

# Micronutrients in Women's Health and Immune Function

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Lawrence J. Machlin's contributions to elucidating the roles of nutrients in optimizing human health included the support of research in the areas of women's health and immune function. Several essential nutrients have been shown to affect women's health throughout the different life stages. Symptoms of premenstrual syndrome affect the vast majority of menstruating women, and calcium supplementation significantly reduces physical and emotional symptoms. Premenstrual syndrome in fact might be a predictor of osteoporosis induced by low calcium intake. Periconceptional multivitamin supplementation has reduced the risk of serious birth defects, premature delivery, and low birth weight by 50% and improved maternal health during pregnancy. Micronutrients of particular importance for prevention of adverse pregnancy outcomes are folic acid, zinc, and iron. However, if the preterm delivery is caused by preeclampsia, then data suggest that calcium supplementation and high doses of vitamins C and E significantly reduce that risk. Well-controlled studies consistently have shown that calcium supplementation, with or without vitamin D, significantly reduces the risk of hip fracture. Antioxidants such as vitamins C and E have been shown to reduce the risk of fracture in women smokers. As in the rapidly growing embryo, the immune system includes rapidly multiplying cells whose functions are dramatically affected by an individual's micronutrient status. Multivitamins have been shown to enhance many aspects of immune response, and antioxidant micronutrients consistently have been found to enhance lymphocyte-proliferative responses and skin-test responses, especially in the elderly. *Nutrition* 2001;17:858–867. ©Elsevier Science Inc. 2001

**KEY WORDS:** vitamin E, vitamin C, calcium, folic acid, b-carotene, PMS, fractures

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## INTRODUCTION

Lawrence J. Machlin was a true renaissance scientist with broad interests in the physiologic and preventive roles of vitamins, minerals, and other bioactive molecules in human health.<sup>1–5</sup> I had the privilege of working with Larry as a colleague and peer on a collaborative project to determine whether vitamin E affected the immune functions of laboratory animals.<sup>6–11</sup> Larry never mentioned that he was the leading expert in vitamin E; he simply pointed me to several of his seminal papers on the requirements of vitamin E for muscle integrity, reproductive success, and cardiovascular muscle function and a number of other key references. Larry cochaired two New York Academy of Sciences (NYAS) symposia on vitamin E. My “bibles” were the two annals that Larry coedited after those meetings in 1981 and 1988.<sup>12,13</sup>

The results of our initial collaboration were very exciting and I was asked to join the Vitamin Research group at Roche. I then had the experience of working for Larry, as he was my supervisor for more than a decade at Roche. We worked together to move the laboratory findings for vitamin E and other antioxidants into clinical studies, and we were fortunate to work on the carotenoids as that science was just getting started.<sup>14–29</sup> In the almost 20 y that we worked together, Larry and I coauthored 13 peer-reviewed papers and were coauthors of the text, *Vitamin Intake and Health*.<sup>26</sup> Larry also edited his *Handbook of Vitamins* published in 1984 and issued the revised edition in 1991.<sup>30,31</sup> In 1986, he cochaired the NYAS conference on vitamin C.<sup>27</sup> Larry's favorite NYAS meeting that he

cochaired and named was “Beyond Deficiency: New Views on the Function and Health Effects of Vitamins,” held in 1991 and published in 1992.<sup>28</sup> At the same time, Larry, as Director of the Department of Clinical Nutrition at Roche, spearheaded collaborations with more than 100 academic researchers worldwide, and we provided clinical supplies (actives and matched placebos) specially formulated for numerous intervention studies, funding, labeled and purified vitamins and carotenoids for analytics and stable isotope studies, and technical expertise and advice.

Larry also introduced me to the world of editing professional reference books and I worked closely with him to revise his classic *Handbook of Vitamins*.<sup>30,31</sup> In addition, Larry opened the doors for me to enter the area of women's health with Roche's involvement in the unfolding story of folic acid and the prevention of serious birth defects.<sup>32–36</sup> As Director, Larry set the supportive agenda and strongly encouraged me to organize the two NYAS symposia on “Micronutrients and Immune Functions” held in 1989<sup>37</sup> and “Maternal Nutrition and Pregnancy Outcome” held in 1992.<sup>34</sup> When Larry retired, he remained active as a consultant until the day he passed away and thus continued to contribute to our knowledge of the importance of antioxidant vitamins, carotenoids, and many other bioactive molecules in preventing disease and optimizing health. The true legacy of Larry's encouragement, enthusiasm, and dedication to scientific advancements in the area of micronutrients and optimizing human health may never be fully told because he gave so much to the researchers in this field.

After I left Roche and began research involving calcium and vitamin D, Larry was as interested in these new findings as he was in any of the studies with the antioxidants. Most importantly for me on a personal level, throughout the almost 20 y I knew him, Larry was a true mentor. Larry and his dear wife, Ruth, became close friends to me and my husband and we shared many life events including the births of our grandchildren and vacation photographs of natural wonders. As my tribute to Larry, this paper

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Date accepted: June 10, 2001

highlights many of the findings of the past decade on the critical roles of the essential nutrients in women's health and immune function.

## WOMEN'S HEALTH

The studies that are highlighted in this overview were chosen mainly because of the involvement of Larry, myself, or both of us in the initiation, development, and/or expansion of the scientific knowledge base. *Women's health* is a term that covers many topics; this review is limited to nutritional aspects of premenstrual syndrome (PMS), pregnancy outcomes including reduction in birth defects, preterm and low-birth-weight (LBW) outcomes, pre-eclampsia, and osteoporosis.

### Premenstrual Syndrome

The term *premenstrual syndrome* refers to a cluster of mood, physical, and cognitive symptoms that occur during the luteal phase of the menstrual cycle and subside with the onset of menstruation. As many as 80% of women of reproductive age may experience premenstrual emotional and physical changes.<sup>38</sup> Up to 40% of women of reproductive age experience premenstrual symptoms sufficient to affect their daily lives to some degree, and 3% to 5% experience severe impairment in a disease state known as *premenstrual dysphoric disorder*.<sup>39</sup> Symptoms differ across individuals; the most common symptoms are fatigue, irritability, abdominal bloating, breast tenderness, and labile mood with alternating sadness and anger, and moodiness or depression.<sup>38</sup>

A wide variety of strategies for PMS have been proposed. For women with mild symptoms, education, supportive counseling, and general self-care measures such as increased exercise and adoption of a healthful diet might be sufficient.<sup>40</sup> For those with severe symptoms, a variety of drugs may be helpful. For many women, however, neither lifestyle change nor the use of drugs is an entirely satisfactory approach to PMS.

