PROPER: a pilot study of the Role of Riboflavin Supplementation for the Prevention of Preeclampsia.

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***Abstract*—** Preeclampsia remains a mayor obstetrical threat worldwide, but recently it has been shown that it could be a predictable and preventable disease. We have published that riboflavin (Vitamin B_2) deficiency seems to be a possible risk factor for preeclampsia in developing countries. Here, we present some results of a pilot study, conducted in Venezuela, examining the usage of high dose riboflavin supplementation for the prevention of preeclampsia.

414 women were enrolled since around 20 weeks of gestation and were treated with 15 mg/d riboflavin supplementation or placebo until delivery. After a loss to follow up of 38.4%, due to political and social problems in Venezuela during the enrolment and follow up time frame of the study, results from 255 women were available for evaluation. Although no statistical difference was achieved related to the development of preeclampsia between groups, high dose riboflavin supplementation led to an approximate 75 percent ($p < 0.05$, RR 4.17 [95% CI 0.87-20,02]) decrease in the number of cases of severe preeclampsia. Women in the riboflavin group who developed any hypertensive disorder of pregnancy had statistically significant lower maximum diastolic blood pressures than corresponding women in the placebo group (diastolic BP 93,27 mmHg vs. 100,19 mmHg, $p = 0,05$). Unfortunately, The study suffered from a significant lost in follow-up, and did not have sufficient statistical power to detect a difference between groups; however, we consider the results encouraging in supporting further studies into the use of riboflavin, or other vitamins or antioxidants, to prevent preeclampsia.

***Keywords*—** antioxidants, preeclampsia, pregnancy induced hypertension, prevention of preeclampsia, riboflavin.

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I. Introduction

PREECLAMPSIA is a serious public health problem, according to the World Health Organization (WHO), and is one of the leading causes of maternal and perinatal morbidity and mortality worldwide, particularly in underdeveloped countries. [1] Preeclampsia increases four to fivefold the risk of preterm birth[2,3]. This disorder affects 3-5% up to 13% of pregnant women, and the incidence among high risk women could be up to 20%.[2] Eclampsia, Abrutio placentae, intravascular disseminated coagulation, HELLP syndrome, acute renal failure, stroke, hepatic failure, long term hypertension, renal failure, intrauterine growth retardation, preterm labour, fetal asphyxia are some if the frequent complications of preeclampsia. While in industrialized countries, neonatal intensive care units improve the chances for preterm babies and close monitoring of preeclamptic mothers allows for an expectant management of preeclampsia, such options do not exist, or are very restricted, for the majority of mothers in the developing world.[2] The only known cure is delivery of the placenta. Recent advances in the molecular understanding of preeclampsia[4] have allowed us a better insight into its pathophysiology, but therapeutic options are still very limited. Very recently, 2 significant advances in the field have opened an optimistic dimension into minimizing its impact as a public health problem: Preeclampsia could be a predictable[4] and a preventable[5] syndrome. But these approaches are not yet available worldwide. The predictive laboratory tests are still limited to some places in the industrialized countries and mostly for research purposes and not yet a clinical tool. On the other hand, unfortunately, aspirin could be a contraindicated medication in those places where the problem of preeclampsia is worse (endemic areas for dengue, malaria, yellow fever and others). Prevention of preeclampsia clearly remains a highly desirable aim to improve public health, especially in the developing world. The use of vitamins C and E, antioxidants, and any other approach for the prevention of preeclampsia has been disappointing. That is why nowadays the problem keeps being with the same magnitude, but

although better control is improving the quality of health in developed countries women in underdeveloped ones preeclampsia is the first cause of morbidity and mortality.

We have reported our findings from pregnant women in Zimbabwe, where women with riboflavin deficiency had a significantly increased risk for developing preeclampsia[6]. The reason for this increased risk is not entirely understood, but several metabolic changes proposed for the pathogenesis of preeclampsia include a strikingly high number of riboflavin-dependent reactions; among them are: trophoblast invasion, endothelial dysfunction by reactive oxygen species, impairment of progesterone synthesis, mitochondrial dysfunction, and reduction of placental nitric oxide synthase activity. [6]

