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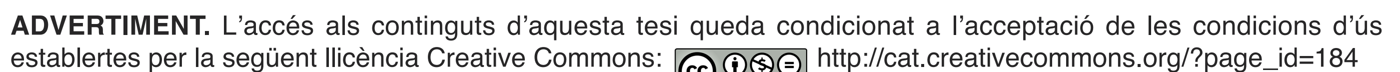

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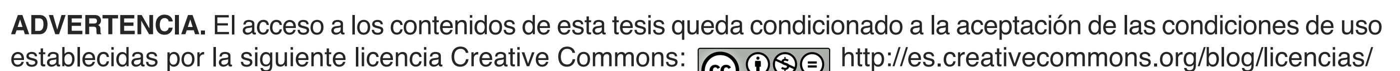

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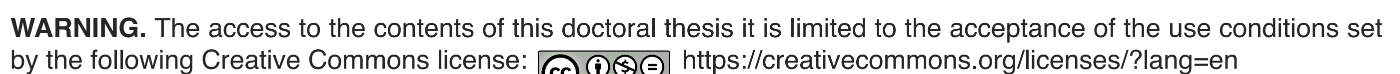

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Universitat Autònoma de Barcelona

Metabolomic analysis of oxidative stress and inflammation biomarkers during chemotherapy treatment in pregnant women with breast cancer and their offspring

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2020

Faculty of Medicine
Department of Biochemistry and Molecular Biology

Metabolomic analysis of oxidative stress and inflammation biomarkers during chemotherapy treatment in pregnant women with breast cancer and their offspring

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Doctoral Thesis

Doctoral Program in Biochemistry, Molecular Biology and Biomedicine

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Tutor: Dr. Simó Schwartz Jr

2020

"Education is the most poweful weapon which you can use to change the Word"
Nelson Mandela

"I need to believe that something extraordinary is possible"
A beautiful mind

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I. LIST OF ABREVIATIONS

$^1O_2$: singlet oxygen

2-dG: 2’-deoxyguanosine

3-chlorotyrosine: 3-Cl-Tyr

3-nitrotyrosine: 3-NO_2-Tyr

4-M-U-b-D-N- triacetylchitotrioside: 4-Methylumbelliferyl β-D-N,N’,N’’-triacetylchitotrioside

4-MU: 4-Methylumbelliferone sodium salt

8-OHdG: 8-hydroxy-2'-deoxyguanosine

AA: Arachidonic acid

AECC: Asociación Española Contra el Cáncer

AGEs: Glycoxidation end products

AnC: Anthracycline

Arg: Arginine

ATM: ATM serine/threonine kinase

ATP: Adenosine triphosphate

BC: Breast cancer

BCT: Breast conservation therapy

BHT: Butylhydroxytoluene

BRCA1: BRCA1 DNA repair associated

BRCA2: BRCA2 DNA repair associated

BSA: Bovine serum albumin

CBB: Coomassie brilliant blue

CH_3OH: Methanol

CH_3CN: Acetonitrile

ChT: Chitotriosidase

CHEK2: Checkpoint kinase 2

COX: Cyclooxygenase

CTX: Chemotherapy

DCIS: Ductal carcinoma in situ

DNA: Deoxyribonucleic acid

DTNB: Drabkin's reagent, 5,5'-Dithiobis (2-nitrobenzoic acid)

DTPA: Diethylenetriaminepentaacetic acid

ECIS: European Cancer Information System

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

EMT: Epithelial mesenchymal transition

eNOS: Nitric oxide synthase

ER: Oestrogen receptor

ESI-: Negative electrospray ionization

FISH: Fluorescence in situ hybridization

G: Gram

GA: Gestational age

GC: Glucocorticoid

GDM: Gestational diabetes mellitus

GH: Growth hormone

GPx: Glutathione peroxidase

GR: Glutathione reductasa

GSA: Glutathione sulfonamide

GSH: Glutathione

GSSG: Glutathione disulfide

H_2O_2: Hydrogen peroxide

H_2O_q: Milli-Q Water

Hb: Haemoglobin

HCl: Hydrochloric acid

HCOOH: Formic acid

HER2: Human epidermal growth factor receptor 2

HOCl: Hypochlorous acid

HPLC: High performance liquid chromatography

HPO_3: Metaphosphoric acid

IDC: Invasive ductal carcinoma

IHC: Immunohistochemistry

ILC: Invasive lobular carcinoma

INE: National Statistics Institute

IS: Internal standard

IsoF: Isofuran

IsoP: Isoprostane

IUGR: Intrauterine growth restriction

KH_2PO_4: Potassium phosphate monobasic

KOH: Potassium hydroxide

LA: Linoleic acid

LASSO: Least absolute shrinkage and selection operator

LCIS: Lobular carcinoma in situ

LC-MS/MS: Liquid Chromatography coupled to tandem Mass Spectrometry

LDL: Low-density lipoprotein

$LOO^{\bullet}$: Lipoperoxyl radical

LOOH: Hydroperoxide

LOQ: Limit of quantification

Lys: Lysine

MasCS: Mammary stem cells

MDA: Malondialdehyde

Meta-tyrosine: m-Tyr

MPO: Myeloperoxidase

MRM: Multiple reaction monitoring

mtDNA: Mitochondrial DNA

NaOH: Sodium hydroxide

nDNA: Nuclear DNA

NEM: N-ethylmaleimide

NeuroF: Neurofuran

NeuroP: Neuroprostane

NO: Nitric oxide

NO_2: Nitrogen dioxide

NOX: NADPH oxidases

$O_2^{-\bullet}$: Superoxide anion radical

O_2: Oxygen

O_3: Trioxygen

$OH^{\bullet}$: Hydroxyl radical

$ONOO^-$: Peroxynitrite

Ortho-tyrosine: o-Tyr

OS: Oxidative stress

P: Percentile

PABC: Pregnancy associated breast cancer

PALB2: Partner and localizer of BRCA2

Para-tyrosine: p-Tyr

PBS: Phosphate-buffered saline

PCA: Principal component analysis

PE: Preeclampsia

PG: Prostaglandin

Phe: Phenylalanine

PLA_2: Phospholipase A_2

PlGF: Placental growth factor

PPROM: Preterm premature rupture of the membrane

PR: Progesterone receptor

Pro: Proline

PTB: Spontaneous preterm birth

PTEN: Phosphatase and tensin homolog

PTX: Paclitaxel

PUFA: Polyunsaturated fatty

QC: Quality control

RBC: Red blood cells

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

RT: Radiotherapy

SGA: Small for gestational age

SH: Sulfhydryl

SOD: Superoxide dismutase

SPE: Solid phase extraction

STK11: Serine/threonine kinase 11

TBA: 2-Thiobarbituric acid

TEP: 1,1,3,3-Tetramethoxypropane

Thr: Threonine

TP53: Tumour protein p53

TXN: Taxanes

UV: Ultraviolet

VEGF: Vascular endothelial growth factor

Wk: Week

XO: Xanthine oxidase

Yr: Year

II. ABSTRACT

ABSTRACT

Introduction:

Pregnancy-associated breast cancer (PABC) is generally defined as breast cancer diagnosed during pregnancy or one-year following postpartum. Chemotherapy administration during the second and third trimester is often accompanied by increasing levels of reactive oxygen species (ROS), but it remains unclear whether increased levels of oxidative stress are responsible for the onset of many obstetric complications.

Objective:

Pregnant women with breast cancer undergoing chemotherapy treatment during pregnancy and their offspring may have an increase in the production of oxidative stress and inflammation products that may account for the increased risk of maternal and perinatal complications observed in these cases.

Therefore, we sought to investigate chemotherapy-induced oxidative stress and inflammation before and after treatment and at delivery in mothers and their offspring.

Material and methods:

For this purpose, we examined multiple oxidative stress, inflammation and antioxidant defence markers in PABC patients treated with anthracyclines and paclitaxel. The study included seventeen PABC patients whose blood samples were withdrawn prior to treatment, before each chemotherapy cycle and at labour. Moreover, we monitored the cumulative effects of chemotherapy treatment. Additionally, we also examined blood samples of ten non-pregnant breast cancer (non-PABC) patients before and after anthracyclines treatment. Blood samples from sixteen healthy pregnant women were analysed as a control pregnancy. We also assessed chemotherapy-induced oxidative stress and inflammation of neonates exposed to chemotherapy in utero. To do so, we collected cord blood samples and urine samples from the newborns within the first 24h after birth. Cord blood and urine samples from neonates born to healthy pregnant women were also included as controls.

Results:

Overall, our data showed that PABC patients exhibited similar levels of oxidative stress and antioxidant defence markers as compared to pregnant controls before treatment with chemotherapy. However, we observed that PABC women have increase levels of chitotriosidase (*P*=0.004), a marker of inflammation. Following chemotherapy, some changes in oxidative stress markers and inflammation markers were found: chitotriosidase was reduced after paclitaxel as compared to healthy pregnant women (*P*=0.018); YKL-40 was decreased after paclitaxel as compared before treatment (*P*=0.047) and protein-SH groups were reduced as compared to healthy pregnant women following anthracyclines but then were again increased after paclitaxel (*P*=0.010 and *P*=0.012 respectively).

The accumulative effect of chemotherapy treatment was analysed by comparing the baseline levels with those obtained at delivery, and our findings demonstrated that chemotherapy exposure during pregnancy increased the DNA and protein damage in PABC patients, as shown by an increase in 8-OHdG/2dG ratio and o-Tyr/Phe ratio, respectively (*P*=0.031; *P*=0.032).

In addition, we found significant increased levels of three metabolites involved in lipid peroxidation: 55-F_{2t}-IsoP+5-*ep*i-5-F_{2t}-IsoP (*P*=0.031), 15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$-IsoP (*P*=3.72e^{-03}) and 10-F_{4t}-NeuroP (*P*=0.031); as well as, $PGF_{2\alpha}$ (*P*=0.026) metabolite of inflammation and antioxidant defence GSH/GSSG ratio (*P*=4.79e^{-03}) biomarkers compared to controls at delivery. However, these differences were not observed regarding DNA and protein damage.

Regarding non-PABC patients, they displayed elevated levels of DNA and protein damage markers (8-OH-dG/2dG ratio; *P*=1.52e^{-03} and m-Tyr/Phe ratio, *P*=7.88e^{-04}respectively) but reduced antioxidant capacity (GSH/GSSG ratio, *P*=5.67e^{-06}) compared to PABC patients.

On the other hand, neonates exposed to chemotherapy in utero generally displayed lower levels of oxidative stress and inflammation markers than neonates born to healthy women. Exceptionally, we found increased plasma levels of 15-F_{2f}-IsoP (*P*=8.05e^{-03}) and urine levels of GSA (*P*=0.016) compounds. Lastly, we found a positive correlation between 15-*epi*-15-F_{2t}-IsoP and 15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11-$PGF_{2\alpha}$ + 2,3-dinor-15-

$F_{2\alpha}$-IsoP (*R*=-0.55, *P*=0.049 y *R*=-0.68, *P*=0.011 respectively) in neonates with intrauterine exposure to chemotherapy and the corresponding mother.

Conclusions:

In summary, our data show that the administration of chemotherapy during pregnancy is significantly associated with a disruption of redox balance. Remarkably, our results indicate that the antioxidant capacity of PABC patients was able to counteract the chemotherapy-induced oxidative stress, as well as levels of inflammation. Moreover, PABC patients and their offprints exhibited similar levels of oxidative stress and antioxidant defence than healthy pregnant women their neonates. Therefore adverse effects in maternal and neonatal outcome reported secondary to chemotherapy treatment not seem to be caused by increase oxidative and inflammatory pathways, or if increased, they were counteracted by the redox system.

RESUM:

Introducció:

El càncer de mama associat a l'embaràs (PABC en anglès) és definit com a càncer de mama diagnosticat durant l'embaràs o un any després del part. La quimioteràpia administrada durant el segon i tercer trimestre de l'embaràs normalment està associada amb alts nivells d'espècies reactives d'oxigen (ROS en anglès) tot i que no està esclarit si aquest augment d'estrès oxidatiu és responsable de l'aparició de molts problemes obstètrics.

Objectiu:

Dones embarassades amb càncer de mama tractades amb quimioteràpia durant l'embaràs així com els seus fills, poden patir una elevada producció d'estrès oxidatiu i inflamació que poden explicar l'augment del risc de desenvolupar complicacions maternes i perinatals observades en aquests casos.
Per tant, vam investigar l'estrès oxidatiu i inflamació associat amb l'administració de quimioteràpia abans i després de cada tractament, així com en el moment del part en dones amb PABC i els seus fills.

Material i mètodes:

Per aconseguir el nostre objectiu, vam examinar diferents marcadors d'estrès oxidatiu, inflamació i defensa antioxidant en dones amb PABC tractades amb antraciclines i paclitaxel. En aquest estudi es va obtenir sang de disset pacients amb PABC abans del tractament, abans de cada cicle de quimioteràpia i al part. A més, vam monitoritzar l'efecte acumulatiu del tractament. Addicionalment, vam obtenir sang de deu dones amb càncer de mama no embarassades (non-PABC) abans i després del tractament amb antraciclines així com setze mostres de dones embarassades sanes durant l'embaràs i al part. Paral·lelament, vam analitzar també l'estrès oxidatiu i inflamació en els nounats que van rebre quimioteràpia en l'úter a partir de sang de cordó umbilical i d'orina recollida durant les primeres 24 h de vida. Mostres de cordó i d'orina de nadons nascuts de mares sanes es van incloure en l'estudi com a controls.

Resultats:

En general, els nostres resultats van mostrar que les pacients amb PABC presentaven nivells semblants d'estrès oxidatiu i de defensa antioxidant abans de l'inici amb quimioteràpia que les controls gestants. Això no obstant, les pacients amb PABC mostraven un augment en l'activitat chitotriosidasa (*P*=0.004) en comparació amb les dones sanes gestant, la qual ens indica inflamació. A conseqüència de la quimioteràpia, vam observar diferents canvis en els biomarcadors d'estrès oxidatiu i inflamació: l'activitat chitotriosidasa va es reduïa després del tractament amb paclitaxel en comparació amb les dones sanes embarassades (*P*=0.018); l'activitat de YKL-40 va disminuir també respecte els nivells d'abans de la quimioteràpia (*P*=0.047) i els grups de proteïnes-SH van ser significativament reduïts després del tractament amb antraciclines per tornar a incrementar seguidament amb el tractament amb paclitaxel en comparació amb els controls embarassades (*P*=0.010 i *P*=0.012 respectivament).

L'efecte acumulatiu del tractament amb quimioteràpia es va analitzar comparant els nivells basals amb els obtinguts al part. El nostre estudi va demostrar que l'exposició a quimioteràpia durant l'embaràs provoca un augment de dany a l'ADN i de proteïnes a les pacients amb PABC tal com es pot veure en els nivells elevats de les ràtios 8-OHdG/2dG i o-Tyr/Phe respectivament (*P*=0.031; *P*=0.032). A més, vam observar nivells significativament elevats en tres metabòlits associats a peroxidació lipídica: 5-F_{2t}-IsoP+5-*epi*-5-F_{2t}-IsoP (*P*=0.031), 15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$-IsoP (*P*=$3.72e^{-03}$) i 10-F_{4t}-NeuroP (*P*=0.031), així com $PGF_{2\alpha}$ (*P*=0.026) marcador d'inflamació i la ràtio GSH/GSSG (*P*=$4.79e^{-03}$) marcador de defensa antioxidant en comparació amb els controls al part. Aquestes diferencies no van ser observades analitzant els biomarcadors de dany a l'ADN i de proteïnes.

Pel que fa a les pacients amb càncer de mama però no embarassades, presentaven nivells elevats dels biomarcadors de dany a l'ADN i de proteïnes (8-OH-dG/2dG ràtio; *P*=$1.52e^{-03}$ i m-Tyr/Phe ràtio, *P*=$7.88e^{-04}$ respectivament), però el biomarcador de defensa antioxidant (GSH/GSSG ràtio, *P*=$5.67e^{-06}$) estava reduït en comparació amb les pacients amb PABC.

D'altra banda, els nounats exposats a quimioteràpia a l'úter presenten generalment nivells més baixos d'estrès oxidatiu i marcadors d'inflamació que els nounats nascuts de dones embarassades sanes. Excepcionalment, vam detectar un increment en els nivells plasmàtics de 15-F_{2T}-IsoP (*P*=8.05e^{-03}) i els nivells urinaris de GSA (*P*=0.016). Per últim, vam observar una correlació positiva entre els nivells de 15-*epi*-15-F_{2t}-IsoP y 15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$-IsoP (*R*=-0.55, *P*=0.049 i *R*=-0.68, *P*=0.011 respectivament) mesurats en sang de cordó de nounats exposats a quimioteràpia en l'úter matern amb la sang obtinguda de les mares corresponents al part.

Conclusions:

En resum, el nostre estudi mostra que l'administració de quimioteràpia durant l'embaràs està significativament associada amb una alteració de l'estat redox. No obstant això, els nostres resultats indiquen que la capacitat antioxidant de les pacients amb PABC és capaç de contrarestar l'estrès oxidatiu i la inflamació induïts per la quimioteràpia. A més, les pacients amb PABC i els seus fills presenten nivells d'estrès oxidatius generalment similars amb els obtinguts en dones sanes embarassades i els seus fills. Per tant, les complicacions maternes i neonatals reportades a l'estudi secundàries al tractament amb quimioteràpia semblen no ser causades per un augment de les vies oxidatives i inflamatòries però de ser-hi, van ser contrarestats pel seu sistema redox.

RESUMEN:

Introducción:

El cáncer de mama asociado al embarazo (PABC en inglés) es definido como cáncer de mama diagnosticado durante el embarazo o un año después del parto. La quimioterapia administrada durante el segundo y tercer trimestre del embarazo normalmente está asociada con altos niveles de especies reactivas de oxígeno (ROS en inglés) aunque no está del todo claro si este aumento de estrés oxidativo es responsable de la aparición de varios problemas obstétricos.

Objetivo:

Mujeres embarazadas con cáncer de mama tratadas con quimioterapia durante el embarazo así como sus hijos, pueden presentar elevados niveles de estrés oxidativo e inflamación que explicarían el aumento del riesgo de desarrollar complicaciones maternas y perinatales observadas en estos casos.

Por lo tanto, quisimos evaluar el estrés oxidativo e inflamación inducido por la quimioterapia antes y después de cada tratamiento y en el parto en mujeres con PABC y sus hijos.

Material y métodos:

Para conseguir nuestro objetivo, examinamos diferentes marcadores de estrés oxidativo, inflamación y defensa antioxidante en mujeres con PABC tratadas con antraciclinas y paclitaxel. En este estudio se tomó sangre de diecisiete pacientes con PABC antes del tratamiento, antes de cada ciclo de quimioterapia y al parto. Además, monitoreamos el efecto acumulativo del tratamiento durante el embarazo. Adicionalmente, recogimos sangre de diez mujeres con cáncer de mama no embarazadas (non-PABC) antes y después del tratamiento con antraciclinas así como dieciséis muestras de mujeres embarazadas sanas durante el embarazo y el parto. Paralelamente, analizamos también el estrés oxidativo e inflamación en los niños que recibieron quimioterapia en el útero a partir de sangre de cordón y de orina recogida durante las primeras 24 horas de vida. Muestras de cordón y de orina de bebés nacidos de madres sanas fueron también incluidas en el estudio como controles.

Resultados:

En general, nuestros resultados mostraron que las pacientes con PABC exhibían niveles similares de estrés oxidativo y de defensa antioxidante antes del inicio del tratamiento quimioterapia que las controles gestantes. Sin embargo, las pacientes con PABC tenían aumentada la actividad chitotriosidasa (*P*=0.004) en comparación con las mujeres sanas gestantes, la cual nos indica inflamación. A consecuencia de la quimioterapia, observamos diferentes cambios de biomarcadores de estrés oxidativo e inflamación: la actividad chitotriosidasa fue reducida después del tratamiento con paclitaxel en comparación con las mujeres sanas embarazadas (*P*=0.018); la actividad de YKL-40 disminuyó también en comparación con los niveles de antes de la quimioterapia (*P*=0.047) y los grupos de proteínas-SH fueron significativamente reducidos después del tratamiento con antraciclinas para volver a incrementar seguidamente con paclitaxel en comparación con los controles embarazadas. (*P*=0.010 y *P*=0.012 respectivamente).

El efecto acumulativo del tratamiento con quimioterapia se analizó comparando los niveles basales con los obtenidos en el parto. Nuestro estudio demostró que la exposición a quimioterapia durante el embarazo provoca un aumento de daño al ADN y de proteínas en las pacientes con PABC tal como se aprecia en los ratios 8-OHdG/2dG y o-Tyr/Phe respectivamente (*P*=0.031; *P*=0.032). Además, encontramos niveles significativamente elevados en tres metabolitos asociados peroxidación lipídica: 5-F_{2t}-IsoP+5-*ep*i-5-F_{2t}-IsoP (*P*=0.031), 15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$-IsoP (*P*=3.72e^{-03}) y 10-F_{4t}-NeuroP (*P*=0.031), así como el biomarcador de inflamación, $PGF_{2\alpha}$ (*P*=0.026) y el ratio de defensa antioxidante, GSH/GSSG (*P*=4.79e^{-03}) en comparación con las controles en el parto. Sin embargo, estas diferencias no fueron encontradas al analizar la oxidación del ADN y de proteínas en ambos grupos.

En cuanto al análisis de mujeres con cáncer de mama no embarazadas, mostraron niveles elevados de biomarcadores de daño en el ADN y proteínas (ratios 8-OHdG/ 2dG ratio; *P* =1.52e^{-03} y m-Tyr/Phe ratio; *P*=7.88e^{-04} respectivamente), pero una capacidad antioxidante reducida (GSH/GSSG ratio, *P*=5.67e^{-06}) en comparación con las pacientes con PABC.

Por otro lado, los recién nacidos expuestos a quimioterapia en el útero presentaron generalmente niveles más bajos de biomarcadores de estrés oxidativo e inflamación que los recién nacidos de mujeres embarazadas sanas. Excepcionalmente, encontramos incrementados los niveles plasmáticos de 15-F_{2T}-isoP (P=8.05e^{-03}) y los niveles urinarios de GSA (P=0.016). Por último, encontramos una correlación positiva entre los niveles de 15-*epi*-15-F_{2t}-IsoP y 15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$-IsoP (R=-0.55, P=0.049 y R=-0.68, P=0.011 respectivamente) medidos en sangre de cordón de recién nacidos expuestos a quimioterapia en el útero materno con la sangre obtenida de las madres correspondientes al parto.

Conclusiones:

En resumen, nuestro estudio muestra que el tratamiento con quimioterapia durante el embarazo está significativamente asociado con una alteración del estado redox. Sin embargo, nuestros resultados indican que la capacidad antioxidante de las pacientes con PABC fue capaz de contrarrestar el estrés oxidativo y la inflamación inducidos por la quimioterapia. Además, las pacientes con PABC y sus hijos exhibían niveles de estrés oxidativos generalmente similares con los obtenidos en mujeres sanas embarazadas y sus hijos.

Por tanto, las complicaciones maternas y neonatales reportadas al estudio secundarias al tratamiento con quimioterapia parecen no ser causadas por un aumento de las vías oxidativas e inflamatorias aunque si fuese el caso, hubiese sido contrarrestados por su sistema redox.

III. INTRODUCTION

1. EPIDEMIOLOGY AND ETIOLOGY OF BREAST CANCER

1.1 Epidemiology

Breast cancer (BC) is the most common malignancy diagnosed in women worldwide, and the second most frequent cancer overall. Approximately about 2.1 million women were diagnosed in 2018 with a crude rate of 55.2 cases per 100,000 women based on data from GLOBOCAN. However, an estimate of 626,679 women, which accounts for 16.6 cases per 100,000, died for breast cancer[1]. Unfortunately, the global incidence of breast cancer is likely to continue to increase owing to the ageing population, population growth, risk factors exposition (e.g., obesity and smoking) and early detection.

According to GLOBOCAN 2018, 32,825 new cases were diagnosed in Spain, which accounts for 138.3 cases per 100,000 women. Although survival rates are continuously raising, 6,421 women died in 2018, which compromises 27.2 cases per 100,000 women **(Figure 1)**[1]. A recent analysis published by 'La Sociedad Española de Oncología Médica' (SEOM) estimates that nearly 32,953 new cases of breast cancer would be diagnosed in 2020[2]. If true, it would follow the trend of increasing the breast cancer incidence every year in Spain.

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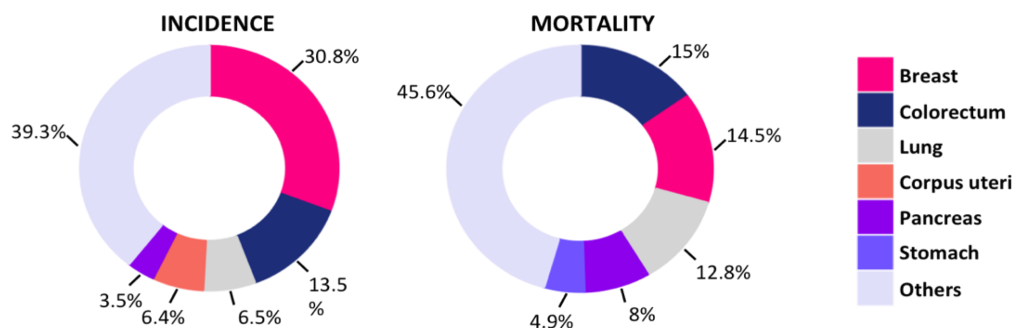


Figure 1. Incidence and mortality of cancer in Spanish women in 2018. Source of data obtained from GLOBOCAN[1].

Precisely, the augmentation of new cases over time is illustrated in **Figure 2.A** showing the incidence rates from 1999 to 2019. Likewise, **Figure 2.B** shows the mortality rates from those years, confirming the improvement of breast cancer survival over the years.

Regarding the appearance of breast cancer in women of reproductive age (15-39 years), the incidence and mortality have decreased for years constituting the group of women with lower cases in both rates **(Figure 2)**.

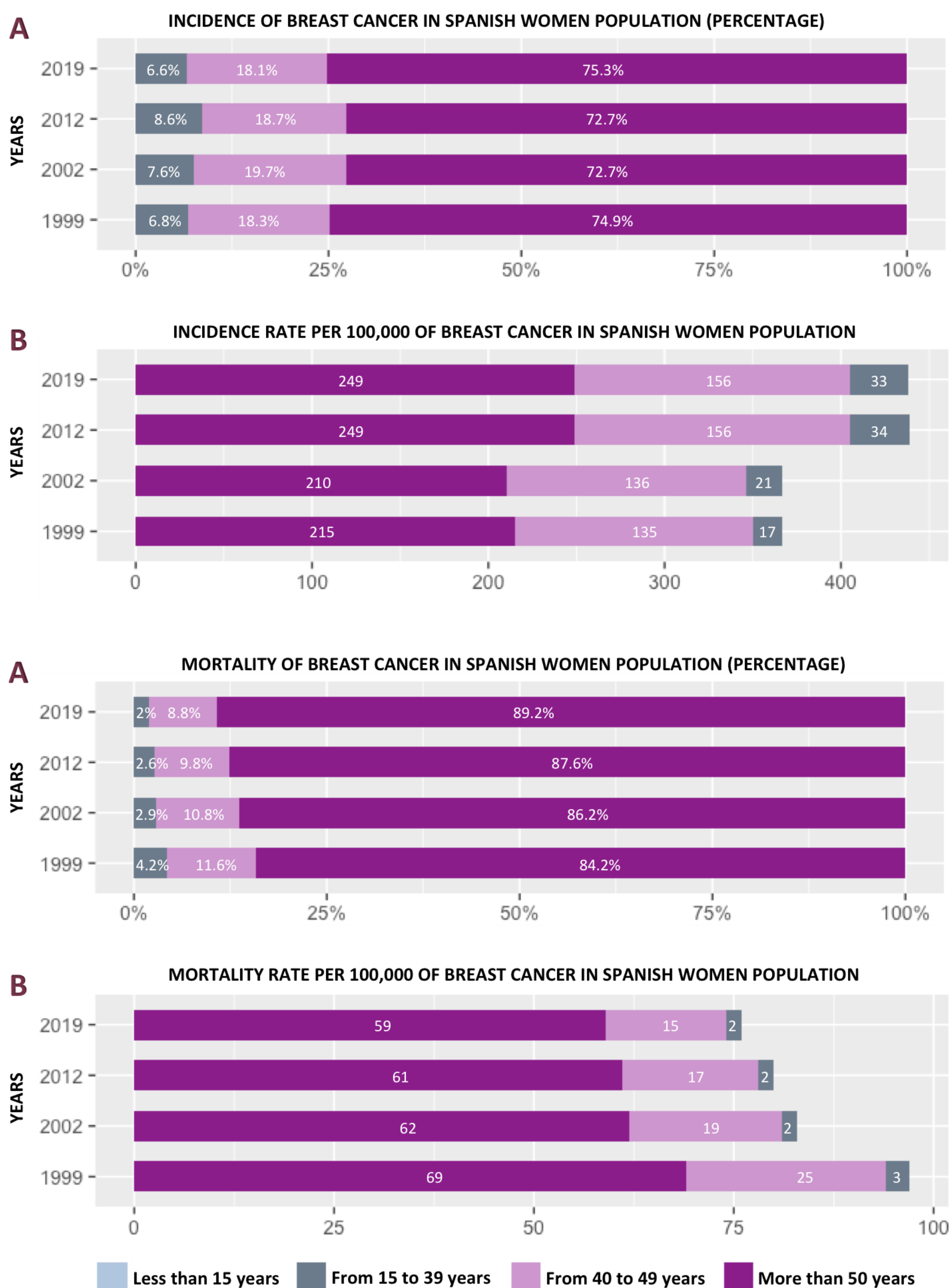


Figure 2. Incidence (A) and mortality (B) of breast cancer in Spanish women population from 1999 to 2012. Data from 1999 to 2002 only available of 15 provinces: Albacete, Asturias, Balearic Islands, Basque Country, Canary Islands, Castellón, Ciudad Real, Cuenca, Girona, Granada, La Rioja, Murcia, Navarra and Tarragona. Source of data from 1999 to 2002 obtained from European Cancer Information System (ECIS)[3] and from 2012 to 2019 obtained from 'Asociación Española Contra el Cáncer' (AECC)[4].

