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Esta es la **versión de autor** del artículo publicado en:
This is an **author produced version** of a paper published in:

Placenta 47.11 (2016): 37-45

DOI: https://doi.org/10.1016/j.placenta.2016.08.090

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Maternal plasma antioxidant status in the first trimester of pregnancy and development of obstetric complications

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This work was supported by Universidad Autónoma de Madrid; Multidisciplinary Research Project (CEMU, 2013-10) and Ministerio de Economía y Competitividad (FEM2012-37634-C03-01 and FEM2015-63631-R).

The authors report no conflict of interest.

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To appear in the print issue: Figure 2

Short version of title: Association between low plasma antioxidant status in first trimester of pregnancy and development of obstetric complications.

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Abstract

Introduction: Oxidative stress is present in pregnancy complications. However, it is unknown if early maternal antioxidant status could influence later development of complications. The use of assisted reproduction techniques (ART) is rising due to the delay of first pregnancy and there is scarce information on its influence on oxidative balance. **Objective**: To assess the possible relationship between maternal plasma antioxidant status in first trimester of gestation with later development of pregnancy complications, evaluating the influence of ART and nutrition. **Methods**: Plasma from 98 healthy pregnant women was obtained at week 10, nutrition questionnaires filled and women were followed until delivery. We evaluated biomarkers of oxidative damage (carbonyls, malondialdehyde-MDA), antioxidants (thiols, reduced glutathione, phenolic compounds, catalase, superoxide dismutase activities) by spectrophotometry/fluorimetry and melatonin (ELISA). Antioxidant status score (Antiox-S) was calculated as the computation of antioxidants. Diet-antioxidants relationship was evaluated through multiple correspondence analysis. **Results**: Melatonin and carbonyls exhibited a negative correlation. No difference in oxidative damage was found between groups, but Antiox-S was significantly lower in women who developed complications. No differences in oxidative damage or Antiox-S were found between ART and no-ART pregnancies. High consumption of foods of vegetable origin cluster with high plasma levels of phenolic compounds and with high Antiox-S score. **Conclusions**: In early normal gestation, low plasma antioxidant status, assessed through a global score, associates with later development of pregnancy complications. Larger

population studies could help to determine the value of Antiox-S as predictive tool and the relevance of nutrition on maternal antioxidant status.

Key words: antioxidants, obstetric complications, plasma, pregnancy, biomarker.

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Introduction

The age of first pregnancy has been gradually increasing in industrialized countries [1-3]. Advanced maternal age reduces fertility, increasing the need of assisted reproduction techniques (ART) and it is associated with a rise in the development of obstetric complications, particularly pre-eclampsia, hypertension and gestational diabetes [1,4]. In addition to immediate consequences for maternal health, these complications also have negative long term effects for the offspring, programming the development of cardiometabolic diseases [5].

The identification of biomarkers in early pregnancy is desirable in order to prevent or decrease the impact of gestation-related pathologies. Several potential biomarkers, including inhibin-A, pregnancy associated plasma protein A (PAPP-A), placental growth factor (PlGF) and placental protein 13 (PP-13) [6] as well as several miRNAs [7] have been evaluated in the first and second trimester of pregnancy. However, low predictive values have been found when these biomarkers were considered individually [6] and a multiple marker approach has been suggested [7]. In addition to the above mentioned biomarkers, oxidative stress-related molecules could be explored, based on the fact that pregnancy complications have been associated with oxidative damage [8-10]. Reactive oxygen species (ROS) play an important role in gestation, but in excess, they might override the antioxidant systems and contribute to oxidative damage. An important ROS elevation takes place between weeks 10^{th}-12^{th} of gestation, when oxygen tension rises steeply within the placenta as the maternal circulation is established. Under physiological conditions, this is matched with an increase of the main endogenous antioxidants -superoxide

dismutases (Cu/Zn and Mn SODs), catalase, glutathione (GSH) and glutathione peroxidase (GP_X) [11,12]. However, if there is an inadequate production of antioxidants, oxidative balance is lost and oxidative damage might contribute to the development of pregnancy disorders [11,12].

Another important endogenous antioxidant during pregnancy is melatonin. This hormone is secreted by the pineal gland related to the duration of darkness and it is also synthesized by the placenta throughout pregnancy [13,14]. Melatonin diffuses through biological membranes and exerts direct ROS scavenging effects and also increases expression of other endogenous antioxidants [15-17].This hormone seems to be essential for successful gestation and has an important role in embryo-fetal development [15,18]. Moreover, melatonin treatment has been demonstrated to reduce placental oxidative stress in complications associated with placental insufficiency [18,19].

Oxidative status is not only determined by endogenous antioxidants, but it is also modulated by diet-derived compounds. Fruits, vegetable and beverages are the major source of antioxidants in the diet, particularly phenolic compounds and vitamins [20] which contribute to the global pool of antioxidants. In the context of pregnancy it has been demonstrated that consumption of a mediterranean-style dietary pattern –rich in fruits, vegetables and cereals- during periconceptional period reduces the risk of hypertension-related pregnancy disorders [21]. Melatonin and its precursor tryptophan are also present in some food items, being universal in plants and it is also a natural compound found in milk [22]. Due to its biological activity and bioavailability, it has been proposed that the consumption of foods containing melatonin may improve human health [23].