The desire for safe and effective non-drug alternatives has prompted many women to consider the use of nutrient-containing dietary supplements for PMS. Numerous supplements have been advocated for this purpose; however, there are relatively few well-controlled trials that have carefully examined the effects of nutrition supplements on PMS symptoms.

A single double-blind trial evaluated the effects of vitamin E supplementation in PMS.<sup>41</sup> In that trial, 41 women with PMS received 400 IU/d of vitamin E or placebo for three cycles; significant improvements in some affective and physical symptoms were observed in the vitamin E group. Unlike most other PMS studies, there was no effect seen in the placebo group. No further trials have been reported. It would be of interest to investigate vitamin E again in a new trial with more rigorous, up-to-date methods of subject selection and symptom assessment.

Vitamin B6 has been advocated widely for PMS, but evidence of its efficacy is inconsistent. Of the 25 published trials, only 12 were positive.<sup>42,43</sup> In a number of the positive trials, the dosage was above the tolerable upper intake level advocated by the National Academy of Science's Food and Nutrition Board. Women who wish to use vitamin B6 should be cautioned not to exceed the accepted safety limit of 100 mg/d.<sup>44,45</sup>

Supplements containing long-chain fatty acids have been examined for the reduction of PMS. Evening primrose oil is the only such product tested for efficacy. Controlled studies of this supplement have had inconsistent results, with the most rigorous studies showing no evidence of a beneficial effect.<sup>46,47</sup>

In contrast to the above-mentioned nutrients, there is a long history of scientific examination of the link between calcium status and the menstrual cycle. A 1930 study<sup>48</sup> showed that plasma calcium levels were lower during the premenstrual period than during the week after menstruation. In 1989, a small (33 partici-

pants) randomized cross-over trial<sup>49</sup> found a significant reduction in premenstrual symptoms after supplementation with 1000 mg/d of elemental calcium (as calcium carbonate). In 1993, decreased symptoms of premenstrual and menstrual distress were reported when women received diets containing 1336 mg/d as opposed to 587 mg or calcium.<sup>50</sup>

The promising results of these preliminary studies served as the impetus for the largest clinical trial for PMS using a dietary supplement in the United States.<sup>51</sup> In that trial, 466 women with rigorously diagnosed PMS received 1200 mg/d of elemental calcium (as calcium carbonate) or placebo for three menstrual cycles. By the third cycle, those receiving calcium showed an overall 48% reduction in symptom scores compared with a 30% reduction in the placebo group. All of four symptom factor scores (negative affect, water retention, food cravings, and pain) were significantly improved by calcium supplementation.

The amount of calcium administered in the trial was well within accepted safety limits. The upper intake level for calcium (i.e., the maximum intake known to be safe) has been set at 2500 mg/d.<sup>52</sup> Results from the US Department of Agriculture's (USDA's) 1994 Continuing Survey of Food Intakes by Individuals showed that, among menstruating women (age 12 to 50 y), the mean daily intake of calcium ranged from 607 to 809 mg, suggesting that most of the population at risk for PMS is not receiving the recommended intake level of calcium.<sup>53</sup> Therefore, because most women consume far less than 1000 mg/d of calcium from food, they would not exceed the safety limit if they added 1000 to 1200 mg/d of supplemental calcium to their normal dietary intakes. Calcium, unlike some other supplements tested for PMS efficacy, is safe even for women who might become pregnant.

There is evidence that abnormalities in calcium and vitamin D regulation contribute to the causation of PMS and that PMS is linked to other disorders associated with inadequate calcium intake such as osteoporosis. A study that compared women with established vertebral osteoporosis with control subjects found that the risk of osteoporosis was higher among those with histories of PMS.<sup>54</sup> Another study found evidence of reduced bone mass in women with PMS as compared with asymptomatic controls.<sup>55</sup> Thus, PMS might serve as a clinical marker of low calcium status, perhaps reflecting an underlying abnormality in calcium metabolism, and it may serve as an early warning sign to young women of a possible increased risk of osteoporosis. The use of calcium supplements therefore might benefit women with PMS by reducing their current symptoms and promoting better bone health in later life.

The totality of evidence indicates that, of all nutrition supplements examined for effects on PMS symptoms, only calcium has consistent efficacy, with intake levels that are below the upper intake level, has a plausible mechanism of action, and might even be a marker of low intake in women at risk for osteoporosis.<sup>56</sup>

### Pregnancy Outcomes: Birth-defect Prevention

The two leading causes of infant mortality are birth defects and premature birth.<sup>57,58</sup> One of the most important discoveries of the 20th century is that micronutrients can prevent many serious birth defects and reduce the risk of premature and LBW birth outcomes. Many of the initial observations concerning the link between diet and serious birth defects were made in the United Kingdom. Of particular note were the observations of Smithells et al. who noted that women who gave birth to infants without craniums and brains (anencephaly) or infants with holes in their spines (spina bifida) also had poor dietary habits and were from areas of poverty.<sup>59,60</sup> Of great concern was the fact that women with one affected pregnancy were at about a 10-fold increased risk of having a second infant with the same neural-tube defect (NTD). Smithells et al. requested permission to initiate a placebo-controlled, double-blind study using a simple multivitamin that contained vitamin C and

several B vitamins including 0.36 mg of folic acid. They received permission to do an intervention study with the supplement and found an approximately 70% reduction in recurrence in the women who took the supplement during the periconceptional period, i.e., before conception (about 1 to 3 mo) and during the early months of their pregnancies.

Other data from subsequent studies pointed to a strong link between folic-acid supplementation and the reduction in recurrent NTD. The definitive placebo-controlled, double-blind study was published in 1991 by the Medical Research Council (MRC) and led by Dr. Nicholas Wald.<sup>61</sup> In that study, recurrence of NTD was reduced by 70% when a folic-acid supplement containing 4 mg was taken during the periconceptional period; additional supplementation with some of the B vitamins did not confer further reduction in NTD. Even though no other types of birth defects were reduced in the supplemented groups, that study clearly showed that a simple B vitamin, folic acid, could prevent NTD, a major cause of infant death and disability.