Nutritional deficiencies have frequently been hypothesized to contribute to the risk of preeclampsia, and a particular focus has been on the role of antioxidant vitamins, calcium, magnesium and others.[8] Unequivocal evidence for oxidative stress has been shown in the pathophysiology of preeclampsia. Despite the disappointing results of several clinical trials and meta-analysis conducted to prevent preeclampsia with these vitamins, [9, 10] no consensus has been established and the final word seem that has not been said yet. Nutritional deficiencies could help to explain the increased risk for preeclampsia of some populations with a monotonous diet[6] and the seasonal changing pattern in the incidence of preeclampsia which we have observed in developing countries.[11] Our German team carried out a clinical trial in a rural area in Burkina Faso (West Africa) evaluating the prevention of preeclampsia with riboflavin supplementation.[12] The study concluded that there was no statistical significant difference on the incidence of preeclampsia among women who received riboflavin supplementation compared with those who received placebo. Fawzi et al. [13] reported a 38% reduction in the risk of developing pregnancy-induced hypertension among Tanzanian women who received a multivitamin supplementation which included 20 mg of riboflavin. This author concluded in that study that "multivitamin supplementation reduced the incidence of low birth weight and small for-gestational-age births but had no significant effects on prematurity or fetal death and that multivitamins should be considered for all pregnant women in developing countries".

We therefore decided to conduct a randomised clinical trial with a double-blind, placebo-controlled design, to test whether riboflavin supplementation can help to prevent or ameliorate cases of pregnancy-induced hypertension or preeclampsia among Venezuelan women assisted by the Program for the Prevention of Preeclampsia.[14, 15]

II. Material and Methods

The study was conducted following the design described elsewhere.[16] Ethical approval was granted by the Ethical Committee of the Ministry of Health, Mérida State. All women were enrolled and were assessed, treated and followed up in the settings of the Program for the Prevention de Preeclampsia;[14, 15] a Venezuelan semi-governmental organization that runs a public health program against preeclampsia in Mérida state, in Venezuela. The study took place during 2002 to 2005. Every women who attended to every antenatal care clinic in Mérida state (approximately 20,000 women), was classified as being at low or high risk for preeclampsia. High risk for preeclampsia included: nulliparity, previous preeclampsia, obesity, chronic hypertension, diabetes, Africans and descendents, familiar history of hypertension or preeclampsia, twin pregnancy, systemic erythematous lupus. An expert system[17-19] developed specially for this program was also used to classify the women and to allocate them in a trial group. For this trial women were enrolled at 20 weeks of gestation or before, if they met either of the inclusion criteria: (1) primigravidity or (2) multigravidity with previous history of hypertensive disorders of pregnancy. Women with chronic hypertension, or any other risk factor mentioned above were also excluded, as well as women from centre other than Mérida or El Vigía. Women were informed about the purpose and requirements of the research. Details were explained to them in Spanish by a native speaker physician or a physician fluent in the language. We only included women from Mérida and El Vigía because the German physicians were who mainly took care of blood samples, medical records and collect the blood samples at the Laboratory of Chemistry of the University of Los Andes. Signed informed consent was obtained from each subject.

Pregnancy induced hypertension was diagnosed if diastolic blood pressure rose to more than 90 mmHg or systolic blood pressure to more than 140 mmHg, after the 20th week of gestation. Preeclampsia was diagnosed if hypertension in pregnancy was recorded in the presence of a proteinuria ≥300 mg/l according to a dipstick test. Blood pressures were taken by a nurse or a physician with the patient sitting on a chair. Severe preeclampsia was defined as: diastolic blood pressure higher than 110 mmHg, if the patient suffered eclamptic episodes or if neurologic symptoms were recorded, such as visual disturbances, increased reflexes and irritability.

Consecutive numbers had been attributed randomly to allocate women either to the riboflavin or placebo group according to a system of block randomisation which ensured that in every 10 women enrolled there would be five who received each of the two regimens. Patients were attributed study numbers consecutively as they were enrolled in the study. Sealed containers containing either riboflavin or placebo had those study numbers printed on them and were distributed to the women at each of the visits which ensured double blind administration of the study medication. Women were examined regularly every four weeks until 36 weeks and weekly thereafter until delivery. Those women who failed a visit or wasn´t compliant with the treatment where also excluded.

Plasma samples were obtained during each clinic visit and at delivery. They were centrifugated, frozen and stored at -20C. Determination of flavin adenine dinucleotide (FAD) was performed by HPLC on a C18 Sep-Pak cartridge (Waters) and

internal FAD standard (Sigma) according to standard procedures.[9] Levels of Vitamins A, B2, E were also determined and we will present this data in another paper, but some results are shown in our results.