1.2 Etiology

Accumulations of genetic and/or epigenetic modifications are known to play an essential role to understand the complexities of the cancer biology[5–7]. However, the exact mechanism by which normal cells acquire malignant properties cannot be longer attribute solely by the specific traits that pose cancer cells to initiate carcinogenesis. However, instead, researchers must focus on the tumour microenvironment that surrounds them.

During the past decades, different authors have described the ability of malignant cells to acquire survival, proliferation and dissemination capacity. Alterations of the cellular homeostasis may lead to genomic instability allowing the acquirement of these malignant properties.

As already mentioned, genomic instability promotes random mutations. Approximately 5-10% of breast cancers are associated with a family history, although this may vary depending on ethnicity and across countries[8,9]. BRCA1 and BCRA2 are genes with high-penetrance that act as tumour suppressors and encode proteins whose function are preventing cells from proliferate and divide uncontrollably. These genes also interact with other proteins to repair damaged DNA. Nowadays, beyond BRCA1 and BRCA2, other genes linked to DNA repair are screened to establish the inherited breast cancer risk such as ATM, CHEK2, PALB2, PTEN, STK11 and TP53[10,11]. Nevertheless, the incidence of genomic instability, which promotes spontaneous mutations, accounts for 90-95% of all cases[9]. Anywise, breast cancer is generally considered a genetic disease independently if it is originated either by inherited or acquitted genetic mutations as both follow common pathways to active oncogenes or silence tumour suppressor genes.

Additionally, lifestyle and environmental factors are as well responsible for the pathogenesis and progression of breast cancer[12–14]. Changes in reproductive factors including advanced maternal age for first childbirth, nulliparity, early menarche, never having breastfed, late-onset menopause, early introduction of breast exploration and mammography screening are likely to increase the incidence of breast cancer over the years.

Some other identified risk factors for breast cancer are obesity, physical inactivity and alcohol and drug addiction. The estimation of these modifiable risk factors is approximately 20% of all breast cancer worldwide, although it could be lessened by promoting a healthy lifestyle[12].

2. PATHOLOGY OF BREAST CANCER

2.1 Histopathology Type Classification

Breast cancer is a heterogeneous disease that given its complexity is classified into different groups according to its histological appearance[15,16]. Over the years, pathologists have associated particular morphological and cytological patterns with distinctive clinical implications. This circumstance has allowed designing specific therapies to improve survival significantly.

Nowadays, there are many types of breast cancer recognised. However, the group of tumours that growths in the epithelial cells lining organs and tissues called carcinomas are especially relevant because they constitute practically the majority of breast cancer **(Figure 3)**. Even so, other less common types of breast cancer can be originated in non-epithelial cells[17].

2.1.1 Non-invasive Breast Cancer

Cancer is considered to be non-invasive when the uncontrolled growth of malignant cells remains inside its original place *('in situ')*. Thus, this group of abnormal cells have not invaded other tissues inside the breast or beyond. However, unlike benign tumours, these cancerous cells can still mutate to acquire the ability to spread[18].
Commonly, non-invasive breast cancer type starts to grow in the cells of the lobules or the ducts.

- **Ductal Carcinoma In Situ (DCIS)**: is the confined proliferation of malignant epithelial cells inside the milk duct in the breast. These cells, apart from having an unquestionable cancerous capacity, present cellular and nuclear atypia and can extend through the walls of the ducts into the nearby breast tissue. This means that, although it does not exist a particular pattern for recognizing the subsequent development of invasive carcinoma, women with DCIS have a higher risk than those without it. Consequently, DCIS requires immediate treatment, even if it is not life-threatening[19–21].

- **Lobular Carcinoma In Situ (LCIS)**: is a non-invasive proliferation of abnormal cells in the milk-producing glands of the breast (named, lobules). LCIS rarely presents a palpable lump, and most of the time is detected incidentally when a biopsy is performed on the breast for another purpose. As it happens in DCIS, suffering from LCIS is not a guarantee of developing invasive carcinoma at some point in the future but instead constitutes a risk factor. Still, most of the time does not require treatment after the biopsy, although a close long-term follow-up and regular BC screenings are mandatory[22,23].

2.1.2 Invasive Breast Cancer

Breast cancer confers the status of being invasive when malignant cells from where it has initially developed, split out into any healthy surrounding tissue. Furthermore, these breast cancer cells may immigrate to different parts of the body through lymphatic and circulatory systems. Once breast cancer is segregated and expanded, producing new tumours in other organs and tissues, it can finally be said that the disease has evolved to metastasis. Generally, breast cancer tends to spread towards bones, lungs, liver and brain. The new metastatic tumour is still breast cancer as the abnormal cells are the same than the ones in the primary tumour[18,24].
Most breast cancers are invasive.

- **Invasive Ductal Carcinoma (IDC):** is originated in the proliferative malignant cells of the milk ducts once having adopted the natural ability to break through the wall of the duct and invades the surrounding breast tissue. Progressively, the IDC may be able to extend (metastasize) to other parts of the body, including organs through lymph nodes and bloodstream. Over 70-80% of all invasive carcinomas are IDC, which is identified as a heterogeneous group of tumours[25,26].

- **Invasive Lobular Carcinoma (ILC)**: is identified as breast cancer that begins inside the milk-producing glands and invades the stroma. Equally, as it happens with IDC, the cancerous cells can be infiltrated into the blood and lymph vessels of the breast and spread to other areas of the body, causing metastasis. ILC is the second most common type of invasive carcinoma, constituting around 5%-15% of all of them[27,28].

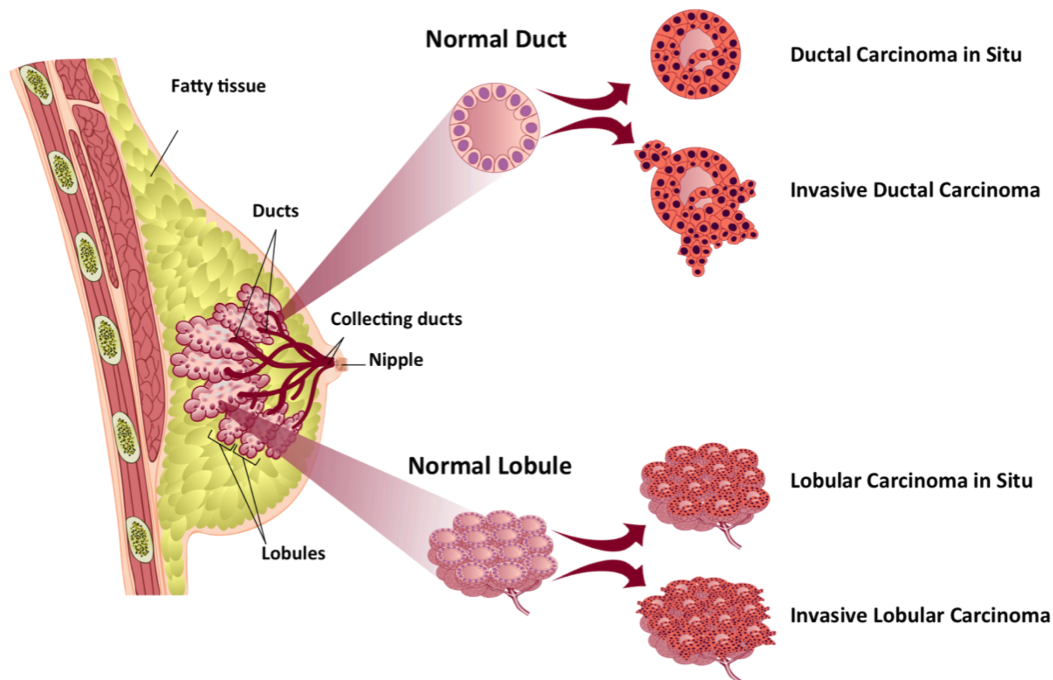


Figure 3. Common types of Breast cancer. This figure is original and designed by Sandra Martinez.

2.1.3 Other less common types of Breast Cancer

Aside from the most frequent types of breast cancer listed previously, there are some less common particular types that each usually constitutes fewer than 5% of all breast cancers. However, the oncologist needs to acquire sufficient wise to identify these atypical forms of breast cancer in order to provide a specific treatment.

Inflammatory breast cancer which produces breast swelling and redness; *Paget's disease of the breast*, which causes changes to the nipple; *phyllodes tumour* which begins in the connective tissues and *angiosarcoma*, which develops in the cells that line the walls of blood vessels or lymph vessels. These are some of the rare forms of breast cancer commonly studied in the past few years[29,30].

2.2 Histopathology Grade Classification

The grade of breast cancer describes the behaviour of the malignant cells reflecting aggressiveness and prognosis of the tumour.

Currently, some available scoring systems determine the grade of the tumour, being the Nottingham Histologic Score system one of the most used by pathologists. This system

takes into consideration three concepts: cellular differentiation, nuclear pleomorphism and mitotic activity[31].

- **Grade 1 (scoring 3-5):** malignant cells are well differentiated.
- **Grade 2 (scoring 6-7):** malignant cells are moderately differentiated.
- **Grade 3 (scoring 8-9):** malignant cells are poorly differentiated.

Grading the tumour according to its cellular appearance, is fundamental to determine treatment options for breast cancer patients. The closer the similarity between breast cancer cells to normal cells, the better the prognosis. Once these abnormal cells reach the status of not well differentiated, they tend to grow and divide more quickly to invade other tissues complicationg signficintaly the prognosis [15].

2.2 Molecular Classification

The presence or the absence of hormonal receptors, oestrogen and progesterone, together with the expression of Human Epidermal Growth Factor Receptor 2 (HER2/neu), is evaluated meticulously using tissue from biopsy or surgery. The identification of the hormone receptors status and HER2/neu status of the tumour provides relevant information related to the aggressiveness of cancer cells and the responsiveness to endocrine therapy or other treatments[32,33].

- **Oestrogen Receptor (ER):** is a protein mostly present in the nucleus of the epithelial cells although a small fraction can be found in the cytoplasm. The hormone oestrogen binds to ER triggering the activation of different pathways that promote cell growth.
- **Progesterone Receptor (PR):** is a protein that acts as a nuclear receptor for its ligand, the hormone progesterone. Like ER, PR is expressed in normal mammary epithelial cells inducing cell proliferation.

- **HER2/neu:** is an oncogene that encodes the HER2 protein. This protein is manifested in normal breast cell to control proliferation, division and reparation.

Breast cancer tumour is considered ER and/or PR positive if >10% of malignant cells showed intranuclear positivity[34]. HER2 is overexpressed if the score obtained by immunohistochemistry (IHC) test is +3 or positive by fluorescence in situ hybridization (FISH) test when the results of IHC are unclear (score of +2)[35].

2.3 Stage

Determine the stage of the disease is relatively complicated and depends on diverse influential parameters including tumour size, tumour type, lymph node involvement and invasion of other parts of the body (metastasize)[36].

Breast cancer stage is usually classified into a scale from I to IV being I describing non-invasive cancer and IV describing metastasis **(Figure 4)**.

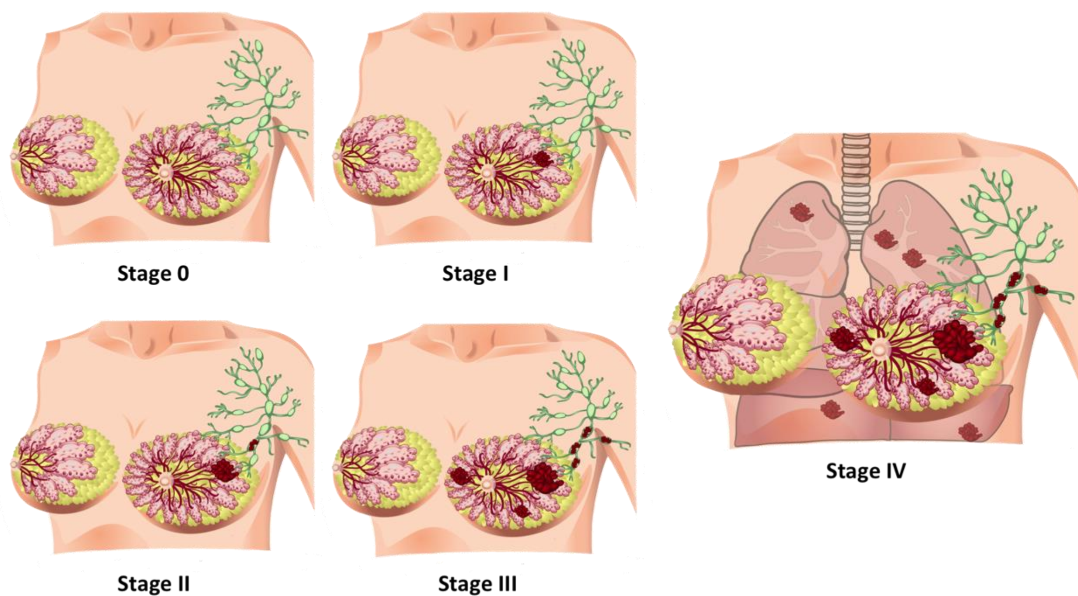


Figure 4. Breast cancer stages. Stage 0: abnormal cells presents in the ducts but have not spread to the surrounding tissue. **Stage I:** the tumour is in small are of the breast tissue or it may spread close the breast. **Stage II:** the tumour is in the breast or lymph nodes are involved or both. **Stage III:** the tumour has spread to lymph nodes nearby to the breast or to the skin or to the chest wall. **Stage IV:** the tumour has spread to other parts of the body[37]. This figure is original and designed by Sandra Martinez.

3. PREGNANCY ASSOCIATED BREAST CANCER

3.1 Definition and epidemiology

Cancer diagnosed during pregnancy is an extremely rare condition with an estimated frequency of one case in 1,000 pregnant women[37]. Breast cancer, along with cervical cancer, haematological cancer and melanoma is the most common malignant tumours diagnosed during pregnancy.

Pregnancy-associated breast cancer (PABC) is commonly defined as breast cancer diagnosed during pregnancy or within the first year postpartum. Some authors, however, have elevated the range up to two-five years mainly to cover the entire breastfeeding period[38–41]. Nowadays, the occurrence of PABC in Western countries is estimated to be at a range of one in 3,000 to one in 10,000 of all pregnancies with an average patient between 32 and 38 years of age and the median age at 33-34 years[42,43]. While the majority of breast cancers are compromised during perimenopausal and menopausal periods[3-4], PABC constitutes around 2.6% to 7% of all breast cancer diagnosed in women less than 45 years of age[44–46]. Unfortunately, the incidence of PABC is expected to rise in the forthcoming years due to more women delaying pregnancy in these countries[47,48].

According to the National Statistics Institute (INE), 59.3% of Spanish women were aged less than 25 years when they gave birth to their first child in 1999. However, this may have changed sine 66.6% of births accounts for mothers aged 25 years and over based on data from 2018[49]. This phenomenon is a reflection of an increasing proportion of women that choose childbearing after their thirties **(Figure 5)**.

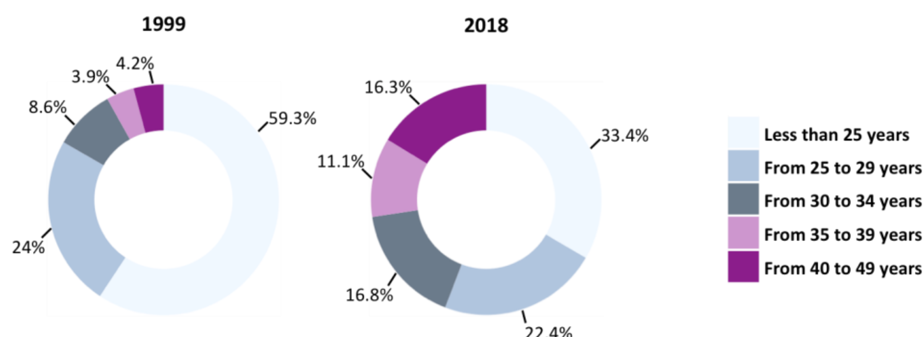


Figure 5. Percentage of first-born live births by age group of mother in Spanish population in 1999 and 2018. Source of data obtained from INE.

In 2018, 79.2% of women from 25 to 29 years did not enter into the world of motherhood, according to the Spanish fertility survey made by INE[49]. As expected, these results differ from those obtained in the same fertility survey accomplished in 1999. In that period, 70.4% of women were childfree by the age between 25 and 29 years[49]. The situation of childfree declines slightly as women age increases in both periods. For instance, 27.8% of women between 35 and 39 years did not have children in 2018, which contrasts sharply with the 12.3% obtained in 1999 **(Figure 6)**.

Consequently, the rising trend of breast cancer in pregnancy is understandable since both risk factors, the continuous increase of breast cancer incidence over the years and the fact of women choosing to postpone childbearing, make it more likely.

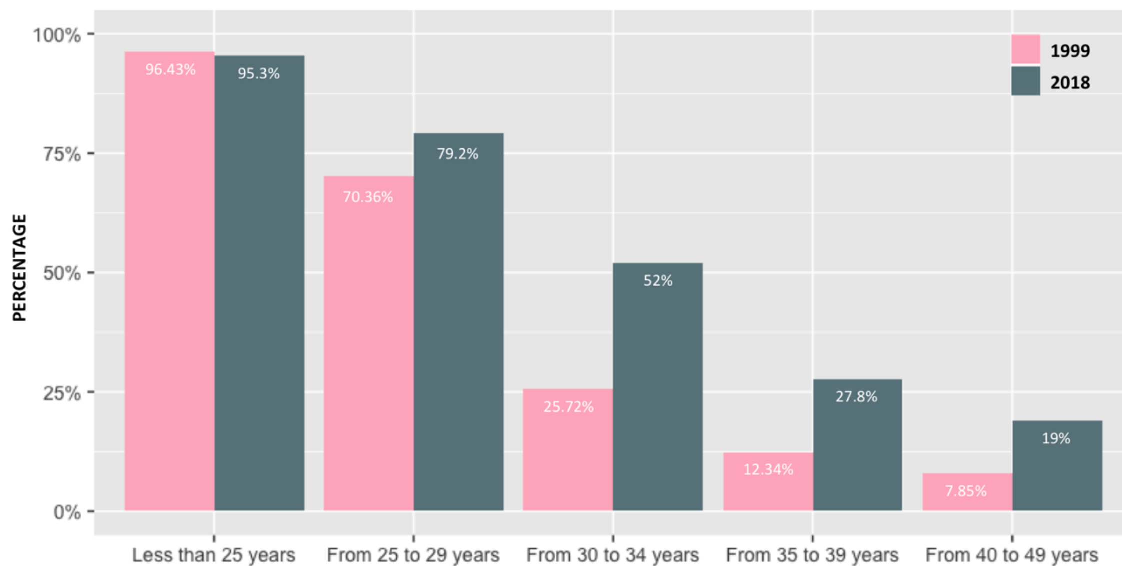


Figure 6. Percentage of childfree distributed by age group of Spanish women population in 1999 and 2018. Source of data obtained from INE.

3.2 Diagnosis

Pregnant women experiment physiological changes associated with pregnancy including rapid breast hypertrophy, especially notorious in the first eight weeks and glandular and ductal hyperplasia[50,51].

These adjustments frequently cause to neglect the early signs and symptoms of breast cancer. As a consequence, women with PABC commonly present more aggressive tumours, are often diagnosed at an advanced stage (larger tumour mass, higher grade,

lymphovascular invasion, frequently nodal involvement and micro-metastases) and have poorer prognosis[43,52–54].

The clinical presentation of PABC is usually a palpable painless lump mostly detectable during self-examination[42,50,55]. Although around 80% of all suspicious masses found during gestation are benign such as adenomas, fibroadenomas or lipomas, a further test must be done if the lesion persists more than two weeks[38,52,56]. Therefore, determine an accurate diagnosis of PABC is a considerable challenge for most of obstetrics and oncologists.

As with non-PABC patients, triple assessment (history/examination, imaging and cytology/histology) is the proper method of accurately diagnosing signs of rare breast disturbance in pregnancy.

3.3 Histological and molecular characteristics

The histology of PABC is usually described in several publications as similar to non-PABC patients when they are matched by age[38,57,58]. Whereas the incidence of DCIS is equally common among patients independently pregnancy association, LCIS exceptionally appears in women with PABC[59]. Unfortunately, the diagnosis of breast cancer is usually made when the tumour has reached the status of invasive. IDC is the primary histological type of breast cancer diagnosed during pregnancy, which accounts for 75-90% of all cases following by ILC type[43,60,61]. In addition, inflammatory breast cancer, although in less proportion than the others, it is highly prevalent among PABC women[43,46,59].

In general, PABC women present higher histological grade in comparison with non-PABC patients but a similar proportion as for non-pregnant age-matched women. This fact is supported by multiple studies, which the incidence of histological grade 3 diagnosis ranges from 60-75% of all PABC cases approximately[52,62–64].
Regarding hormone receptors, PABC has been frequently reported to present a trend for decreased ER-positive and PR-positive tumours[38,60,65]. Because of pregnancy is a period of high levels of circulating oestrogen and progesterone, the ER and PR can be saturated and give falsely negative results, especially using ligand-binding assays[66,67].

In light of improving the accuracy of determination of hormone receptor expression, IHC assays have emerged to assess the adequate hormone receptor status in PABC[38,68,69]. Other studies, however, have suggested that the difference in ER and PR expression between PABC and non-PABC women is the consequence of the down-regulation of hormone receptors as an effect of the pregnancy[61,70]. Additionally, BRCA mutations are often linked to ER-negative and/or PR-negative carcinomas, and precisely these mutations are over-expressed in PABC women[71,72].

HER2/neu status in PABC has been assessed in a few studies, and although the results differ, the majority of them have shown a significant overexpression of HER2/neu compared to age-matched controls[61,73,74].

Concerning the risk of metastasis in PABC patients, is generally described higher than the non-PABC women due to the delay of diagnosis associated with the disease, to the biological effects of pregnancy or a combination of both[42,43,75]. Indeed, Zemlickis *et al.* found that the risk of metastasis was over 2.5 times higher in PABC subjects than their age-matched control group[76].

3.4 Treatment

PABC is a challenging situation when it comes to deciding the appropriate treatment since the welfare of both the mother and the foetus must always be a priority. In general, the treatment guidelines indicated that PABC patients do not differ from those for non-pregnant patients[77,78]. However, additional measures may be implemented to protect the foetus, even rescheduling or modifying the treatment if its wellbeing is compromised. Besides tumour size and stage, women with PABC have an extra factor affecting treatment decision, the gestational age. As a consequence, the final choice of treatment needs to be discussed within a multidisciplinary team that should include gynaecologists specialized on risky pregnancy and oncologists and subsequently with the patient[79–81].

Pregnancy termination is not considered a treatment, as there is no evidence that improves maternal survival[52,66,82]. However, the possibility of an abortion and how to deal

with the extraordinary situation of facing deadly while being pregnant must remain as a unique patient choice and thus, should be respected[83].

3.4.1 Surgery

Surgery is frequently the first treatment for early breast cancer, as it is not contraindicated during pregnancy, although the risk of spontaneous abortion increases if it is performed during the first trimester[48,60,78]. Additionally, the physicians must establish appropriate postoperative analgesia because the pain caused by the surgery, may induce premature labour[82,84].

Modified radical mastectomy is the preferred treatment of choice for PABC over breast conservation therapy (BCT) because it does not need for postoperative radiotherapy and involves axillary management[50,85,86]. Even though, BCT with axillary lymph node dissections may be a suitable surgical option when the diagnosis of PABC is made by the end of second and in the third trimester as postponing radiotherapy treatment has acceptable effects on the maternal prognosis[50,85,87]. Preceding BCT with postpartum radiation, neoadjuvant chemotherapy (CTX) is administered. Oppositely, adjuvant chemotherapy is usually prescribed after mastectomy[55,88,89].

3.4.2 Chemotherapy

The indication of chemotherapy during pregnancy for breast cancer has been the subject of several scientific researches[63,90–92]. Some of them have evaluated the protective effect of the placenta barrier in contact with some specific chemotherapeutic agents indicating low levels in foetal blood[93,94]. At certain gestational age, the expression of drug-extruding transporters in the placenta, including P-glycoprotein confers to the foetus an extraordinary resistance against the effects of chemotherapy [50,77].

Chemotherapy may be administered either as neoadjuvant therapy (pre-operative) or as adjuvant therapy (postoperative). Neoadjuvant chemotherapy is mainly given to PABC women that present locally advanced disease in order to reduce the size of the tumour and achieve operability[56,88,95].

As for the chemotherapy guidelines, PABC patients should follow the same chemotherapy -based regimens as for non-PABC patients but after the first trimester. Given that most of the chemotherapeutic agents are designed to destroy dividing cells, it is understandable to avoid their administration during periods of embryogenesis and organogenesis[60,84,96]. Elevated risk of malformations (14-25%) and miscarriage are associated with an exposition to chemotherapy during the first trimester[67,78,82,97]. However, it can be offered from week 14th of pregnancy onwards with a risk of foetal malformation similar to those who have not being exposed to chemotherapy [54,77,79,98].

The treatment is always deferred before birth as the administration of chemotherapy should be avoided after the week 35th or within three weeks of the planned delivery to diminish the risk of haemorrhage and sepsis[82,85,99]. This point is crucial principally for preterm babies that do not have their liver and kidneys fully developed to metabolize or excrete certain drugs quickly, and placenta provides time for foetal drug excretion.

The findings in most reports regarding systemic treatment stipulated for PABC, four cycles of anthracycline-based regimens every three weeks intervals were commonly scheduled with limited maternal and foetal complications[55]. Although maternal exposure to anthracyclines (AnC) may increase the risk of foetal cardiotoxicity, no foetal anomalies were observed in different prospective studies[60,92,100,101].

Taxanes (TXN) are widely used following the last dose of anthracyclines when is too early for a safe delivery[55,77]. The standard regimens are based on 12 cycles scheduled in weekly intervals. Exceptionally PABC patients get to complete four cycles of anthracyclines and 12 cycles of taxanes during pregnancy, mainly when they are diagnosed in the early stage of the disease[55]. Regardless of the dilated experience in the use of taxanes, they have been less extensively evaluated than anthracyclines. Considering that there is limited available data on the use of taxanes for PABC, some authors have shown reluctance in their administration on pregnant women although their efficacy was proven a long time ago and without inducing foetal damage[79,82,102] .

