buildup in the arteries driven by the uptake of cholesterol in artery walls (Medline Plus, 2007). Lipids are not water soluble, and must rely on lipoproteins, produced in the liver and small intestines, in order to be transported in the blood. By combining lipids with lipoproteins, the lipids are able to be transported either to the liver for elimination, or from the liver for the production of steroid hormones, bile salts, and for cell membrane repair. The two major lipoproteins involved in these functions are low-density lipoproteins (LDL), and high-density lipoprotein (HDL); with LDL being responsible

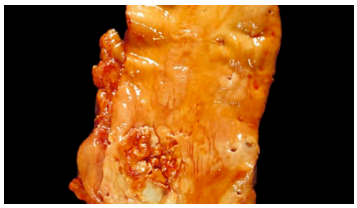

Figure 1. Dissected aorta with atherosclerotic lesions (Ewing, 1972)

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lipid transport from the liver, and HDL being responsible for transport of lipids to the liver for elimination (Tortora & Derrickson, 2006).

Plaque formation begins with the aggregation of excess LDL in the inner layer of arterial walls where the lipoproteins are subject to oxidation. In response, substances released by endothelial and smooth muscle cells attract macrophages that ingest the oxidized LDL forming plaque. So much so, that the macrophages take on a foamy appearance under the microscope (foam cells). Additionally, lymphocytes (T cells) follow the macrophages into the arterial wall and increase the streaks in the arterial walls. Subsequently, the macrophages cause the migration of the middle layer of the artery to the surface of the plaque thus separating it from the bloodstream, but also narrowing the arterial lumen. In the event that the cap over the plaque bursts, the T cells stimulate the foam cells to produce tissue factor (TF), which ultimately leads to blood clot formation and possible obstruction of the coronary artery, resulting in myocardial infarction (MI). Other factors currently being considered by researchers include C-reactive proteins, which bind to damaged cells and assist in phagocytosis, clotting factors, and the amino acid homocysteine that promotes platelet aggregation (Tortora & Derrickson, 2006).

Statins:

In order to treat atherosclerosis, scientists have focused their attention on reducing the amount of cholesterol available in the bloodstream for oxidation with the use of statin drugs.

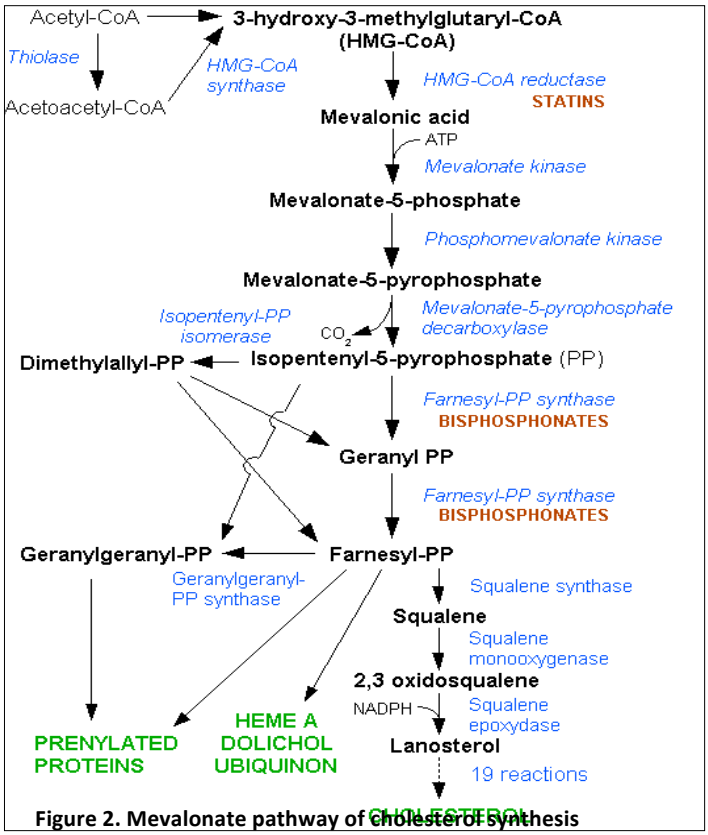

Figure 2. Mevalonate pathway of cholesterol synthesis

(Chemistry Daily, 2007)