A total number of 414 women were enrolled and were planned to be followed until delivery. Clinical examination was conducted and serum samples were drawn at four week intervals. Through a double blinded block randomisation procedure performed by a neutral study coordinator; and inclusion of identical looking placebo tablets, neither the participants nor the treating physicians were aware of the treatment which the patient received until the end of the trial. The study medication was produced exclusively for this trial. Women were randomized to receive either 15 mg riboflavin per day, or an identical looking placebo tablet, from 20 weeks gestation onwards. Both were distributed in pre-labelled and sealed containers (bottles). This starting date for the supplementation was chosen for practical reasons (many women do not register with the obstetric services prior to 20 weeks of gestation), and to coincide with the second wave of trophoblast invasion where riboflavin deficiency might play a pathogenic role[3]. No toxicity is known for riboflavin and no upper limit is defined and 15mg/day were chosen to represent a feasible high dose supplementation (10 times the minimum intake). Main outcome parameters included the incidence of hypertensive disorders of pregnancy as well as plasma concentration of FAD (flavin adenine dinucleotide) and serum markers of nutritional status, such as vitamin B1, B6, B12, folic acid, homocysteine and the detection of MTHFR mutation.. Statistical analysis was obtained using the Statistical Package for the Social Sciences (SPSS, version 10.0) software. P-values ≤0.05 (using t-tests to compare means and chi-squared tests for over-all numbers) were considered significant. The preliminary calculations implied a prevalence of 10% of preeclampsia in the study cohort. Original power calculations suggested that with a statistical power of 90% and an assumed loss to follow-up of 20% a minimum of 1500 subjects needed to be enrolled to achieve an authoritative result.

During the study period severe political and social problems took place in Venezuela, thus the study failed to meet these enrolment goals and therefore is considered as a pilot study only; and in need of further confirmation.

III. Results

Of the 414 enrolled women; 209 were randomised to receive riboflavin and 205 placebo. Characteristics of the patient groups are shown in Table I. Due to loss in follow up, only 255 could be considered for statistical analysis of the main outcome data shown in Table II.

TABLE I
Summary of baseline patient characteristics at enrolment

	Riboflavin (n=209)			Placebo (205)		
Loss to follow-up (%)	39.2			37.6		
n=414	Mean (SD)			Mean (SD)		
Age [years]	24.2 (5.9)			24.9(6.8)		
Gravidity: median (range)	1 (0-11)			1 (0-10)		
Parity: median (range)	0 (0-7)			0 (0-8)		
Gestational age [weeks]	18.3 (5.7)			17.7 (6.5)		
BMI [kg/m^2]	24.4 (4.5)			24.0 (4.6)		
BP systolic [mmHg]	105 (15)			105 (12)		
BP diastolic [mmHg]	64 (10)			64 (9)		
Marital status (%)	Married	with partner	Single	Married	with partner	Single
	23.4	47.8	27.3	26.8	45.4	25.4
Level of education (%)	no school	primary school	secondary school and academic	no school	primary school	secondary school and academic
	1.0	12.4	86.5	0.5	12.6	86.8

BP=blood pressure, BMI=body mass index, n=number of cases

Table II
Main outcome parameters

	Riboflavin (n=127)		Placebo (n=128)		
n=255	Mean	standard deviation	Mean	standard deviation	Significance
Duration of pregnancy [weeks]	38.4	3.5	38.9	2.8	p=0.194
Birthweight [g]	3130	602	3158	416	p=0.673
Preeclampsia/ Eclampsia	7 (5.5%)		11 (8.6%)		p=0.237
Patients with any hypertensive disorder: BP sys max [mmHg]	142	19	149	23	p=0.206
Patients with any hypertensive disorder:: BP dia max [mmHg]	93	9	100	9	p=0.027
Pregnancy induced hypertension	5 (3.9%)		7 (5.5%)		p=0.829
Severe Preeclampsia/ Eclampsia	2 (1.6%)		8 (6.3%)		p=0.053

BP=blood pressure, max=maximum, n=number of cases, p=level of significance

TABLE III
Frequency distribution of the different degrees of severity of hypertensive disorders of pregnancy of patients PROPER study patients

	Pregnancy induced hypertension (PIH) %		Preeclampsia (PE) and Eclampsia %	
	Mild	Severe	Mild	Severe
All patients	5.3		8.0	
	4.7	0.7	4.4	3.7
Venezuela	5.4		8,1	
	4.6	0.8	3.8	**4,2**
Tanzania	4.9		7,3	
	4.9	0.0	7.3	**0,0**
Sign. Level	n.s.	n.s.	n.s.	**p < 0,01**

ns - Not significant

The loss to follow-up in the entire cohort was 38.4%. The main reason for the loss to follow-up was a general strike in Venezuela, which took place during the study period, and which led to a virtual shutdown of public life from December of 2002 to February of 2003 and slowed down the public health system for several months.