Despite the apparent safety of chemotherapy for PABC, there remains a concern since pregnancy complications including preterm delivery, low birth weight, intrauterine

growth restriction (IUGR) and transient leukopenia among others, have been documented[96,98,103]. However, various observational studies have not found any critical disorders in neonates concerning chemotherapy exposition in uterus[77,92,102,104].

3.4.3 Radiotherapy

Radiotherapy is always delayed after delivery because of the risk of neonatal complications such as IUGR, mental retardation, induction of childhood malignancy and even foetal death[52,60]. Unfortunately, there are too little available data to withdraw this assumption, although some literature has reported successful cases of radiotherapy treatment for PABC with neonates born healthy. Retrospective analyses have suggested a worsening prognosis when radiotherapy is delayed more than three months[52,105]. However, if PABC is diagnosed during the second half of gestation, radiotherapy can be postponed after birth without compromising the prognosis[52,61].

3.4.4 Biological and hormonal therapy

- **Trastuzumab:** is a biologically targeted therapy based on monoclonal antibodies that it is widely used to counteract the overexpression of HER2/neu. While it is safely administered together with chemotherapy in non-PABC patients, trastuzumab is contraindicated in pregnancy since can cross the placenta and impair foetal development[106–108]. Oligohydramnios is the major complication associated with trastuzumab administration in pregnancy. The estimated incidence of oligohydramnios is around 33% but may increase with the duration of treatment[82]. Oligohydramnios is mainly attributed by the action of trastuzumab on HER2/neu, which is strongly expressed in the foetal renal epithelium responsible for amniotic fluid production[109]. Fortunately, oligohydramnios seems to be reversible once the administration is suspended without major clinical complications.

- **Tamoxifen:** is an hormal tarfed therapy based on inhibiting the entrance of ER hormes into malignant cells. Unfortunately, hormonal therapy is contraindicated during pregnancy for being associated with severe foetal anomalies such as craniofacial malformations ambiguous genitalia and foetal death[67,110,111].

3.4.5 Antiemetic regimens

Glucocorticoids are frequently administered in pregnancy as first-line antiemetic owing to their powerful immunosuppressive and anti-inflammatory properties. In general, glucocorticoids are avoided in the first trimester but may be safe after for preventing miscarriage and improving neonatal outcomes[112,113]. Additionally, glucocorticoids are also useful to help patients tolerate chemotherapy and relieve pain. Hence, glucocorticoids are used as a palliative treatment rather than as a chemotherapeutic agent that kills cancer cells itself in breast cancer [82,114].

3.4.6 Psychological support

Once PABC is diagnosed, patients need to deal with psychological aspects that are not probably ready to. While facing a wide range of feelings such as anger, fear, anxiety and sadness, they are asked to make a decisive choice: interruption of the pregnancy being aware that may be the last opportunity to enter the motherhood; to accept the therapy chosen by a specialized medical team but assuming possible foetal damage or to decide not to be treated until the end of pregnancy probably worsening their prognosis. Despite the existence of a multidisciplinary team behind PABC patients, the election of one of the three possibilities resides only to the mother's personal decision.

Recent studies highlight the importance of the particular psychological approach and extensive counselling during treatment[78,115,116]. Patients with PABC suffer from an unstable emotional state mostly caused by uncertain prognosis, maternal and neonatal side effects of treatment and insecurity of relapse of the cancer[81,117]. After pregnancy or breast surgery, additional psychological aspects should be included in the follow up of these patients because other concerns may arise, such as sexual desire, infertility, and attractiveness[18,116,118].

3.5 Maternal complications associated with breast cancer

A delay of the diagnostic is a common complication among women with PABC. Unfortunately, most of the masses whether they are not difficult to discover due to hypertrophy and engorgement of the breasts, they are wrongly attributed to physiologic changes related to pregnancy[52,59,119].

Besides, women with PABC usually present more aggressive tumour biology[43,77,78]. For instance, Nguyen, B *et al.* demonstrated significant molecular differences in PABC patients that may explain the hostile clinical behaviour. They reported enrichment of non-silent mutations, high frequency of mucin gene family mutations and mismatch repair deficiency in PABC patients. The real impact of these molecular features remains unknown, although it is tempting to speculate that they may be involved in encouraging tumour progression during gestation[120]. Likewise, Azim HA *et al.* reported that pregnancy might promote the aggressiveness of breast cancer through the augmentation of mammary stem cells (MaSC) number and growth hormone as MaSC presents high levels of growth hormone receptors[98]. On the other hand, pregnancy is considered a pro-angiogenic state in which several angiogenic factors are required for its development. Thus, pregnancy may contribute to cancer development through the formation of these factors, as they are known to contribute to tumorigenesis or to promote tumour[121–123].

So, it is likely to assume that pregnancy may be indeed a negative factor for breast cancer development worsening the disease via promotion of different biological mechanisms.

Breast cancer during pregnancy is treated according to multimodality therapy. Except for radiotherapy, surgical resection and system chemotherapy have been used successfully without apparent damage to the mother[78,103,124]. Recently, many authors have corroborated that mastectomy, and the use of general anaesthesia does not increase the risk of maternal complications substantially [82,83,86]. Conflicting opinions exist regarding the obstetric side effects induced by the administration of chemotherapy during pregnancy[52,125]. While some studies have indicated a similar risk of preeclampsia (PE) in women with PABC than those with a healthy pregnancy, others have stated that chemotherapy through different physiological mechanisms including the effect on the trophoblasts and oxidative stress may induce PE[37,52,96,97]. Moreover, anthracycline-based

regimes are associated with dose- and time-dependent cardiotoxicity although are generally reported safe in pregnant women[77,126].

Chemotherapy dosing in pregnancy should not differ from those administered in non-PABC women, although the physiological changes in pregnancy may cause complications. For instance, alterations of the pharmacokinetic and pharmacodynamics of chemotherapy have been documented affecting principally renal and hepatic clearance and decreasing drug submission[82,85]
Therefore, oncologists must follow the current recommendations and administrate the dose according to actual body weight without modifying the established regimes. However, this decision may increase the risk of developing anaemia or febrile neutropenia[82]. Additionally, PABC patients are needed to receive supportive therapy, as they are more vulnerable to the side effects of chemotherapy, including dizziness, sickness and vomiting.

Women with PABC are more likely to have induced preterm delivery and high risk of caesarean section, although vaginal delivery is the desirable method[80,99,127]. However, the timing and mode of delivery are always carefully determined based on the obstetrical opinion to ensure both the mother and the neonate safety[103,122]. Recognised cancer complications such as sepsis, severe morbidity and the risk of thromboembolic events are found increased in women with PABC. These affections are even more presented at the moment of delivery as a consequence of chemotherapy treatment. Cytotoxic drugs are widely associated with myelosuppression, which may cause maternal haemorrhage at the moment of birth. Hence, chemotherapy should be postponed at least three weeks before the expected date of delivery[66,77,79,126].

With regard to lactation, it is not recommended during chemotherapy treatment as it has been demonstrated that anthracyclines, among other chemotherapeutic agents, are excreted in breast milk[54,57,128]. Breastfeed either after conservative breast surgery or chemotherapy are feasible although the patients are more likely to experience a decrease of the breast milk volume[50,57,90].

Finally, it is essential to highlight the fertility-related issues that suffer from PABC women. Preservation of fertility is a complicated decision, and this issue should be addressed prior to the initiation of chemotherapy because of the high probability of amenorrhea[59,88,117,128]. Approximately only 4%-7% of women with breast cancer history manage to get pregnant again but at expenses of increasing the risk of recurrence, especially within the first two years of relapse[129]. Moreover, exist evidence of birth complications such as preterm birth and neonatal low birth weight related to previous chemotherapy treatment[129].

3.6 Perinatal complications associated with breast cancer

Indeed, the management of PABC should mimic the established treatments for non-PABC as much as possible. However, a complete comprehension of the perinatal risks associated with the exposition of cytotoxic drugs is necessary before the initiation of chemotherapy [78].

As previously mentioned, hormone or endocrine therapy is avoided during pregnancy. Trastuzumab is associated with a risk augmentation of oligohydramnios, which may trigger to preterm labour, foetal morbidity and mortality while tamoxifen is related to birth defects[107,110].

Chemotherapy, however, is not contraindicated in pregnancy, although its administration is restricted to specific circumstances. During the first trimester, chemotherapy treatment should be avoided due to the high risk of miscarriage, perinatal death and congenital malformations, as it coincides with the period of organogenesis. Some studies have set the occurrence of teratogenic effects associated with chemotherapy up to 17%[85,130]. On the other hand, the administration of cytotoxic drugs onward the second trimester is considered relatively safe for the foetus even though most of them can cross the placenta. Whereas anthracyclines may affect the foetal heart, taxanes may attack the peripheral nervous system[131,132]. Therefore, it is understandable the existence of great concern regarding perinatal toxicity related to chemotherapy even after organogenesis[66].

IUGR and small for gestational age (SGA) are the most common adverse complications associated with the exposition of chemotherapy *in utero*[77,115]. Both conditions may put

the neonate at risk of specific health problems such as birth and perinatal morbidity, perinatal mortality and growth impairment late in life[133–135]. Different large case series of PABC patients under chemotherapy treatment have estimated the rate of SGA between 7-9% respect to 0-4% in those who did not receive any chemotherapy during pregnancy[97,104].

Likewise, there is a high frequency of prematurity (<37 weeks) in neonates of mothers who had undergone chemotherapy treatment for breast cancer according to different publications. Delivery at term should be accomplished precisely to avoid prematurity, as it has been associated with impaired cognitive development[77,90,92]. Other perinatal complications associated with the administration of chemotherapy during the second and third trimester are myelosuppression and low-birth weight[77,82,85]. However, neonates with low weight at birth tend to recover, reaching average values in the first month of infancy[90].

Although the exact mechanisms in which chemotherapy may trigger most of the perinatal complications remain unknown, neonates exposed to chemotherapy *in utero* are generally reported to born healthy and with a low rate of complications[77,82,92,102] .

Apart from the perinatal complications associated with chemotherapy exposition described above, other adverse outcomes may not become apparent until later in life. Some long-term effects already linked to chemotherapy include gonadal dysfunction, germ-cell mutagenesis, teratogenicity in subsequent generations, and impaired physical and neurologic development[66,115,131]. However, most of the few studies evaluating the long-term effect of children after prenatal exposure to chemotherapy found healthy development and average cognitive ability within the standards of the general population.

Nevertheless, further follow-up should always be required to establish the potential long-term effects of the exposition to chemotherapy in perinatal period[92,103,131,136].

Besides, although placenta metastasis during pregnancy is an infrequent occurrence, it may affect the foetus prognosis when the metastasis is not limited to the intervillous space[137]. Thus, histological evaluation of the placenta should be immediately required after delivery[82,130].

3.7 Prognosis

Historically, PABC patients have been mistakenly mistreated as a result of the terrible prognosis associated with. Furthermore, abortion was used as a therapeutic option due to the persistent belief that pregnancy trigged the poor prognosis through hormonal changes[138]. Recently, the majority of authors agree with the fact that termination of pregnancy does not seem to improve prognosis or survival[82]. Several factors, as the delay of diagnosis and maternal age, have been classified as indicators of poor prognostic in women with PABC. Precisely, women younger than 35 years of age usually suffer from considerably aggressive tumours[82,139,140].

On the other hand, pregnancy could serve as an independent prognostic factor for poor survival in women with PABC through the action of different physiological changes, which may induce further aggressive tumour biology[43,77,78].

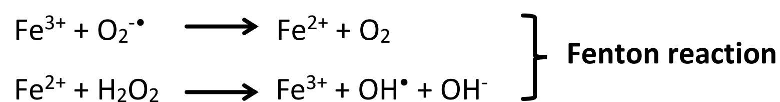
Likewise, retardation in breast cancer diagnosis highly increases the risk of worse prognosis. To be more precise, a delay of one month in the diagnosis of PABC from the first symptoms may increase the risk of axillary involvement by 0.9% to 1.8%, and it could reach the risk by 5.1% if the delay is of 6 months[141]. Unfortunately, the average delay of breast cancer diagnosed in pregnancy range from 1-2 up to 5-7 months, although some authors even lengthen the period of diagnosis up to 15 months[42,43,55,88].

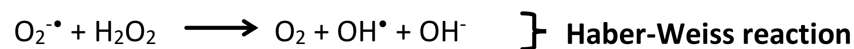
Although all these factors may explain the more advanced size of tumours and more advanced stage, conflicting opinions regarding the worse prognosis of women with PABC still exist[41]. For instance, Azim HA Jr *et al.* found a correlation between poor prognosis and women with PABC setting the risk of death higher than 40% in comparison with non-PABC women. Nevertheless, after further adjustment for age at diagnosis, the mortality slightly decreased, leading to suggest that age rather than the stage is a crucial factor[142]. However, the majority of studies show the opposite results revealing similar prognosis when PABC and non-PABC patients are matched for age and disease stage. These authors concluded that equivalent disease-free and overall survival was found in women with PABC than those with non-PABC[75,104,111].

Unfortunately, due to the apparent conflict of the current data, a better understanding is still necessary to elucidate whether the delay either of the diagnosis or treatment or biological changes in pregnancy or a combination of all factors may contribute to worse prognosis[50,85].

4. OXIDATIVE STRESS

Oxidative stress (OS) is traditionally defined as an imbalance between the production of free radicals and natural antioxidant capacity[143,144]. Free radicals are highly reactive molecules that bear an unpaired electron and function as secondary messengers. Reactive oxygen species (ROS) are free radicals generated mainly from each intermediate stage from the biological reduction of molecular oxygen to water in the mitochondrial respiratory chain[145,146]. As a consequence of the aerobic environment, four single electrons are transferred progressively to oxygen (O_2), however, sometimes the reduction is incomplete and generates superoxide anion radical, $O_2^{-\bullet}$ (one-electron reduction), hydrogen peroxide, H_2O_2 (two-electron reduction) and hydroxyl radical, $OH^{\bullet}$ (three-electron reduction)[147,148]. Both oxygen radicals and non-radicals (HOCl, O_3, H_2O_2 and $^1O_2$) - *which may be later converted into free radicals*- are considered ROS. For instance, H_2O_2 can reach the cytosol and generate $OH^{\bullet}$ by crossing the mitochondrial membranes and interacting with transition metals iron via the Fenton reaction. H_2O_2 can also react with $O_2^{-\bullet}$ to form $OH^{\bullet}$ by Haber-Weiss reaction[144,149].





Similarly, reactive nitrogen species (RNS) are a family of nitrogen-containing free radicals majority derived from nitric oxide (NO) and nitrogen dioxide (NO_2) under hypoxic conditions. Non-radicals, including peroxynitrite ($ONOO^-$) among others, is also included in the family of RNS[150,151].

The mitochondrial respiratory chain is the principal endogenous non-enzymatic source of ROS. Some publications have estimated that less than 5% of oxygen is reduced to form $O_2^{-\bullet}$ ("primary" ROS) which can further generate other ROS ("secondary" ROS)[152–154]. Paradoxically, the reactions that make possible these transformations are usually part of the physiological response trying to neutralize these free radicals.

Recently, other authors have suggested that the production of ROS derived from mitochondrial respiration are lower than previously reported, drawing attention to further sources of ROS production[146,152,155]. Additional endogenous sources of ROS include the endothelial nitric oxide synthase (eNOS), NADPH oxidases (NOX), xanthine oxidase (XO), myeloperoxidase (MPO), cytochrome p450 enzymes and cyclooxygenase (COX)[146].

In addition to the endogenous sources of ROS, the antioxidant defence can also be challenged by exogenous sources, including ultraviolet (UV), alcohol, tobacco, drugs, environmental pollutants **(Figure 7)**[155,156].

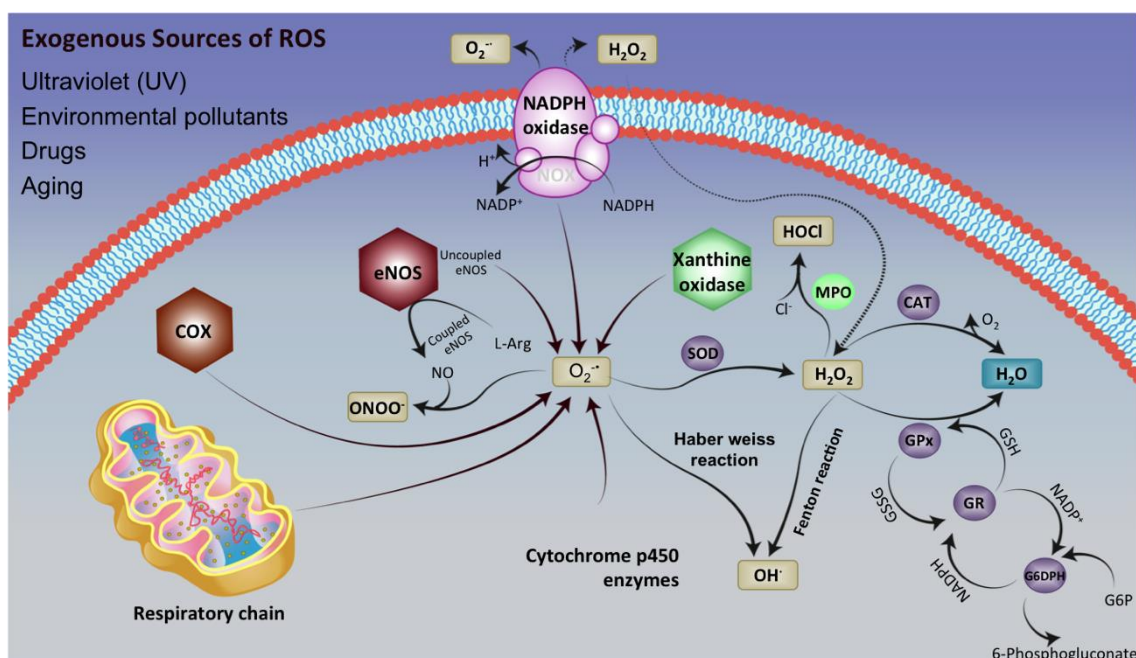


Figure 7. Mechanisms of ROS generation and detoxification. Endogenous sources of superoxide radical anion ($O_2^{-\bullet}$) are produced through the activation of NADPH oxidase, endothelial nitric oxide NO synthase (uncoupled eNOS), cyclooxygenase (COX), Xanthine oxidase and cytochrome p450 enzymes and via mitochondrial respiratory chain. Exogenous sources of ROS include ultraviolet (UV), environmental pollutants, drugs or aging.
$O_2^{\cdot-}$ can be dismuted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) or can be transformed to peroxynitrite ($ONOO^-$) through eNOS. H_2O_2 can form hypochlorous acid (HOCl) via myeloperoxidase (MPO). Hydroxyl radical (OH^-) can be generated by haber Weiss reaction and Fenton reaction. H_2O_2 is physiologically neutralized by the action of glutathione peroxidase (GPx) and the catalase (CAT) enzymes. This figure is original and designed by Sandra Martinez.

ROS play a dual biological role depending on their concentrations[153]. While low ROS levels might be beneficial as they participate in various normal biological processes, excess of ROS can damage cellular lipids, proteins, or DNA[155,157,158]. Oxidative stress might be

reversible depending on the extent of ROS actions. Furthermore, similar deleterious effects may be induced as a result of a deficiency of the antioxidant mechanism to defence the organism against ROS[159]. Therefore, it is essential to maintain the redox homeostasis through different mechanisms that neutralize oxidative stress.

Direct measurement of ROS levels represents a significant challenge for the investigators, given the lack of accurate methods for the analysis[160]. Alternatively, oxidative stress can be measured indirectly by monitoring levels of DNA/RNA damage, lipid peroxidation, and protein oxidation/nitration. These oxidative modifications are more stable and less reactive, hence have prolonged half time than ROS[158,161].
Similarly, carbohydrates can also be a source of oxidation products, although in less proportion, under an extreme oxidative stress environment.

- **Proteins:** the side chains of all amino acid residues are the principal targets of ROS, in particular, cysteine and methionine residues are the most susceptible to be oxidised by their action[162,163]. Currently, many oxidative changes in proteins have been used as biomarkers of oxidative stress such as tyrosine isomers although protein carbonyl is still the most common used[161,164–166]. Protein carbonyl is usually formed as a result of the reaction between hydroxyl radicals and amino acid side chains being lysine (Lys), arginine (Arg), proline (Pro) and threonine (Thr) the most residues targets[167–169].

- **Lipids:** polyunsaturated fatty acid residues (PUFA) of phospholipids, in particular linoleic (LA) and arachidonic acids (AA), are incredibly susceptible to be oxidised by hydroxyl and peroxyl radicals. Once oxidative stress initiates lipid peroxidation, lipoperoxyl radical ($LOO^{\bullet}$) is formed and triggers a chain reaction, giving rise to lipid radicals and lipid hydroperoxides (LOOH)[170,171]. LOOHs generate more ROS and decompose to oxidative stress second messengers[147]. The formation of these reactive compounds and the lipid peroxidation can compromise the membrane fluidity and permeability disrupting abruptly the integrity of the cell.
  Malondialdehyde (MDA) is the final product of lipid peroxidation in cell membranes and low-density lipoproteins (LDL), and it is commonly used as a biomarker of

oxidative damage to lipids[172–174]. Likewise, isoprostanes (IsoPs) are prostaglandin-like compounds generated non-enzymatically through the oxidation of AA. IsoPs are widely measured in almost all biological fluids, including plasma and urine. Besides their easy detection, they are considered more reliable biomarkers of lipid peroxidation than MDA[175,176].

- **DNA:** ROS destroy the deoxyribose backbone and the base of DNA formatting a wide variety of DNA adducts including 8-hydroxyguanosine (8-OHdG), the most sensitive index of DNA damage and frequently used biomarker of oxidative stress. In particular, guanine is oxidized and forms 8-OHdG, which causes transverse mutations A:T to CC or G:C to T:A[177–180].
  Mitochondrial DNA (mtDNA) has unique characteristics that include lack of introns and histones, limited DNA repair and above all proximity to the place of ROS generation that made it more susceptible to be attacked by ROS than nuclear DNA (nDNA)[181].

- **Carbohydrates:** although they are commonly less attacked by oxidative stress, advanced glycation end products (AGEs) are formed by the non-enzymatically glycation of proteins (amino groups of Lys and Arg) though the reduction of free carbonyl groups of carbohydrates[154,156].

4.1 OXIDATIVE STRESS: Antioxidant defence system

The complex system of antioxidant detoxifying mechanisms is majority designed to provide protection for biological sites against the physiological rate of ROS and repair the damage that they may cause[154,160]. Therefore, a disturbance in the balance between pro-oxidant and antioxidant defences in favour of pro-oxidant not only depends on their production but also the cellular antioxidant ability. A significant number of antioxidants species (non-enzymatic antioxidants) in addition to antioxidant enzymes have been described in living cells, both having the same purpose of preventing and reducing the extent of oxidation induced by ROS[154,182,183].

- **Enzymatic antioxidant:** superoxide dismutase (SOD), catalase (CAT) glutathione peroxidase (GPx), glutathione reductase (GR) etc.

- **Non-enzymatic antioxidant:** glutathione (GSH), vitamin C, vitamin D, vitamin E etc.

Various antioxidant species such as GSH and antioxidant enzymes including SOD and GPx are highly present on both sides of the mitochondria, as this organelle constitutes the main source of ROS. SOD, CAT and GPx are considered the first-line antioxidant enzymes defence owing to their ability to neutralize ROS directly[184]. SOD dismutase two $O_2^{-\bullet}$ anions into H_2O_2 and oxygen. CAT (peroxisomes) and GPx are antioxidant enzymes responsible for detoxification of H_2O_2 to water. GPx is also capable of reducing organic peroxides ($ROO^{\bullet}$), including LOOH oxidizing GSH. The oxidized glutathione, glutathione disulphide (GSSG) is again reduced by GR in the presence of NADPH **(Figure 3)**[144,185].

GSH is the major non-enzymatic antioxidant in living cells since it is very abundant in the cytosol, nuclei and mitochondria. Inside the nucleus, GSH is responsible for stabilizing the redox homeostasis of crucial protein sulfhydryl, which are very important for the reparation of DNA and cell expression[144,186]. GSH is oxidized via GPx to form GSSG, which is reduced again through GPx using NADPH as a cofactor. GSH and its oxidized form GSSG, constitutes the ratio GSH/GSSG, fairly used as an indicator of oxidative stress since low ratio GSH/GSSG has been implied in molecular damage[147,153,187].

4.2 OXIDATIVE STRESS: Inflammation

In the process of inflammation, elevated levels of ROS in particular H_2O_2 are produced due to an increment of oxygen consumption when recruited mast cells and leukocytes generate a ‘respiratory burst’ on the focus of damage[188]. Alternatively, inflammatory cells can also recruit other inflammatory cells to give rise to even more ROS by producing soluble mediators[189,190]. This vicious circle can damage the surrender cells, and after a long period of exposition, several pathological processes may be developed, including carcinogenesis[191–193].

Free radicals generated during the oxidative burst also include $O_2^{-\bullet}$ and NO, which may react in conjunction to form the potent oxidizing molecule, $ONOO^-$.

- **Prostaglandins**: are a group of biomarkers of stress-induced inflammation originated by the catalization of COX from AA. COX-1 is the isoform expressed constitutively in living cells, whereas COX-2 is mainly induced in the inflammatory process and pathological diseases such as cancer. Nevertheless, both isoforms are capable of synthesisising prostaglandins during inflammation[194,195].

  Under prolonged oxidative stress related to inflammation, ROS induce the expression of different inflammatory mediators that subsequently lose the AA from the plasma membrane via phospholipase A2 (PLA2). After the release of AA, it is metabolized by the action of COX **(Figure 8)**.

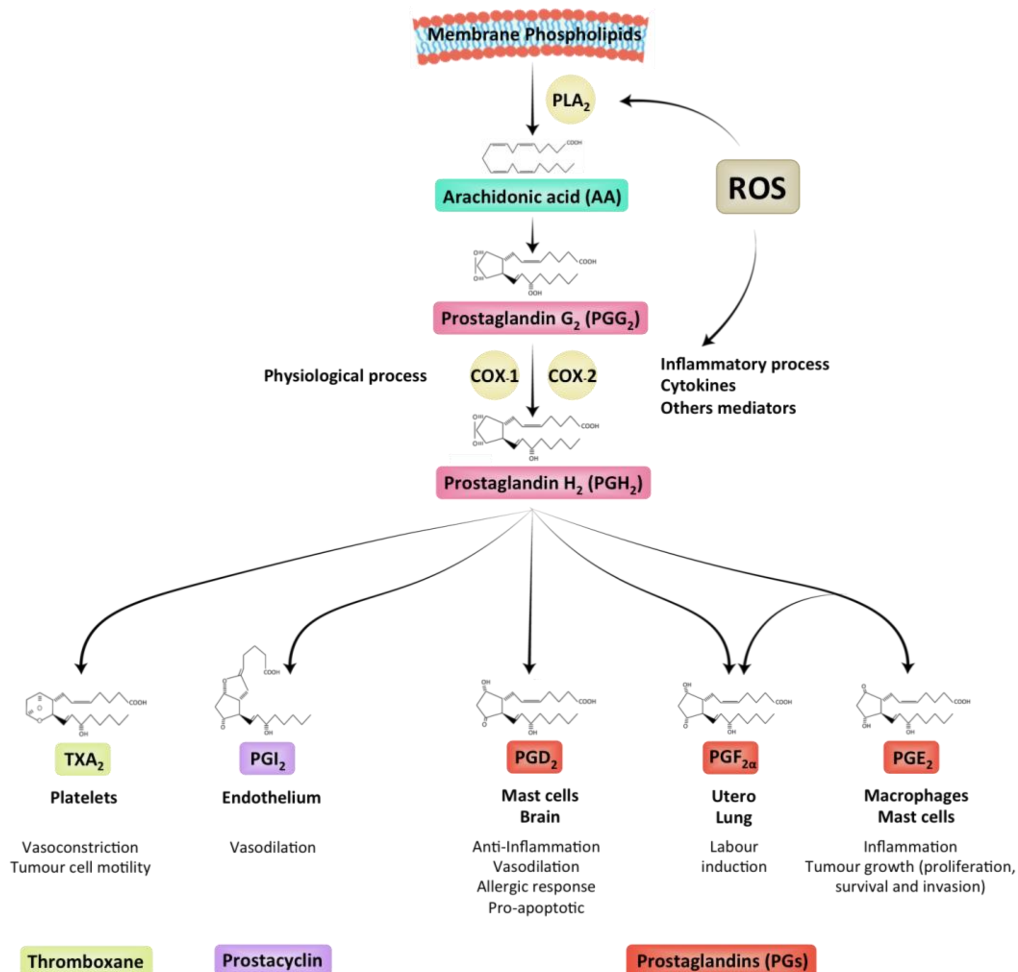


Figure 8. **Prostaglandins generation via inflammatory response induced by ROS.** This figure is original and designed by Sandra Martinez.