The studies evaluating oxidative-related biomarkers in pregnancy complications have been performed in the last trimester of pregnancy, when the complication has already been diagnosed [8,9]. At this stage, redox balance alterations might be consequence of the pathology itself [24]. In normal pregnancies, adequate levels of maternal antioxidants during the first trimester of gestation seem to be important to counteract early ROS elevation [11,12]. Therefore, we hypothesize that a low maternal antioxidant status in the first trimester of pregnancy can play a role in the later development of an obstetric complication. To test our hypothesis, we have evaluated several plasma biomarkers of oxidative status in a group of normal pregnant women at 10^{th} week of gestation. The complex chain reaction nature of ROS and antioxidants makes difficult to assign a prevalent role to a particular molecule and the calculation of a score (based on the computation of individual biomarkers) has been proposed as a better strategy to evaluate global redox state [25]. We have previously adapted methods for quick assessment of the main oxidative status biomarkers in small plasma volume and applied this score to assess global oxidative status in patients [26] [27] and experimental animals [28]. In the present study we have calculated a similar global score of antioxidant status (Antiox-S) and have evaluated its relationship with obstetric complication development. Finally, since diet-derived antioxidants might play a modulator role on global redox balance, we also aimed to assess the relationship between maternal nutrition and plasma antioxidant status.

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Material and Methods

1. Population of study

We recruited 98 healthy pregnant women at the Obstetrics and Gynecology Service from La Paz University Hospital (Madrid, Spain). Exclusion criteria were women with present or previous cardiovascular disease or risk factors. Women entered the study at 9 weeks of gestation. At this time they signed an Informed Consent , the use of ART was recorded and the women filled two questionnaires regarding their socio-economic status and dietary habits. A blood sample was obtained at 10 weeks of gestation to determine biochemical and hematological parameters and to assess plasma biomarkers of oxidative status. The women were followed-up at the Obstetrics and Gynecology Service until delivery and the development of maternal severe or minor complications was recorded. Severe complications included gestational diabetes (glucose levels over 140 mg/dl in the glucose challenge test), preeclampsia (blood pressure over 160/110 mm Hg with proteinuria or thrombocytopenia after 20 weeks of gestation), gestational hypertension (blood pressure over 160/110 mm Hg, without features of preeclampsia) and miscarriage. Minor complications included *Hyperemesis Gravidarum*, skin lesions and anemia.

1.1. Ethical approval.

The study was performed in accordance with the Declaration of Helsinki regarding studies in human subjects and it was approved by Hospital La Paz and the Universidad Autónoma de Madrid Ethical Committees. The women participated in the study anonymously and voluntarily signed an Informed Consent.

2. Blood sample

At 10 weeks of gestation blood samples were extracted from 8:00 to 9:00 a.m. by venipuncture in Vacutainer® tubes following the protocols established by the medical staff in charge. One sample was used to assess hemoglobin, glucose, cholesterol and triglycerides by the Clinical Laboratory Service of the Hospital as well as other routine parameters requested by the Obstetrics and Gynecology Service. A second sample was obtained in Vacutainer® tubes containing lithium heparin and separation gel. From this blood sample plasma was obtained by centrifugation (2100 g, 15 min at 4ºC) within a maximum of 2h after extraction. Thereafter, it was immediately aliquoted and stored at -80ºC until use.

3. Plasma biomarkers of oxidative status.

3.1. Total protein carbonyls.

Plasma protein carbonyls were assessed by the 2,4-dinitrophenylhydrazine-based assay [29] adapted to a microplate reader [26]. The protein carbonyl concentration was determined using extinction coefficient of 2,4-dinitrophenylhydrazine (ε=22,000 M/cm) and expressed as nmol/mg of protein. Protein content was assessed by Coomassie-blue-based microtiter plate assay, according to manufacturer's instructions (Bio-Rad). The absorbance was measured at 370 nm in a microplate reader (Synergy HTMultimode; BioTek).

3.2. Protein-bound malondialdehyde.

The concentration of plasma MDA was measured by a spectrophotometric method detecting Thiobarbituric Acid (TBA) reactive substances as previously described in [30]. Briefly, the plasma samples were incubated with TCA, EDTA, SDS and BHT, followed by addition of TBA and boiled in a water bath at 100ºC for 30 min. After cooling, the mixture was centrifuged at 10,000 g and the

absorbance was measured at 532 nm and compared with a standard curve of 1,1,3,3-tetrathoxypropane.

3.3. Reduced glutathione (GSH).

Plasma GSH was assessed by a fluorimetric method based on the reaction with o-phthalaldehyde [31] and adapted to a microplate reader [26]. Fluorescence was measured at 360±40 nm excitation and 460±40 nm emission wavelengths. GSH concentration in the samples was expressed as μmol/mg of protein.

3.4. Total thiols.

Plasma thiols were assessed by a modification of the method based on 5,5′-dithiobis(2-nitrobenzoic acid) assay [29] adapted to a microplate reader, as previously described [26]. The absorbance was measured at 412 nm and thiol content was expressed as nM GSH/mg of protein.

3.5. Superoxide anion scavenging activity (SOSA):

Superoxide ($O_2^{\cdot-}$) scavenging activity quantification was assessed by SOSA assay based on the inhibition of luminescence emitted by coelenterazine (CTZ) when is oxidized by $O_2^{\cdot-}$ [32], adapted to a microplate reader [27,32]. SOSA values were quantified by comparing the luminescence inhibition of each sample with a SOD activity standard curve (0–4 U/ml) and expressed asmU SOD/mg of protein. SOSA assay evaluates all the plasma antioxidants capable to eliminate $O_2^{\cdot-}$ and has been used as global measure of superoxide dismutase (SOD) activity [27,28].

3.6. Catalase activity.

Catalase activity was assessed by Amplex Red catalase assay (EnzChek Myeloperoxidase Assay Kit with Amplex Ultra Red reagent; Invitrogen). Catalase activity was expressed as U/mg of protein.

3.7. Melatonin.

To assess melatonin levels, plasma was first evaporated to dryness with an evaporator centrifuge (Speed Vac SC 200;Savant, USA). The residues were dissolved in distilled water and melatonin levels were determined by a competitive enzyme immunoassay kit (Melatonin ELISA; IBL International, Germany) according to manufacturer's instructions. The kit is characterized by an analytical sensitivity of 1.6 pg/ml and high analytical specificity (lowcross-reactivity). Melatonin was expressed as pg/ml.