There was a tendency to a reduced incidence of all hypertensive disorders of pregnancy (pregnancy-induced hypertension and preeclampsia) among women receiving riboflavin supplementation (9.5% vs. 14.1% in the placebo group; p=0.34) and the number of preeclampsia cases tended to be lower in women receiving riboflavin compared with women receiving placebo (Table II, Table III and Fig. 1), though these tendencies failed to reach the level of statistical significance (p > 0,05). However, the incidence of severe preeclampsia was significantly (p < 0,05 in chi-square test) lower in patients receiving riboflavin compared with patients receiving placebo (Table II and Fig 1). We observed two women with severe preeclampsia (1.6%) in the study group vs. eight (6.3%) in the placebo group (odds ratio 4,17 [95% CI 0,87-20,02]), including 1 case of eclampsia. The number needed to treat was 22.

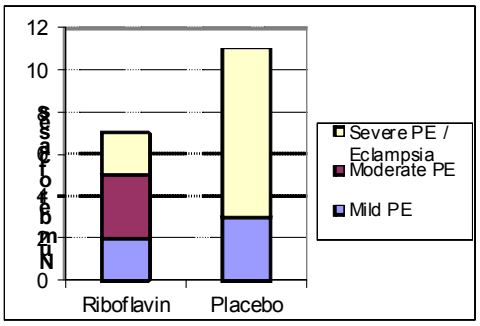

Fig 1: Frequency and severity of preeclampsia

Women who developed a hypertensive disorder of pregnancy (preeclampsia or pregnancy-induced hypertension, Table II) while receiving riboflavin supplementation had significantly slightly lower maximum diastolic blood pressures, compared with the placebo group (93 mmHg vs. 100 mmHg; p = 0.027).

Serum levels of flavin adenine dinucleotide (FAD) , one of the active coenzyme metabolites of riboflavin, were higher in women who received riboflavin supplementation when compared to patients of the placebo group (310 vs. 255ug/l, respectively, at 36 weeks gestation). This may be considered a compliance control, though results failed to reach statistical significance. Plasmatic levels of FAD in newborns were not influenced by the mother's intake of riboflavin or placebo (395 vs. 385 ug/l; n.s.), Fig. 3.

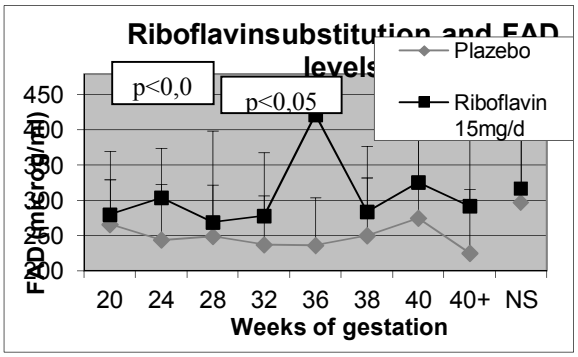

Fig 2: FAD levels with Riboflavin during pregnancy

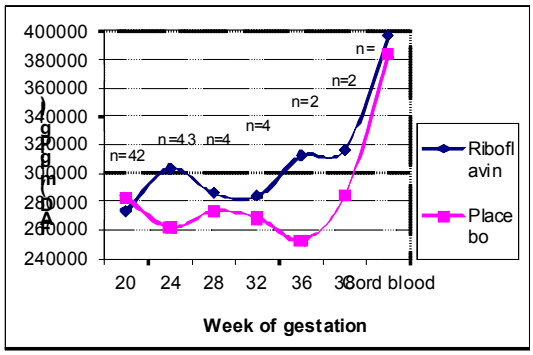

Fig 3: FAD levels during pregnancy in women and in the blood cord in the newborns– study group vs. placebo