4.3 OXIDATIVE STRESS: Cancer

Oxidative stress is widely linked to cancer initiation and progression by disrupting vital biological functions and subsequently causing somatic mutations and neoplastic transformations[152,196]. Although ROS predominantly induce permanent DNA damage, increasing genome instability, also stimulates cell proliferation, activates cell invasion and metastasis, angiogenesis and suppresses apoptosis[147,197,198].

Carcinogenesis is usually divided into three stages. Some experimental evidence has suggested that ROS may play an essential role in all of them[143,152,163].

- **Initiation**: ROS may increase the accumulation of oxidative DNA lesions, which induce irreversible changes in the genome, significant for tumour initiation.

- **Promotion**: ROS can acquire essential features to act as promoters of various processes, including signalling pathways modulation and physiological mechanisms aberration. Also, ROS stimulates cell proliferation at low concentrations and may inhibit the action of cellular antioxidant defence systems.

- **Progression**: ROS may stimulate angiogenesis in tumour cells and tumour-infiltrated immune cells (i.e., macrophages). The role of angiogenesis in cancer is well established as lead to malignant cells spread to distant sites by creating new blood vessels from pre-existing vessels. Moreover, oxidative stress modulates pathways involved in cell adhesion, migration and degradation of extracellular matrix (ECM) proteins, which are crucial for tumour cells to become metastatic and colonize other tissues.

However, both ROS and RNS not always exert pro-oncogenic actions, especially when they are in excessive concentrations[183]. For example, whereas ROS can promote senescence, apoptosis, necrosis and restrain angiogenesis, RNS can impair cell proliferation and inhibit tumour growth[198].

As previously explained, ROS is characterized by its potential dual effect on living cells. In a matter of cancer, under oxidative stress, low levels of ROS promote cell proliferation and survival, while moderate levels induce cell differentiation[153,155,197].

On the other hand, excessive amounts of ROS have a negative impact in tumorigenesis as long as the cell viability is disrupted; if it is not then, it may contribute to cancer progression **(Figure 9.A)**.

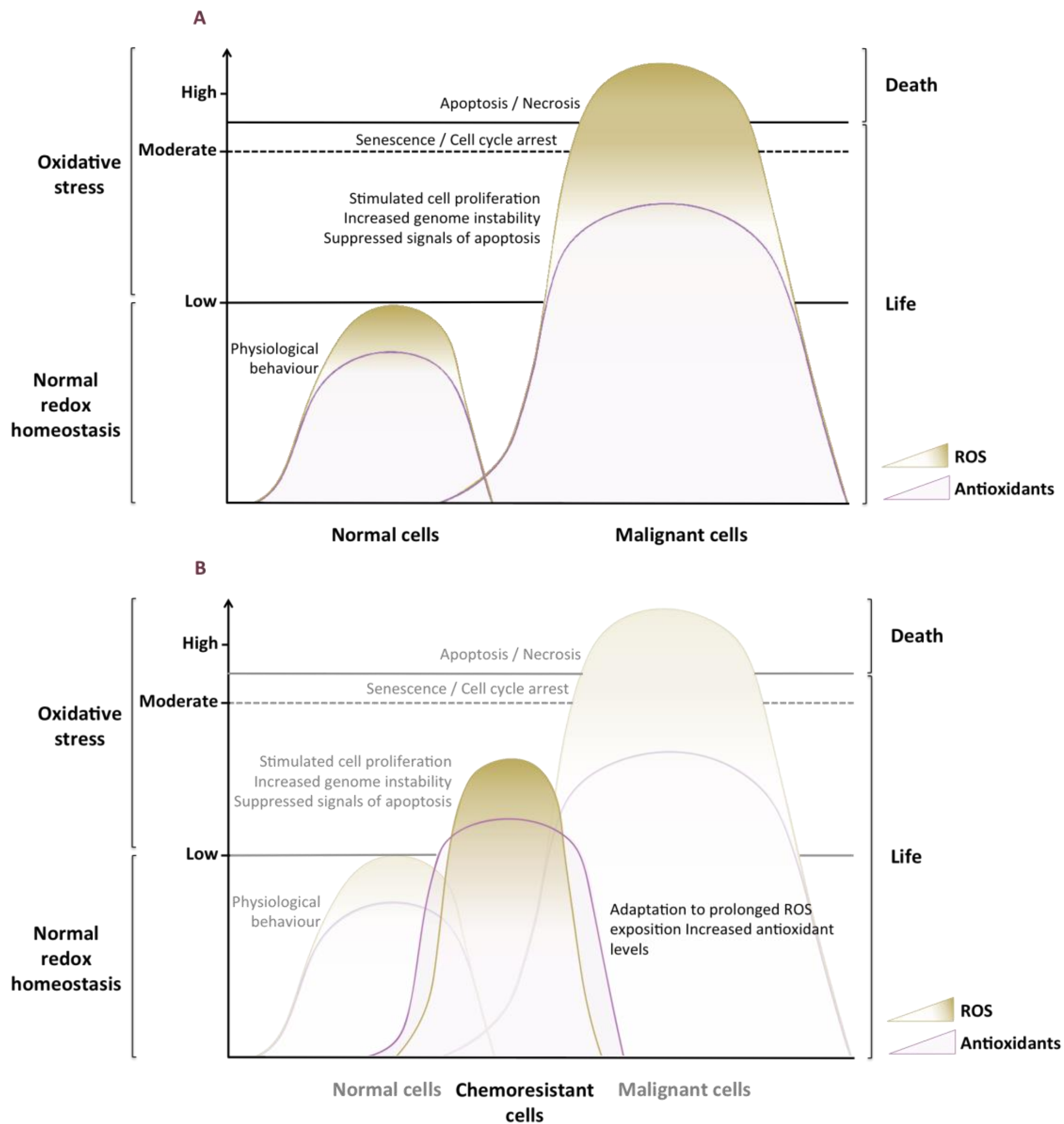


Figure 9. **ROS and antioxidant defence levels in normal cells and malignant cells (A) and in chemoresistant cells (B).** Under normal physiological conditions the balance between ROS and antioxidant mechanisms is maintained. Malignant cells increased ROS production enough to disrupt the balance to promote tumorigenesis but below the toxic threshold. Once further ROS is generated, the cells go into apoptosis/necrosis. Chemoresistant cells are adapted to the redox state by increased the expression of endogenous antioxidants. This allows the malignant cells to survive under long periods exposition to ROS and also creates resistance against anticancer agents. This figure is original and designed by Sandra Martinez.

Currently, more than 100 oxidised DNA products have been described in various types of cancer being 8-OHdG the most extensive biomarker for DNA lesion studied to date[153,199]. Although there is a strong connection between DNA mutations and cancer, other mechanisms induced by ROS such as lipid peroxidation and deficient antioxidant are also involved in the stimulation of cancer development[153,200,201]. Therefore, various biomarkers of oxidative stress and antioxidant mechanisms can be measured to link ROS and carcinogenesis.

4.3.1 OXIDATIVE STRESS: Breast Cancer

As regards breast cancer, there are described many different mechanisms in which oxidative stress may play a specific role in the development of the disease.
Some studies have found elevated concentrations of 8-OHdG in breast cancer tissues[159,179,202,203]. For instance, Okoh, *et al.* suggested that ROS may intervene in the early phases of carcinogenesis as 8-OHdG was found specifically increased in tissue from early-stage tumours[204]. Likewise, lipid peroxidation has been involved in breast carcinogenesis, although the exact mechanism is still not fully understood[205]. Tas F *et al.* found that elevated levels of lipid peroxidation in breast cancer tissue were accompanied by an inadequate antioxidant defence. CAT was found lower than in the reference tissue, but SOD and GPX activities were higher[206]. Therefore, in cancerous tissue, SOD may react with O_2 to form H_2O_2, but this cannot be wholly detoxified into H_2O as the lack of proper levels of CAT. As a result, a high amount of OH^- is produced exert its oxidizing function.

Additionally, endogenous and synthetic oestrogens can cause chromosomal alterations and DNA damage in the genome of breast cells by inducing oxidative stress through the generation of ROS from instable compounds[163,204,207]. Alternatively, antioxidant enzymes activity may be affected by an oestrogen receptor-dependent mechanism, giving rise an overproduction of oxidative stress induced by neglecting antioxidant defences[208]. However, some authors do not support this scenario and have reported increases rather than decreases levels of antioxidant enzymes[201,209,210].

Recently, stromal cells have assumed an essential role in the transformation of breast cells into an invasive and metastatic phenotype[15,197,211]. Indeed, in several experimental models, stromal cells and tumour cells have worked together to create a positive environment for tumour development[212,213]. While stromal cells secret growth factors, matrix-degrading enzymes and energy substrates, tumour cells are able to secret soluble factors needed for the activation of stromal cells[152]. ROS may be involved in the paracrine signalling between malignant cells and stromal cells.
On the other hand, epithelial-mesenchymal transition (EMT), which is a biological process, can participate in the obtainment of the invasive and metastatic phenotype. In the course of EMT, several morphological modifications occur and most of them are compatible with tumour development such as invasiveness, cytoskeletal reorganization, and mobility augmentation. Although the exact mechanisms are not well described, EMT and oxidative stress seem to directly interact together to promote cancer malignancy through various signalling pathways[152,163,207].

Inflammation may also participate in the promotion of breast cancer development as many studies have suggested that tumours usually trigger an inflammatory response. Indeed macrophages often found inside many tumours although their action (tumour growth suppression or metastasis promotion) remains uncertain and may differ from the type of cancer[123,198].

Despite these abilities of ROS to trigger different mechanism that favours a pro-oncogenic environment; further deeply investigations should be done to establish if the reason behind the presence of ROS in cancer cells is a cause or a consequence of tumorigenesis. Yet, it is undeniable the extremely importance of oxidative stress in cancer by any means.

4.4 OXIDATIVE STRESS: Chemotherapy

Considering that oxidative stress has been linked to almost every single step of carcinogenesis, it is understandable that some investigations have focus on the production of further ROS as a consequence of chemotherapy treatment[214].

Chemotherapy drugs interfere with the ability of tumour cells to uncontrollably divide and reproduce, ergo, destabilize the mitotic and metabolic process in order to induce apoptosis even if that means to affect surrounding normal cells and tissues[187,215,216]. Consequently, some mild and severe side effects are associated with their administration such as nausea, vomiting, bone marrow suppression, hypotension and heart failure[217].

In general, chemotherapeutic agents contribute to attack tumour cells by disrupting ROS homeostasis as a consequence of their anticancer activity.

Anthracycline-induced oxidative stress regulate processes that involve cellular hypertrophy in cardiac cells, remodelling of the ECM structure, impaired contractile cardiac function and programmed cardiac cell death[217]. Although the molecular mechanism underlying anthracycline-induced cardiotoxicity remains controversial, oxidative stress has been postulated as its potential promoter[187,217,218]. Unfortunately, the combination of anthracyclines with other drugs, in particular with paclitaxel, has been proved to enhance the risk of cardiotoxicity[219,220]. Taxanes (e.g., paclitaxel) lead to block mitotic spindle formation and apoptosis. Although taxanes generate fewer levels of oxidative stress than anthracyclines, they may promote the formation of $O_2^{-\bullet}$ by releasing cytochrome c from mitochondrial respiratory chain[152,196,221].

One of the major concerns of chemotherapy treatment is the susceptibility of patients to suffer chemoresistance that may cause a reduction of the effectiveness and lead to the failure of therapy. Although the precise mechanisms underlying chemoresistance remain poorly understood, this phenomenon has been associated with a disrupted cellular redox balance as a result of a long period exposition to ROS **(Figure 9.B)**[214,221].

4.5 OXIDATIVE STRESS: Pregnancy

The human placenta is of the hemochorial type, very rich in mitochondria and deeply vascularised. Therefore, it is reasonable to assume that following these characteristics; there is a highly mitochondrial respiratory chain activity that further favour ROS production[222,223]. Nevertheless, the balance between ROS and antioxidant protection is not disrupted since the response of antioxidant defences is equivalent and compensates the increase of ROS generation[135].

Initially, the placenta is characterized by having low O_2 levels, which stimulates normal cell proliferation and placental angiogenesis[224,225]. As a result, angiogenic factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) can be regulated transcriptionally and post-transcriptionally[226]. The absence of established maternal intraplacental circulation is responsiblefor this hypoxia environment. Towards the end of the first trimester, this is finally accomplished increasing O_2 threefold and, with it, so does mitochondrial mass that leads to a burst in ROS[224,227].
Consequently, the placenta is capable of adapting to this change by modulating other factor and producing further antioxidant mechanisms. Maintaining the redox homeostasis is essential to foetal development; otherwise, pregnancy complications may occur[135,228].
Most of the studies related to oxidative stress and antioxidant defence in uncomplicated pregnancies showed increased levels of IsoPs and 8-OHdG biomarkers in samples from the third trimester and early stage of delivery[223,229–231]. However, the levels were restored after six to eight weeks of postpartum. Similarly, Djordjevic, A *et al.* found the antioxidant activity (GPx and SOD) incremented in the third trimester of pregnancy[232].

Conversely, when an excessive production of ROS and defects in maternal antioxidant defence mechanisms exist, systemic oxidative damage in placental tissue may occur, submitting in danger both mother and foetus[121,135,224,233]. During the past few decades, different studies have evaluated the damage caused by increased levels of oxidative stress during pregnancy establishing several pathologies involved including PE, spontaneous abortion, gestational diabetes mellitus, IUGR among others **(Figure 10)**.

- **Gestational diabetes mellitus (GDM):** occurs when healthy pregnant women become intolerant to glucose for the first time. Usually is developed around the second half of pregnancy and may increase the risk of fetal macrosomia, perinatal mortality and diabetes mellitus type II for the mother[234,235]. Although some lipid peroxidation and protein oxidation biomarkers have been found elevated in GDM women, others recently did not find differences in both oxidative stress biomarkers and antioxidant capacity[234]. Therefore, to day the results are inconsistent, and there is still a controversy about which biomarkers of oxidative stress are the best to use in clinical practice. Longitudinal studies that monitoring the course of pregnancy are needed to examine the real implication of oxidative stress in the onset and progression of GDM.

- **Intrauterine growth restriction (IUGR):** is a gestational disorder characterized by the failure of the foetus to full growth in the womb. IUGR is diagnosed in neonates whose estimated weight is less than the 10th centile for gestational age[233]. IUGR negatively affects the foetus since the risk of perinatal mortality and mobility is incremented and other pathologies such as hypertension, cardiovascular disorders and renal diseases may appear later in life. Oxidative stress may play an important role in IUGR. Whereas lipid peroxidation and protein oxidation levels have been found elevated in IUGR pregnancies, antioxidant defences were deficient in various studies leading to correlate IUGR and increased oxidative stress[134,236,237].

- **Preeclampsia (PE):** is a pregnancy-specific disorder principally characterized by abnormal placentation, overproduction of maternal inflammatory vascular mediators and hypertension after twenty weeks of pregnancies in women without previous cardiovascular pathologies. These symptoms may be accompanied by other conditions, including proteinuria or end-organ dysfunction[238]. PE may lead to the development of other pathologies, such as IUGR, premature delivery, and is considered the first cause of maternal death affecting around 5% to 7% of all pregnancies worldwide[233,239]. Determine the etiology and pathogenesis of PE are relatively complicated because the exact mechanisms underpinning the disease remains mostly unknown[240]. However, oxidative stress may mediate placental

insufficiency through the generation of further ROS and inflammatory mediators triggering an augmentation of protein and DNA oxidation and lipid peroxidation.

Considerable scientific evidence has described abnormal trophoblastic invasion and uterine spiral arteries remodelling as a result of an excessive placental hypoxia[241,242]. In pregnancies complicated by PE, the levels of biomarkers of oxidative stress, in particular lipid peroxidation is exaggerated; meanwhile, the antioxidant capacities are compromised[243,244]. In addition, the 8-OHdG biomarker was also found elevated in women with PE[245,246].

Opposite to normal pregnancy in which an environment with vasodilatory prostaglandins is favoured, in PE a vasoconstrictive state is induced. Reflecting of this condition is the fact that levels of isoprostanes and prostaglandins such as $PGF_{2\alpha}$ instead of PGE are increased[241]. Moreover, PE is associated with an imbalance in the angiogenic ratio sFlt-1/PLGF in favour of sFl1-1, which is proportional to the adverse pregnancy outcomes[240,247].

- **Spontaneous miscarriage:** is considered an intentional pregnancy termination that occurs when the foetus weighs less than 500g being the genetic or chromosomal abnormalities the first cause. Miscarriage usually occurs during the first trimester of pregnancy being the incidence around 25% and having as the major cause some genetic or chromosomal abnormalities in the embryo[228,241]. However, some authors have suggested that increased levels of ROS or a loss of antioxidant defence levels may produce premature disruption of maternal placental perfusion[222,242]. The burst of oxidative stress prior 10-11 weeks of gestation leads to the abrupt arrival of oxygenated blood and deteriorates the syncytiotrophoblast causing spontaneous miscarriage and recurrent pregnancy loss. Moreover, these high levels of oxidative stress in an early stage of gestation may also induce other modifications in cell functions that favour pregnancy loss such as angiogenesis, matrix remodelling and endocrine function[233,241].

- **Preterm premature rupture of the membrane (PPROM) and spontaneous preterm birth (PTB):** oxidative stress contributes to placental membrane disruption stimulating the onset of labour at term[240]. So it is likely to assume that ROS may be

accountable for some cases of PPROM (defined as a spontaneous rupture in the chorioamnion before the 37 weeks of gestation) especially, in which infections can be involved[225,241]. Immune cells can generate ROS in order to kill bacteria and then attack the placental membranes. Likewise, oxidative stress has been postulated as the cause of cellular ageing in PPROM and PTB (<37 weeks) since foetal telomere length decreased[233]. Analysis of antioxidant defence in cord blood from PTB and amniotic fluid samples from women with PPROM demonstrated decreased levels when compared to healthy and term pregnancies[121,248]. Dutta, *et a*l. also found similar results when analysed the oxidative stress and antioxidant enzymes in amniotic fluid from women with PPROM and PTB. However, PPROM samples showed greater DNA damage and lowered antioxidant enzymes in comparison with PTB samples[249].

Numerous neonatal complications are related to PPROM and PTB. Respiratory distress syndrome, sepsis, bronchopulmonary dysplasia and intraventricular haemorrhage are usually associated with PPROM[250] whereas infant morbidity and mortality are related to PTB[233].

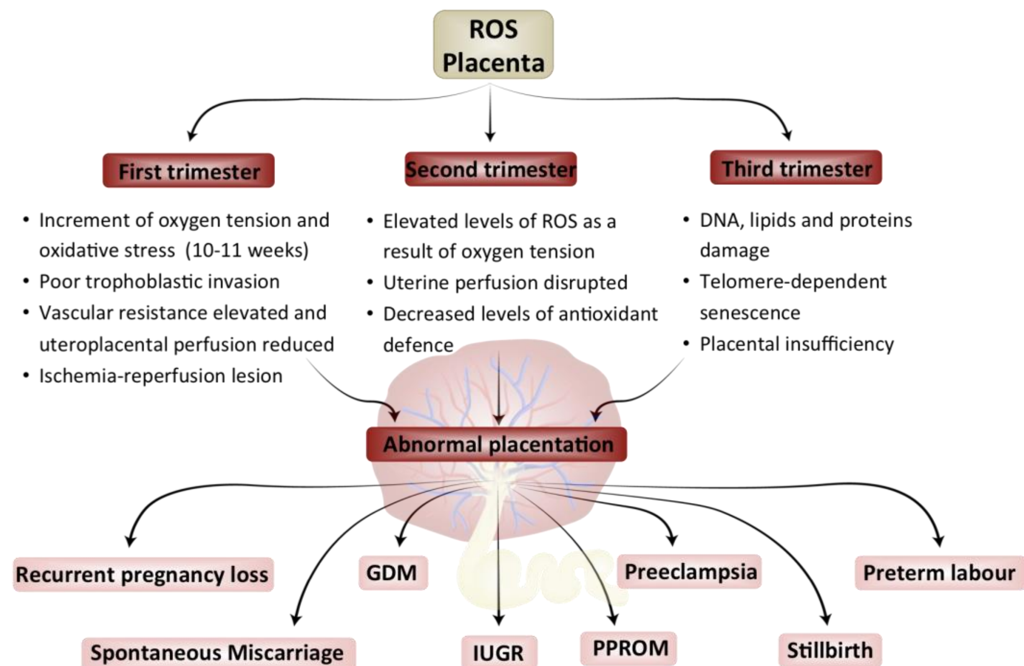


Figure 10. Pregnancy disorders associated with abnormal placentation induced by oxidative stress. This figure is original and designed by Sandra Martinez.

5. JUSTIFICATION OF THE STUDY

The potential role of oxidative stress in several biological and pathological processes is undeniable. Pregnancy, cancer and the administration of antineoplastic drugs have individually proved to deregulate redox homeostasis and induce inflammation in several studies but not when these sources of oxidative stress occur at the same time.

Considering this fact, it is likely to assume that PABC women undergoing chemotherapy in pregnancy generate further oxidative stress since they face different origins of ROS production. Additionally, PABC patients are usually associated with worse tumour biology and subsequently, worse prognosis. However, the role of oxidative stress in this situation has not been documented despite oxidative stress have been closely linked to cancer.
Likewise, chemotherapy may lead to perinatal complications. However, little information is available, and most of the studies are contradictory with regard to the effect of chemotherapy exposition in utero and its safety.

IV. HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

Women with breast cancer and their offspring under chemotherapy treatment during pregnancy, may have an increase production of reactive oxygen species and inflammation products that may account for the increased risk perinatal complications observed in these cases.

OBJECTIVES OF THE STUDY

- To get insight of oxidative stress status and inflammation biomarkers in women with breast cancer and their offspring treated with anthracyclines and/or paclitaxel during pregnancy

Secondary objectives:

- To evaluate maternal and perinatal complications in PABC patients.
- To monitor oxidative stress, inflammation and antioxidant defence in blood from PABC patients according to chemotherapy type (anthracyclines or paclitaxel).
- To assess changes of oxidative stress as well as inflammation and antioxidant defence biomarkers in PABC patients before chemotherapy treatment and at labour.
- To analyse levels of oxidative stress, inflammation and antioxidant defence biomarkers prior to chemotherapy treatment in PABC and non-PABC patients.
- To assess levels of oxidative stress, inflammation and antioxidant defence biomarkers in cord blood from neonates exposed to chemotherapy *in utero* compared to neonates born to healthy women.
- To compare oxidative stress, inflammation and antioxidant defence biomarkers in maternal blood at labour and cord blood from neonates exposed to chemotherapy *in utero.*

V. MATERIALS AND METHODS

1. STUDY DESIGN

Multicentre prospective case-control study conducted in three Spanish hospitals, the *Vall d´Hebron University Hospital* (Barcelona), the *Son Espases University Hospital* (Palma de Mallorca) and the *La Fe University and Polytechnic Hospital* (Valencia) between 2013 and 2019. Eligible participants were divided into different studies according to their characteristics, origin and objectives of the study **(Figure 10)**.

- **Pregnant women diagnosed with breast cancer:** eligible patients were women with primary or recurrent breast cancer, who were treated according to the standard protocol (*Vall d´Hebron University Hospital).*
- **Control pregnant women group:** healthy pregnant women attending prenatal care (*Vall d´Hebron University Hospital* and the *Son Espases University Hospital)*.

- **Non-pregnant patients diagnosed with primary or recurrent breast cancer**: women with breast cancer treated with anthracycline-based therapy and without other pathologies diagnosed (*Vall d´Hebron University Hospital)*.

- **Neonates born to mothers diagnosed with breast cancer during pregnancy:** neonates exposed to various cycles of chemotherapy *in utero* (*Vall d´Hebron University Hospital)*.

- **Control neonates:** neonates born to healthy women who did not present any clinical disease during gestation (*Vall d´Hebron University Hospital, Son Espases University Hospital* and the *La Fe University and Polytechnic Hospital).*

The exclusion criteria were termination of pregnancy and chromosomal anomalies or cardiopathies diagnosed prior chemotherapy initiation.

To evaluate the proposed objectives, we designed two studies:

- **STUDY I:** analysis of different biomarkers of oxidative stress, inflammation and antioxidant defence through pregnancy in women diagnosed with PABC before initiation chemotherapy treatment, during treatment and at delivery **(Table 1)**.

- **STUDY I.A:** blood samples from PABC women were obtained prior to chemotherapy initiation (basal sample), before and after each cycle of chemotherapy , during routine blood test analysis and at delivery.
- **STUDY I.B:** blood samples from PABC women were obtained prior to chemotherapy initiation (basal sample) and at delivery.

Blood samples from healthy pregnant women during pregnancy and at labour as well as non-pregnant women with breast cancer before and after anthracyclines were additionally included in the study to evaluate the impact of breast cancer and chemotherapy treatment in pregnancy.

- **STUDY II:** analysis of different biomarkers of oxidative stress, inflammation and antioxidant in cord blood and urine from neonates with chemotherapy exposure *in utero* obtained at birth and within the first 24h of life respectively **(Table 2)**. Blood and urine samples from control neonates were also measured to assess the effect of chemotherapy *in utero*.

We additionaly compared the levels of oxidative stress, inflammation and antioxidant defence from cord blood with those form maternal plasma at birth (study I.B).

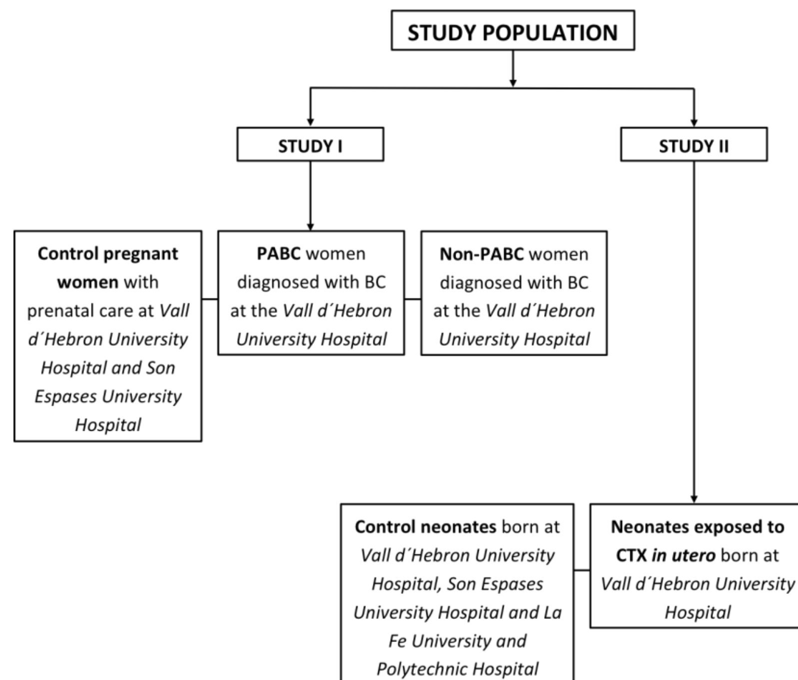


Figure 11. Flow chart illustrating the study design according to the population included in the studies.