In the 1960's (Endo, 1992) scientists discovered that when cholesterol is completely eliminated from the diet, the liver takes over and produces up to 82% percent of the required cholesterol. However, when cholesterol is added to the diet the synthesis of cholesterol is almost completely suppressed. They discovered that the production or suppression of cholesterol was in response to modulation by HMG-CoA reductase which modulates the inhibition of the Mevalonate pathway of cholesterol synthesis (Figure 2). Through modulating this enzyme they were able to alter the production of cholesterol. Thus was born the first statin drugs. However, there are medical researchers who challenge the claim that statin drugs actually lower the risk factors for myocardial infarction (MI). In a scathing review of studies purporting to show that by lowering

cholesterol with statin drugs mortality rates are decreased, Kauffman (Kauffman, 2007) cites a study carried out in New York City of a group of 2,277 people with the median age of 76 who were studied for a period of ten years. What he found was that "The chance of dying was twice as great in subjects with the *lowest* quartile of total cholesterol (TC) or LDL-C levels, compared with those in the highest quartile". Kauffman goes on to cite another study which shows that for men aged 35-57 "all cause" mortality rates increased for those with a TC of <170 mg/dL and the risk increased further as TC decreased to <140 mg/dL to the same levels seen for subjects with extremely high cholesterol >300mg/dL. Kaufmann flatly states "Serum TC level is not even predictive of cardiovascular disease (CVD) in men over the age of 47". Kauffman is not alone in challenging the use of statins; the British Medical Journal (Ravnskov et al., 2006) also challenges its widespread use, safety and efficacy. This raises serious questions about how the medical community is facing the challenge of CAD.

Oxidation:

While statins focus on reducing TC as a remedy to the formation of atherosclerosis, there is another very important juncture in the formation of atherosclerosis that has been the focus of research, namely oxidative stress caused by free radicals and reactive oxygen species (ROS). ROS and free radicals are injurious to lipids, DNA, and proteins if left unchecked by the bodies' native defense mechanisms (Davis, 1995). What are ROS and free radicals and why are they so important?

Examples of ROS include hydrogen peroxide (H_2O_2), hypochlorous acid (HClO). Free radicals include superoxide anion ($\cdot O_2^-$) and hydroxyl radical (·OH) (Davis, 1995). One source of the free radical superoxide anion ($\cdot O_2^-$) is that produced by the NADH/NADPH oxidase in macrophages during the uptake of LDL (Runge, 1999). The superoxide is formed during the electron transport chain (ETS) when the reduction of coenzyme Q in complex III forms the unstable Q·− radical which can leak electrons directly to oxygen instead of cascading down the rest of the ETS thus forming superoxide radicals (Holbrook, 2000).

Considering that 1% - 15% of O_2 respired by mammals undergoes the superoxide anion ($\cdot O_2^-$) state (McGraw-Hill's Access Science), there is also ample opportunity to form undesired side reactions. An example of a side reaction causing oxidative stress results when superoxide reacts with nitric oxide (NO) to form peroxynitrite ($\cdot O_2- + \cdot NO \rightarrow ONO_2-$). Peroxynitrite then reacts as a strong oxidizing agent with lipids, DNA, and proteins. Superoxide and NO individually can both be efficiently removed from the body. Superoxide reacts with hydrogen in the presence superoxide dismutase (SOD) to form oxygen and hydrogen peroxide ($2\cdot O_2- + 2H^+ \rightarrow O_2 + H_2O_2$), which can then be converted by catalase to water and oxygen. NO is rapidly removed by its diffusion through tissues into red blood cells. However, when compared with SOD, the kinetics and thermodynamics during a reaction of NO and superoxide favor the formation of peroxynitrite - resulting in the inevitable formation of a highly reactive oxidizing agent. Peroxynitrite acts as a potent oxidizing agent towards LDL which then becomes the target for scavenger cells and the formation of foam cells. Various studies have also shown that LDL can be oxidized by peroxynitrite even in the presence of endogenous lipophilic antioxidants (Pacher et al., 2007).