Much work is ongoing to elucidate the mechanisms by which folic acid prevents this incomplete closure of the neural tube. One hypothesis concerns the exceptional requirement of the embryo for folic acid during cell duplication because the vitamin is required for the synthesis of nucleic acids and proteins; another theory concerns the presence of higher-than-normal levels of the amino acid, homocysteine, that might preferentially destroy neural-tube cells. There appears to be an inverse relation between serum levels of folic acid and homocysteine.<sup>57</sup>

The studies that found that folic acid prevented recurrence of NTD cannot be underestimated, but the more prevalent event is the first occurrence of the NTD. In 1992, Czeizel and Dudas published the results of their impressive study showing that a prenatal multivitamin supplement containing 0.8 mg of folic acid not only reduced NTD by more than 90% but also halved the rate of all serious major birth defects in the group taking the supplement compared with the placebo group.<sup>62</sup> That study involved nearly 5000 pregnancies and the results were greater than ever expected. Cardiovascular birth defects, which occur at a rate 5 to 10 times than NTD, were reduced by 60% and there were fewer cleft-lip and cleft-palate defects, kidney defects, and limb reductions. The prenatal supplements were taken during the periconceptional periods and throughout the pregnancies, and Roche provided those supplements and the matched placebos.

In addition to the significant reductions in several classes of birth defects, the supplemented group had a 40% increase in multiple births (twins) and the women had easier pregnancies, with significantly less morning sickness. During the preconceptional period, the supplemented group's menstrual cycles became more regular, the time taken to become pregnant was shorter than in the placebo group, and there was a 7% increase in the rate of conception.<sup>57</sup> Moreover, based on a health-economics analysis, we found that the health benefits of periconceptional supplementation for all U.S. women of childbearing potential are highly cost effective, and certainly the reduction in pain and suffering for the child and family was immeasurable.<sup>63</sup>

It is important to note that the prenatal multivitamin supplement used by Czeizel and Dudas contained 12 vitamins (including 800  $\mu\text{g}$  of folic acid, 4  $\mu\text{g}$  of vitamin B12, 2.6 mg of vitamin B6, 100 mg of vitamin C, and 15 IU of vitamin E). In the British MRC trial on the prevention of NTD recurrence, that multivitamin contained only eight of the vitamins and did not include vitamins E and B12; it contained only 40 mg of vitamin C and 1 mg of vitamin B6. Even though it contained 4 mg of folic acid (five times the level in the trial by Czeizel and Dudas), there was no decrease in cardiovascular birth defects in the group taking folic acid alone or the one taking folic acid plus the multivitamins in the MRC trial. Cardiovascular birth defects were not decreased in the group taking the multivitamins without folic acid as compared with the placebo group.<sup>61</sup>

The supplement used in the trial by Czeizel and Dudas is more

comparable to the typical one-a-day type of multivitamin/mineral supplement sold in the United States than the supplement used in the MRC trial. Thus, it is not surprising that survey data from the United States associated lowered cardiovascular birth-defect risk with periconceptional use of multivitamin/mineral supplements.<sup>57</sup>

Unfortunately, there are few women (about 20%), even today, who take multivitamins during the periconceptional period, before they have confirmed their pregnancies.<sup>64</sup> In an editorial in the *New England Journal of Medicine*, Oakely suggested that the right advice to American women is that they eat the best diets possible and also take multivitamins containing folic acid to ensure that the birth-defect preventive level of folic acid is consumed daily.<sup>65</sup> This advice was given even after the U.S. Food and Drug Administration initiated its policy for the fortification of enriched grain products and flour with folic acid. This level, which raises folic-acid intakes on average by 100  $\mu\text{g}/\text{d}$ , might not be sufficient to prevent birth defects responsive to folic acid. Further verification of the need to take 400  $\mu\text{g}$  of folic acid was provided by a recent study from China.<sup>66</sup> There was a four-fold reduction in NTD in the high-risk area and a 40% reduction in a lower-risk area when women anticipating pregnancy took 400  $\mu\text{g}$  of supplemental folic acid during their periconceptional periods.

### **Prevention of LBW and Premature Birth**

LBW and preterm delivery often occur simultaneously. Preterm delivery is defined as birth after fewer than 37 wk of gestation; very-preterm delivery is defined as fewer than 33 wk of gestation. LBW is defined as less than 2500 g and very LBW is less than 1500 g. Of all LBW infants born, 60% to 70% are also preterm. Preterm delivery associated with LBW is the second leading cause of infant hospitalization and the second leading cause of infant mortality, after birth defects.<sup>67</sup> LBW is second only to cardiovascular birth defects in annual hospitalization costs, exceeding \$2.5 billion/y.<sup>63</sup> The vast majority of these costs is borne by public funds and the affected mothers and children often are the least equipped to deal with this event. Preterm births are more prevalent in teenagers, those with less than a high-school education, and those with the lowest incomes.

Scholl et al.<sup>36</sup> in a prospective case-control study found that pregnant women in Camden, New Jersey who took prenatal multivitamins during their first trimesters had four-fold reductions in very preterm births and two-fold reductions in preterm births. Even if supplementation began in the second trimester, there were significant two-fold reductions in very-preterm and preterm births. The risk of very-LBW outcomes (which was highly correlated with preterm delivery) was dramatically reduced by six- to seven-fold when prenatal multivitamins were taken during the first two trimesters. LBW also was reduced significantly with supplementation. Those results were found in women who were at high risk for preterm or LBW outcomes: they were poor, teenagers, and many had low weight gains during pregnancy. Prenatal supplements significantly increased iron and folate status but did not alter serum zinc levels. Low iron and/or folate status had been associated with increased risk of preterm and LBW. Zinc-containing multivitamins also reduced preterm births in an intervention study.<sup>68</sup>

Links recently have been made between maternal diet during pregnancy, preterm birth, and cardiovascular disease in offspring at least 50 y after their births.<sup>69,70</sup> We will have to wait to see whether women who used supplements before and during pregnancy have children who, at middle age, have fewer chronic diseases such as cardiovascular disease. Thus, a program that provides multivitamins containing folic acid to all women of childbearing potential before and during their entire pregnancies probably will have far greater consequences than the immediate effects of reduction of birth defects or preterm births. The full effects of reducing adverse birth outcomes in developed nations might not be realized until those children reach their senior years.