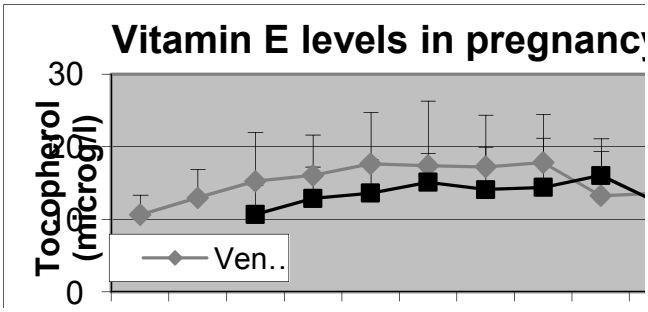

Fig 4: Vitamin E levels during pregnancy – study group vs. placebo

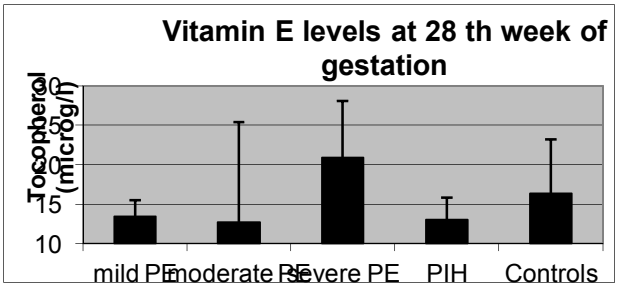

Fig 5: Vitamin E levels at 28th week of pregnancy in those women who developed hypertensive disorders of pregnancy PE: Preeclampsia, PIH: Pregnancy induce Hypertension

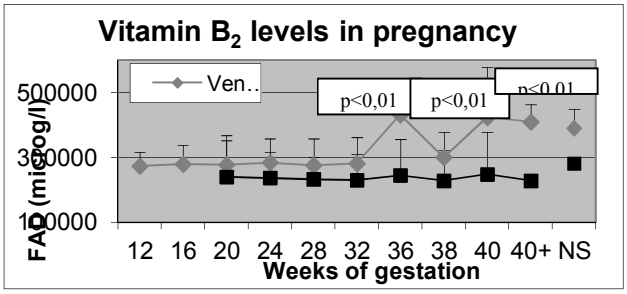

Fig 6 : Vitamin B2 levels during pregnancy – study group vs. placebo

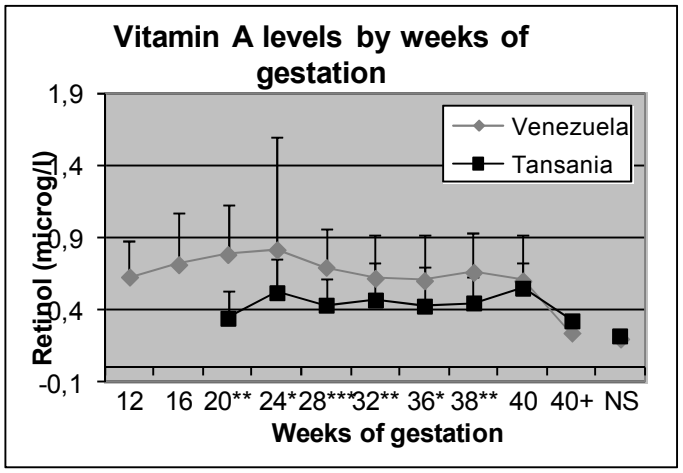

Fig 7 : Vitamin A levels during pregnancy – Venezuelan vs. Tanzanian patients. * p<0,05 ** p<0,01 *** p<0,005

Figures 2, 3, 4, 5, 6 and 7 present the different levels of FAD and vitamins A, B2, E in our patients in Venezuela (study and control groups) and Tanzania from 12 weeks of gestation every 4 weeks until delivery. The graphics show the p value when there was statistical significance. We can see that the Venezuelan women are, in general, better nourished and the levels of vitamins are slightly higher than those women from Tanzania. Interestingly we can observe that levels of vitamin E in severe preeclampsia cases seems to be higher but we didn´t obtain statistical difference. Detailed data of this laboratory results are being submitted for another publication.