Another source of oxidation is the Hydroxyl radicals (•OH) that are produced in the iron catalyzed Haber-Weiss reaction. The Haber-Weiss reaction (Koppenol, 2001) was first discovered in the

early 1930's by Fritz Haber and Josef Weiss and proceeds as follows: Recalling that 1%-15% of O_2 respired by mammals undergoes the superoxide anion (·O2−) state, we can propose the following reactions:

$$Fe_3^+ + \bullet O_2^- \rightarrow Fe_2^+ + O_2,$$

Followed by the Fenton reaction:

$$Fe_2+ + H_2O_2 \rightarrow Fe_3^+ + OH^- + \bullet OH,$$

For a net reaction of:

$$\bullet O_2^- + H_2O_2 \rightarrow \bullet OH + HO^- + O_2.$$

Hydroxyl radicals are short lived in vivo, (Approx 10^{-9} s) and are highly reactive (Yan et al., 2005). They also cannot be enzymatically removed because of its short half life and tends to react extremely quickly with whatever is in its vicinity including: carbohydrates, nucleic acids, lipids, and amino acids.

The limitations of this process are inherent in that it requires Iron III in order to proceed, and the blood plasma protein Transferrin is very effective at scavenging free iron, making it unavailable for reduction. However, if there is a buildup of iron either due to dietary Iron overload or any other multiple of diseases, it can outstrip the ability of Transferrin to perform properly and results in the production of free radicals (Fouad, 2008).

Antioxidants:

As the oxidation of lipids form a key step in the formation of atherosclerosis, it should also lead to the conclusion that "antioxidants" offer protection against lipid oxidation. However, the more research that is being done, the more it becomes clear that exogenous sources of antioxidants have questionable effects in vivo. The field of antioxidants is very broad and can be the subject of many papers, but just to scratch the surface; antioxidants can be broken down into metabolite and enzymatic categories as well as lipid soluble and water soluble. The difference between them is their mechanism as well as site of action. For example: lipid soluble antioxidants act in cell membranes, and water soluble antioxidants act in the blood plasma and cytoplasm. Lipid soluble antioxidants include vitamin E and coenzyme Q (Q_{10}). Water soluble antioxidants include ascorbic acid (vitamin C), glutathione, lipoic acid, uric acid, and polyphenols (Flavonoids, tannins). Enzymatic antioxidants include the previously mentioned SOD, catalase, as well as glutathione among others (Sies, 1997).

Vitamin E is a potent antioxidant and some research has shown that it can regulate HMG-CoA reductase (Pal et al., 2003; Parker, 1993). Vitamin E is the name for a group of four tocopherols and four tocotrienols, of which the most interesting to scientists is the α-tocopherol variety because of its high bioavailability. Vitamin E is the main lipid-soluble antioxidant in the body and it works in cell membranes where it prevents the propagation of free radical reactions (Herrera & Barbas, 2001).

Because vitamin E is lipid soluble, when absorbed vitamin E is packaged in Chylomicrons (large lipoprotein particles) that are produced in the absorptive cells in the small intestine. They are then

secreted via exocytosis into lacteals of the lymphatic system and delivered into the circulatory system at the juncture of the thoracic duct and the left subclavian where they are transported to the liver. At the liver, lipoprotein lipase (LPL) induces the unloading of some of the chylomicrons into the extrahepatic tissue and the remainder chylomicrons transports the tocopherols into the liver. Here, "α-tocopherol transfer protein" incorporates α-tocopherol into very low density lipoprotein (VLDL) the excess of which is excreted into the bile duct. The α-tocopherol binded VLDL is then transported into circulation where it is converted to LDL again by the action of LPL. The α-tocopherol is then taken up by the endothelial cell membrane by the uptake of LDL via cell receptors (Herrera & Barbas, 2001).