In developing countries, premature births are an even more serious problem. Another problem is intrauterine growth retardation that is often coincident with premature birth but also can occur with term delivery. DeOnis et al.<sup>71</sup> reviewed the 12 nutritionally based interventions that have been examined for their potential to reduce the incidence of intrauterine growth retardation and often preterm birth and LBW. Only one intervention, supplementation that balanced protein and energy during pregnancy, significantly reduced the risk of LBW. The researchers also suggested that many of the micronutrient supplementations they reviewed are likely to prove beneficial, and larger cohorts are required to assure their efficacy. These micronutrients were vitamin D, folic acid, zinc, calcium, magnesium and iron. Ramakrishnan et al.<sup>72</sup> also extensively reviewed observational and intervention studies from developing and developed countries that examined the role of micronutrients in optimizing pregnancy outcomes. In developing countries, one of every five infants are LBW (20%) versus a rate of 6% in developed countries. Moreover, in developing countries, most LBW infants are carried to term. Thus, intrauterine growth retardation is a significant problem in developing countries and affects the potential physical and most likely the mental growth of the child throughout its lifetime. Low maternal micronutrient intake of zinc, calcium, magnesium, vitamin A, vitamin C, and possibly B vitamins, copper, and selenium has been associated with premature birth and LBW. Deficiency of iodine, folate, and/or iron also has been linked to adverse pregnancy outcomes.

Since the publication of those reviews, two key papers have been published from large, well-controlled intervention studies in developing countries. West et al.<sup>73</sup> examined, in a placebo-controlled trial, the effects of weekly supplementation with the recommended dietary allowance of preformed vitamin A or  $\beta$ -carotene in more than 20 000 pregnant women in Nepal. They found a significant, 40% reduction in maternal mortality in the supplemented group. It is important to note that the women were provided the supplements before conception and throughout their pregnancies. Interventions were a once-a-week supplement of 20 000 IU of vitamin A as retinol or 42 mg of  $\beta$ -carotene or placebo.  $\beta$ -Carotene appeared more effective than the preformed supplement of retinol. Low  $\beta$ -carotene status has been reported in cases of preeclampsia in women from developing countries and, as discussed below, the antioxidant potential of  $\beta$ -carotene (versus the much lower antioxidant potential of retinol) may have been involved in reducing the incidence of maternal mortality in that study.

The second critical study involved more than 1000 pregnant women with human immunodeficiency virus (HIV) from Tanzania.<sup>74</sup> In that placebo-controlled trial, the pregnant women received placebo;  $\beta$ -carotene and preformed vitamin A; a multivitamin containing vitamins B1, B2, B6, B12, niacin, folic acid, and vitamins C and E; or the multivitamin plus  $\beta$ -carotene and retinol (provided by Roche) from 12 to 27 wk of gestation to birth. There were 40% reduction in fetal death, 44% reduction in low birth weight, 39% reduction in very preterm birth, and 43% reduction in small-for-gestational-age outcomes in the groups supplemented with the multivitamin independent of the vitamin A. In addition, mothers taking the multivitamin had significantly heavier babies than those not taking the multivitamin ( $P = 0.01$ ). Even though the supplement did not contain iron, the women in the multivitamin group had significant increases in their hemoglobin levels compared with those not taking the multivitamin. Because the study involved HIV-positive women, the investigators also measured the concentration of total T cells (CD3), T-helper cells (CD4), and T-suppressor cells (CD8). HIV-positive pregnant women who took the multivitamin supplement had significant increases in total T cells due mainly to increases in CD3 cells; CD8 cells also increased. Although vitamin A did not show an effect in this study, it is important to note that the HIV-positive women had low vitamin A status at the onset and the level provided may not have been sufficient or may not have been absorbed sufficiently to show

an effect. Another possibility is that the vitamin A was administered too late in these pregnancies to see its effect. In the study by West et al.,<sup>73</sup> vitamin A supplementation was started before conception. Vitamin A is critical for early embryonic growth. The critical finding in the study by Fawzi et al. was that a multivitamin supplement containing modest doses of micronutrients significantly improved birth outcomes at levels similar to that seen when poor, non-HIV-positive women in the United States used multivitamins during their pregnancies.<sup>58</sup>

### Preeclampsia

Preeclampsia is defined as hypertension and proteinuria beginning during the second half of gestation. Approximately 5% of pregnant women develop this condition during their pregnancies. Preeclampsia is the leading cause of maternal death and accounts for more than 40% of premature births worldwide.<sup>75-77</sup>

Several mechanisms have been proposed for the initiation and progression of preeclampsia, including oxidative damage to the placenta and/or imbalance in blood-pressure regulation.<sup>78</sup> Because of the importance of micronutrients such as vitamins C and E (and  $\beta$ -carotene) as antioxidants and the association of low calcium status and high blood pressure, these micronutrients have been studied in well-designed protocols to determine their effects on preeclampsia.

Fourteen randomized clinical trials that involved the use of calcium supplements during pregnancy to determine effects on maternal blood pressure and preeclampsia were reviewed by Bucher et al.<sup>79</sup> They concluded that calcium supplementation (usually 1500 to 2000 mg/d) led to a significant reduction in maternal systolic and diastolic blood pressures and 62% reduction in the risk of preeclampsia. The investigators recommended that calcium supplementation be provided for all women at risk for preeclampsia. It should be noted that the populations included in that analysis had calcium intakes of about 300 to 500 mg/d, well below recommended intake levels.<sup>80,81</sup>

Two important studies were published after the review. Levine et al.<sup>77</sup> randomized more than 4500 pregnant women to 2000 mg/d of calcium or placebo and found no difference in the rate of preeclampsia between groups (about 7%). However, those women were not at increased risk for preeclampsia and they had dietary calcium intakes of over 1100 mg/d. In a recent reevaluation of the data concerning calcium and risk of preeclampsia, DerSimonian and Levine<sup>76</sup> found that calcium supplementation did not decrease the risk of preeclampsia, but for high-risk pregnancies, calcium supplementation appeared to decrease the risk significantly. High-risk pregnancies include teens, women with preexisting hypertension, and women carrying multiple fetuses.

