

## Summer Day/Night Gender Differences in Serum Total Antioxidant Capacity as a Methodological Pitfall in Human Antioxidant Research

\*Armando L. Morera-Fumero<sup>1,2</sup>, Pedro Abreu-Gonzalez<sup>3</sup>, Lourdes Fernandez-Lopez<sup>1,4</sup>, Estefanía Díaz-Mesa<sup>5</sup> & Maria del Rosario Cejas-Mendez<sup>5</sup>

### Abstract

Background: A dysregulation of the oxidant-antioxidant system has been described in several medical conditions. The total antioxidant capacity (TAC) is one measure of the antioxidant capacity of a system. Circadianity is a biological characteristic of hormones such as melatonin or cortisol. There is little information about TAC circadian rhythm in healthy subjects. Objective: Assessing if healthy subjects present day/night serum TAC changes. Methods: Blood of 48 men and 49 women were drawn at 12:00 and 00:00 hours in summer. Serum TAC was measured by the ABTS radical cation technique. TAC results are expressed as mmol of trolox/L (mean  $\pm$  SD). Results: Men had higher serum TAC concentrations at midday than midnight ( $0.88 \pm 0.18$  vs.  $0.75 \pm 0.19$ ,  $p < 0.001$ ). Women did not have a day/night difference in TAC concentrations (12:00:  $0.78 \pm 0.19$  vs.  $0.76 \pm 0.19$ ,  $p$ : NS). Conclusions: Methodological pitfalls may be committed if those gender day/night differences are not taken into account when researching about this biological parameter.

**Keywords:** total antioxidant capacity; oxidant-antioxidant system; circadian rhythms; gender; biological markers; methodology

<sup>1</sup> Departamento de Medicina Interna, Dermatología y Psiquiatría. Facultad de Medicina. Universidad de La Laguna (ULL). 38071. La Laguna. Santa Cruz de Tenerife. Islas Canarias. <sup>2</sup> Consultoria Psiquiátrica. Santa Cruz de Tenerife. Islas Canarias. <sup>3</sup> Departamento de Ciencias Médicas Básicas: Unidad de Fisiología. Facultad de Medicina. Universidad de La Laguna. La Laguna. Santa Cruz de Tenerife. Islas Canarias. <sup>4</sup> Sociedad para la Investigación y Asistencia en Salud Mental. Santa Cruz de Tenerife. <sup>5</sup> Servicio de Psiquiatría. Hospital Universitario de Canarias, Ofra. Santa Cruz de Tenerife. Spain.

## 1. Introduction

The result of a bioanalysis relies on the accuracy of laboratory techniques, including aspects of sensitivity and specificity among other parameters. Sometimes the "source" from where the biological sample has been extracted (human subject) is not considered as a source of variability in the results.

Researching on human biological markers is a difficult task because several biological variables present data that depend on demographic and individual factors such as gender, ethnic group or chronotype, among others (Yue et al., 1989; Morera-Fumero et al., 2013). Therefore, high inter individual and low intra individual variability have been reported in biological measures (Yue et al., 1989; Cope et al., 2013). Free radicals (FRs) are compounds with unpaired electrons or an open shell configuration that may have positive, negative, or zero charge. Depending on the atom placed at its core, the radical can be described as oxygen, carbon, nitrogen or metal centered radicals (IUPAC, 1997). The unpaired electrons cause radicals to be highly reactive chemical molecules.

FRs have been involved in biological processes such as lipid peroxidation, the intracellular killing of bacteria by phagocytic cells and the alteration of the electron transport system in mitochondria (Pourova et al., 2010). The human body is continuously producing FRs and an excess of FRs can produce oxidative damage. The oxidative stress due to FRs production can be counteracted by enzymatic and non-enzymatic antioxidant systems (Sies, 2007). FRs has been used as markers of many medical conditions, affecting several specialties such as endocrinology (Maureen-Jepkorir et al., 2015), oncology (Il'yasova et al., 2009) or obstetric (Hassan et al., 2013) among others. Blood concentrations of specific antioxidants can be measured individually as well as global measures of antioxidants are also possible. The total antioxidant capacity (TAC) is a well-known global antioxidant measure.

Synonymous of TAC are TAA (Total Antioxidant Activity), TAOP (Total Antioxidant Power), TAS (Total Antioxidant Status) and TAR (Total Antioxidant Response) (Erel, 2004a; Erel 2004b). In this paper, the term TAC will be used to refer to the total antioxidant capacity and the rest of acronyms will be used as the original investigators used them in their own researches. Circadian and seasonal rhythms of biological measures have been pointed as sources of methodological variability (Morera and Abreu, 2007; Morera et al., 2009; Morera-Fumero et al., 2013).