3.8. Food-derived antioxidants.

Folin-Ciocalteu assay, modified to remove protein interference, was used to assess food-derived antioxidants, mainly phenolic compounds and ascorbic acid [33]. Absorbance was measured at 760 nm and the results were expressed as mg gallic acid equivalent (GAE) per litre (mg GAE/L).

3.9. Calculation of the global score of antioxidant status (Antiox-S).

A global antioxidant score (Antiox-S) was calculated for each subject taking into account the antioxidant biomarkers measured in plasma (GSH, total thiols, SOSA, catalase activity and phenolic compounds). The calculation uses the statistical methodology previously described, which normalizes and standardizes each of the parameters of interest, enabling to sum parameters of different units [26,28]. Firstly, we analyzed the normality of each antioxidant biomarker assessed (*j*) through the Kolmogorov–Smirnov test. Those

169 parameters that did not exhibit a normal distribution were normalized through a
170 logarithmic transformation (log *j*). Once normalized, the parameter was
171 standarized for each subject (*i*). For this, the mean (M_j) and standard deviation
172 ($STDV_j$) were calculated taking into account all the data in the population
173 studied and the standardized parameter (Z_{ij}) was calculated as follows:

174 $Z_{ij} = (X_{ij} - M_j)/STDV_j$, where X_{ij} is the raw normalized parameter for each subject.

175 Antiox-S was then calculated for each woman as the sum of standardized
176 antioxidants. The advantage of this method is that every subject has an
177 individualized score at a time point. Antiox-S is statistically treated as any other
178 parameter, evaluating differences between groups (complicated versus no
179 complicated pregnancies, ART versus No-ART).

180 *4. Statistical analysis.*

181 Data are expressed as mean±S.E.M. Student's *t* test was used to assess
182 statistical differences between groups in those parameters that followed a
183 normal distribution and Mann Withney test for those which did not fit a normal
184 distribution. χ^2-test was used for the analysis of the relationship between
185 qualitative variables. Statistical significant level was established at $p<0.05$.

186 A multivariant logistic regression analysis was used to evaluate the influence of
187 age and ART on pregnancy complications.

188 Multiple Correspondence Analysis was performed to examine the relationship
189 between several nominal variables in a multidimensional space. This analysis
190 generates plots that graphically illustrate the underlying relationship between
191 categories of these variables. Optimum dimensional reduction was used to

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192 assess the relationship between variables using the discriminant dimension as

193 *α* Cronbach.

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Results

Population of study. In the studied sample (n= 98), 63 women had spontaneous pregnancies and 35 had ART pregnancies, the majority of which were derived from *in vitro* fertilization techniques. The analyzed population had an average age of 34.5±0.5 years. Women on ART had a significantly higher average age compared to non-ART (ART=36±0.7 years; non-ART=33.9±0.6 years; p=0.027).

Regarding socio-economic aspects, 91.6% of the women reported being employed. Regarding income level, the women were classified as low-middle if they earned between 600-1500€/month and upper if they had an income above 1500 €/month, according to the Spanish population income level [34]. With respect to the level of studies, 74.7% of the total population reported a higher degree.

64.3% of the women had normal pregnancies and 35.7% developed any type of complication (severe 13.7% and 22.0% minor). Severe complications were distributed as follows: 4.4% preeclampsia, 4.4% gestational diabetes, 2.2% gestational hypertension and 2.7% miscarriage.

A significantly higher prevalence of pregnancy complications was found in women with a lower educational level compared to women with higher studies. No significant differences were found regarding other socio-economic aspects evaluated (civil status or income level) or the intake of the different food categories evaluated (Table 1).

The incidence of complications was significantly higher in ART pregnancies compared to non-ART (ART=53.1%; NO-ART=31.0%; p<0.04; χ^2 test). Applying a logistic regression model we found that the probability to have a pregnancy

218 complication was 2.79 times larger in ART pregnancies (p=0.041 with a
219 confidence interval of 1.04-7.49).

220 *Biochemical parameters*.

221 Blood hematological and biochemical parameters at 10th week were all within
222 the normal range. Triglyceride levels were higher in ART pregnancies compared
223 to non-ART. Triglycerides were also higher in women who developed a
224 pregnancy complication compared with those with normal pregnancy (Table 2).

225 There was no statistical difference in plasma carbonyl groups comparing normal
226 pregnancies with those that developed an obstetric complication (Figure 1A), or
227 between ART and non-ART pregnancies (Figure 1B). Similarly, we did not
228 detect statistical differences in MDA levels between complicated versus non
229 complicated pregnancies (Figure 1C) or between ART and no-ART pregnancies
230 (Figure 1D).

231 We did not detect statistical difference in the levels of diurnal melatonin
232 comparing normal pregnancies with those that developed an obstetric
233 complication (Figure 2A), or between ART and non-ART pregnancies, although
234 there was a tendency towards lower melatonin levels in ART pregnancies
235 (Figure 2B). A negative and significant correlation was found between melatonin
236 and carbonyl groups (n=80;r=-0.303; p=0.01).

237 Individual plasma antioxidants, except SOSA, tended to be lower in women who
238 developed a maternal complication, but we did not detect statistical differences
239 between them (Table 3). However, the global score Antiox-S was significantly
240 lower in women who developed a complication compared with normal
241 pregnancies. The group with severe complications tended to have a lower

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Antiox-S, but we did not find a statistical significance, probably due to the low sample size (Figure 2C). We did not detect statistical differences in Antiox-S comparing ART with non-ART pregnancies (Figure 2D).