Table 1. Biomarkers of oxidative stress, inflammation and antioxidant analysed in **STUDY I**

STUDY DESIGN		BIOMARKERS
STUDY I	**STUDY I.A** *Vall d´Hebron University Hospital*	MDA Protein Carbonyl GSH SH-protein groups Chitotriosidase YKL-40
	STUDY I.B *La Fe University and Polytechnic Hospital*	GSH GSSG Cysteine (Cys) Cystine (Cyss) ortho-Tyrosine (o-Tyr) meta-Tyrosine (m-Tyr) Phenylalanine (Phe) 3-Nitro-Tyrosine (3NO_2-Tyr) 3-Cloro-Tyrosine (3Cl-Tyr) para-Tyrosine (p-Tyr) Glutathione sulfonamide (GSA) Isoprostanes (IsoPs) Isofurans (IsoFs) Neuroprostanes (NeuroPs) Neurofurans (NeuroFs) dihomo-Isoprostanes (dihomo-IsoPs) dihomo-Isofurans (dihomo-IsoFs) Prostaglandins (PGs)

Table 2. Biomarkers of oxidative stress, inflammation and antioxidant analysed in **STUDY II**

STUDY DESIGN	BIOMARKERS
STUDY II *University and Polytechnic Hospital*	GSH GSSG Cys Cyss o-Tyr m-Tyr Phe $3NO_2$-Tyr 3Cl-Tyr p-Tyr GSA IsoPs IsoFs NeuroPs NeuroFs dihomo-IsoPs dihomo-IsoFs PGs

See **Suppl. table S1** and **S2** for additional description of all biomarkers analysed in both studies.

1.1 Recruitment process

All patients diagnosed with breast cancer during pregnancy were attended by each member of a multidisciplinary team constituted exclusively for this relatively rare event. This team was integrated by a different specialist including oncologists, obstetricians and surgeons. The treatment plan was designed for each patient based on the histological type, stage and gestational age at the moment of the diagnosis. The main purpose was to achieve the best oncological results for the mother while minimizing the risk of foetal damage related to maternal treatment.

PABC patients were treated with anthracycline-based regimens and taxanes during the second and third trimesters of their pregnancy.

The chemotherapy regimens used were as follows:

Anthracyclines (21-day intervals)

- **FAC_{50}:** fluorouracil 1.000 mg/m^2, doxorubicin 60 mg/m^2, cyclophosphamide 600 mg/m^2
- **AC:** doxorubicin 60 mg/m^2, cyclophosphamide 600 mg/m^2
- **EC:** epirubicin 90 mg/m^2, cyclophosphamide 600 mg/m^2

Taxanes (weekly)

- **Paclitaxel** (PTX): 80 mg/ m^2

The antiemetic regimens administrated before each cycle of chemotherapy to battle against the short-term side effects of the treatment are described below:

- **1st cycle:** fortecortin 10 mg (endovenous administration), dexchlorpheniramine 5 mg (oral administration) and ranitidine 50 mg (oral administration).
- **2nd cycle:** fortecortin 8 mg (endovenous administration), dexchlorpheniramine 5 mg (oral administration) and ranitidine 50 mg (oral administration).
- **3rd cycle and onward:** fortecortin 4 mg (endovenous administration), dexchlorpheniramine 5 mg (oral administration) and ranitidine 50 mg (oral administration).

Additionally, antiemetic regimens prescribed the following days of chemotherapy to prevent delayed emesis were ondansetron 8 mg 12h during two days and dexchlorpheniramine and dexamethasone when necessary. Subsequently, the medication was adjusted individually according to the reported symptoms of each patient.
Other standard systemic therapies contraindicated in pregnancy such as tamoxifen and trastuzumab were postponed after childbirth.

Additionally, all non-PABC patients breast cancer were treated at 21-day intervals with anthracycline-based regimens.

Oncological information included details about the patient's age at diagnosis, histological type and grade, clinical stage, oestrogen and progesterone receptor status, overexpression HER2/neu, genetic status and surgery before chemotherapy. The participants also provided consent for obtaining clinical and obstetric records for the study.

Additionally, relevant perinatal data regarding prematurity, birth weight and Apgar (1min and 5min) and maternal complications were included.

1.2 Population size

Acknowledging that breast cancer is rarely diagnosed during pregnancy, we estimated the inclusion of approximately fifteen PABC patients along the recruitment period. Moreover, we expected to include a similar number of age-matched non-PABC patients and age-matched control pregnant women.

1.3 Ethical considerations

The study protocol was approved by the Ethics Committee of the Vall d'Hebron University Hospital (PR(AMI) 83/2012). All PABC and non-PABC patients and control pregnant women expressed their written informed consent for participation in the study.

1.4 Financing

The study has been funded by *Carlos III Health Institute* through the project AES PI15/02252 Co-funded by European Regional Development Fund/European Social Fund "Investing in your future".

2. MATERIAL AND METHODS

2.1 Sample preparation

Venous blood samples (5mL) from PABC, non-PABC and control women were drawn after a period of fasting and processed within one hour. Blood was collected at least into two EDTA-containing tubes (for whole blood, RCB and plasma samples) and two heparin-containing tubes, for obtaining plasma samples. Plasma and RBCs were isolated by centrifugation at 1,500 x *g* for 5 minutes at 4ºC, and sample aliquots were immediately stored at -20ºC or -80ºC until experimental analysis. Exceptionally, one aliquot of RCB always was washed three times in isotonic saline (NaCl 0.9%) and stored at 4ºC.

Cord blood samples from neonates of PABC-N and Control-N group were obtained at birth and collected into heparin-containing (for plasma samples) and EDTA-containing (for RBCs and plasma samples) tubes. Plasma and RBCs were separated from cord blood by centrifugation at 1,500 x g for 5 minutes at 4ºC, and sample aliquots were immediately stored at -80ºC until assayed.
Urine samples were obtained within 24h after birth under sterile conditions by adding gauzes into the diapers and stored shortly after at -80ºC until further analysis.

The experimental analysis was performed in two different institutions according to the type of biomarker measured **(see Table 1 and 2)**.

2.2 Biomarker quantification employing spectrophotometry, fluorometry and high-performance liquid chromatography (HPLC)

Biomarkers of oxidative stress, antioxidant defence and inflammation were determined in the collected plasma and RCBs samples employing different methods at biochemistry and molecular biology research centre for nanomedicine (*Vall d´Hebron University Hospital*).

Reagents

Drabkin's reagent, 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), potassium phosphate monobasic (KH_2PO_4), ethylenediaminetetraacetic acid (EDTA), metaphosphoric acid (HPO_3), GSH, 1,1,3,3-Tetramethoxypropane (TEP), butylhydroxytoluene (BHT), 2-Thiobarbituric acid (TBA), diethylenetriaminepentaacetic acid (DTPA), 4-Methylumbelliferyl β-D-N,N',N''-triacetylchitotrioside (4-M-U-b-D-N-triacetylchitotrioside), glycine, 4-Methylumbelliferone sodium salt (4-MU), citric acid monohydrate, sodium phosphate dibasic, tris base and 5 N Sodium hydroxide (NaOH) were purchased from Sigma Chemical Co. St Louis, MO, USA. 2 mg/mL bovine serum albumin (BSA), coomassie brilliant blue (CBB) and acetonitrile acid (CH_3CN) (HPLC grade) were from Fisher Chemical, Waltham, MA, USA. Butanol (HPLC grade), 5 M hydrochloric acid (HCl), glacial acetic acid (HPLC grade) and absolute ethanol were obtained from Panreac, Barcelona, Spain. 30% BRIJ 35 solution and milli-Q water ($H_2O_{mili\text{-}Q}$) were purchased from Millipore Bedford, MA, USA. Sodium acetate and absolute methanol were from Merck, Darmstadt, Germany and VWR respectively, Radnor, PA, USA.

Material

Reax Top vortex mixer was from Heidolph (Schwabach, Germany). 5911 centrifuge was from Kubota (Bunkyo-ku, Tokyo, Japan). CT15RE refrigerated centrifuge was from Eppendorf Himac Technologies (Hitachinaka, Ibaraki, Japan). U-3210 spectrophotometer was from Hitachi (Chiyoda City, Tokyo, Japan). HT2 microplate reader was from Anthos Labtec (Heerhugowaard, Netherlands). Biochrom Asys UVM340 microplate reader from Biochrom (Cambridge, UK). Twinkle LB970 microplate fluorimeter was from Berthold Technologies (Bad Wildbad, Germany). Waters 2695 system from Waters (Milford, MA, USA).

2.2.1 Determination of GSH by spectrophotometry

GSH content was measured in RBC using the enzymatic method detailed by Anderson[251].

Reagents preparation

- **6% HPO_3**

  6 g of HPO_3 in 100 mLH_2O_d

- **0.1M KH_2PO_4 - 5mM EDTA buffer, pH 7.4**

  13.609 g of KH_2PO_4 and 1.901 g of EDTA were dissolved in 800 mL of H_2O_d. The pH was adjusted with 5 N NaOH until achieving pH 7.4. Additional H_2O_d was added to reach 1 L

- **10 mM DNTB**

  0.0984 g of DTNB was dissolved in 25 mL of dilution buffer. The solution was divided into 12 aliquots of 2 mL and stored at -20ºC

- **1 mM GSH standard stock**

  0.03073 g of GSH was dissolved in 100 mL of dilution buffer. The solution was divided into 50 aliquots of 2 mL and stored at -20ºC

Standard preparation

Table 3. Preparation of the standard curve points of GSH

	Points of the standard curve (µM)	6% HPO_3 (µL)	GSH standard (µL)
P1	500	200	200 of 1mM GSH standard
P2	250	200	200 of point P1
P3	125	200	200 of point P2

Procedure

The analysis of haemoglobin (Hb) was made from whole blood on the same day of blood extraction. A volume of 10 µL of each sample was added to 2.5 mL of Drabkin's solution (fume hood). After mixing, they were further incubated at room temperature for 15

minutes. The absorbance of the samples was measured at 540 nm on a spectrophotometer using the Drabkin's solution as a blank. All samples and standard curve were analysed in triplicate.

The amount of Hb was internally calculated by the spectrophotometer based on a standard that follows the equation:

$$\mathbf{X\ g/dl} = (A5_{40nm} \times 36.83) - 1{,}347$$

The analysis of GSH was performed using the aliquot of washed RBCs stored at 4ºC within 48h after blood collection. RBCs were mixed in 6% metaphosphoric acid (1:4 ratio) to prevent the oxidation of GSH and were centrifuged at 21,000x *g* for 15 minutes (4 ºC). The supernatant was collected and diluted 1/5 in dilution buffer. Subsequently, 100 µL of the sample, 785 µL of dilution buffer and 25 µL of 10mM DTNB were pipetted into each cuvette and were gently mixed. After incubation in complete darkness, at room temperature for 5 minutes, the absorbance of the samples was measured at 420 nm on a spectrophotometer. The standard curve was prepared following the same indications but with an established concentration of GSH **(Table 3)**. All samples and standard curve were analysed in duplicate.

The concentration of GSH in RBC was determined from the standard curve and expressed as µmol/g Hb once normalized to the concentration of Hb.

2.2.2 Determination of protein-SH groups by spectrophotometry

Plasma SH-protein groups were measured by the method described by Hu[252]. Plasma protein levels were obtained from Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) using BSA as standard and essentially based on the method designed by Bradford[253].

Reagents preparation

- **10 mM DNTB**

  0.04 g of DNTB was diluted in 10 mL of absolute methanol (fume hood). Only stable for up two weeks when stored at 4 ºC

- **0.25 M Tris base – 20 mM EDTA buffer, pH 8.2**

  3.028 g of tris base and 0.8324 g of EDTA were diluted in 100 mL of H_2O_d. The pH was adjusted with 5 M HCl until achieving pH 8.2.

Standard preparation

Table 4. Preparation of the standard curve points of BSA

	Points of the standard curve (mg protein/mL)	H_2O_d (µL)	BSA standard (µL)
P1	1.500	50	150 of 2mg/mL BSA standard
P2	1.000	100	100 of 2mg/mL BSA standard
P3	0.750	100	100 of P1
P4	0.500	100	100 of P2
P5	0.250	100	100 of P4
P6	0.125	100	100 of P5
P7	0.025	100	25 of P6
P8	0.000	200	-

Procedure

Plasma proteins were determined from EDTA or heparin plasma stored at -20ºC. Samples were centrifuged at 21,000 x *g* for 5 minutes (4 ºC). The supernatant containing protein fraction was collected and diluted 1/150 in H_2O_d and subsequently mixed. After the dilution, 5 µL of the diluted sample and 250 µL of CBB were added into each well of a 96-well plate. The points of the standard curve were prepared in parallel as previously described, but with a concentration range from 0.0 to 1.5 mg/mL of BSA **(Table 4)**. Finally, the 96-well plate was shaken for 30 seconds, incubated at room temperature for 10 minutes and further reading.

The absorbance of each well was measured at 595 nm in a plate reader and the standards curve was used to determine the concentration of plasma proteins expressed as µg/µL. All samples, and standard curve points were analysed in triplicate.

Plasma total SH groups were determined from EDTA plasma stored at -80ºC. Samples were centrifuged at 21,000 x *g* for 5 minutes (4 ºC). The supernatant was collected and diluted 1/5 in Tris-EDTA buffer in a final volume of 200 µL followed by addition of 10 µL of

10 mM DTNB and 800 µL of absolute methanol (fume hood). After incubation at room temperature for 15 minutes with the sample-containing tubes capped, the samples were centrifuged at 21,000 x *g* for 5 minutes. The absorbance was measured at 412 nm on a spectrophotometer using 750 µL of the supernatant.
Blank samples were prepared with H_2O_d and processed exactly as samples were. All samples and blank sample were analysed in triplicate.

Total SH groups were calculated used the following formula:

$$\mathbf{X\ \mu M} = (\text{Absorbance sample} - \text{Absorbance blank}) \cdot 1487$$

The concentration of plasma protein-SH groups was obtained after the normalization of total-SH groups for total protein to avoid changes in plasma protein content. Plasma protein-SH groups were expressed as µmol/mg.

2.2.3 Determination of MDA by HPLC

MDA was measured in plasma using the method explained by Fukunaga[254] and with minor modifications previously detailed[255].

Reagents preparation

- **4 µM TEP**

TEP solution (mM)	TEP (µL)	Solute (µL)
10 mM TEP	61.5 µL of TEP	25 mL of 40% ethanol
100 µM TEP	100 µL of 10mM TEP	9.9 mL of $H_2O_{mili\text{-}Q}$
4 µM TEP	800 µL of 100 µM TEP	19.2 mL of $H_2O_{mili\text{-}Q}$

The solution was divided in 20 aliquots of 1 mL and stored at -20ºC.

- **5% BHT**

0.5 g of BTH was dissolved in 10mL of absolute ethanol.

- **2 M acetic acid – 2 M acetate buffer, pH 3.5**

  2 M acetic acid: 1.5mL of glacial acetic acid (HPLC) in 100 mL of H_2O_{miliQ} (fume hood)

  2 M sodium acetate: 3.28 g of sodium acetic in 100 mL of H_2O_{miliQ} mL

  The pH of 2M acetic acid was adjusted with 2M sodium acetate until achieving pH 3.5.

- **TBA solution**

  0.3 g of TBA and 0.03932 of DTPA in 100 mL of 2 M acetic acid – acetate buffer pH 3.5.

  The solution was divided in 22 aliquots of 4.5 mL and stored at -20°C.

- **70% CH_3CN**

  150 mL of H_2O_{miliQ} in 350 mL of CH_3CN (HPLC grade). Sonicated for 15 minutes.

Standard preparation

Table 5. Preparation of the standard curve points of TEP

	Points of the standard curve (µM)	H_2O_{miliQ}	TEP standard (µL)
P1	4.000	-	1000 of 4 µM TEP
P2	2.000	500	500 of P1
P3	1.000	500	500 of P2
P4	0.500	500	500 of P3
P5	0.250	500	500 of P4
P6	0.125	500	500 of P5

Procedure

The analysis of MDA was made from EDTA plasma stored at -20°C. A volume of 100 µL of each sample was diluted in 240 µL TBA solution followed by the addition of 10 µL of 5% BHT and mixed. After incubation at 95°C for 45 minutes, 250 µL of butanol was added, and tubes were mixed for 1 minute and centrifuged for 10 minutes at 1,500 x *g* to perform lipid extraction. Finally, 100 µL of each supernatant containing MDA-TBA fraction were placed into special tubes for HPLC. The tubes were careful capped ensuring no bubbles remained inside. The points of the standard curve were prepared as described in **Table 5**. Quality control (QC) was prepared from adding an equal volume of each sample expected to analyse, meanwhile, a blank sample was prepared from $H_2O_{mili\text{-}Q}$. QC and

blank sample were processed as exactly as samples were. A blank sample was always placed first to be read by the instrument followed by the standard points.

Procedure for HPLC analysis

The amount of MDA was measured as its MDA-TBA adduct after reversed-phase isocratic HPLC separation and expressed as µM.

Chromatographic separation of the MDA-TBA adduct was achieved employing a Waters Symmetry $C_{18.5}$ stainless steel column (4.6 x 150 mm, 10 µm). The analytical column was protected by a Waters Guard-Pak precolumn packed with the same material. The mobile phase used was $CH_3CN:H_2O$ 7:3 v/v solution. A volume of 20 µL of plasma MDA-TBA was injected, and the flow-rate was 1.0 mL/minute at room temperature. Retention time was set at 7 minutes. The TBA-MDA complex was monitored by fluorescence detection, with excitation at 515 nm and emission at 553.

2.2.4 Determination of ChT activity by fluorometry

ChT enzyme assay was based on the method published by Hollak[256] but including a few modifications implemented by Comabella[257].

Reagents preparation

- **0.2 M Phosphate 0.1M citrate buffer pH 5.2**

  0.1M citric acid monohydrate: 2.1 g of citric acid monohydrate in 100 mL of H_2O_d

  0.2M Sodium phosphate dibasic: 2.84 g of Sodium phosphate dibasic in 100 mL of H_2O_d

  70 mL of 0.1M citric acid monohydrate was mixed with 80 mL 0.2M Sodium phosphate dibasic. The pH was adjusted until achieving pH 5.2

- **22 µM M-U-T-A-chitotriosidase substrate**

  1 mg of 4-M-U-b-D-N- triacetylchitotrioside was mixed with 57.84 mL of .2M Phosphate 0.1M citrate buffer pH 5.2. Sonicated for 5 minutes.

- **100 mM Glycine-NaOH buffer pH 10.4**

  7.507 g of glycine was dissolved in 800 mL of H_2O_d. The pH was adjusted with 5N NaOH until achieving pH 10.4. Additional H_2O_d was added to reach 1 L.

- **1 mg/mL 4-MU Stock solution**

  25 mg of 4-MU was dissolved in 25 mL of glycine buffer. Stored at -20°C

- **1000 ng/mL 4-MU working solution**

  50 µL of 4-MU Stock solution was dissolved in 50 mL of glycine buffer. The solution was divided into 25 aliquots of 2 mL and stored at -20°C

Standard preparation

Table 6. Preparation of the standard curve points of 1000 ng/mL 4-MU working solution

	Points of the standard curve (ng 4-MU/mL)	Glycine buffer (µL)	1000 ng/mL 4-MU working solution (µL)
P1	400	600	400
P2	200	800	200
P3	100	900	100
P4	50	950	50
P5	25	975	25
P6	10	990	10
P7	5	995	5
P8	0	1000	0

Procedure

ChT activity determination was performed from EDTA plasma stored at -20°C. The experimental analysis was started with incubating 5µL of undiluted plasma with 100 µL of 22 µM M-U-T-A-chitotriosidase fluorogenic substrate for 15 and 30 minutes at 37°C. After the incubation, 1 mL of 100mM Glycine-NaOH buffer, pH 10.4 was used to stop the reaction helped by mixing well each sample. The points of the standard curve were prepared as described in **Table 6**.

The fluorescence was read on 365 nm excitation and 450 nm emission on a fluorimeter. ChT activity was measured in duplicate. Blank samples were prepared in duplicate from H_2O_d and processed as exactly as samples were.

Plasma ChT activity was expressed as nmol/hr/mL based on the following formula:

15 minutes: (fluorescence sample mean – fluorescence blank) · 4.46 = **X_1 nmol/hr/mL**

30 minutes: (fluorescence sample mean – fluorescence blank) · 2.23 = **X_2 nmol/hr/mL**

The concentration of plasma Cht activity was finally obtained using the highest value between the results in 15 and 30 minutes.

Samples with ChT activity lower than 4 nmol/mL/h were considered to be from ChT-deficient individuals and were subsequently excluded from the study group. In this study, plasma ChT activity was below 4 nmol/mL/h in three women of the PABC group.

2.2.5 Determination of protein carbonyl groups by spectrophotometry

Plasma protein carbonyls were determined by derivatising with dinitrophenylhydrazine (DNP). The protein-bound DNP was measured by enzyme-linked immunosorbent assay (ELISA) using the standard BioCell PC test kit method (Biocell Corp, Auckland, New Zealand).

Reagents preparation

All reagents were provided by the test kid and prepared before use according to the indications of the manufacturer.

Standard preparation

Table 7. Standard curve points prepared from serum albumin with five different concentrations of hypochlorous acid-oxidized protein calibrated colourimetrically

	Points of the standard curve (nmol/mg)	Tube colour
P1	0.95	Red/pink
P2	0.60	ºBlue
P3	0.40	Green
P4	0.22	Orange
P5	0.10	Purple
P6	0.00	White/clear

Procedure

The measure of plasma protein carbonyls was made from EDTA plasma stored at -20ºC. A volume of 10 µL of plasma was diluted in 40 µL of DNP solution. After mixing, samples were further incubated at room temperature for 45 minutes, followed by adding 5 µL of each sample to a new 1.5 mL tube containing 1 mL of EIA buffer. Following by a brief mix, 200 µL of each sample in EIA buffer was added to the assigned ELISA-plate wells. The ELISA-plate was covered with sealing tape and left overnight at 4ºC. The next morning, the ELISA-plate was washed with EIA buffer five times using 300 µL per well followed by adding 250 µL of diluted blocking solution per well. After incubation at room temperature for 30 minutes, the ELISA-plate was again washed with EIA buffer as described above and 200 µL of diluted anti-DNP-biotin-antibody was added per well. The ELISA-plate was incubated at 37ºC for 1 hour and washed again with EIA buffer as described above. After adding 200 µL of diluted streptavidin-HRP per well, the ELISA-plate was washed one last time as described above.
Finally, 200 µL of chromatin reagent was added per well to achieve colour development. The reaction was stopped after 5 minutes by adding 100 µL of stopping reagent per well. The absorbance of the samples was read at 450 nm shortly after stopping the reaction on a spectrophotometer. QC samples were provided by the manufacturer and processed as described for plasma samples.
The standard curve was prepared following the same indications but with an established concentration of serum albumin and hypochlorous acid-oxidized protein **(Table 7)**.
The concentration of the carbonyl content of the samples was calculated from a standard curve and expressed as nmol/mg protein.

2.2.6 Determination of YKL-40 by spectrophotometry

Plasma YKL-40 was determined using a specific commercial two-site, sandwich-type, ELISA assay manufactured by Quidel Corporation (San Diego, CA, USA).

Reagents preparation

All reagents were provided by the test kid and prepared before use according to the indications of the manufacturer.

Standard preparation

Table 8. Standard curve points prepared from YKL-40 purified from osteosarcoma MG-63 cells in a buffered solution with stabilizer and sodium azide (0.1%) as a preservative

	Points of the standard curve (ng/mL)
P1	300
P2	200
P3	100
P4	50
P5	20
P6	0

Procedure

The measure of plasma protein carbonyl was made from EDTA plasma stored at -20ºC. Within 30 minutes, 20 µL of each sample was added to each well of the coated strips. Subsequently, 100 µL of capture solution was added to each well with enough force to be sure that the samples were perfectly mixed. After incubating the samples for 1h at room temperature, they were washed four times with 250 µL of 1X wash buffer blotting the strips dry on paper towels after the last wash. Again the samples were incubated for 1h at room temperature after adding 100 µL of reconstituted enzyme conjugate to each well. A series of four washed with 250 µL of 1X wash buffer was made as described above, and 100 µL of working substrate solution was added to each well. Finally, the samples were incubated for 1h at room temperature followed by the addition of 100 µL of stop solution to each well based on the same pattern time intervals as it was employed to add the working substrate solution.

The absorbance of the samples was read at 405 nm within 15 minutes of stopping the reaction on a spectrophotometer. All samples were prepared in duplicate.

The standard curve was prepared following the same indications but with an established concentration of YKL-40 **(Table 8)**.

The levels of YKL-40 in plasma was determined from a standard curve and expressed as ng/mL.

2.3 Biomarker quantification employing Liquid Chromatography coupled to tandem Mass Spectrometry (LC-MS/MS)

Biomarkers of oxidative stress, antioxidant defence and inflammation were determined in the collected plasma, RBC and urine samples employing two LC-MS/MS methods at the Health Research Institute La Fe (*La Fe University and Polytechnic Hospital*).

Reagents and Standards

N-ethylmaleimide (NEM) (≥ 98%), phosphate-buffered saline (PBS), potassium hydroxide (KOH) and β-Glucuronidase from *E. Coli* were obtained from Sigma-Aldrich Quimica SA (Madrid, Spain). Methanol (CH_3OH) (LC-MS grade), CH_3CN (LC-MS grade) and heptane (analytical grade) were from J.T. Baker, (Phillipsburg, NJ, USA). 98% w/w formic acid (HCOOH) and ethyl acetate (analytical grade) were purchased from Panreac (Barcelona, Spain). $H_2O_{mili\text{-}Q}$ was from Millipore purification system.

Analytical standards of 8-OHdG, 2-dG, m-Tyr, o-Tyr, p-Tyr, 3NO2-Tyr, 3Cl-Tyr and Phe (>96% w/w purity) were from Sigma-Aldrich (St Louis, MO, USA). GSH, GSSG, Cystine and Cysteine (>96% w/w purity) and as well as individual internal standards (IS), cystine-D4 and cysteine-D2 (>96% w/w purity) were obtained from Cayman Chemical Co (Ann Arbor, Michigan USA). Other IS employed such as 8-OHdG$^{13}C^{15}N$, Phe-D5 and p-Tyr-D2 were obtained from Toronto Research Chemicals (Toronto, Canada), CDN Isotopes (Pointe-Claire, Canada) and Cambridge Isotope Laboratories (Tewksbury, USA) respectively. Isotopically labelled compounds purities were >98% w/w. GSA was synthesized and purified following the indications published by Hardwood et al[258].

Commercially available standards 2,3-dinor-15-F_{2t}-IsoP, 15-keto-15-E_{2t}-IsoP, 15-keto-15-F_{2t}-IsoP, 15-*epi*-15-F_{2t}-IsoP, 15-E_{2t}-IsoP, 15-F_{2t}-IsoP (IsoPs), PGE_2, $PGF_{2\alpha}$, 2,3-dinor-11β-$PGF_{2\alpha}$, 11β-$PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ (PGs) and 1a,1b-dihomo-$PGF_{2\alpha}$ (dihomo-PG) with ≥95% w/w of purities were purchased from Cayman Chemical Co (Ann Arbor, Michigan USA). Deuterated IS with purities ≥98% and incorporation ≥99% deuterated form (d1-d4);<1%d_0, $PGF2\alpha$-d4 and 15-F2t-IsoP-d4 were obtained as well from Cayman Chemical Co (Ann Arbor, Michigan USA). Additional analytical standards employed included: 5-F_{2t}-IsoP, 5-*epi*-5-F_{2t}-IsoP, 15-*epi*-2,3-dinor-15-F_{2t}-IsoP (F_{2t}-IsoPs), 4-F_{4t}-NeuroP, 4-*epi*-4-F_{4t}-NeuroP, 10-*epi*-10-F_{4t}-NeuroP, 10-F_{4t}-NeuroP, 14(*RS*)-14-F_{4t}-NeuroP (F_{4t}-NeuroPs), 4(*RS*)-ST-Δ^5-8-NeuroF (F_{4t}-NeuroF), 17-F_{2t}-dihomo-IsoP, 17-*epi*-17-F_{2t}-dihomo-IsoP, *ent*-7(*RS*)-F_{2t}-dihomo-IsoP (F_{2t}-dihomo-IsoPs) and 17(*RS*)-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF, 7(*RS*)-ST-Δ^8-11-dihomo-IsoF (F_{2t}-dihomo-IsoFs) were synthesized at the Institut des Biomolecules Max Mosseron (Montpellier, France) with purities ≥99%[259,260].

Materials

SPE-96 well plates (Discovery® DSC-18, 100 mg) were from Sigma-Aldrich (St. Louis, MO, USA). Vortex mixer was from VelpScientifica (Usmate, Italy). Centrifuge Biocen22R was from OrtoAlresa (Madrid, Spain). Thermomixer HLC from Ditabis (Pforzheim, Germany). Speed vacuum concentrator (mi Vac) was from Genevac LTD (Ipswich, UK). 96-well sample plates (Acquity UPLC 700 µL) were from Waters (Barcelona, Spain). Acquity-Xevo TQS system was from Waters (Milford, MA, USA).