Another study of interest examined the blood pressure of children of mothers who took calcium during their pregnancies to prevent preeclampsia. Belizan et al.<sup>82</sup> found that at age 7 y the group of children whose mothers had taken 2000 mg/d of calcium during their pregnancies had significantly lower systolic blood pressure. The greatest antihypertensive effect was seen in the overweight children.

Oxidative stress is another mechanism that has been examined in preeclampsia. Vitamin E is the major lipid-soluble antioxidant in serum and vitamin C is the major water-soluble antioxidant in the blood. Wang et al.<sup>83</sup> found and Jain and Wise<sup>84</sup> confirmed that serum lipid peroxide levels are significantly higher and serum vitamin E levels significantly lower in women with preeclampsia than in women with normal pregnancies.

Chappell et al.<sup>75</sup> enrolled 283 pregnant women at risk for preeclampsia in a placebo-controlled trial using daily supplements of vitamin C (1000 mg) and vitamin E (400 IU). There was a significant, 61% reduction in risk of preeclampsia in the antioxidant-supplemented group. The investigators hypothesized that antioxidants stabilize the maternal endothelium and placenta

and thus reduced preeclampsia risk. They found that the plasma marker for endothelial activation and the index for placental dysfunction decreased significantly in the supplemented group. The levels of the antioxidants used in the study were well above national recommended intake levels; therefore, the study also provides important data concerning the safety of high doses of antioxidants during pregnancy.

### **Osteoporosis**

More than 6 million U.S. adults, mainly women, have osteoporosis<sup>85</sup> which is defined as having bones that are two standard deviations below the peak bone mineral density in young adults.<sup>85,86</sup> Osteoporosis is a known risk factor for hip fracture.<sup>87,88</sup> There are nearly 300 000 annual hip fractures in the United States. Those with hip fractures have increased risk of institutionalization and death.<sup>89</sup>

Supplemental calcium with or without vitamin D has been shown to reduce the risk of hip fractures.<sup>90–92</sup> Three placebo-controlled, double-blind studies have shown that calcium supplementation (with or without vitamin D) significantly reduce the risk of hip fracture in individuals older than 50 y. The studies were mainly in women, although one included men. Bendich et al.<sup>93</sup> conducted a meta-analysis from the risk-reduction data presented in those papers and arrived at a Mantel–Haenzel combined relative-risk estimate of 0.53 (95% confidence interval = 0.31–0.90) for hip fractures. The pooled relative risk for all non-vertebral fractures, including hip, was 0.61 (95% confidence interval = 0.46–0.80). Thus, in the studies, there was a 47% reduction in the risk of hip fracture in those individuals who took supplemental calcium at levels from 500 to 1200 mg/d for up to 3.4 y. There was an additional benefit of a 39% reduction in all types of non-vertebral fractures.

New data indicate that vertebral fractures also are reduced in many older women who supplement with 1200 mg/d of calcium. In a placebo-controlled, double-blind study in women older than 60 y who consumed less than 1000 mg/d of calcium, Recker et al.<sup>94</sup> found that supplementation significantly reduced the risk of spine fractures, especially in women with histories of bone fractures.

The National Institutes of Health and the National Academy of Sciences recommend that postmenopausal women not taking estrogen-replacement therapy should consume 1500 mg/d of elemental calcium.<sup>80,81</sup> The benefit of estrogen-replacement and other antiresorptive therapies for osteoporosis prevention are predicated on the daily consumption of 1000 mg of calcium.<sup>95</sup> However, data from a telephone survey of a representative sample of U.S. households showed that the average daily intake of dietary calcium falls far short of the minimum recommended daily amount of 1000 mg. The telephone survey found that only half of the adults 60 to 94 y drank one glass of milk, which provides 300 mg of calcium, every day.<sup>96</sup> LeBoff et al.<sup>97</sup> measured the vitamin D and calcium status of postmenopausal women with hip fractures and found that 50% had deficient vitamin D levels and more than 80% had low calcium levels. Because vitamin D is required for calcium absorption, the researchers suggested that the low calcium status is linked to the low vitamin D status. Thus, these data suggest that individuals at risk for hip and other fractures should increase their intakes of calcium and vitamin D.

Recent data have suggested that fracture risks are actually greater in men when they are young than in age-matched women and that this risk changes as women age. Singer et al.<sup>98</sup> documented the incidence of fractures in individuals 15 to 94 y of age in Edinburgh, Scotland. They reported that between the ages of 15 and 49, men had 2.9 times the fractures as age-matched women; fractures of the wrist began to increase in women at age 40 y, before menopause, and women older than 60 y had 2.3 times the risk of fractures as men. Although the study did not examine nutrition factors, other studies have linked increased risk of frac-

tures in young adults with low intakes of calcium and other micronutrients and low sun exposure (and consequently, low vitamin D status), as found in Scotland.

In addition to calcium, antioxidant status has been associated with hip fracture risk. Lifestyle factors, such as smoking, that decrease antioxidant status also increase the risk of hip fracture. Melhus et al.<sup>99</sup> found a three-fold increased risk of hip fracture in women who were current smokers and had the lowest intakes of vitamin E or C compared with non-smoking women with the highest antioxidant intakes. If the smokers had the lowest intakes of both vitamins, the odds ratio for hip fracture increased to 4.9.

Smoking, independent of antioxidant status, increases the risk of hip fracture, possibly because of its association with decreased calcium absorption.<sup>100</sup> To complete the circle of life events, Jones et al.<sup>101</sup> found that maternal smoking during pregnancy resulted in their children having shorter stature that was linked to lower bone mass. The children of smoking mothers may well be at greater risk for osteoporosis because their bones never accumulate the bone mass needed to prevent that disease in later life. Importantly, infant bone mass increases if mothers are supplemented with calcium during pregnancy. Koo et al.<sup>102</sup> found that total bone mineral content was significantly greater in infant children born to mothers supplemented with 2000 mg/d of calcium during pregnancy than in women in the placebo group who consumed less than 600 mg/d of calcium.