Once in the cell membrane, α-tocopherol can act as a terminator to lipoprotein oxidation chain reaction as follows:

Where "I" is the initiator and "LH" is the fatty acid and L• is formed from the fatty acid

$$I + LH \rightarrow L\bullet + IH \quad [1]$$

Propagation continues as follows:

$$L\bullet + O_2 \rightarrow LOO\bullet \quad [2]$$

$$LOO\bullet + LH \rightarrow LOOH + L\bullet \quad [3]$$

(See Figure 3)

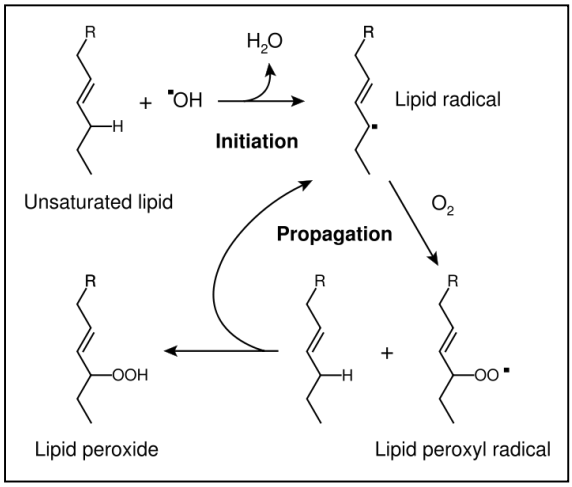

Figure 3. The free radical mechanism of lipid peroxidation

Termination occurs when tocopherol "TO" breaks the chain reaction:

$$LOO\bullet + TOH \rightarrow LOOH + TO\bullet \quad [4]$$

The tocopheroxyl radical then reacts with another peroxyl radical to form tocopheryl quinine (Q_{10}) and thereby arresting the chain reaction (Wolf, 2005).

As with almost every system in the body, vitamin E does not function in isolation but is part of an antioxidant network in which vitamin E can be synthesized from vitamin C and thiol redox cycles involving glutathione and lipoic acid. (See attachment). Vitamin C has pro-oxidant properties as well because of its ability to reduce metal ions via the Fenton reaction which can be a source of oxidative stress. However, this is thought to be of minor significance as compared with its antioxidant properties (FREI, 1999).

On the other front, research has shown that vitamin E, specifically tocotrienols, can modulate HMG-CoA reductase similar way to that done by statins as discussed previously. (Parker et al., 1993)

A number of studies showed this effect in mammals. In one study hyperlipidemic pigs given a diet rich in tocotrienols showed a marked decrease in plasma concentrations of cholesterol, apolipoprotein B (required in the formation of LDL), thromboxane B2 and platelet factor 4(both required for blood coagulation), indicating a protective effect on endothelium and platelet aggregation (Qureshi et al., 1991). There have been some conflicting results in similar trials in humans; however, those may be attributed to plasma levels of tocotrienols and not to the efficacy of the compound (Packer et al., 2001). It would thus seem that by ingesting vitamin E one would be able to take concrete measures in arresting the propagation of atherosclerosis.

However, a recent Meta analysis by The Cochrane Collaboration - of studies proving the remedial effects of vitamin E as well as other antioxidants - has not supported those results. Not only did the ingestion of exogenous antioxidants not prove to be beneficial but has also shown to be detrimental. The study included 232,550 participants who were "randomised to antioxidant supplements (beta-carotene, vitamin A, vitamin C, vitamin E, and selenium) versus placebo or no intervention". "A total of 17,880 of 136,023 participants (13.1%) randomised to antioxidant supplements and 10,136 of 96,527 participants (10.5%) randomised to placebo or no intervention died. In the analyses of the trials with low risk of bias, beta-carotene, vitamin A, and vitamin E *significantly increased mortality*." The study flatly states that there is *no evidence* to support antioxidant supplementation. This study brings into question the widespread use of antioxidants as a remedial and/or prophylactic compound for oxidative diseases (Bjelakovic et al., 2008).