2.3.1 Biomarkers of oxidative damage to DNA, proteins, antioxidant defence and inflammation quantification by LC-MS/MS

The first method included redox pairs and metabolites from the transsulfuration pathway (i.e. GSH, GSSG, glutathione sulfonamide (GSA), cystine, cysteine) as well as biomarkers of oxidative damage to proteins (meta-tyrosine (m-Tyr), ortho-tyrosine tyrosine (o-Tyr), 3-nitro-tyrosine ($3NO_2$-Tyr), and 3-chloro-tyrosine (3Cl-Tyr)) and their precursors (para-

tyrosine tyrosine (p-Tyr) and phenylalanine (Phe)), and a biomarker of oxidative damage to DNA and its precursor (8-OHdG and 2'-deoxyguanosine (2-dG), respectively)[258,261–263].

Reagents preparation

- **10 mM NEM / 50 mM NEM**

  g (NEM) were obtained from $10 \cdot 10^{-03}$ / $50 \cdot 10^{-03}$ mM · 125.13 g/L · X of PBS, being X the number of samples to be tested.

- **IS (working solution)**

  2 mL of IS was prepared by mixing appropriate volumes of 5 µM 8-OHhdG-C13, 10 µM p-Tyr-D2, 50 µM Cystine-D4, 5 µM Cysteine-D2 and 10 µM Phe-D5 solutions in H_2O:CH_3CN 97:3 v/v with 0.1% HCOOH solution. The aliquots obtained were stored in capped amber vials at -20°C.

- **Standard (working solution)**

  2 mL of standard was prepared by mixing appropriate volumes of 50 µM GSH-NEM, 20 µM GSA, 20 µM Cystine, 200 µM Cysteine-NEM, 100 µM GSSG, 5 µM 8OhdG, 5 µM m-Tyr, 10 µM 2-dG, 20 µM 3I-Tyr, 10 µM o-Tyr, 10 µM 3NO2-Tyr, 20 µM 3Cl-Tyr, 500 µM p-Tyr and 500 µM Phe solutions in H_2O:CH_3CN 97:3 v/v with 0.1% HCOOH solution. The aliquots obtained were stored in capped amber vials at -20°C. For each experiment one aliquot was used for preparing the calibration standards as shown in **Table 9**.

- **H_2O:CH_3CN 97:3 v/v with 0.1% HCOOH**

  3 mL of CH_3CN was diluted in 97 mL of $H_2O_{mili\text{-}Q}$ and 100 µL of HCOOH

Standard preparation

Standards were prepared by serial dilution of the working solution. The final concentrations were the result of mixing 65 µ of each with 5 µL of IS before LC-MS/MS analysis.

Table 9. Preparation of the standard solutions according to their individual concentrations.

	POINTS OF THE STANDARD CURVE [NM]							$H_2O:CH_3CN$ 97:3 v/v with 0.1% HCOOH [µL]	Standard working solution [µL]
	GSH-NEM	GSA, Cystine, 3Cl-Tyr	Cysteine-NEM	GSSH	8-OHdG, m-Tyr	2-dG, o-Tyr, $3NO_2$-Tyr	p-Tyr, Phe		
P1	9285.71	3714.29	37142.86	18571.43	928.57	1857.14	92857.14	400	100 of working solution
P2	6964.29	2785.71	27857.14	13928.57	696.43	1392.86	69642.86	25	75 of P1
P3	4642.86	1857.14	18571.43	9285.71	464.29	928.57	46428.57	100	100 of P1
P4	2321.43	928.57	9285.71	4642.86	232.14	464.29	23214.29	100	100 of P3
P5	1160.71	464.29	4642.86	2321.43	116.07	232.14	11607.14	100	100 of P4
P6	580.36	232.14	2321.43	1160.71	58.04	116.07	5803.57	100	100 of P5
P7	290.18	116.07	1160.71	580.36	29.02	58.04	2901.79	100	100 of P6
P8	145.09	58.04	580.36	290.18	14.51	29.02	1450.89	100	100 of P7
P9	72.54	29.02	290.18	145.09	7.25	14.51	725.45	100	100 of P8
P10	36.27	14.51	145.09	72.54	3.63	7.25	362.72	100	100 of P9
P11	18.14	7.25	72.54	36.27	1.81	3.63	181.36	100	100 of P10
P12	9.07	3.63	36.27	18.14	0.91	1.81	90.68	100	100 of P11
P13	4.53	1.81	18.14	9.07	0.45	0.91	45.34	100	100 of P12

Procedure for Plasma samples

Plasma samples were thawed on ice, homogenized on a Vortex® mixer for 10 seconds, and 100 μL of plasma was added to 100 μL of 10 mM NEM solution in PBS. A volume of 5 μL of 5 μM IS solution was added followed by 600 μL of cold (4 ^{o}C) methanol. Samples were centrifuged at 10,000 x *g* at 4 ^{o}C for 10 minutes, and 600 μL of supernatant were collected and evaporated to dryness on a miVac centrifugal vacuum concentrator. After that, sample extracts were reconstituted in 70 μl of H_2O:CH_3CN 97:3 v/v with 0.1% HCOOH solution followed by centrifugation at 10,000 x *g* at 4 ^{o}C for 10 minutes.
Supernatants were withdrawn and injected into the LC-MS/MS system (for the detection of low abundance compounds) and diluted (10 μL + 90 μL) with H_2O:CH_3CN 97:3 v/v with 0.1% HCOOH solution before analysis (for the detection of high abundance compounds).

Procedure for RBC samples

RBCs were thawed on ice and homogenized on a Vortex® mixer for 10 seconds. A volume of 100 μL of RBCs was added to 350 μL of 50 mM NEM solution in PBS. Subsequently, samples were frozen (-80 ^{o}C) and defrosted (room temperature) three times for cellular lysis, followed by the addition of 5 μL of 5 μM IS solution and centrifugation at 10,000 x *g* at 4 ^{o}C for 10 minutes. After centrifugation, 300 μL of supernatant was mixed with 900 μL of cold (4 ^{o}C) methanol. Samples were again centrifuged at 10,000 x *g* at 4 ^{o}C for 10 minutes and 900 μL of supernatant was collected and evaporated to dryness on a miVac centrifugal vacuum concentrator from Genevac LTD (Ipswich, UK). Sample extracts were reconstituted in 70 μl of H_2O:CH_3CN 97:3 v/v with 0.1% HCOOH solution. Extracts were centrifuged at 10,000 x *g* at 4 ^{o}C for 10 minutes. Finally, supernatants were withdrawn and injected into the LC-MS/MS system (for the detection of low abundance compounds) and diluted (1 μL + 200 μL) with H_2O:CH_3CN 97:3 v/v with 0.1% HCOOH solution before analysis (for the detection of high abundance compounds).

Procedure for Urine samples

Urine samples were thawed on ice, homogenized on a Vortex® mixer for 10 seconds and centrifuged at 15,000 x *g* at 4 ^{o}C for 10 minutes. Supernatants were withdrawn and diluted (90 µL + 10 µL) in IS solution before being injected into the LC-MS/MS system A QC sample was prepared by mixing 5 µL of each study sample.

Procedure for Quality Control and Blank samples and Standard solutions

A QC sample was prepared by mixing 5 µL of each study sample. The QC samples, as well as a blank extract, prepared with H_2O:CH_3CN 97:3 v/v with 0.1% HCOOH solution, were processed as described for plasma, RBC or urine samples but in triplicate. They were injected in random order. The standard solutions were prepared on each measurement day from the standard working solution by serial dilution in H_2O:CH_3CN 97:3 v/v with 0.1% HCOOH solution **(Table 9)**.

Procedure for LC-MS/MS analysis

LC-MS/MS analysis was carried out on an Acquity-Xevo TQS system from Waters (Milford, MA, USA) operating in positive electrospray ionization mode (ESI^+) using the following instrumental conditions: capillary voltage 2.0 kV, source temperature 150 ^{o}C, desolvation temperature 380 ^{o}C and nitrogen cone and desolvation gas flows of 150 and 800 L h^{-1}, respectively. Chromatographic separation of the studied metabolites was achieved employing a Waters BEH C_8 reversed-phase column (2.1 x 100 mm, 1.7 µm). A binary mobile phase gradient H_2O (0.1%v/v HCOOH):CH_3CN (0.1%v/v HCOOH) with a total runtime of 6.0 minutes was run as follows: from 0.0 to 1.25 minutes 1% v/v CH_3CN (0.1%v/v HCOOH) (i.e mobile phase channel B); between 1.25 to 4.75 minutes conditions were held constant at 1%B; from 4.75 to 5.0 %B increased up to 98%; between 5.0 to 5.1 conditions were held constant at 98%B: from 5.1 to 6.0 followed by the return to initial conditions (i.e 1%B). Flow rate, column temperature and injection volume were set at 400 mL min^{-1}, 55 ^{o}C, and 4 µL, respectively.

MS detection was carried out operating in multiple reaction monitoring (MRM) mode with the acquisition parameters displayed in **Table 10**.

Biomarkers were quantified employing linear regression lines with 1/x weighting from the standard signals normalized with an IS. Concentrations found <LOQ or peaks with a retention time shift >0.05 minutes in comparison to a standard solution were discarded. Blank samples and solvent blanks were analysed at the beginning of the analytical batch and repeatedly along with the batch in order to check for column carry-over and cross-contamination. The signal intensity of the QC sample was used to detect deviations inaccuracy and/or precision. An analysis batch was accepted if at least 75% of the values found for the QC samples were within ±25% of their respective nominal values.

For data acquisition and processing, MassLynx 4.1 and QuanLynx 4.1 software (Waters, Milford, MA, USA) were employed, respectively. Biomarker concentrations detected below the limit of quantification (LOQ) were replaced by ½ LOQ prior to data analysis. Linear or quadratic response curves were calculated for each analyte correcting with peak areas of their corresponding IS.

Table 10. Mass spectrometric parameters and chromatographic windows selected for biomarkers of oxidative stress

Analyte	m/z parent ion	CONE [v]	CE [ev]	*m/z* daughter ion	*Internal standard*
Phe	166.10	20	20	91.00	Phe-D_5
Phe-D_5	171.50	30	20	125.00	-
m-Tyr	182.10	20	10	91.00	p-Tyr-D_2
o-Tyr	182.10	20	10	91.00	p-Tyr-D_2
p-Tyr	182.10	20	10	91.00	p-Tyr-D_2
p-Tyr-D_2	184.10	20	10	138.10	-
3Cl-Tyr	216.00	30	15	170.00	p-Tyr-D_2
3NO_2-Tyr	227.10	25	10	181.00	Phe-D_5
Cystine	241.20	20	15	120.00	Cystine-D_4
Cystine-D_4	245.00	20	15	199.00	-
Cysteine	247.10	30	20	158.10	Cysteine-D_2
Cysteine-D_2	249.00	30	15	232.00	-
2dG	268.00	25	15	152.00	8-OHdG^{13}C-^{15}N
8-OHdG	284.00	30	15	168.00	8-OHdG^{13}C-^{15}N
8-OHdG^{13}C-^{15}N	287.00	30	15	171.00	-
GSA	338.00	45	25	155.10	p-Tyr-D_2
GSH	433.10	25	20	201.00	Phe-D_5
GSSG	613.20	50	35	355.00	p-Tyr-D_2

2.3.2 Biomarkers of oxidative damage to lipids and inflammation quantification by LC-MS/MS

The second method covered a panel of various biomarkers of oxidative damage to lipids and inflammation [264–267].

<u>*Reagents preparation*</u>

- **15% KOH**

  g (KOH) were obtained from (15% ·X mL of H_2O_{miliQ})/100% being X the number of samples to be tested.

- **β-Glucuronidase solution**

  10 mg of β-Glucuronidase from *E. Coli* in 1 mL of H_2O_{miliQ}

- **H_2O_{miliQ} (0.1% HCOOH)**

  100 ml H_2O_{miliQ} were mixed with 100 µL of HCOOH

- **20 µM IS (working solution)**

  2 mL of IS was prepared by mixing appropriate volumes of 20 µM of $PGF_{2\alpha}$-d4 and 20µM of 15-F_{2t}-IsoP-d4. The aliquots obtained were stored in capped amber vials at -20ºC.

- **Standard (working solution)**

  1 mL of standard was prepared by mixing adequate volumes of the individual stock solutions followed by evaporation to dryness and dissolution in H_2O:CH_3OH 85:15 v/v with 0.1% HCOOH. The aliquots obtained were stored in capped amber vials at -20ºC. For each experiment one aliquot was used for preparing the calibration standards as shown in **Table 10**.

- **H_2O:CH_3CN 97:3 v/v with 0.1% HCOOH**

  3 mL CH_3CN was diluted in 97 mL H_2O_{mili-Q} and 100 µL HCOOH

- **H_2O:CH_3OH 85:15 v/v with 2.8% HCOOH**

  3 mL CH_3OH was diluted in 97 mL H_2O_{mili-Q} and 100 µL HCOOH

- **H_2O:CH_3OH 85:15 v/v with 0.1% HCOOH**

3 mL CH_3OH was diluted in 97 mL H_2O_{mili-Q} and 100 µL HCOOH

Standard preparation

Creatinine standards were also prepared by serial dilution of the working solution **(see Table 11)** only to normalize urine samples.

Table 11. Preparation of the standard curve points of creatinine

	Points of the standard curve [nM]	H_2O_{miliQ} [µL]	Creatinine standard [100 mg/dL]
P1	20	800	200 of 100 mg/dL Creatinine standard
P2	10	500	500 of P1
P3	5	500	500 of P2
P4	2.50	500	500 of P3
P5	1.25	500	500 of P4
P6	0.63	500	500 of P5
P7	0.31	500	500 of P6

Standards were prepared by serial dilution of the working solution. The final concentrations were the result of mixing 57 µ of each with 3 µL of IS before LC-MS/MS analysis.

Table 12. Preparation of the standard solutions according to their individual concentrations

	Points of the standard curve (µM)	H_2O:CH_3OH 85:15 v/v with 0.1% HCOOH [µL]	Standard (working solution) [µL]
P1	4750.00	-	200 of 5 µM working solution
P2	3325.00	30	70 5 µM Mix
P3	2375.00	100	100 of P1
P4	1187.50	100	100 of P3
P5	593.75	100	100 of P4
P6	296.88	100	100 of P5
P7	148.44	100	100 of P6
P8	74.22	100	100 of P7
P9	37.11	100	100 of P8
P10	18.56	100	100 of P9
P11	9.28	100	100 of P10
P12	4.64	100	100 of P11
P13	2.32	100	100 of P12

Procedure for Plasma samples

Plasma samples were thawed on ice, homogenized on a Vortex® mixer for 10 seconds, and 100 µL of plasma was added to 100 µL of 15% KOH for hydrolysis followed by incubation at 40 ^{o}C for 30 minutes with gentle agitation in a thermomixer. After the addition of 3 µL of 20 µM IS, hydrolysed samples were diluted with 697 µL of H_2O:CH_3OH 85:15 v/v with 2.8% HCOOH solution, homogenized and placed on ice for 10 minutes followed by centrifugation at 16,000 *x g* at 4 ^{o}C for 10 minutes.

Solid-phase extraction (SPE) employing Discovery® DSC-18 SPE 96-well plates was performed as follow: SPE cartridges were conditioned with 1 mL of CH_3OH and 1 mL H_2O_{miliQ} (0.1% v/v HCOOH, pH 3). Subsequently, 900 µL of each sample was loaded into their corresponding SPE wells. Each well was rinsed with 1 mL of H_2O_{miliQ} (0.1% v/v HCOOH, pH 3) and 500 µL of heptane during the washing steps. Cartridges were dried with room air, and sample extracts were eluted with 100 µL ethyl acetate four times followed by evaporation using a miVac centrifugal vacuum concentrator from Genevac LTD (Ipswich, UK). Finally, sample extracts were dissolved in 60 µL of H_2O:CH_3OH 85:15 v/v with 0.1% HCOOH solution.

Procedure for Urine samples

Urine samples were thawed on ice, homogenized on a Vortex® mixer for 10 seconds. The glucuronide conjugates were hydrolysed by adding 10 µL of 10 mg/mL β-Glucuronidase from E.coli to 600 µL of sample to deconjugate glucuronides of IsoPs. The mixture was then incubated at 37 ^{o}C for 90 minutes. Afterwards, a volume of 3 µL 20 µM IS was added to hydrolysed samples followed by dilution in 297 µL of H_2O:CH_3OH 85:15 v/v with 0.1% HCOOH solution. Before clean-up and pre-concentration of the samples, they were centrifuged at 16,000 x g at 4 ^{o}C for 10 minutes. SPE was accomplished as described previously for plasma samples.

Creatinine levels were quantified following the modified Jaffe method implemented in the DetectX® urinary creatinine detection kit from Arbor Assays (Ann Arbor, MI, USA) according to the manufacturer's instructions. In brief, 50 µL of each urine sample was

centrifuged at 16,000 x g at 4 °C for 10 minutes. A volume of 20 µL of supernatant was collected and diluted in 80 µL of H_2O_{mili-Q}. Afterwards, 50 µL of diluted samples were mixed in 100 µL of DetectX® Creatinine Reagent pipetted into each well. Finally, samples were incubated at room temperature for 30 minutes in agitation using a thermomixer, The absorbance of each well was measured at 490 nm in a plate reader, and the standards curve **(Table 11)** was used to determine the amount of creatinine in urine expressed as mg/L. Standard and blank sample, prepared by replacing urine with H_2O_{mili-Q}, were processed as described for urine samples. The standard curve points were analysed in duplicate.

Procedure for Quality Control and Blank samples and Standard solutions

A volume of 5 µL of each study sample was mixed to prepare QC samples whereas blank extracts were prepared with H_2O:CH_3CN 85:15 v/v with 0.1% HCOOH solution both in triplicate. QC samples and blank extracts were processed as described for plasma or urine samples and randomly injected.

The standard solutions were prepared prior each experimental day from the standard working solution by serial dilution in H_2O:CH_3CN 97:3 v/v with 0.1% HCOOH solution **(Table 12)**.

Procedure for LC-MS/MS analysis

Samples were processed according to a validated method on the same LC-MS/MS system as described for the first method.

Acquity-Xevo TQS system using negative electrospray ionization (ESI^-) was employed for LC-MS/MS analysis. Instrumental conditions were determined as follows: capillary voltage 2.9 kV, source temperature 150 °C, desolvation temperature 395 °C and nitrogen cone and desolvation gas flows of 150 and 800 L h^{-1}, respectively. Flow rate, column temperature and injection volume were set at 450 mL min^{-1}, 45 °C, and 9 µL, respectively. Chromatographic separation of the studied metabolites was achieved employing a Waters BEH C_8 reversed-phase column (2.1 x 100 mm, 1.7 µm). A binary mobile phase gradient H_2O (0.1%v/v HCOOH):CH_3CN (0.1%v/v HCOOH) with a total runtime of 7.0

minutes was run as follows: from 0.0 to 0.1 minutes 15% v/v CH_3CN (0.1%v/v HCOOH) (i.e mobile phase channel B); from 0.1 to 5.0 minutes %B increased up to 40%; from 5.0 to 6.0 minutes %B increased up to 75%; between 6.0 to 6.15 conditions were held constant at 75% B followed by the return to initial conditions (i.e 15% B) between 6.15 to 6.25 minutes. Conditions were maintained for 0.75 minutes for system re-equilibration.

MS detection was carried out operating in MRM mode with the acquisition parameters displayed in Table 12. The quantification of biomarkers was carried out by employing linear regression lines with 1/x weighting from the standard signals normalized with an IS. Concentrations found <LOQ or peaks with a retention time shift bigger than ± 0.05 minutes in comparison to a standard solution were not further accepted.

Blank samples and solvent blanks were analysed at the beginning of the sample batch, after a high concentration standard and repeatedly along with the batch in order to check for column carry-over and cross-contamination. The signal intensity of the QC sample was employed to detect deviations inaccuracy and/or precision. An analysis batch was accepted if at least 75% of the values found for the QC samples were within ±25% of their respective nominal values.

MassLynx 4.1 and QuanLynx 4.1 software were used for data acquisition and processing, respectively. Biomarker concentrations detected below LOQ were replaced by ½ LOQ prior to data analysis. Linear or quadratic response curves were calculated for each analyte correcting with peak areas of their corresponding IS.

All compounds detected in urine samples were normalized by creatinine and expressed as nmol/g creatinine.

Table 13. Mass spectrometric parameters and chromatographic windows selected for biomarkers of lipid peroxidation and inflammation

Analyte	*m/z* parent ion	CONE [v]	CE [ev]	*m/z* daughter ion	Internal standard
5-F_{2t}-IsoP + 5-*epi*-5-F_{2t}-IsoP	352.20	35	30	115.00	$PGF_{2\alpha}$-d_4
15-*epi*-2,3-dinor-15- F_{2t}-IsoP + 2,3-dinor-11β-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$ -IsoP	325.27	40	13	237.00	15-F_{2t}-IsoP-d_4
4-F_{4t}-NeuroP + 4-*epi*-4-F_{4t}-NeuroP	377.32	20	19	271.12	$PGF_{2\alpha}$-d_4
10-*epi*-10-F_{4t}-NeuroP	377.32	10	19	153.00	$PGF_{2\alpha}$-d_4
10-F_{4t}-NeuroP	377.32	10	19	153.00	15-F_{2t}-IsoP-d_4
14(*RS*)-14-F_{4t}-NeuroP	377.32	50	19	204.89	$PGF_{2\alpha}$-d_4
4(*RS*)-ST-Δ^5-8-NeuroF	393.60	40	35	123.19	$PGF_{2\alpha}$-d_4
17-F_{2t}-dihomo-IsoP + 17-*epi*-17-F_{2t}-dihomo-IsoP	381.30	20	25	337.15	$PGF_{2\alpha}$-d_4
***ent*-7(*RS*)-F_{2t}-dihomo-IsoP**	381.30	50	25	142.98	$PGF_{2\alpha}$-d_4
17(*RS*)-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF	397.40	20	31	155.02	$PGF_{2\alpha}$-d_4
7(*RS*)-ST-Δ^8-11-dihomo-IsoF	397.40	40	25	201.03	$PGF_{2\alpha}$-d_4
15-keto-15-F_{2t}-IsoP	351.00	35	25	289.00	$PGF_{2\alpha}$-d_4
1a,1b-dihomo-$PGF_{2\alpha}$	381.30	20	25	337.15	15-F_{2t}-IsoP-d_4
15-F_{2t}-IsoP	353.00	35	30	193.00	15-F_{2t}-IsoP-d_4
15-E_{2t}-IsoP	351.00	35	30	271.00	$PGF_{2\alpha}$-d_4
11β-$PGF_{2\alpha}$	353.00	35	30	193.00	15-F_{2t}-IsoP-d_4
15-*epi*-15-F_{2t}-IsoP	353.00	35	30	193.00	15-F_{2t}-IsoP-d_4
6-keto-$PGF_{1\alpha}$	369.00	40	35	245.00	15-F_{2t}-IsoP-d_4
$PGF_{2\alpha}$	353.00	35	30	193.00	$PGF_{2\alpha}$-d_4
15-keto-15-E_{2t}-IsoP	349.00	40	30	113.00	$PGF_{2\alpha}$-d_4
PGE_2	351.00	35	30	271.00	$PGF_{2\alpha}$-d_4
$PGF_{2\alpha}$-d_4	357.00	40	30	197.00	-
15-F_{2t}-IsoP-d_4	357.00	40	30	197.00	-

2.4 Statistical analysis

Previous data analysis, the concentrations obtained from all biomarkers (except for those corresponding to lipid damage, GSA and PGs quantification) were normalised using Min-Max scaling with a range between 1 and 2. Statistical analysis was performed employing R version 3.3.3 (2017-03-06). The package ggplot2 version 3.1.0 (CRAN repository) and was installed in R for the development of doughnuts and bars charts and boxplots. In addition, corrplot version 0.84 (CRAN repository) was installed for Spearman correlation analysis.

Continuous variables were expressed as mean ± standard deviation or medians with the interquartile range depending on underlying distributions. Shapiro-wilk test was used to evaluate whether samples came from a normally distributed population. Student's t-test was applied when samples followed a normal distribution, whereas Wilcoxon rank-sum test was used when samples did not meet the assumption of normally distributed data. Student's t paired and paired Wilcoxon signed-rank test were used for dependent data. A two-tailed p-value less than 0.05 was considered significant. *P value <0.05, **P value < 0.01, ***P value < 0.001.

Heatmaps were used to illustrate the clustering of those differentiating biomarkers across the comparative groups. Principal component analysis (PCA) was additionally employed for score plots formation to visualize the distribution of the significant groups.

A multivariate logistic regression analysis was employed to establish an association between patient status (disease-free or metastatic) with other "clinical variables" including reproduction technique (yes/no), hormone receptor (positive/negative), HER2/neu (positive/negative), parity (nulliparous/parous), gestational age at delivery (pre-term/full-term) and clinical stage (stage I+ stage II/stage III+ stage IV).

To evaluate associations between clinical stage (stage I+ stage II/stage III+ stage IV) and status (disease-free or metastatic) with the levels of each metabolite analysed in PABC patients before chemotherapy and at labour respectively, we conducted a univariate logistic regression model. In addition, a univariate logistic regression was used to find associations between the mode of delivery and each metabolite measured from PABC

patients and neonates exposed to chemotherapy *in utero* at birth. A multivariate logistic regression analysis was not employed when the data from metabolites presented missing values (study IA); or when de the number of independent/explanatory variables (number of metabolites) were higher than the number of observations (patients), such us in the study IB.

Multivariate linear regression was employed to describe associations between each metabolite measured in PABC patients before chemotherapy with the following independent variables: patient status (disease-free or metastatic) and reproduction technique (yes/no), hormone receptor (positive/negative), HER2/neu (positive/negative), parity (nulliparous/parous), gestational age at delivery (pre-term/full-term) and clinical stage (stage I+ stage II/stage III+ stage IV). The evaluation for an association between birth weight and the levels of each metabolite analysed in PABC patients and neonates exposed to chemotherapy *in utero* at birth was made using a univariate linear regression model.

The correlations between the plasma and RBCs levels of each metabolite measured before chemotherapy, following anthracyclines and at labour in PABC patients (study IA); the plasma and RBCs levels of each metabolite measured before chemotherapy and at labour in PABC patients (study IB) and the plasma, RBCs and urinary levels of each metabolite measured in neonates exposed to chemotherapy (study II) with their antioxidant capacitu (GSH/GSSG) was determined by Spearman correlation analysis.

VI. RESULTS

STUDY I: *Evaluation of oxidative stress, inflammation and antioxidant defence biomarkers in PABC patients undergoing chemotherapy treatment during pregnancy*

1.1 Demographic characteristics of the study population

A total of seventeen pregnant (PABC) and ten non-pregnant women diagnosed with breast cancer (non-PABC) at the Vall d´Hebron University Hospital were enrolled in this study. The median age of PABC patients and non-PABC patients was 38 years (mean, 36.91 years; range, 26–44 years) and 41 years (mean, 38.88 years; range, 27–47 years) respectively.

Sixteen healthy pregnant women with a median age of 34 years (mean, 33.66 years; range 24–45 years) were admitted in the study as a pregnant control group. All of them were in their second and third trimester with a median gestational age of 30.6 weeks (mean, 30.3 weeks; range 23.5–41 week) at the moment of the inclusion.

1.2 Oncologic characteristics of the study populations

PABC women were at least treated at 21-day intervals with chemotherapy containing anthracycline-based regimens during the second and third trimester of their pregnancy. In particular, four patients received two cycles of anthracyclines, two patients, three cycles, eight patients, four cycles, two patients, five cycles and one patient six cycles. Paclitaxel was subsequently administrated in seven PABC patients weekly. One patient received two cycles of paclitaxel, one patient four cycles, two patients, five cycles, two patients, eight cycles and the last patient ten cycles.

Non-PABC women received four cycles of anthracycline-based chemotherapy administered at 21-day intervals.