Several biological variables of the oxidant-antioxidant status, such as, malondialdehyde (MDA) and melatonin (MLT) have circadian and seasonal rhythms (Morera and Abreu 2006; Morera and Abreu, 2007). The information available about TAC circadian rhythms in humans is scarce. A circadian rhythm of serum TAS levels, with significantly higher levels at night (01:00 h) compared to daytime levels (13:00 h), has been reported in healthy subjects (Benot et al., 1999). No significant differences between 12:00 and 00:00 h serum TAS levels have been reported in healthy subjects (Morera et al. 2007). Because the information about circadian TAC concentrations is very limited and there is no consensus on it, the aim of this research is to study if healthy subjects present summer day/night changes in serum TAC levels.

## **2. Methods**

Ninety-seven healthy subjects (48 men and 49 women) were recruited among students and staff of the University of La Laguna (ULL). The study was carried out in accordance with the Helsinki declaration and the Ethic and Investigation Committee of the ULL approved the research protocol. Written informed consent was obtained from all subjects after full explanation of the study.

The following variables that may affect the oxidant-antioxidant system were considered as exclusion criteria: 1) Taking drugs of abuse, 2) Presence of acute or chronic physical illness, 3) Pregnancy, 4) Following a vegetarian diet, and 5) Taking vitamin supplements.

Body mass index (BMI), age, gender, smoking status (yes/no), and the number of pieces of fruit, plates of fish, meat and salads per week were controlled. The study was carried out in July during one week-end in order to minimize the interference with the subjects working life. The volunteers arrived at the School of Medicine at 11:00 h and laid in bed from 11:00 to 12:00 h when the first blood sample was drawn. Subjects were instructed to have a light breakfast not later than 09:00 h.

After the first blood extraction, the subjects were free to go home until 23:00 h when they had to be back at the School of Medicine. The second blood sample was drawn at 00:00 h after having laid in bed for one hour. Subjects were instructed to have a light dinner not later than 20:30 h. Because artificial light has been reported to affect nocturnal TAS concentrations (Benot et al., 1999), all the night samples were extracted in an environment with a 4 lux light intensity (dark environment). In order to avoid light contamination, the eyes of each subject were covered with a black sleep mask. Nocturnal samples were drawn with the help of a small torch of red light (20 lux) pointing at the forearm of the subject.

The rationale to lay in bed one hour before blood extraction was to allow the subjects to relax in order to minimize the psychological and physical stress they may have had (Buyakhatipoglu et al., 2010). All blood samples were extracted by venepuncture. After each blood extraction, blood was placed in vacutainer tubes without anticoagulant. Blood was allowed the formation of a clot at room temperature during 15 minutes and then was centrifuged at 3000 rpm during 10 minutes.

Serum samples were aliquoted in eppendorf tubes and kept frozen at  $-70^{\circ}\text{C}$  until analysis. Healthiness of the volunteers was ensured through a general haematological, biochemical and urine analysis.

TAC was measured to evaluate the antioxidant capacity. Serum TAC was analysed by the formation of the ABTS radical cation (Miller et al., 1993), with commercially available kits (Antioxidant Assay kit, SIGMA, Madrid, Spain). The principle of the antioxidant assay is based on the formation of a ferryl myoglobin radical from methamyoglobin and the hydrogen peroxide, which oxidizes the ABTS (2, 2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid]) to produce a radical cation, ABTS.+ a soluble chromogen that is green colour.

This can be determined spectrophotometrically at 415 nm in a microplate spectrophotometer reader (Benchmark Plus, Bio-Rad, and Hercules, CA, USA). Antioxidant compounds suppress the production of the radical cation in a concentration dependent manner and the colour intensity decreases proportionally. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid), a water-soluble vitamin E analogue, serves as a standard (Miller et al., 1993). The intra- and inter-assay coefficients of variation (CV) were 6.96 % and 9.13 %, respectively. The results are expressed as mmol of Trolox/L. All serum samples were analysed the same day and by the same analyst, who was blind with respect to the characteristics of the samples.

Data were analysed with the 15th version of the Statistical Package for Social Sciences (SPSS, Illinois, Chicago, USA). Comparison of two quantitative variables was carried out by means of paired or independent t test. Chi-square was applied to analyse qualitative variables associations. All statistical tests were two-tailed and their significance level was set at 0.05. Quantitative data are presented as mean  $\pm$  standard deviation (S.D.).

### 3. Results

Table 1 shows the demographic and dietary characteristics of the sample. The only variable that significantly differed between men and women was the mean number of salads per week. Women ate significantly more salads than men.