IV. Discussion

Prevention of preeclampsia remains a desirable goal for obstetric practice, particularly for countries with limited resources. Aspirin has been shown to be a preventable tool for preeclampsia; [5] but in endemic areas for dengue, malaria, yellow fever, etc. (as the case of Venezuela) massive distribution of aspirin could be contraindicated. Nutritional intervention or supplementation with non-toxic compounds such as water soluble vitamins would constitute a highly attractive modality for such a significant medical problem. We studied the impact of high dose (15mg/d) riboflavin supplementation on the incidence and severity of preeclampsia

in a high risk population (nulliparous women and those who have developed preeclampsia previously). Our findings are in line with the results of a recent study[12], which failed to find a reduction in the overall number of preeclampsia or pregnancy-induced hypertension cases in patients recruited from a rural area in Burkina Faso who had been randomly selected to receive either riboflavin supplementation or placebo. In the present study, however, a reduction in the number of women with severe preeclampsia was demonstrated. It is possible that Neugebauer et al.[12] missed such an effect in their study based on a limited follow-up. This could explain the relatively low preeclampsia rate in their study (3.6%) compared to reported frequencies of 12-16% for similar patients population[21] and could imply that many diagnoses may have been missed. Although our study suffered a high loss to follow-up rate, the distribution of lost patients was even in each group and there was no statistical significance difference in any baseline data to presume that this effect was a confounder. Recording of patients' health data could be assured for all the 255 evaluated cases, the above described "Program for the Prevention of Preeclampsia (PPP)" supplied us with an optimal infrastructure to conduct this trial. Most of the lost to follow-up cases were excluded because they missed one or two month of treatment or blood samples collection. On the other hand, African and Venezuelan women are very different in ethnical, nutritional and cultural factors. It is likely that the nutritional situation in rural Burkina Faso is worse than in urban Venezuela. Actually plasmatic levels of riboflavin in our cohort (both groups) were high. It is possible that riboflavin supplementation given in a situation of empty body storages and many other nutritional deficiencies may not have been sufficient to show an effect on the reduction of the disease.

The mechanism by which riboflavin supplementation could contribute to the amelioration of a clinical course of preeclampsia or pregnancy-induced hypertension is still unclear, although several lines of thoughts exist. One mechanism may be the lowering of homocysteine levels, because hyperhomocysteinaemia have frequently been observed in cases of preeclampsia, although a causal relation has not been proven[22]. Published data support the hypothesis that folate, vitamin B12 and B6 lower homocysteine levels in hyperhomocysteinaemic women.

Previously, we have reported a high prevalence (70% overall) of heterozygous (55%) or homozygous (15%) methylenetetrahydrofolate reductase (MTHFR) polymorphisms in the same population of this study. Significantly higher homocysteine levels in homozygous [9.9mcmol/L] vs. heterozygous patients [8.6 mcmol/L, $p<0.05$)] or normal genotype [8.3 mcmol/L, ($p<0.01$)] was observed. In women with heterozygous or homozygous methylenetetrahydrofolate reductase (MTHFR) polymorphisms in which homocysteine accumulates due to an enzymatic defect, riboflavin supplementation as well as folate supplementation has been reported to help lower homocysteine levels by providing coenzyme functions. The coenzyme functions of riboflavin metabolites, FAD and FMN are also at the heart of a second hypothesis. Nitric oxide (NO), an important vasodilatator in the small vessel vasculature, is produced by nitric oxide synthase (NOS). All three isoforms of this enzyme (eNOS (endothelial), nNOS (neural) and iNOS (inducible form) depend on FAD or FMN as coenzymes and riboflavin deficiency could result in hypertension via a reduction of NOS activity and relatively lower NO levels in the vasculature[24].

Chappell et al. [8] initially found hints of a possibly successful prevention of preeclampsia in high risk cases through administration of high doses of antioxidative vitamins E and C; similar results were presented by our group[25]. These reports sparked new hopes for the feasibility of preeclampsia prevention by nutritional supplementation. Unfortunately, this finding could not be verified in a larger prospective trial conducted by the same group of researchers[26]. A recent meta-analysis by the Cochrane Library[27] also failed to demonstrate preventative benefit in 566 women supplemented with vitamin E. Many other studies agree with these results.

V. Conclusion

This randomized, placebo controlled, prospective pilot study of riboflavin supplementation showed a trend towards fewer cases of severe preeclampsia in the riboflavin group and lower blood pressures in women who did develop preeclampsia during riboflavin supplementation. Despite the limited sample size caused by a high drop-out rate, we feel that Vitamin B_2 supplementation may carry a promise for the prevention of preeclampsia. Larger studies, including other populations are needed to investigate this possibility.

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