Women with PABC presented more advanced disease at diagnosis with tumours of higher grade and had a worse immuno-histological phenotype with a higher frequency of negative hormone receptors and higher frequency of HER/neu overexpression than non-PABC patients. Only 29.41% of PABC patients had a tumour of grade II, whereas the majority of non-PABC patients were diagnosed with that grade (70%). Grade III was the

most common histological grade accounting for 64.71% of all cases in contrast with the 30% of non-PABC patients. Likewise, PABC patients showed advanced clinical stages. Four patients had stage I (23.53% IA), four had stage II (23.53% IIA; 29.41% IIB), three had stage III (5.88% IIA; 11.76% IIIB), and only one had stage IV (5.88%). Opposite, three and seven of non-PABC patients had stage I (30% IA) and stage II (50% IIA; 20% IIB) respectively.

Hormonal receptors status differs from both groups. Although most of PABC patients presented positive ER and negative PR receptors, four had negative ER receptor (47.06%), and seven had positive PR receptor (45%). However, all non-PABC patients were positive for ER and PR receptor. Despite eight PABC patients (47.06%) and two non-PABC patients (20%) showed HER2/neu overexpressed, usually was negative in both groups (52.94% and 80% respectively).

Clinical genetic testing for inherited breast cancer risk was performed in all candidates that required it. Two different PABC patients were positive for BRCA1 (9%) and PALB2 (9%). On the other hand, a BRCA2 gene mutation was identified in three non-PABC patients, whereas PALB2 gene was found mutated in two patients.

Chemotherapy was the first-line therapeutic option after breast cancer diagnosed either in PABC or non-PABC patients. Neoadjuvant chemotherapy was administered in ten PABC patients (58.82%) and five non-PABC patients (50%). As for surgery, tumorectomy was the first therapeutic option in the remaining PABC patients (71.43%) with sentinel node removed in four cases (66.67%) and axillary lymphadenectomy in two (33.33%). On the opposite, mastectomy was the preferred surgical procedure in non-PABC patients accounting for 80% of all cases whilst only 20% of patients underwent tumorectomy. Among these non-PABC patients, two had axillary lymphadenectomy (40%) and three sentinel node removed (60%).

All these features are summarized in **table 14**.

Table 14. Oncologic characteristics of PABC and non-PABC patients

VARIABLE	PABC N=17	PABC %	non-PABC N=10	non-PABC %
Age median	37.55	(26-44)	39.50	(27-47)
Tumour type				
IDC	15	88.24%	9	90%
ILC	2	11.76%	1	10%
Other	0	0	0	0
Histological grade				
I	0	0	0	0
II	5	29.41%	7	70%
III	11	64.71%	3	30%
NA	1	5.88%	0	0
Clinical staging				
IA	4	23.53%	3	30%
IB	0	0	0	0
IIA	4	23.53%	5	50%
IIB	5	29.41%	2	20%
IIIA	1	5.88%	0	0
IIIB	2	11.76%	0	0
IV	1	5.88%	0	0
ER status[¥]				
Negative	8	47.06%	0	0
Positive	9	52.94%	10	100%
PR status[¥]				
Negative	10	58.82%	0	0
Positive	7	41.18%	10	100%
HER2/neu overexpression[ζ]				
No	9	52.94%	8	80%
Yes	8	47.06%	2	20%
Genetic status				
BRCA1 positive	2	11.76%	0	0
BRCA2 positive	0	0	3	30%
PALB2 positive	1	5.88%	2	20%
Surgery before Chemotherapy				
No	10	58.82%	5	50%
Yes	7	41.18%	5	50%
Breast surgery				
Mastectomy	2	11.76%	4	80%
Tumorectomy	5	29.41%	1	20%

Continue on next page

Table 14. *Continuation*

VARIABLE	PABC N=17	PABC %	non-PABC N=10	non-PABC %
Axillary surgery				
Axillary lymphadenectomy	2	11.76%	2	40%
Sentinel node	4	23.53%	3	60%
Patient status			-	-
Disease-free	13	76.47%	-	-
Metastatic	4	23.53%	-	-

Abbreviation. NA, not available; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma
¥ ER or PR score >10%
ζ HercepTest positive (+++) or fluorescence in situ hybridization (FISH) positive for HER2/neu

1.3 Demographic and Obstetric characteristics of the study populations

Clinical and obstetric information of PABC patients and controls is listed in **Table 15**.
Most PABC diagnoses were made after the second trimester of pregnancy (mean = 15.70 weeks; range 4-29.5 weeks) allowing not postponing excessively the initiation of chemotherapy treatment (mean= 21.58 weeks; range 13.3-32 weeks).

The mode of conception was principally spontaneous in PABC patients (64.71%), while the remaining four needed assisted conception treatments (35.39%). As a result, four patients were pregnant with twins (23.53%). However, all women from the control group conceived spontaneously and carried one baby (100%).
According to the classification of the mode of delivery, in sixteen out of seventeen PABC patients, labour induction was recommended to facilitate the continuation of chemotherapy treatment as soon as possible (94.12%). Nine underwent vaginal delivery (52.94%) while the remaining eight patients underwent caesarean section (47.06%). On the other hand, eight out of sixteen controls entered labour spontaneously (50%) whereas only four were induced (24%). There was no available data for the remaining controls. Although vaginal delivery was preferred in eight controls (66.67%), elective caesarean section was performed in four (33.33%).
As it can be observed in **Table 15**, the majority of neonates exposed to chemotherapy *in utero* were born prematurity, whereas neonates from control mothers mostly were born full term. The mean gestational age at delivery was 35.80 weeks (range 34-38.5 weeks)

and 39.25 weeks (range, 36.6 to 41.3 weeks) in PABC patients and controls, respectively (*P* value= 1.34e-05).

As a consequence, neonates born to PABC patients had statistically significant lower weight than those from control mothers (*P* value= 1.75e-05). The mean birth weight in neonates exposed to chemotherapy *in utero* was 2356.37 g (range 1315-3350 g) with a mean weight percentile of 24.50 (range 1-99; percentile of one neonate was not available). As a result, one neonate was born with low birth weight (5.88%), and two were SGA (11.76%). Regarding neonates born to controls, the mean birth weight was 3241.86 g (range 2250-3710 g) with a mean weight percentile of 37.38 (range 2-74).

On the other hand, one PABC patients suffered from RCIU and two from PPROM, whereas among women included in the control group, one of them was diagnosed with RCIU. Also, one neonate was diagnosed with transient leukopenia at birth.

Table 15. Clinical and obstetric information of PABC and control women

	PABC		**Control**	
CHARACTERISTIC	*N*=17	%	*N*=16	%
Maternal age (yr)	36.91 ± 4.93	(26-44)	33.66 ± 5.51[Ω]	(24-45)
GA at diagnostic (wk)	15.70 ± 7.50	(4-29.5)	-	-
GA first dose (wk)	21.58 ± 5.91	(13.3-32)	-	-
Conception				
Spontaneous	11	64.71%	13	81.25%
Assisted	6	35.39%	0	-
NA	-	-	3	18.75%
Parity				
Nulliparous	13	76.47%	12	75%
Parous	4	23.53%	1	6.25%
NA	-	-	3	18.75%
Mode of delivery				
Spontaneous	1	5.88%	8	50%
Induced	16	94.12%	4	25%
Vaginal delivery	9	52.94%	8	66.67%
Caesarean section	8	47.06%	4	33.33%
NA	-	-	4	25%

Continue on next page

Table 15. *Continuation*

	PABC		Control	
CHARACTERISTIC	*N*=17	%	*N*=16	%
GA at delivery (wk)***	35.80 ± 1.54	(34-38.5)	39.23 ± 1.70Ω	(36.6-41.3)
Preterm	13	76.47%	1	6.25%
Term	4	23.53%	11	68.75%
Birth weight (g)***	2356.37 ± 447.99	(1315-3350)	3222.69 ± 436.11Ω	(2250-3710)
Birth weight (p)	24.50 ± 32.38	(1-99)	36.36 ± 26.23Ω	(2-85)
1 min Apgar	8.64 ± 0.58	(7-9)	9	(9)
5 min Apgar	9.58 ± 0.86	(7-10)	10	(10)
Low birth weight	1	5.88%	0	-
SGA	2	11.76%	0	-
RCIU	1	5.88%	1	8.33%
PPROM	2	11.76%	0	-
Transient leukopenia	1	5.88%	0	-

Abbreviation. GA, gestational age; yr, year; wk, weeks; g, gram; p, percentile;
Ω Data available of twelve women from control group
Statistical analysis was made by Student's t test. **P* ≤0.05; ***P* ≤0.01 ****P* ≤0.001

1.4 Toxicity and Obstetric complications associated with chemotherapy treatment

Side effects caused by the administration of chemotherapy were registered after each cycle of treatment. Except for one patient that suffered from mucositis grade 2, the remaining toxicities were classified as grade 1. Toxicities associated with chemotherapy treatment are shown in **Table 16**.

Asthenia was the most frequent complication registered in PABC patients (64.71%) followed by mucositis (35.39%) and nausea and constipation (29.41%). Itching, anaemia, neutropenia, heartburn, gastritis, and diarrhoea (23.53%) were also recurrent in PABC patients. Chemotherapy-induced anaemia diagnosed in four patients was treated with oral iron supplements and intravenous iron. Others less common complications were diarrhoea and vomiting (17.65%) and xeroderma, thrombocytopenia, fever, sinus tachycardia, conjunctivitis and anorexia (5.88%). Oppositely, non-PABC patients had barely side effects induced by the administration of chemotherapy. Nausea was the principal complication (60%) succeeded by asthenia and constipation (20%) and neutropenia, gastritis and vomiting (10%).

Some obstetric complications were also registered in PABC patients including gestational diabetes mellitus, suspicion of foetal anaemia and vaginal bleeding (11.76%) and as previous reported, preterm labour (76.47%) **(Table 17)**.

Table 16. Complications related to chemotherapy reported in PABC and non-PABC patients

	PABC		Non-PABC	
TOXICITY	*N*=17	%	*N*=10	%
Itching	4	23.53%	0	-
Nausea	5	29.41%	6	60%
Xeroderma	1	5.88%	0	-
Anaemia	4	23.53%	0	-
Neutropenia	4	23.53%	1	10%
Thrombocytopenia	1	5.88%	2	20%
Asthenia	11	64.71%	0	-
Mucositis	6	35.39%	0	-
Diarrhoea	3	17.65%	0	-
Heartburn	4	23.53%	2	20%
Constipation	5	29.41%	1	10%
Gastritis	4	23.53%	1	10%
Vomiting	3	17.65%	0	-
Fever	1	5.88%	0	-
Sinus tachycardia	1	5.88%	0	-
Conjunctivitis	1	5.88%	0	-
Anorexia	1	5.88%	0	-

Table 17. Obstetric complications related to chemotherapy reported in PABC

	PABC		Controls	
COMPLICATION	*N*=11	%	*N*=16[ψ]	%
Gestational diabetes mellitus	2	11.76%	0	-
Suspicion of foetal anaemia	2	11.76%	0	-
Vaginal bleeding	2	11.76%	0	-
Prematurity	13	76.47%	1	8.33%

[ψ] Data available data of twelve women from control group

STUDY I.A: *Effect of anthracyclines and paclitaxel treatment on oxidative stress, inflammation and antioxidant defence biomarkers in PABC patients during pregnancy*

Blood was withdrawn from seventeen PABC women before starting chemotherapy treatment, prior administration of each cycle of chemotherapy (anthracyclines and paclitaxel) and at delivery. Blood was also withdrawn during routine appointments (non-CTX). Unfortunately, full monitoring was not possible in one patient. Besides, the representative blood samples of some cycles of chemotherapy were not available **(Suppl. table S3)**.

Furthermore, blood from eleven out of sixteen controls (control-P) with a median gestational age of 29.6 weeks (mean= 29 weeks; range 23.5-33.3 weeks) was drawn while undergoing a routine pregnancy blood test at the *Vall d´Hebron University Hospital* **(Figure 12)**.

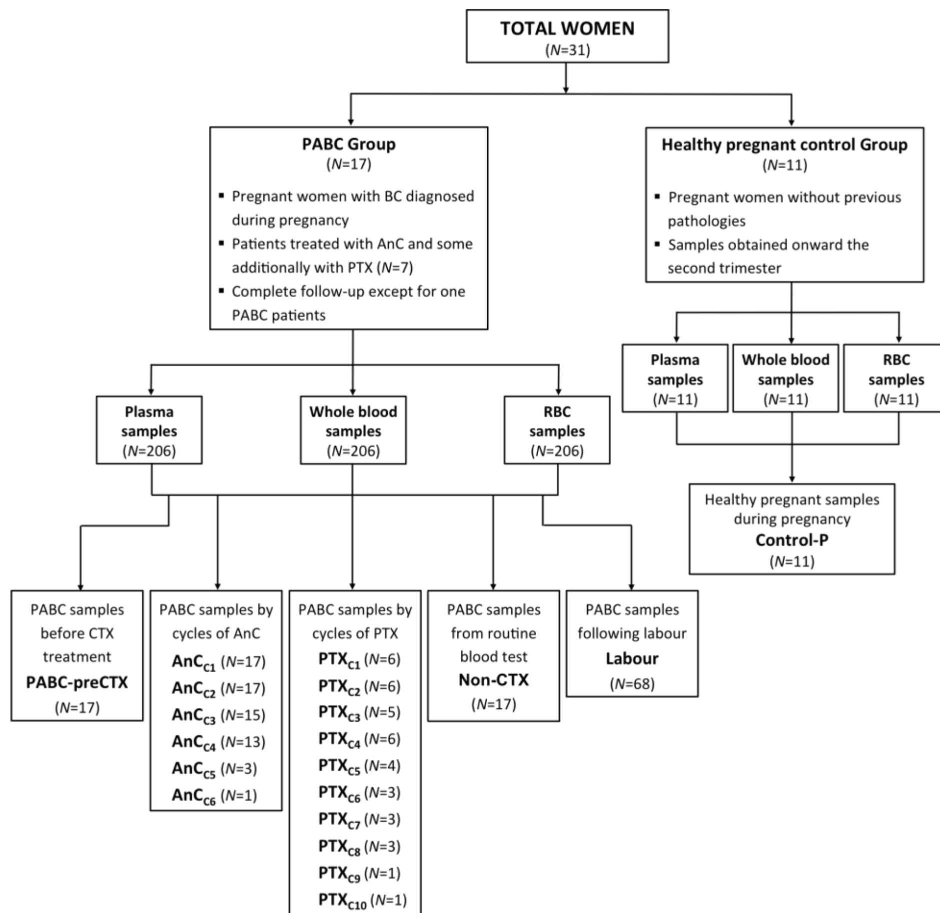


Figure 12. STUDY IA flow chart describing all participants of the study and the origin and type of the samples.

Different biomarkers of lipid peroxidation, protein damage, inflammation and antioxidant defence were measured at the *Vall d´Hebron University Hospital* **(see Table 1)**. Nevertheless, data of all biomarkers measured in every point of the study were not available. **Table 18** displays the missing test according to each patient.

Table 18. Biomarkers with data missing in each patient

	AnC_{C1} ***(26.5 wk)***	Non-CTX ***(26.5 wk)***	AnC_{C2} ***(26.5 wk)***				
PABC-1	GSH	GSH	GSH				
	AnC_{C1} ***(13.2 wk)***	Non-CTX ***(14.6 wk)***	Non-CTX ***(21 wk)***	Labour ***(35.6 wk)***			
PABC-2	GSH	GSH	YKL-40	GSH			
	Non-CTX ***(28.7 wk)***	AnC_{C3} ***(26.5 wk)***	Non-CTX ***(28.7 wk)***	Labour ***(35.6 wk)***			
PABC-3	Protein carbonyl	GSH	GSH	Protein-SH groups			
	AnC_{C3} ***(23.3 wk)***	Non-CTX ***(23.6 wk)***	AnC_{C6} ***(32.5 wk)***	Non-CTX ***(33.7 wk)***			
PABC- 4	GSH	GSH	Protein carbonyl; GSH	GSH			
	AnC_{C1} ***(31 wk)***	Non-CTX ***(32.7 wk)***	AnC_{C2} ***(34.6 wk)***				
PABC-5	Protein carbonyl; GSH	GSH	Protein carbonyl				
	AnC_{C1} ***(14.3 wk)***	AnC_{C2} ***(17.1 wk)***	Non-CTX ***(18.2 wk)***	PTX_{C3} ***(27.7 wk)***			
PABC-6	GSH	GSH	GSH	GSH			
	Non-CTX ***(25.5 wk)***	AnC_{C4} ***(27.5 wk)***	PTX_{C1} ***(28.5 wk)***	PTX_{C2} ***(29.5 wk)***	PTX_{C5} ***(32.5 wk)***	PTX_{C7} ***(34.5 wk)***	PTX_{C8} ***(35.5 wk)***
PABC-7	Protein-SH groups	Protein-SH groups	Protein-SH groups	Protein-SH groups	Protein carbonyl; GSH	GSH	Protein-SH groups
	AnC_{C1} ***(27.6 wk)***	AnC_{C1} ***(30.4 wk)***	AnC_{C1} ***(33.3 wk)***	Labour ***(36.5 wk)***			
PABC-8	Protein-SH groups	Protein-SH groups	GSH	MDA			
	PTX_{C1} ***(31.6 wk)***						
PABC-9	GSH						
	AnC_{C2} ***(25.4 wk)***	AnC_{C4} ***(32.3 wk)***					
PABC-10	GSH	GSH					
	AnC_{C1} ***(30.7 wk)***	Non-CTX ***(32 wk)***					
PABC-13	GSH	GSH					
	AnC_{C3} ***(30.7 wk)***	AnC_{C4} ***(32 wk)***					
PABC-14	GSH	GSH					

Continue on next page

Table 18. *Continuation*

	AnC$_{C1}$ *(21.5 wk)*	Non-CTX *(22.4 wk)*	AnC$_{C5}$ *(33.5 wk)*	Non-CTX *(36.2 wk)*	Labour *(36.3 wk)*			
PABC-16	YKL-40	YKL-40	MDA	MDA	MDA; GSH			
	AnC$_{C2}$ *(19.2 wk)*	Non-CTX *(20 wk)*	AnC$_{C3}$ *(22.2 wk)*	Non-CTX *(23.2 wk)*	Non-CTX *(25.1 wk)*	Non-CTX *(32.1 wk)*	Non-CTX *(34.2 wk)*	Labour *(34.3 wk)*
PABC-17	MDA	MDA	MDA	MDA	MDA	All	All	All except GSH

As detailed before (see material and methods section), three of PABC patient were a ChT-deficient individual. Therefore ChT analyses were only performed in fourteen PABC patients.

A.1 Effect of chemotherapy on the levels of oxidative stress, inflammation and antioxidant defence biomarkers by cycles of treatment

A.1.1 Oxidative damage to lipids: MDA

The levels of MDA during treatment with chemotherapy along pregnancy were unstable as some patients had their levels of MDA incremented or decreased following chemotherapy days. Eight patients showed an increase of MDA levels after the first cycle of chemotherapy while eight of them had the levels decreased instead. However, PABC-6 and PABC-8 did not have blood withdrawn in between both cycles.

Among patients who received both types of therapy, three of them suffered an increment of MDA levels during paclitaxel treatment. On the opposite, the levels were decreased during paclitaxel treatment in one patient while in two patients were similar to those obtained along with anhracyclines.
According to the MDA detected at birth, five patients showed an elevation of the levels respect to those measured before the first cycle of chemotherapy, eight patients presented a decrease, and one patient had similar levels. Data of the delivery was missed in two patients.

On the other hand, the levels of MDA measured in control-P group differed from those detected in PABC group in the majority of the patients. Precisely, the levels were in some of them higher and others lower than in control-P group.

Figure 13 illustrates the evolution of MDA levels along pregnancy in PABC patients included in the study. Control-P group is represented by the mean of MDA levels measured in eight women (0.66 µM ± 0.31).
As regards the analysis of MDA levels in all healthy pregnant women enrolled in control-P group, there was not a concordance between MDA levels and gestational age (**Figure 19.A)**.

A.1.2 Oxidative damage to proteins: Protein carbonyl

As shown in **Figure 14**, protein carbonyl levels were inconsistent among PABC patients despite the trend to increase during chemotherapy treatment along with pregnancy in many cases. Data from the first cycle of chemotherapy was not available in two patients.

The levels of protein carbonyl were elevated in six patients after the first cycle of chemotherapy, whereas the levels of eight patients were the opposite. Only one patient showed similar protein carbonyl levels before and after first cycle of chemotherapy. On the other hand, six patients presented a decrease of protein carbonyl levels following paclitaxel treatment, while two of them showed increase levels. As for PABC-15 patient, the levels of protein carbonyl were incremented during both therapies.

Although most of PABC patients suffered from a diminution of protein carbonyl levels at delivery in comparison with those measured before chemotherapy treatment, six patients showed an increment of protein carbonyl levels at birth. Data of the delivery was missed in the remaining patient.

When comparing control-P group (mean, 0.13 nmol/mg protein ± 0.12; *N*=9) with every PABC patient, we generally observed similar protein carbonyl levels among them **(Figure 14)**. On the other hand, the analysis of protein carbonyl in all women included in the control group showed that the levels were incremented in two controls whose gestational age were the most advanced among all **(Figure 19.B)**.

A.1.3 Inflammatory mediators: Cht and YKL-40

The measurement of ChT levels in ten PABC patients showed a tendency to drop after the first cycle of chemotherapy **(Figure 15)**. Alternatively, two patients presented elevated levels of ChT, whereas the remaining two had similar concentrations as before chemotherapy initiation. Nonetheless, blood samples in between cycle one and two of anthracyclines were not obtained in those patients with elevated levels of ChT.

According to the results, the ChT levels were adversely affected by the treatment with anthracyclines and paclitaxel. Samples measured before and after therapy (non-CTX) showed lower levels of ChT than those obtained from days of chemotherapy administration (anthracyclines and paclitaxel). Additionally, PABC patients were less affected by therapy with paclitaxel since the levels of ChT were higher than those acquired along with anthracyclines treatment.

On the other hand, the levels of ChT measured in labour were majority dropped in comparison with those detected before the beginning of chemotherapy treatment. Nine patients presented decreased levels, whereas three patients showed the opposite results. As for the remaining two patients, they had similar levels.

As for the levels of ChT detected in control-P group, they were mostly decreased in comparison with PABC patients. Control-P group is presented as the mean of ChT levels analysed in ten women (25.11 nmol/hr/mL ± 12.29).

Regarding the levels of YKL-40 measured along with chemotherapy treatment, they were decreased in twelve patients after the first cycle. On the opposite, YKL-40 levels were elevated in three patients while one presented similar concentrations. Unlike the results obtained in ChT analysis, the levels of YKL-40 were differently affected among patients as a consequence of the administration of anthracyclines and paclitaxel. Whereas some patients presented lower levels of YKL-40 before and after chemotherapy treatment along with pregnancy, others showed the contrary. Likewise, the YKL-40 levels measured during paclitaxel therapy were similar among cycles in most patients and were not increased following anthracyclines treatment.

In addition, levels of YKL-40 measured in labour were decreased in eleven patients, and elevated in three patients respect to those obtained prior chemotherapy initiation. The remaining patient presented similar levels.

Following the analysis of YKL-40 in the control-P group, we observed discrepancies with most of PABC patients. Basically, the levels of YKL-40 were increased or decreased indistinctly among PABC patients compared to control-P group.

The YKL-40 levels in PABC patients during pregnancy and control-P group (mean, 9.05 ng/mL ± 3.33; *N*=10) are showed in **Figure 16**.
The levels of ChT and YKL-40 measured in the control group were not associated with the gestational age as they were inconsistent along with pregnancy **(Figure 19.C** and **19.D)**.

A.1.4 Antioxidant defence: protein-SH groups and GSH

Information about GSH levels before chemotherapy initiation was not available in five patients. In general, the levels of GSH were incremented in most of the patients following chemotherapy treatment along with pregnancy. Despite only six patients showed higher GSH levels after the first cycle of chemotherapy than before, some of the remaining patients had later raised their levels. Nevertheless, the levels of GSH measured during both anthracyclines and paclitaxel treatments were similar between them except for two patients.

On the other hand, GSH levels quantified in labour were elevated in six patients while were dropped in five patients in comparison with those levels measured previous treatment initiation.

As regards the levels of GSH in control-P group, the were fairly similar to those measured in nearly all PABC patients. Despite this trend, some of them showed decreased levels in comparison with control-P group.
The progression of GSH concentrations in PABC patients during pregnancy is exhibited in **Figure 17**. Control-P group is showed as the mean of GSH levels detected in ten healthy pregnant women (33.18 µmol/g Hb ± 8.94).

The impact on the levels of protein-SH groups as a consequence of chemotherapy treatment during pregnancy was contradictory among patients **(Figure 18)**. Although many patients presented low levels of protein-SH groups along with therapy, others showed the opposite effect. At the beginning of the therapy, the levels of protein-SH groups were decreased after the first cycle in ten patients whereas were incremented in seven patients. In addition, the levels of protein-SH groups did not differ excessively from both types of chemotherapy. Exceptionally, PABC-7 patient showed a progressive increase in the levels of protein-SH groups during chemotherapy treatment. However, the levels of protein-SH groups measured in PABC-15 patient along pregnancy were progressively decreasing.

Contrasting GSH levels measured in labour, the levels of protein-SH groups were dropped in eight patients while were elevated only in four in comparison with those levels obtained previous therapy. The remaining patient showed similar concentrations in labour in both situations.

Concerning the levels of protein-SH groups detected in control-P group (mean, 25.17 µmol/mg ± 11.67; *N*=11), they were mainly increased compared to PABC patients **(Figure 18)**.

As for the control group, levels of GSH varied regardless of gestational age while the levels of protein-SH groups tended to decrease as gestational age increased **(Figure 19.E** and **19.F)**.

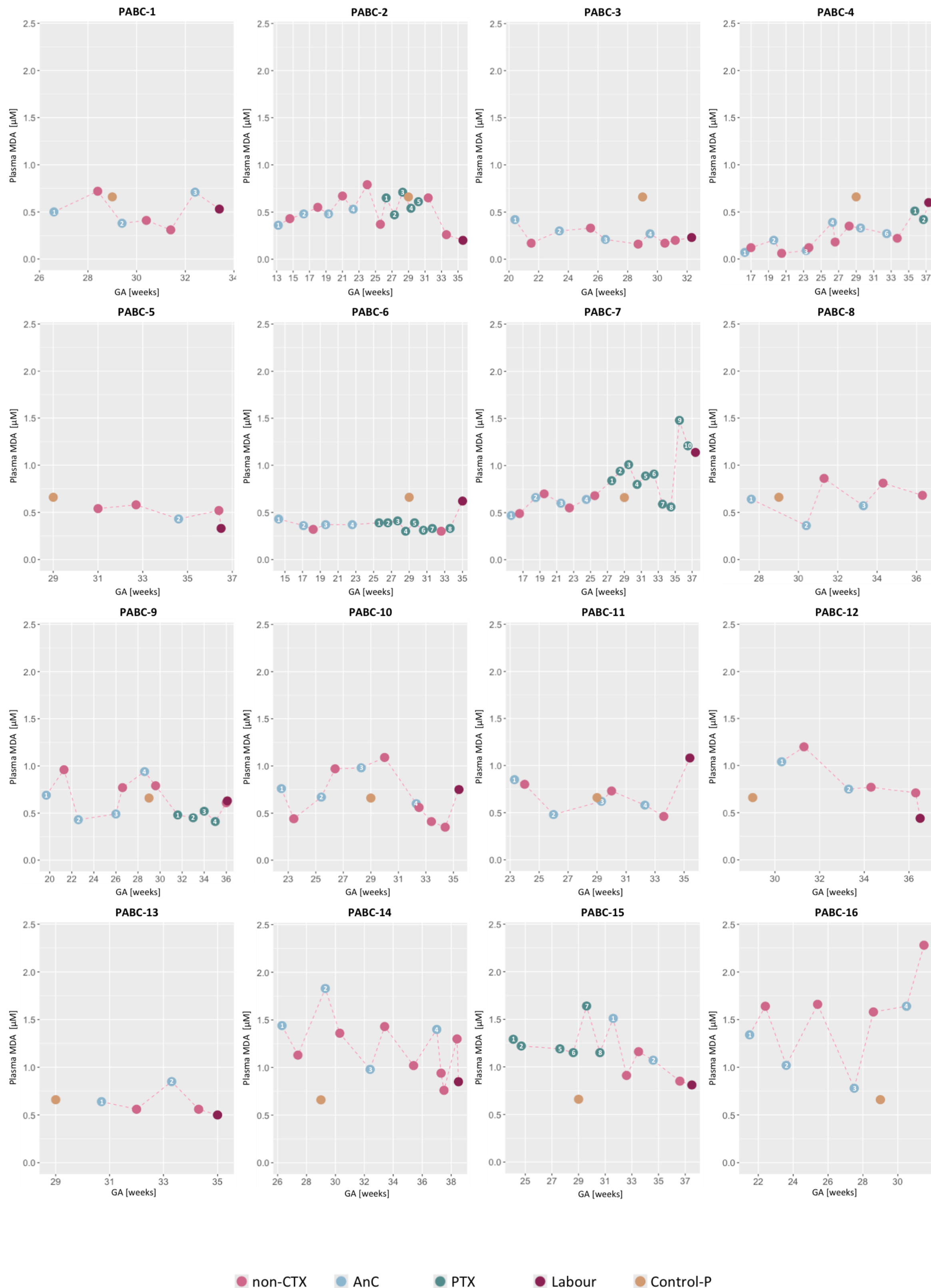


Figure 13. Dotplots of MDA levels measured along pregnancy in each patient included in PABC group in comparison with control group. Samples were obtained during routine appointments (non-CTX), before administering anthracycline-based regimens (AnC) or paclitaxel (PTX) and in labour (Labour). Control-P group is represented by the mean of MDA levels (0.66 µM ± 0.31; *N*=8) and gestational age (29 weeks).