	Whole sample	Men	Women	P
Gender (men/women)	97	48	49	NA
Age (mean±sd)	35.3±9.7	34.2±9.1	36.4±10.2	0.43
Body mass index (mean±sd)	24.3±3.8	25.2±3.3	23.4±4.1	0.17
Smoking status (yes/no)	35/64	17/34	18/30	0.14
Fruits (mean±sd)	9.3±9.4	11.3±11.7	7.7±5.7	0.20
Fish (mean±sd)	1.8±1.1	1.6±0.9	2.0 ±1.1	0.19
Salads (mean±sd)	3.4±2.1	2.8±2.1	4.3 ±2.0	0.02
Meat (mean±sd)	1.5±0.7	1.6±0.8	1.4±0.6	0.22

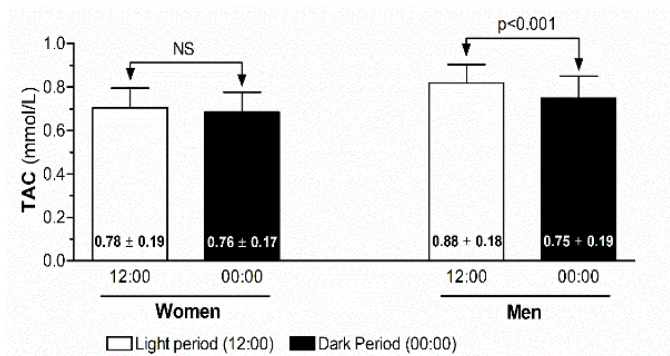
**Table 1: Demographic and dietary characteristics of the whole sample and by gender**

Comparison of serum TAC concentrations at 12:00 and 00:00 h showed that serum TAC concentrations at 12:00 were significantly higher than at 00:00 h ( $0.83\pm 0.19$  vs.  $0.75\pm 0.18$ ,  $p < 0.001$ ). Because this is the first time that this specific difference is described, the next statistical study carried out was a stepwise multiple regression analysis in order to know which of the studied variables may affect that circadian difference.

In the stepwise method each variable is entered in sequence and its value assessed (if  $p < 0.05$  is included, if  $p > 0.1$  is excluded). If the addition of one variable contributes to the model is retained, but then all other variables are re-tested to check if they are still contributing to the success of the model. If they do not contribute significantly, they are removed.

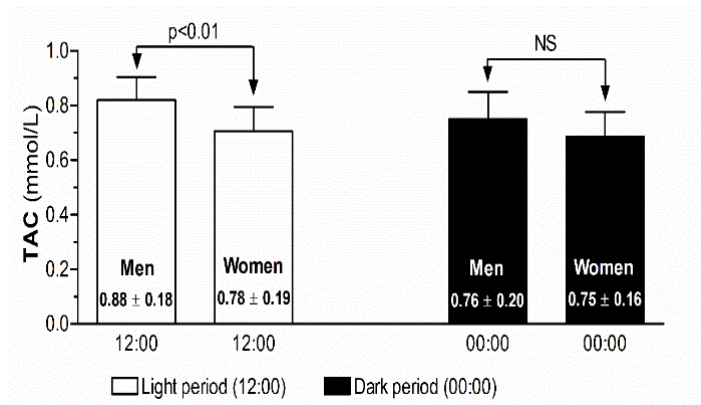
In our model serum TAC concentrations at 12:00 and 00:00 h acted as dependent (criterion) variables. Age, BMI, number of pieces of fruit/dishes of fish/salads/meat eaten per week, gender and smoking status acted as independent (predictors) variables. The only variable that significantly predicted serum TAC levels at 12:00 h (Beta: 0.265,  $p < 0.009$ ) was gender. The rest of the variables did not add significant prediction to TAC levels at 12:00 h. The stepwise multiple regression analysis did not elicit any significant predictive variable for serum TAC levels at 00:00 h.

In order to control the effect of gender on serum TAC concentrations, we again compare the levels of serum TAC between 12:00 and 00:00 h in men and women separately. Figure 1 shows that there was no circadian change of serum TAC concentrations in women, while men had significantly higher TAC levels at midday than midnight.



**Figure 1: Comparison of serum TAC concentrations between midday and midnight by gender. NS: non-significant**

Figure 2 shows the comparison of serum TAC levels by gender between 12:00 and 00:00 h. Men had significant higher serum TAC levels than women at 12:00 h. Men and women had similar serum TAC levels at 00:00 h.



**Figure 2: Comparison of serum TAC concentrations by gender between midday and midnight. NS: non-significant**

#### 4. Discussion

Our research reports for the first time an important finding, a day/night change of serum TAC concentrations with higher levels at midday than midnight, but this difference is circumscribed only to men. As far as we know, two publications specifically address the question of studying day/night differences in blood TAS concentrations. Benot et al. (1999) published the first paper. The authors reported significant higher serum TAS levels at 01:00 than at 13:00 h.