The relationship between diet and antioxidants was determined with a multiple correspondence analysis. We found that a high intake of fruits and vegetables keeps relationship with higher plasma levels of phenolic compounds and with high Antiox-S. Similarly, diets deficient in fruits and vegetables are grouped with low levels of these antioxidant compounds (Cronbach α= 0.56; Figure 3A).

On the other hand, low level of milk consumption (weekly intake) is grouped with lower levels of plasma melatonin and higher carbonyls (Cronbach α= 0.41; Figure 3B).

Discussion

The age of first pregnancy is rising in industrialized societies, increasing the possibility to develop pregnancy complications. Therefore, it would be desirable to detect women at risk at early stages of pregnancy. Since oxidative stress is associated with several obstetric complications, we aimed to assess if plasma antioxidant status of the mother in early gestation has a relationship with later development of a complication, and thus, could be of predictive value. The increase in the age of first pregnancy is also linked to the need of assisted reproduction and, therefore, we have also evaluated if ART modifies oxidative balance. The main findings of the present study are that: 1) the development of a pregnancy complication is associated with low plasma global antioxidant status (evaluated thought a score) in the first trimester of gestation; 2) the use of ART does not affect early antioxidant status and 3) there is an association between fruit and vegetable consumption and exogenous antioxidants in plasma as well as between milk intake and plasma melatonin levels.

The women included in our study were over the optimum reproductive age - established between 20 and 30 years old [35] and even slightly above the average of the Spanish population [34]. This is likely due to the fact that our study included a group of women using ART, which were older than the group with spontaneous gestation. Our data reflects the overall tendency in Spain, showing that the age of first pregnancy has been increasing in the last three decades [1,34], similarly to the trend in other industrialized countries [2,3,36]. Among other reasons, this is due to the social evolution leading to some important changes regarding the role of women in society and in the traditional family; including a gradual increase in the access to employment, particularly in

women with higher education, as shown by European statistics [37]. Despite the important incorporation of women to employment, statistical data show that they still encounter important difficulties to conciliate family with work life, being this one of the main reasons to postpone pregnancy. Our population of study lies within this category, i.e. over 90% of the women had a job.

In association with the increase in maternity age, the prevalence of pregnancy complications has been rising in the last 2 decades, being 2-9% for gestational diabetes [38], and 2-8% for preeclampsia [39,40]. Similar values were found in our population of study. As previously described [41], we observed a higher prevalence of pregnancy complications in women using ART. This was also demonstrated when adjusted by age, suggesting that it is not related to the fact that women using ART were older. We also found that the percentage of complications was higher in women with lower educational level. We suggest this could be related to the fact that women with higher studies might be more concerned about health habits and have a better life style behavior.

At 10 weeks of pregnancy, when plasma biomarkers were assessed, the women included in this study were healthy, with no signs of obstetric complication and had normal biochemical and hematological parameters. Despite the fact that triglyceride levels were within the normal range, they were significantly higher at 10 week in women who developed a complication. Other studies have reported that women with preeclampsia exhibit an elevation of plasma triglycerides in the third trimester of pregnancy [10]. Therefore, the possibility that triglycerides could be an early biomarker of pregnancy complications deserves further attention. The detection of early predictive biomarkers for gestational complications is of great interest since it would help

303 to prevent or decrease the impact of these pathologies. Several molecules
304 detectable in plasma have been proposed to be altered in the first and second
305 trimesters of pregnancy, mostly associated with preeclampsia. Among others a
306 recent metaanalysis suggests that a low PlGF in early gestation seems to be
307 the best predictive biomarker of preeclampsia [6]. However, these and other
308 authors point out that measurement of a single biomarker might not be
309 appropriate and suggest that a multiple approach -including several plasma
310 biomarkers or the combination of a plasma biomarker with clinical parameters-
311 are a better approach [6,7]. A similar idea lies behind the use of Antiox-S
312 proposed in the present study. This score is based on multiple plasma
313 antioxidants, which enables to assess the global antioxidant status of an
314 individual. We focused on oxidative status, since gestational complications,
315 particularly those related to placental insufficiency, have been associated with
316 oxidative stress [8-10]. Oxidative stress is a disbalance between production and
317 elimination of ROS. Since ROS are short lived molecules their excess is usually
318 quantified by their products of oxidation, which are more stable. In a situation of
319 oxidative stress, the first targets are the lipids, followed by proteins and finally,
320 the DNA, which is more protected against damage [42]. Increased lipid
321 peroxidation, assessed by the plasma levels MDA have been reported at the
322 end of pregnancy in women with preeclampsia [9,10]. Furthermore, increased
323 levels of carbonyls (a biomarker of protein oxidative damage), have also been
324 reported in the placenta [43] and in plasma [9]. However, these studies have
325 been conducted at the end of pregnancy when preeclampsia has already been
326 diagnosed. It has been pointed out that the presence of a potential oxidative
327 damage marker when a disease is already established, does not ensure that

the molecule is a predictive biomarker, since it can be the consequence of the disease itself [24]. Based on the above mentioned relationship between MDA and carbonyl levels with placental insufficiency, we postulated the possibility that they can also by early biomarkers of the disease, and serve as diagnostic tools of pregnancy complications. However, in the present study, conducted in healthy pregnant women, MDA or carbonyl plasma levels were not elevated in women who later developed a complication. Therefore, we can conclude that oxidized proteins or lipids do not seem to be early biomarkers of obstetric complications. It is unlikely that DNA oxidation products would be an early marker, since DNA undergoes oxidation later than lipids and proteins [42].