Flavonoids:

Increased consumption of fruits and vegetables is associated with reduced incidences of CAD and other disease. The cause of this has been attributed in part, to antioxidant Flavonoids present in these foods. The hypothesis in this case is that because after the consumption of Flavonoids there is a marked increase in the antioxidant properties of blood plasma and it can play a direct role in the prevention of lipid oxidation (Lotitoa & Frei, 2006). A clear example of this can be seen in the French paradox, where despite the French consuming large amounts of red meat that are rich in unsaturated fats they still develop lower instances of death from CAD then Americans. This has been attributed to their consumption of red wine which is rich in Flavonoids (Ferrières, 2004).

Michael Aviram of the Technion faculty of medicine has done extensive research in the field of pomegranate polyphenols (Flavonoids), and has made a very important contribution to the field. Among the points studied three will be focused on: Antioxidant properties of blood plasma, paraoxonase-1 (PON-1)[1] levels, and aortic stenosis - all after the ingestion of PJ. In one study (Aviram, et al., 2000), both healthy human males and atherosclerotic E-deficient (E^0) mouse subjects were fed PJ for variable periods of time. The objective of the study was to determine what effect PJ consumption had on lipoprotein oxidation, aggregation and retention; macrophage atherogenicity; platelet aggregation; and atherosclerosis. The results of the study were striking. In humans, PJ consumption resulted in a decrease in LDL susceptibility to aggregation (-11% ex vivo) and retention, also observed was serum PON-1 increased activity by 20% and that it also had a protective effect against LDL oxidation. Human plasma in

[1] Paraoxonase1 (PON1) is an anti-inflammatory enzyme located on HDL.

one case showed a decrease in lipid plasma peroxidation of ≤33%. In E^0 mice, a 90% decrease in LDL oxidation was observed as well as a 20% decrease in the uptake of LDL by peritoneal macrophages. Also observed was a 44% decrease in atherosclerotic lesions and foam cells as compared with water fed mice. Aviram et al., attributes the protective antioxidant effect of PJ to increased cellular glutathione content, and states that the reduction of atherosclerotic lesions is directly related to the antioxidant properties of PJ. Another study by Aviram of the long term effects of PJ consumption - in human subjects with carotid artery stenosis (CAS) - on carotid lesions and changes in oxidative stress and blood pressure had remarkable results. After 1 year control subjects showed a 9% increase in intima-media thickness (IMT) as opposed to the PJ group that showed a 30% decrease in IMT. PON-1 activity increased by 83%, serum LDL basal oxidative state was reduced by 90%, copper ion induced oxidation was reduced by 59% and total antioxidant status (TAS) increased by 130%. Again, Aviram attributes these results to the antioxidant characteristics of PJ polyphenols (Aviram, et al., 2004).

Jane Higdon, Ph.D. of The Linus Pauling Institute at Oregon State University takes a different tack and claims that because the bioavailability of Flavonoids is very low, its antioxidant properties cannot be attributed to the antioxidant properties proved in vitro. For example, the bioavailability of Flavonoids in vivo is 100-1000 times lower than the bioavailability of vitamin C or glutathione and thus has a very small antioxidant effect. However, it is available in concentrations that effect cell signaling proteins. "Numerous studies in cell culture suggest that flavonoids may affect chronic disease by selectively inhibiting kinases" including those related to growth factors. Higdon hypothesizes that it is this property that protects against CAD by decreasing inflammation, decreasing vascular cell adhesion molecule expression, increasing endothelial nitric oxide synthase (eNOS) activity, and decreasing platelet aggregation (Higdon, 2005). Another study (Lotito & Frei, 2006), attributes the protective effect of Flavonoids not directly to its antioxidant activity, but rather to the consequence of increased uric acid.[2] What is clear from all these studies is that it is not the antioxidant properties of Flavonoids that directly protect against CAD but rather to a whole host of activities including: increased native antioxidants such as glutathione, uric acid, modified cell signaling pathways, increased PON-1 activity, and increased NO levels.