### **Immune Function**

Investigation of the roles of single vitamins on immune function was relatively new in the early 1980s, and I was the fortunate immunologist who joined Larry Machlin in exploring the effects of feeding graded levels of vitamin E on the immune responses of laboratory animals. We prevented testes and muscle degeneration with relatively low levels of dietary vitamin E but needed considerably higher levels (50 times more) for optimal immune responses.<sup>11</sup> We also found that vitamin C protected vitamin E levels in serum and vice versa and the combination of antioxidants provided the best immune responses in another animal model.<sup>8</sup> We were very fortunate to work with the early supplies of Roche  $\beta$ -carotene beadlets. That beadlet processing significantly enhanced the absorption of the nutrient in rats and so we could study  $\beta$ -carotene and canthaxanthin (a carotenoid that lacks vitamin A activity but is very similar in structure to  $\beta$ -carotene) in immune-function models.<sup>15</sup> We showed and others have confirmed that the immunoenhancing functions of  $\beta$ -carotene were independent of its function as a precursor of vitamin A. Although Larry was not listed as an author of that paper, he was my supervisor and very excited by those important results. It is to Larry's credit that he did not require that he be a coauthor on all of the research papers that were developed under his supervision.

When Roche determined that laboratory research on vitamins and carotenoids would be done only in Basel, we began our investigations of the importance of antioxidant vitamins for human immune responses. We visited the USDA Human Nutrition Research Center at Tufts University. With the help of Jeff Blumberg and Simin Meydani and then with Irv Rosenberg, we showed that humans also require higher levels of vitamin E for optimal immune responses than the requirements to protect dietary fat from oxidation.<sup>103</sup> Larry and I shared in the immense satisfaction that the data derived from animal studies were replicated in and thus of great relevance to humans.

Similarly, there have been many confirmations of the importance of  $\beta$ -carotene in enhancing a number of immune responses.  $\beta$ -carotene supplementation has been shown to enhance natural-killer immune cell functions such as the killing of tumor cells<sup>104</sup> and increase the secretion of tumor necrosis factor from human monocytes.<sup>105</sup>  $\beta$ -carotene is the major dietary carotenoid precursor of vitamin A. Vitamin A cannot quench singlet oxygen and has

less antioxidant activity than the  $\beta$ -carotene; however, its importance in clinical medicine<sup>106</sup> and for the immune system is well recognized.<sup>107,108</sup>

Vitamin C, the major water-soluble antioxidant in human serum, was clearly shown to be essential for overall immune responses as measured with the delayed-hypersensitivity skin-test response (DTH). Jacob et al.<sup>109</sup> examined the effects of marginal vitamin C deficiency on immune and other parameters in healthy males. Serum levels of white blood cells and levels of vitamin C in sperm were reduced significantly when the diet contained 5, 10, or 20 mg of vitamin C for 2 mo. DTH responses to seven antigens also were depressed significantly during the period of low vitamin C intake. In fact, when subjects initially consumed 250 mg/d of vitamin C, they responded to 3.3 out of 7 antigens, which were reduced to less than one antigen after only 1 mo at 5 mg/d of vitamin C. Even when intakes were increased back to 250 mg/d for 1 mo, the average number of DTH responses did not increase above one of seven antigens. The robustness of the response, as determined by the diameter of the induration, was 35 mm at baseline, dropped to 11 mm when vitamin C intakes were 5, 10, or 20 mg for 2 mo, and increased to half of the initial level (17 mm) when 60 or 250 mg of vitamin C was consumed daily for 1 mo. Immune function did not return to baseline levels even though vitamin C concentrations in serum and white blood cells returned to baseline levels when vitamin C intake was increased to 250 mg/d.

The vitamin C study used young men as the study population. Cell-mediated immune responses such as DTH have been related to the age-related decline in immune responses.<sup>110</sup> As a consequence, DTH responses to skin-test antigens diminish significantly in the elderly and often can result in complete loss of response to antigen challenge (anergy) in the most immunosuppressed individuals.<sup>111,112</sup> Merrie et al.<sup>110</sup> documented the progressive decline in the number and diameter of skin-test responses to seven test antigens in individuals aged 66 to 82 y with those in individuals aged 25 to 40 y; 35% of those aged 25 to 40 y had positive responses to five of seven antigens, whereas none of those aged 66 to 82 y had five responses. Moreover, only 1.5% of the younger group was anergic (no responses to the seven antigens); 18% of the older group had no responses. In addition to responding to fewer antigens, the older group had approximately half the induration response of the younger group.

Clinical studies have shown that DTH can be used as a predictor of morbidity and mortality in the elderly; e.g., anergic elderly had twice the risk of death from all causes as elderly who responded to the antigens.<sup>113</sup> Moreover, in hospitalized elderly who had undergone surgery for any reason, anergy was associated with a greater than 10-fold increased risk of mortality and a 5-fold increased risk of sepsis.<sup>112</sup> DTH responses also are indicative of morbidity within an age-matched elderly population; those who lived at home and were self-sufficient averaged positive responses to two antigens and indurations of about 8 mm compared with those in nursing homes who were self-sufficient (1.1 response and 4-mm induration) and nursing-home residents who were not self-sufficient (0.5 response and 4-mm induration).<sup>111</sup> Thus, if micronutrient supplements could improve DTH responses in the elderly, the health effects could be very great.<sup>114</sup>

Two placebo-controlled, double-blind studies have found that vitamin E supplementation alone can significantly enhance DTH responses, antibody titers to certain vaccines and proliferative responses, and interleukin-2 activities in the elderly. In a carefully controlled study conducted in a metabolic ward where the daily diet contained approximately recommended daily allowances of all nutrients, vitamin E supplementation (800 IU/d) for 1 mo significantly increased DTH responses of healthy elderly.<sup>103</sup> Vitamin E levels in lymphocytes increased more than three-fold with supplementation and were correlated with enhanced immune responses such as enhanced interleukin-2 production. The group supplemented with vitamin E showed no adverse effects<sup>115</sup>; in fact, the

beneficial effects included enhanced lymphocyte proliferation, decreased production of immunosuppressive prostaglandin E<sub>2</sub>, and decreased levels of serum lipid peroxides. Those subjects consumed meals containing the recommended levels of all nutrients including vitamin E while they were residents of a metabolic ward. Thus, the diets and environments of the placebo and vitamin E groups were virtually equivalent. The researchers concluded, "it is encouraging to note that a single nutrient supplement can enhance immune responsiveness in healthy elderly subjects consuming the recommended amounts of all other nutrients."