The other factor implicated in oxidative stress is the level of antioxidants. In fact, oxidative damage found in preeclampsia has been suggested to be related to an insufficient capacity of endogenous antioxidants to counteract ROS elevation [43,44]. Adequate levels of antioxidants might be particularly important around 10^{th}-12^{th} week of pregnancy when ROS exhibit a large increase, due to the burst of oxygen in the placenta [11]. We found that individual plasma antioxidants tended to be lower in women who developed a maternal complication, but we did not find a statistical difference in any of the measured biomarkers. The complex nature of redox reactions makes difficult to ascribe a prominent role to a particular antioxidant. Therefore, the use of a score integrating several biomarkers has been proposed as a better tool to address oxidative status of an individual, particularly in human populations with high variability [25]. Using a similar approach we found that Antiox-S –a global score integrating all plasma antioxidants- was significantly lower in women who

developed a pregnancy complication, suggesting that this global parameter could be a potential predictive tool to assess pregnancy complications.

ART might also be related to alterations in oxidative status, as demonstrated by the reduction of several serum antioxidants after an *in vitro* fertilization cycle [45]. Therefore, we considered important to investigate the relationship between ART and maternal oxidative status at the end of the first trimester. We found that Antiox-S tended to be lower in ART pregnancies, but it did not reach statistical difference. Therefore, our data do not provide evidence for a negative input of the use of ART on global antioxidant status once pregnancy has been established.

We also found of interest to assess the levels of melatonin, a hormone acting as a direct ROS scavenger and also stimulating other endogenous antioxidants [16], which has gradually gained attention in the field of pregnancy. Recent data indicate that melatonin is a key hormone for successful gestation [13,18] and reduces placental oxidative stress in pregnancy complications[18,19]. We did not found statistical differences in melatonin levels between normal and complicated pregnancies. There was a tendency towards lower melatonin levels in women using ART, but did not reach statistical significance. However, it has to be taken into account that they were older and this hormone decreases with age. Furthermore, we have only addressed diurnal melatonin, due to ethical reasons and it is possible that nocturnal levels might have yielded different results. Despite the fact that we failed to find a significant alteration in melatonin levels at 10 weeks of gestation in women who developed an obstetric complication, the negative relationship between melatonin and carbonyl levels

suggests a possible protective effect of this hormone against protein oxidative damage in pregnancy.

The diet is an important modulator of human health and diets rich in foods of vegetable origin have demonstrated several benefits. For example, consumption during periconceptional period of a mediterranean diet -rich in fruits, vegetables and cereals- reduces the risk of hypertension-related pregnancy disorders [21]. Fruits and vegetables are the major source of antioxidants in the diet, particularly phenolic compounds and vitamins [20]. Therefore we considered of interest to determine their contribution to plasma antioxidant status. We did not find statistical differences in fruit and vegetable consumption comparing women with normal or complicated pregnancy. However, using a multiple correspondence analysis, we detected that a high consumption of foods of vegetable origin cluster with high plasma levels of phenolic compounds and also with high Antiox-S score. The lack of significant differences among groups might be related to the fact that the majority of the women included in the study had a moderate-high fruit and vegetable consumption, as expected from a mediterranean-style diet pattern. We also found that a high milk consumption (daily intake) associate with high levels of melatonin and low levels of carbonyl groups. This association might be related to the milk content in tryptophan, which is the precursor of melatonin [22]. Therefore, we suggest that diet-derived antioxidants might be important in the regulation of antioxidant status and diets rich in foods of vegetable origin are desirable in pregnancy, particularly if endogenous antioxidant systems are deficient.

In conclusion, the present data indicate that the development of pregnancy complications is associated with a low global antioxidant status in early gestation, independent of the use of ART. This might be related to a deficient increase in endogenous antioxidant systems during a critical period of rapid ROS elevation. Our data also suggest that the global antioxidants status may be modulated through the diet and, therefore, nutritional education programs for pregnant woman should be emphasized. An important outcome of the study is the use of a global score, calculated from individual plasma biomarkers, which is a more powerful tool to assess global antioxidant status. Larger population studies will allow establishing the utility of Antiox-S or similar scores as predictive tool of pregnancy complications.

Acknowledgements

This work was supported by Universidad Autónoma de Madrid; Multidisciplinary Research Project (CEMU, 2013-10) and Ministerio de Economía y Competitividad (FEM2012-37634-C03-01 and FEM2015-63631-R).

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Table 1. Socio-economic and dietary habits in women with or without pregnancy complications

			Normal pregnancy (%)	Pregnancy complications (%)
Socio-economic	**Civil status**	Married	64.4	35.6
		Single	68.0	32.0
	Studies	Basic	33.3	66.7*
		Higher	76.9	23.1
	Income	Low-Middle	40.0	60.0
		Upper	69.7	30.3
Dietary habits	**Fruit and vegetables**	Low	5.3	3.4
		Moderate	61.4	79.3
		High	33.3	17.2
	Milk	Moderate	41.7	58.3
		High	68.1	31.9
	Meat or fish	Low	61.5	38.5
		Moderate	58.1	41.9
		High	82.8	17.2
	Pasta or beans	Low	68.3	31.7
		Moderate	61.0	39.0
	Candies	Never	71.4	28.6
		Low	66.7	33.3
		Moderate	53.3	46.7

Data represent percentage in the population. Income level was classified as low-middle (600-1500 €/month) or upper (>1500 €/month). Intake categories were classified as: low (1-2 times/week), moderate (3-6 times/week) and high (1-2 times/day); *p<0.05 when compared to women with higher education studies; χ^2 test.

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Table 2. Hematological and biochemical parameters

	Normal pregnancy	Pregnancy complications	Non-ART pregnancy	ART pregnancy
Hemoglobine *(g/dl)*	13.05±0.15 (40)	13.17±0.13 (27)	13.05±0.14 (49)	13.01±0.17 (24)
Glucose *(mg/dl)*	85.2±1.5 (49)	82.5±1.2 (35)	85.1±1.1 (60)	83±1.8 (32)
Cholesterol *(mg/dl)*	179±3.9 (49)	173±4.8 (35)	173±3.7 (60)	181±4.2 (32)
Triglycerides *(mg/dl)*	84.8±4.3 (48)	108±5.6** (35)	87.8±4 (60)	110.5±7.9** (31)

Mean ± SEM. In parenthesis the number of cases; ***p*<0.001 compared to the respective study group, Student´s *t* test.