Possible Risks of Antioxidants vs. Flavonoids:

So then what really does account for the differences in Flavonoids and for example vitamin E? What may be at play is as follows:

Vitamin E as well as having antioxidant properties is a pro-oxidant as well (Bowry et al., 1992). For example: instead of the termination reaction by tocopherol to the oxidative chain reaction of lipids as a final step:

$$LOO\bullet + TOH \rightarrow LOOH + TO\bullet \quad [4]$$

We instead form

[2] Recent studies have implicated hyperuricemia as being a true risk factor in development of cardiovascular disease (Jing Fang & Michael H. Alderman, 2000). However, nearly half of the bodies' antioxidant activity is from uric acid and in moderation may play a positive role.

$$TO\bullet + LH \rightarrow TOH + L\bullet \quad [5]$$

This will then feed into the chain reaction

$$L\bullet + O_2 \rightarrow LOO\bullet \quad [2]$$

$$LOO\bullet + LH \rightarrow LOOH + L\bullet \quad [3]$$

One study (Bowry, Ingold, & Stocker, 1992) has found that in aqueous solution, strikes between the stable TO• and other radicals were found to take upward of 17 minutes, a time long enough that even the most stable radical will find "`something to do'" i.e. propagate the chain reaction. The termination of this reaction must then rely on two endogenous antioxidants vitamin C, as demonstrated by the thiol redox cycles (Appendix 1), and ubiquinol ($Q_{10}H_2$, a reduced form of Q_{10}) in the electron transport chain. What can be inferred from this is that by ingesting vitamin E the body sets off a cascade of endogenous antioxidants, in order to minimize the *damage* being incurred by vitamin E. While the body does have the mechanisms of scavenging TO• how can making more of them by ingesting vitamin E be advantageous? Essentially there is a delicate balance of electron radicals that are passed down a cascade until they can be neutralized to a non reactive end product, and by adding more electron radicals in the form of α-tocopherol it may have the effect of being counterproductive.

C - reactive protein and PON-1

By contrast, Flavonoids are effective not by acting as antioxidants but through modulating the inflammatory response of oxidized cells.

The relationship between C - reactive protein (CRP) and Flavonoids illustrates this point. CRP is found to be elevated during inflammatory response by the body, specifically, macrophages and T cells release Interleukin-6 (IL-6) as a pro inflammatory to stimulate a response to tissue damage. CRP production is then part of the acute-phase response to most forms of inflammation, infection, and tissue damage. CRP levels by themselves cannot be used as marker for atherosclerosis, but they have been positively correlated with CAD (Pepys & Hirschfield, 2003). Flavonoids have an inverse relationship with CRP. One study using demographic data (Chun et al., 2008) found that the greater consumption of Flavonoid rich foods, the lower the concentrations of serum CRP thus clearly indicating that Flavonoids affect inflammatory response. Another study has also found an inverse relationship between levels of CRP and levels of PON-1 expression. They found that "Higher levels of CRP seem to be generally associated with low levels of PON1 activity" (Macknessa et al., 2006). Which should lead to the conclusion that the atherosclerotic protection associated with Flavonoids is not related to an inherent antioxidant activity but rather to other factors as mentioned.

Another study also found that polyphenols (i.e. Flavonoids) acutely enhanced nitric oxide bioactivity. NO is necessary to maintain vasodilatation - the lack of which is implicated in increased risk of cardiovascular disease (Anter et al., 2004).

Conclusions:

In comparing the protective effects on atherosclerosis associated with statins, vitamin E, and Flavonoids, there are many questions as to the ability of statins and vitamin E to afford real protection against atherosclerosis. The data in many cases implicates these very compounds increased mortality rates. By contrast, Flavonoids have shown themselves able to boost the bodies' native antioxidants including uric acid and glutathione, while at the same time reducing the inflammatory response to the uptake of oxidized lipids. It could be that mother really knew best all along "an apple a day – really does – keep the doctor away". (Apple peals have been found to consist of 40% flavonols by weight (Łata & Tomala, 2007)).

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