Meydani et al.<sup>116</sup> extended their previous findings and examined the effects of 6 mo of supplementation with 60, 200, or 800 IU/d of vitamin E in a placebo-controlled, double-blind study in healthy, free-living elderly. In addition to DTH responses, they determined *in vitro* proliferation and *ex vivo* antibody titers to clinically relevant vaccines. DTH responses increased significantly above placebo levels in all three supplemented groups; the greatest responses were seen in the 200-IU group. *In vitro* proliferative responses were the highest in the 800-IU group. Antibody titers to tetanus were unaffected by vitamin E supplementation, but titers to hepatitis B vaccine were the highest in the 200-IU group.

Pallast et al.<sup>117</sup> investigated the effects of 6 mo of supplementation with vitamin E at doses of 50 and 100 mg daily for 6 mo in healthy older men (aged 65 to 80 y). There was a dose-related trend of increased DTH responses, especially in those subjects with initially low responses, suggesting that those with low vitamin E status might benefit most from vitamin E supplements.

Low serum vitamin E levels also have been seen in individuals with impaired immune responses associated with viral infection. Von Herbay et al.<sup>118</sup> reported that serum vitamin E levels were significantly lower in patients with severe viral hepatitis than in controls and those levels returned to control levels when the hepatitis subsided. These data suggested that hepatitis involves oxidative reactions that consumes vitamin E and as a consequence might decrease potential immune responses to the disease. Comstock et al.<sup>119</sup> found that lower-than-average serum vitamin E levels precede the diagnosis of two autoimmune diseases, rheumatoid arthritis and systemic lupus erythematosus. Low vitamin E status has been associated with the conversion of an avirulent viral strain to a virulent one in an animal model.<sup>120</sup>

Although our initial interest was the determination of the effects of single vitamins such as vitamin E, vitamin C, and  $\beta$ -carotene on DTH responses, we were well aware that the most widely used nutrition supplement was the multivitamin. Multivitamins usually contain 100% of the daily value of vitamins C and E (60 mg and 30 IU, respectively) and can contain 5000 IU of vitamin A as a combination of retinol and  $\beta$ -carotene (where 3 mg of  $\beta$ -carotene is equivalent to 100% of the daily value for vitamin A). The average daily adult dietary intakes are approximately 100 mg for vitamin C, 8 IU for vitamin E, and 1 to 2 mg of  $\beta$ -carotene. Thus, the level of vitamin E in the multivitamin represents about three times the usual intake levels, whereas levels of vitamin C and  $\beta$ -carotene approximate the usual intake levels.<sup>121</sup>

Intake of the one-a-day type of multivitamin/mineral supplement for 12 mo significantly enhanced DTH in healthy elderly compared with the placebo group.<sup>122</sup> There was a less than 10% increase in the number of responses and the level of induration over the 12 mo compared with baseline values in the placebo group and greater than 60% increase in the multivitamin-supplemented group. Because the multivitamin included  $\beta$ -carotene (approximately 1 mg), vitamin E (30 IU), vitamin C (60 mg), all other essential vitamins and several minerals, it is not possible to determine whether the antioxidants or the other components in the multivitamin supplement were responsible for the improved DTH responses. However, the only significant changes in serum micronutrient levels were for vitamin C, vitamin E,  $\beta$ -carotene, folic acid, and vitamin B6.

Pike and Chandra<sup>123</sup> showed an increase in the number of natural-killer cells in 35 healthy elderly (average age of 69 y) who

participated in a 1-y, placebo-controlled, double-blind study involving a multivitamin/mineral supplement that contained 45 IU of vitamin E and 90 mg of vitamin C. There was a significant decrease in T-helper cells in the placebo group that resulted in a significant decline in the helper:suppressor ratio over 1 y, which was not seen in the supplemented group.

In another placebo-controlled intervention trial, elderly were given a multivitamin daily for 1 y that contained approximately eight times the standard level of intake of  $\beta$ -carotene (16 mg). The supplemented group had significantly fewer infections than the placebo group. Responses to influenza vaccine as measured by increased antibody titers also were improved in the supplemented group.<sup>124</sup>

The capacity to survive pneumonia and influenza is significantly reduced in the elderly.<sup>125</sup> Of the 20 000 deaths associated with pneumonia and influenza reported in 1991 in the United States, more than 90% were in persons aged 65 y and older. For bacterial pneumonia, there is a vaccine, but it is only 56% effective in preventing pneumococcal pneumonia, the most common cause of bacterial pneumonia. Currently only 28% of elderly receive pneumococcal vaccination, and 52% receive influenza vaccination. Thus, it is especially important to improve immune responses in this at-risk population because another major consequence of reduced immune responses in the elderly is decreased effectiveness of vaccination (which can be predicted by reduced antibody titer after inoculation). Poor immune responses to vaccines such as the pneumococcal, hepatitis, and/or the flu vaccine can increase the risk of morbidity and mortality, especially in frail elderly.

In addition to aging, there are environmental factors that can suppress immune responses. Fuller et al.<sup>21</sup> studied the effect of  $\beta$ -carotene supplementation on ultraviolet-radiation-induced photosuppression of DTH in 24 young adult males aged 19 to 39 y. They found that exposure to a light source of ultraviolet A and B over 16 d significantly reduced DTH responses in a control group to 39% of the initial values but not in a group given 30 mg/d of  $\beta$ -carotene during the study period. The results were similar when repeated in an elderly population.<sup>126</sup>

Santos et al.<sup>127</sup> found that men participating in the Physician's Health Study who consumed 50 mg of  $\beta$ -carotene on alternate days for an average of 12 y had significantly greater natural-killer cell activity than did controls given placebos. Surveillance by natural-killer cells is considered to be protective against the development of cancer.