Table 3. Plasma antioxidants in women with or without pregnancy complications

	Normal pregnancy (54)	**Pregnancy complications** (32)
Catalase activity *(U Catalase/mg protein)*	0.47±0.08	0.40±0.07
SOSA *(U SOD/mg protein)*	0.60±0.07	0.61±0.08
GSH *(mg GSH/mg protein)*	1.74±0.08	1.52±0.08
Thiol groups *(mM GSH/mg protein)*	0.52±0.03	0.44±0.02
Phenolic compounds *(mg GAE/L)*	255±7.49	241±7.95

Mean ± SEM. In parenthesis the number of cases; Mann Withney U test.

Figure 1

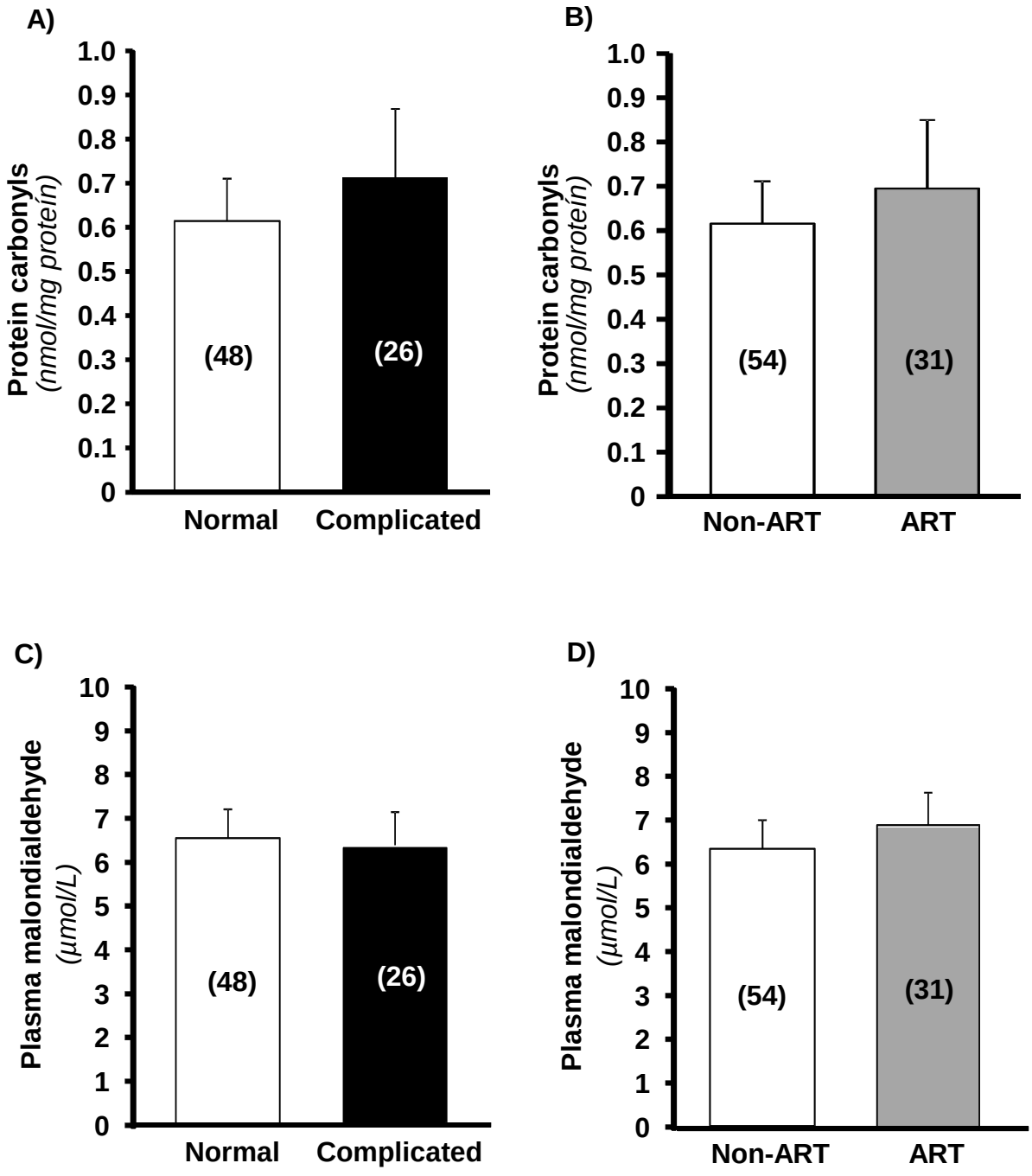
A)
Protein carbonyls
(nmol/mg protein)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
(48)
(26)
Normal
Complicated
B)
Protein carbonyls
(nmol/mg protein)
(54)
(31)
Non-ART
ART
C)
Plasma malondialdehyde
(µmol/L)
10
9
8
7
6
5
4
3
2
1
0
(48)
(26)
Normal
Complicated
D)
Plasma malondialdehyde
(µmol/L)
(54)
(31)
Non-ART
ART