Those results are just the opposite of our results. The authors suggested that the TAS rhythm was related to the MLT rhythm, so when MLT levels were high TAS levels were also high. Our own research group (Morera et al., 2007) published the second paper. In this paper there were no significant differences in serum TAS levels between blood samples taken at 12:00 and 00:00 h. Although no significant differences were found in this research, a tendency to higher serum TAS levels in the morning sample with respect to the midnight sample was reported.



Methodological differences may explain those discrepancies. First, our sample is bigger ( $N_{12:00}$  and  $00:00 = 97$ ) than the sample of Benot et al. (1999) ( $N_{13:00} = 12$  and  $N_{01:00} = 12$ ). Second, our sample was comprised by the same subjects at midday and midnight (paired design) while in the study of Benot et al. (1999) the subjects at 13:00 h were different from the subjects at 01:00 h (independent design). Third, we keep subjects relaxed one hour before blood extraction. In the case of Benot et al. (1999) this information has not been considered or reported in the paper. With respect to the second paper (Morera et al., 2007), the study was carried out on five non-smoker male subjects, a very small sample of subjects. In spite that there were no significant differences between midday and midnight TAC concentrations; however, they presented a tendency without statistical significance.

Regarding the differences in TAC concentrations by gender, this is the first time that has been reported that men had significantly higher TAC levels than women did at midday. Most of the previous researches published on this topic do not specifically analyse differences in TAS levels between men and women but whole samples (Miller et al., 1993; Rice-Evans and Miller, 1994; Woo et al., 1997; Cao and Prior, 1998). However, in a paper published by Erel (2004a), it was reported that there were no significant differences in the TRAP (total antioxidant response) between men and women. On the other hand, two papers reported that men had significantly higher morning serum TAC concentrations than women (Woo et al., 1997; Demirbag et al., 2005). Our morning results are in line with the aforementioned researches.

Woo et al. (1997) considered that the gender difference might stem from the fact that men have higher levels of serum bilirubin and urate concentrations than women do, and both substances are some of the major contributors to the antioxidant status.

A more recent paper linked the gender differences to sexual hormones (Demirbag et al., 2005). Demirbag et al. (2005) found that TAC levels in male subjects correlated positively with testosterone levels while TAC levels in female was positively correlated with estradiol levels.

Men have significantly higher levels of testosterone than women (Van der Meij et al., 2010). On the other hand, testosterone concentrations also have significant differences between day and night. In men, blood testosterone concentrations start rising at approximately 20:00 h reaching its maximum peak at 08:00 h when testosterone levels start to steadily decrease until reaching its minimum at 20:00 h (Bremner et al., 1983). We did not measure TAC hourly but TAC levels at 12:00 h coincide with the increasing slope of testosterone, while TAC levels at 00:00 h coincide with the decreasing slope of testosterone. Another possible explanation for this gender difference may stem from another biological difference between women and men, namely that women have higher estradiol levels than men in similar physiological conditions.

The fact that Demirbag et al. (2005) found a positive and significant correlation in a multivariate analysis between testosterone and TAC concentrations in male subjects ( $\beta=0.560$ ) and between estradiol and TAC concentrations in female subjects ( $\beta=0.818$ ) give grounds to our gender related results.

Age has also been inversely related with TAS/TAC concentrations (Benot et al., 1999; Ziobro and Bartosz, 2003). A positive significant relationship has been reported between TAC and age in men but a lack of correlation between TAC and age has also been reported in women (Demirbag et al., 2005). Bennot et al. (1999) suggested that there was an age decline in the antioxidant capacity of human sera that may be the consequence of an associated reduction in circulating MLT.

Ziobro and Bartosz (2003) reported that young blood donors (18-24 years old) had significantly higher TAC levels than older donors (40-60 years old). We did not find an association between age and TAC levels. Those differences in TAS/TAC age-related results may be due to the differences in age between samples. In the study of Benot et al. (1999) their age's samples ranged from 2 to 89 while in the paper of Ziobro and Bartosz (2003) the age of the sample ranged from 18 to 60. In our study, the age's sample ranged from 18 to 53. In fact, Benot et al. (1999) consider that the main reduction of TAS concentration is produced after the sixth decade of life, so, that may explain the lack of correlation in our sample.

Because the circadian gender difference is the first time that has been described, this difference needs to be replicated. The main limitation of our study is that we did not analyse gender biological markers, such as testosterone and estradiol. However, on the other hand, the fact that the same group of subjects were studied at midnight and midday give consistency to our results. Our results should be considered preliminary because they need to be replicated. Furthermore, it is also necessary to know if the differences between men and women and the day/night change persist in winter.

In conclusion, our research reports two new important results, the day/night difference in male TAC concentrations and the higher midday serum TAC concentrations in men compared to women. Methodological flaws may be committed when those differences are not taken into account when researching on this topic, therefore, the inclusion of that difference in the research protocol is highly recommended. Future research is guaranteed on this field and the addition of biological markers of gender difference is strongly recommended.

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