However, there are still questions concerning  $\beta$ -carotene's functions that were raised as a result of the findings of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC)<sup>128</sup>

and the Beta-Carotene and Retinol Efficacy Trial (CARET).<sup>129,130</sup> In both studies, smokers supplemented with  $\beta$ -carotene had unexpected increased rates of lung cancer. Several hypotheses have been proposed. 1)  $\beta$ -Carotene was oxidized to a carcinogenic molecule. Although there is evidence that oxidized products of  $\beta$ -carotene can be formed and might be carcinogenic,<sup>131</sup> there are no definitive studies indicating that this is the case in vivo. 2)  $\beta$ -Carotene supplementation blocked the absorption of other carotenoids with better antioxidant profiles. There were no data to show that other carotenoid levels were lower in the clinical studies with  $\beta$ -carotene. In fact, in some studies, certain carotenoid levels were increased.<sup>132</sup> 3) A novel hypothesis proposed by this author is that  $\beta$ -carotene supplementation enhanced lung function in smokers and thus permitted more of the carcinogens and reactive oxygen species to reach the smokers' lungs. Data from the Third National Health and Nutrition Examination Survey (NHANES III) associated higher levels of antioxidant nutrients with better lung function in smokers.<sup>133</sup> Similarly, Grievink et al.<sup>134</sup> found that subjects with high levels of plasma  $\beta$ -carotene had substantially higher forced vital capacity and higher forced expiratory volume in 1 s than those with low levels even after adjusting for smoking status and smoking pack years. The end result would be higher rates of lung cancer in individuals who continued to smoke and were supplemented with  $\beta$ -carotene but reduced rates of lung cancer in those who stopped smoking and continued taking  $\beta$ -carotene supplements. Actually, there were lower rates of lung cancer in those who stopped smoking and took  $\beta$ -carotene supplements in the ATBC and CARET studies.<sup>128-130</sup> It behooves the scientific community to understand the causes of the increased cases of lung cancer in smokers supplemented with  $\beta$ -carotene because this same dietary carotenoid reduced the risk of lung cancer in former and non-smokers. Moreover, the beneficial effects of  $\beta$ -carotene on immune functions might be especially important to smokers and serve as an inducement for cease smoking. Ongoing clinical studies, such as the Physician's Health Study II, may provide answers to some of the questions concerning the potential benefits of  $\beta$ -carotene.

## CONCLUSIONS

Lawrence J. Machlin contributed greatly to our understanding of the importance of optimal intakes of micronutrients for enhancing health and reducing the risk of chronic disease. There are several essential nutrients that have been shown to affect women's health throughout their different life stages (Table I). With the com-

TABLE I.

MICRONUTRIENT SUPPLEMENTS AND WOMEN'S HEALTH		
Supplement	Significant effects	Reference
Calcium	Reduction in PMS symptoms	51
	Reduction in preeclampsia in at-risk pregnant women	79
	Maternal supplementation increases bone-mineral content in neonate	102
	Reduction in osteoporosis-related hip fracture	90-92
Folic acid	Prevention of NTD recurrence	61
Folic acid and/or zinc-containing multivitamins	Prevention of NTD and four other major classes of birth defects; reduction in preterm births and LBW birth outcomes	62, 68
Multivitamins	Reduction in risk of preterm birth and LBW birth outcomes	36
	Reduction in fetal death, LBW, very preterm birth, and small-for-gestational-age infants; enhanced immune cell concentrations in HIV-positive pregnant women	74
Vitamin A/ $\beta$ -carotene	Reduction in maternal mortality	73
Vitamins C and E	Reduction in preeclampsia	75

HIV, human immunodeficiency virus; LBW, low birth weight; NTD, neural-tube defect; PMS, premenstrual syndrome.

TABLE II.

MICRONUTRIENT SUPPLEMENTS AND IMMUNE RESPONSES		
Supplement	Significant effects	Reference
Multivitamins	Enhanced DTH in healthy elderly; increased IL-2 production; enhanced NK cell number; maintained helper T-cell number; fewer infections; enhanced antibody titer to flu vaccine	122–124
Vitamin E	Enhanced DTH; immune-cell proliferation; antibody titer to hepatitis B vaccines; increased IL-2 production; decreased PGE <sub>2</sub> production	103, 116, 117
Vitamin C	Enhanced DTH skin-test responses	109
β-carotene	Blocked UV-induced DTH immunosuppression in young and older subjects; enhanced NK-cell function	21, 126, 127

DTH, delayed-hypersensitivity skin-test; IL-2, interleukin-2; NK, natural killer; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; UV, ultraviolet.

mencement of menstruation, requirements for certain micronutrients appear to be critical for the maintenance of good health throughout the menstrual cycle. PMS symptoms affect most menstruating women and carefully controlled studies have indicated that calcium supplementation significantly reduces physical and emotional symptoms. PMS might be a predictor of osteoporosis induced by low calcium intake.

During the past decade, exciting clinical data have clearly shown the value of folic acid and multivitamins containing folic acid in the prevention of serious birth defects. Pregnancy is a time of increased nutrition needs and only recently has it been widely acknowledged that the additional nutrition requirements are needed at or before conception to ensure normal growth of the embryo. Periconceptional multivitamin supplementation reduced the risk of serious birth defects by 50% and improved maternal health during the pregnancy. Prenatal multivitamin supplementation reduced the risk of preterm, LBW births by more than 50% even in the riskiest pregnancies. Micronutrients of particular importance for prevention of LBW are zinc, folic acid, and iron. However, if the preterm delivery is caused by preeclampsia, then calcium supplementation and high doses of vitamins C and E might significantly reduce this risk.

In developing countries, women of childbearing potential face serious pregnancy-associated complications that include the burden of HIV infection. Prenatal multivitamin supplementation significantly reduced the risk of fetal death and improved maternal immune responses. Unfortunately, supplementation did not protect the neonate from maternal transmission of HIV.

As women age, there is an increased risk of osteoporosis and potential fractures. Hip fracture significantly increases the risk of death within the subsequent year and impairs the quality of life of the survivor. Well-controlled studies have consistently shown that calcium supplementation, with or without vitamin D supplementation, significantly reduces the risk of hip fracture in middle-aged and elderly men and women. If the woman smoked cigarettes, her risk of hip fracture is increased. Antioxidants such as vitamins C and E can reduce the risk of fracture in smokers. As more of the population ages, the need to prevent hip and other age-associated fractures increases dramatically.

As with the rapidly growing embryo, the immune system includes rapidly multiplying cells whose functions are dramatically affected by an individual's micronutrient status. Multivitamins have been shown to enhance many aspects of immune response; antioxidant micronutrients consistently have been found to enhance lymphocyte proliferative responses and skin-test responses, especially in the elderly (Table II).

The totality of the research in women's health and immune function continues to confirm the insights of Lawrence J. Machlin, that the value of essential micronutrients goes beyond prevention of vitamin deficiency diseases and extends to optimizing health.

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