Figure 2

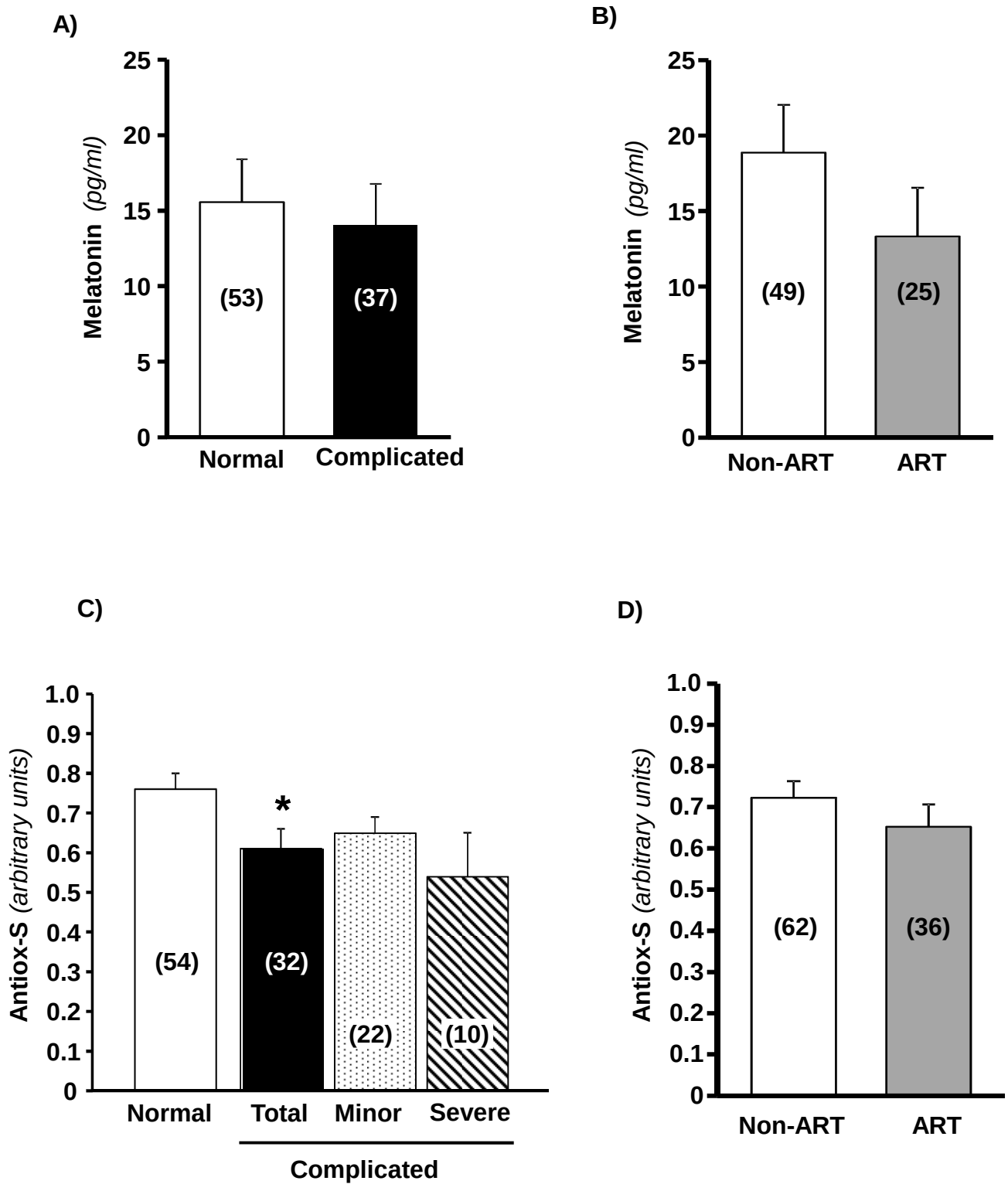
A)
Melatonin (pg/ml)
25
20
15
10
5
0
(53)
(37)
Normal
Complicated
B)
Melatonin (pg/ml)
25
20
15
10
5
0
(49)
(25)
Non-ART
ART
C)
Antiox-S (arbitrary units)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
(54)
(32)
(22)
(10)
Normal
Total
Minor
Severe
Complicated
D)
Antiox-S (arbitrary units)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
(62)
(36)
Non-ART
ART

Figure 3

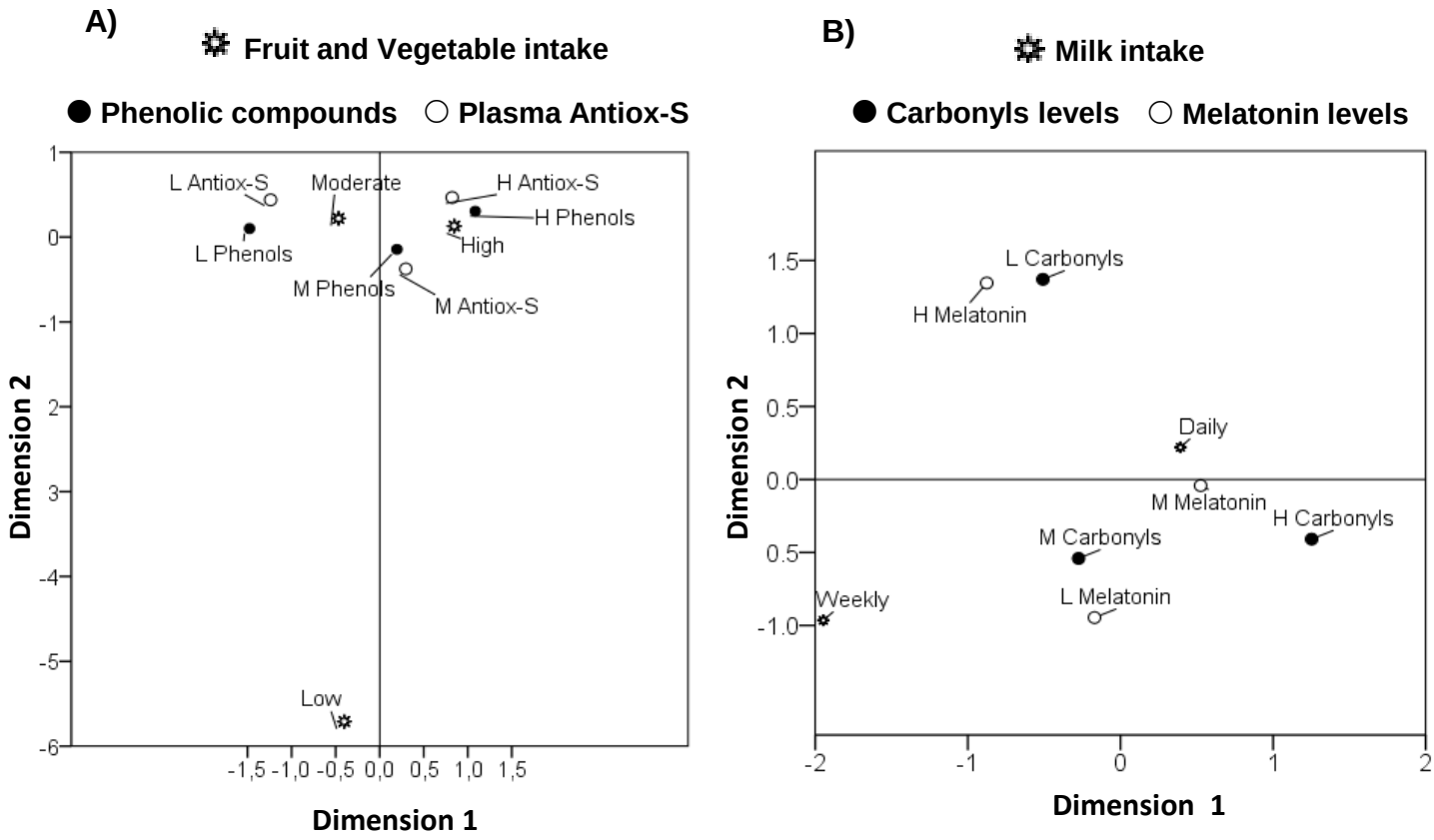
A)
Fruit and Vegetable intake
Phenolic compounds
Plasma Antiox-S
L Antiox-S
Moderate
H Antiox-S
H Phenols
High
L Phenols
M Phenols
M Antiox-S
Low
Dimension 2
Dimension 1
B)
Milk intake
Carbonyls levels
Melatonin levels
L Carbonyls
H Melatonin
Daily
M Melatonin
H Carbonyls
M Carbonyls
Weekly
L Melatonin
Dimension 2
Dimension 1

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Figure legends

Figure 1. Plasma protein carbonyls in normal and complicated pregnancies (A) and in non-ART and ART pregnancies (B). Malondialdehyde levels in normal and complicated pregnancies (C) and in non-ART and ART pregnancies (D). Graphs represent the mean and SEM. The number of cases in each group is shown between brackets. Statistical analysis was performed with Student´s t test.

Figure 2. Plasma melatonin in normal and complicated pregnancies (A) and in non-ART and ART pregnancies (B). Global antioxidant score (Antiox-S) in normal and complicated pregnancies, including total number and broken down into minor and severe complications (C). Global antioxidant score (Antiox-S) in non-ART and ART pregnancies (D). Graphs represent the mean and SEM. The number of cases in each group is shown between brackets. *$p<0.05$ when compared with normal pregnancy group. Statistical analysis was performed with Student´s t test.

Figure 3. Cluster between fruit and vegetable intake, plasma antioxidant score (Antiox-S) and dietary antioxidants (phenolic compounds) (A). Intake levels were categorized as: high (1-2 per day); moderate (3-6 per week) and low (1-2 per week). Cluster between milk intake and plasma melatonin and protein carbonyl levels (Carbonyls) (B). Intake levels were categorized as: high intake (daily) and low intake (weekly). The levels of plasma parameters (carbonlys, melatonin and poliphenols) and Antiox-S were categorized in quartils. 1º quartile represents the lowest levels (L), 2º and 3º quartile represents medium levels (M) and above the 3º quartile represents high levels (H).

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Highlights

- A global score calculated from plasma biomarkers might be used as predictive tool
- Low antioxidant status in early gestation associates with complications development
- Assisted reproduction does not affect early antioxidant status or oxidative damage
- Plasma diurnal melatonin has an inverse relationship with protein oxidative damage
- Intake of vegetables influences plasma oxidative status and could modulate disease

Conflict of Interest Statement

The authors report no conflict of interest.

Authors Contributions

David Ramiro-Cortijo, experimental procedures (plasma antioxidants and carbonyl groups), inclusion of experimental data on data base, interpretation of data.

Teresa Herrera, experimental procedures (melatonin and polyphenols), inclusion of experimental data on data base.

Pilar Rodríguez-Rodríguez, experimental procedures (plasma antioxidants).

Ángel L. López de Pablo, project design, experimental design and data interpretation on oxidative stress parameters.

María de la Calle, project design, pregnant women inclusion in the study, obstetric data collection, data interpretation.

María R. López-Giménez, project design, design of questionnaires and data base, statistical interpretation.

Ana I. Mora-Urda, clinical data collection and inclusion in data base.

Perla Y. Gutiérrez-Arzapalo, blood sample collection and processing.

Rubén Gómez-Rioja, organization of blood sample collection, clinical biochemical data.

Yolanda Aguilera, experimental procedures (polyphenols), interpretation of data.

María A. Martín-Cabrejas, project design, experimental design and interpretation of data on melatonin and polyphenols.

Luis Condezo-Hoyos, experimental design and adaptation of micro methods of oxidative stress.

María C. González, project design, data interpretation oxidative stress.

Pilar Montero, project design, design of nutrition questionnaires and data interpretation.

Bernardo Moreno-Jiménez, project design, social questionnaires, project funding and organization.

Silvia M. Arribas, project design, interpretation of data, manuscript writing.

All authors have seen and approved the final version of manuscript.