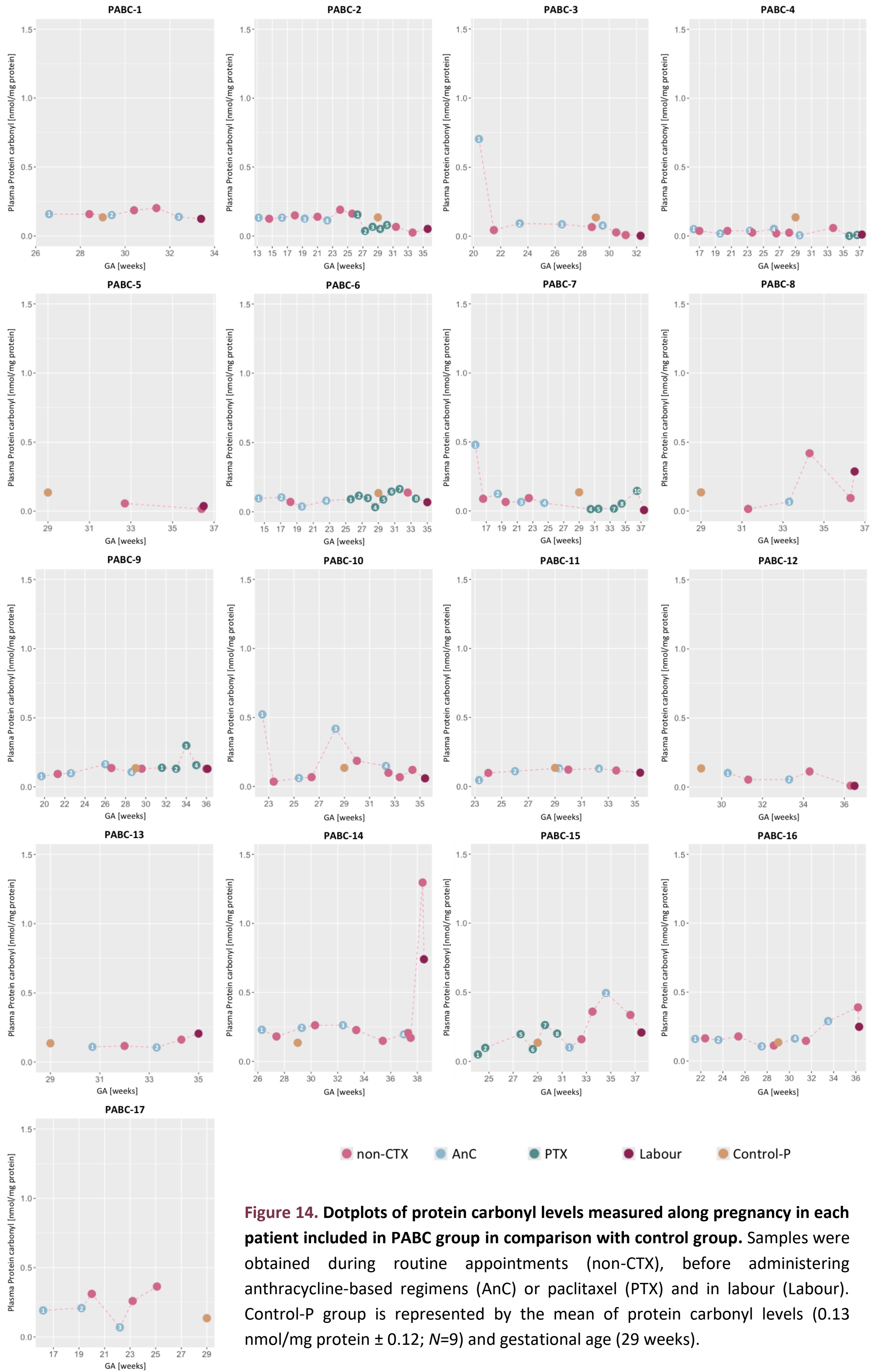


Figure 14. Dotplots of protein carbonyl levels measured along pregnancy in each patient included in PABC group in comparison with control group. Samples were obtained during routine appointments (non-CTX), before administering anthracycline-based regimens (AnC) or paclitaxel (PTX) and in labour (Labour). Control-P group is represented by the mean of protein carbonyl levels (0.13 nmol/mg protein ± 0.12; *N*=9) and gestational age (29 weeks).

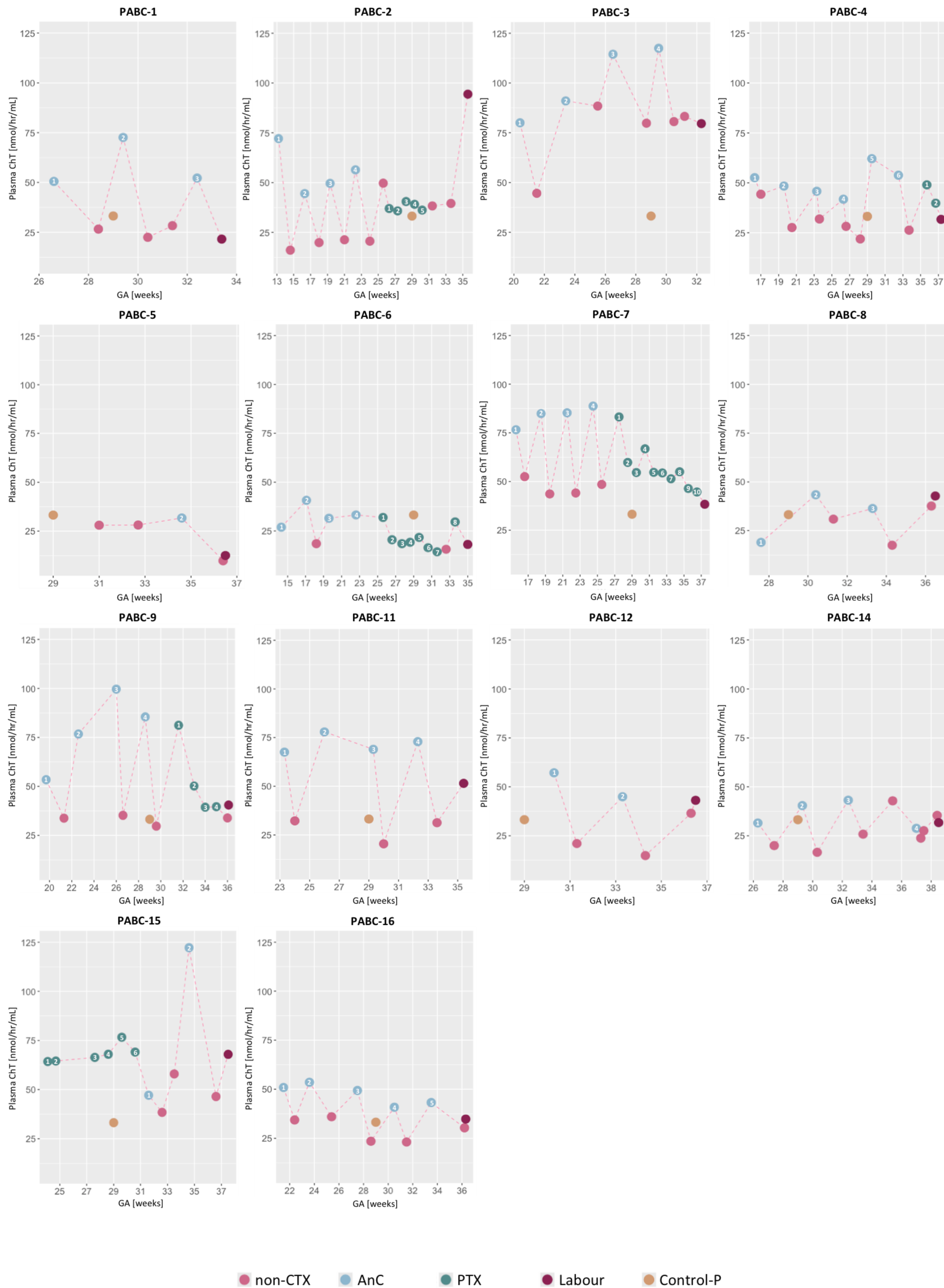


Figure 15. Dotplots of ChT levels measured along pregnancy in each patient included in PABC group in comparison with control group. Samples were obtained during routine appointments (non-CTX), before administering anthracycline-based regimens (AnC) or paclitaxel (PTX) and in labour (Labour). Control-P group is represented by the mean of ChT levels (25.11 nmol/hr/mL ± 12.29; *N*=10) and gestational age (29 weeks).

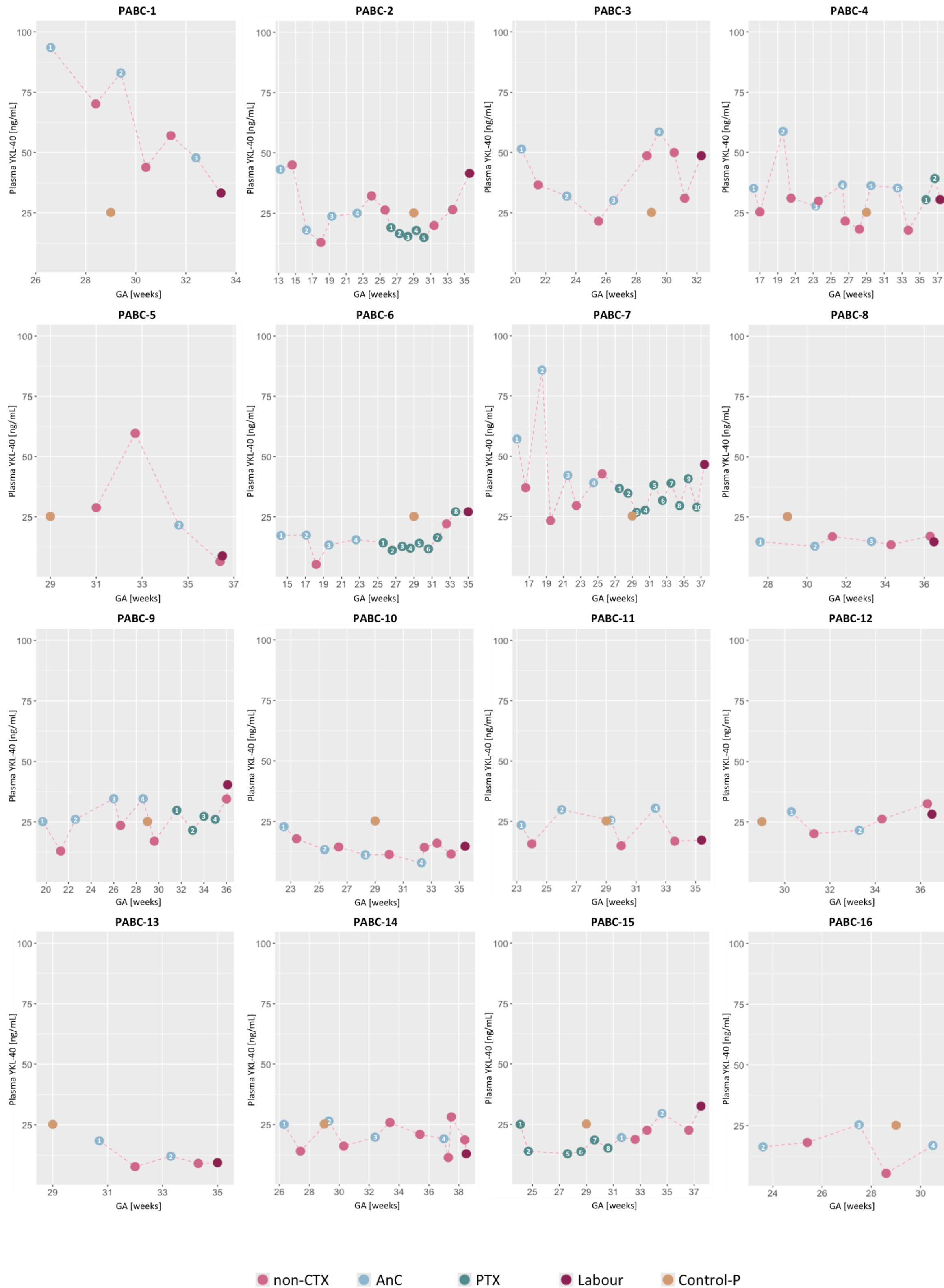


Figure 16. Dotplots of YKL-40 levels measured along pregnancy in each patient included in PABC group in comparison with control group. Samples were obtained during routine appointments (non-CTX), before administering anthracycline-based regimens (AnC) or paclitaxel (PTX) and in labour (Labour). Control-P group is represented by the mean of YKL-40 levels (9.05 ng/mL ± 3.33; *N*=10) and gestational age (29 weeks).

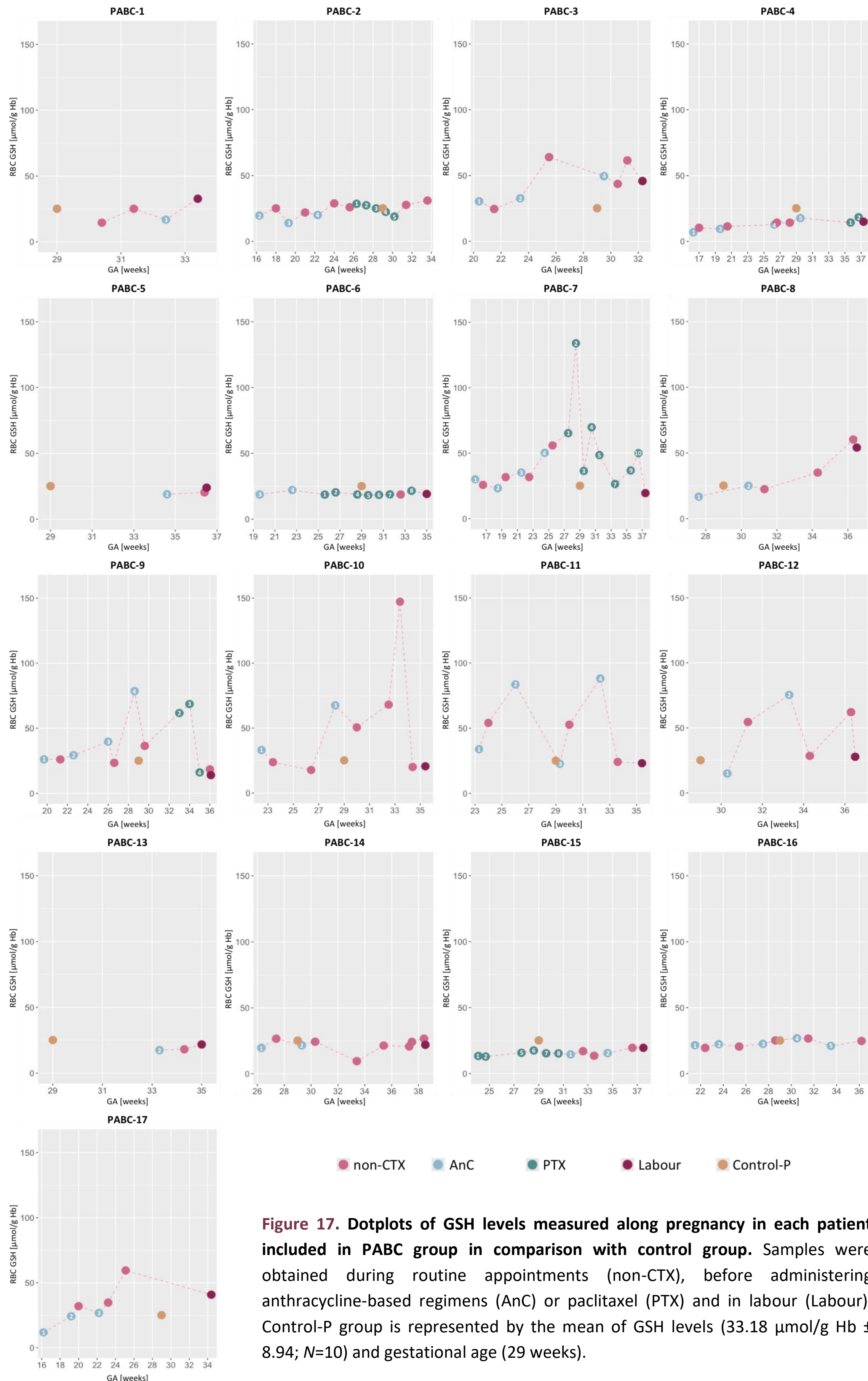


Figure 17. Dotplots of GSH levels measured along pregnancy in each patient included in PABC group in comparison with control group. Samples were obtained during routine appointments (non-CTX), before administering anthracycline-based regimens (AnC) or paclitaxel (PTX) and in labour (Labour). Control-P group is represented by the mean of GSH levels (33.18 μmol/g Hb ± 8.94; *N*=10) and gestational age (29 weeks).

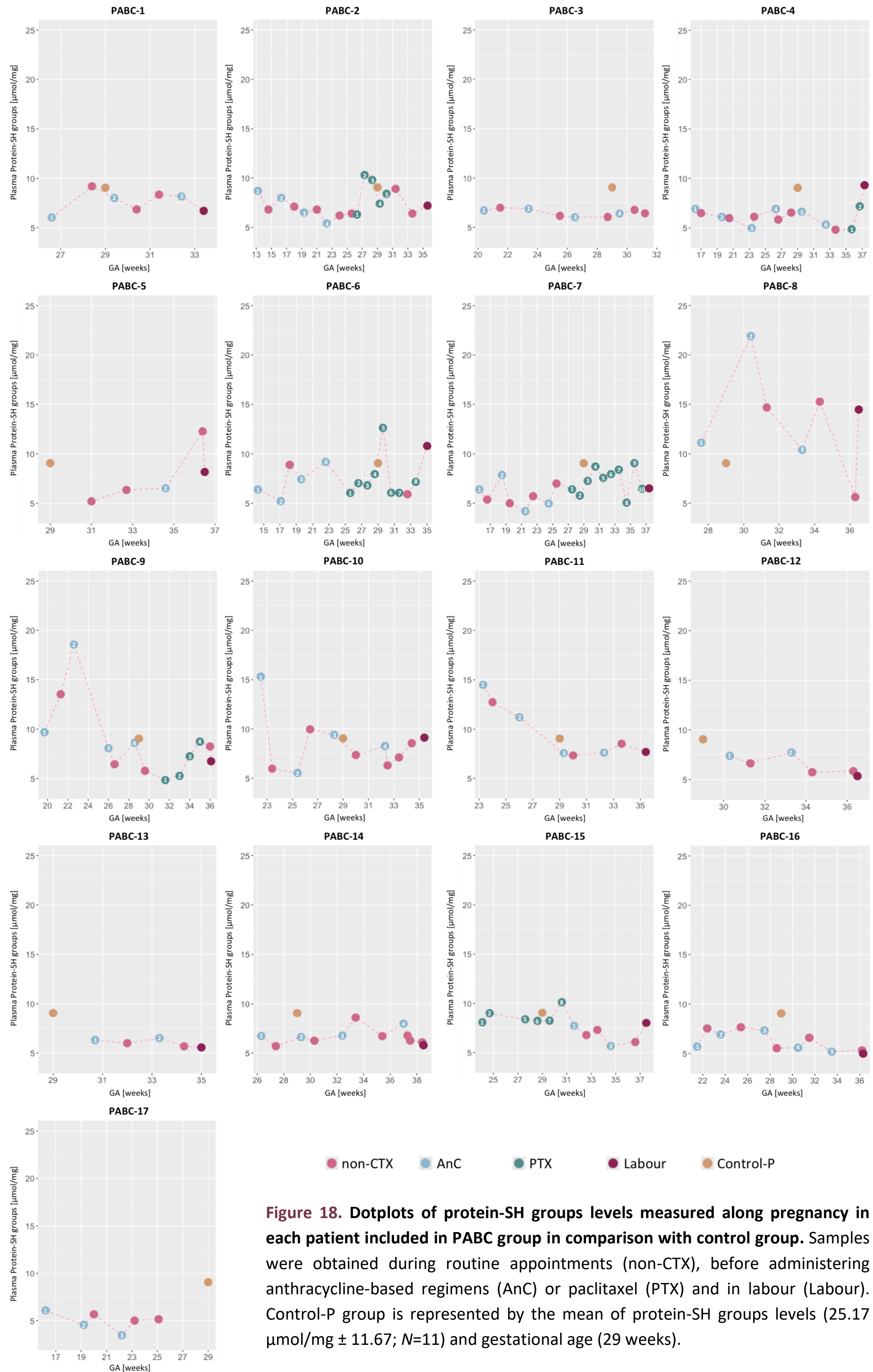


Figure 18. Dotplots of protein-SH groups levels measured along pregnancy in each patient included in PABC group in comparison with control group. Samples were obtained during routine appointments (non-CTX), before administering anthracycline-based regimens (AnC) or paclitaxel (PTX) and in labour (Labour). Control-P group is represented by the mean of protein-SH groups levels (25.17 µmol/mg ± 11.67; *N*=11) and gestational age (29 weeks).

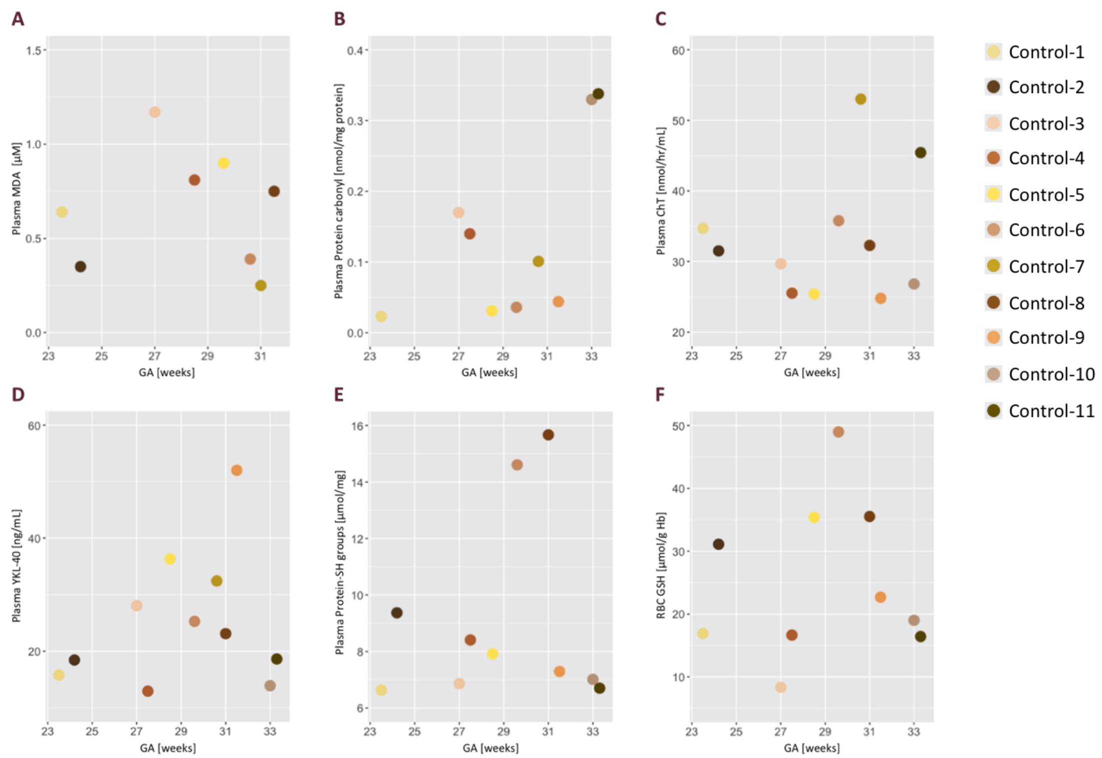


Figure 19. Dotplots of biomarkers of OS, inflammation and antioxidant defence levels measured along pregnancy in each participant included in control-P group. Lipid peroxidation **(A)**, protein damage **(B)**, inflammation **(C and D)** and antioxidant defence **(E and F)**.

Suppl. Table S4 discloses the analysis of the control-P expressed as the mean and ± SD.

A.2 Results of the statistical analyses of the study

As illustrated in **Figure 12**, blood samples obtained from PABC patients were classified in four major study groups and compared among them: before chemotherapy initiation (preCTX), following anthracyclines and paclitaxel (postAnC and postPTX respectively) and lastly at birth (Labour). Additionally, they were compared to blood samples from healthy pregnant women (control-P).

Shapiro-Wilk test was employed to verify whether the study groups were normally distributed. Subsequently, student t-test (parametric test) was used when we assumed that the biomarkers analysed follow a normal distribution among the study groups. Alternatively, the non-parametric wilcoxon rank-sum test performed used when this assumption was in doubt.

Table 19 summarises the levels of the biomarkers analysed among the study groups employing the corresponding statistical test.

Table 19. Metabolite concentrations found in plasma and RBC $^{\Phi}$ samples from all groups of study.

	Control-P	preCTX	*P*	Control-P	postAnC	*P*
Lipid damage	**(*N*=8)**	**(*N*=17)**		**(*N*=8)**	**(*N*=105)**	
MDA [µM]	0.66 ± 0.31	0.75 ± 0.40	n.s.	0.66 ± 0.31	0.67 ± 0.41	n.s.
Protein damage	**(*N*=9)**	**(*N*=15)**	*P*	**(*N*=9)**	**(*N*=104)**	*P*
Carbonyl proteins [nmol/mg protein]	0.13 ± 0.12	0.21 ± 0.20	n.s.	0.13 ± 0.12	0.14 ± 0.15	n.s.
Inflammation	**(*N*=11)**	**(*N*=14)**	*P*	**(*N*=11)**	**(*N*=95)**	*P*
ChT [nmol/hr/mL]	33.18 ± 8.94	52.17 ± 19.48	0.004^{γ}	33.18 ± 8.94	46.17 ± 24.91	n.s.
	(*N*11)	**(*N*=15)**	*P*	**(*N*11)**	**(*N*=102)**	*P*
YKL-40 [ng/mL]	25.17 ± 11.67	34.02 ± 20.51	n.s.	25.17 ± 11.67	26.27 ± 15.36	n.s.
Antioxidant defence	**(*N*=10)**	**(*N*=12)**	*P*	**(*N*=10)**	**(*N*=91)**	*P*
GSH [µmol/g Hb] $^{\Phi}$	25.11 ± 12.29	21.52 ± 9.06	n.s.	25.11 ± 12.29	32.10 ± 21.85	n.s.
	(*N*=10)	**(*N*=17)**	*P*	**(*N*=10)**	**(*N*=110)**	*P*
SH-protein groups [µmol/mg protein]	9.05 ± 3.33	8.06 ± 2.99	n.s.	9.05 ± 3.33	7.25 ± 2.65	0.010^{Ψ}

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Table 19. *Continuation*

	Control-P	postPTX	*P*	Control-P	Labour	*P*
Lipid damage	**(*N*=8)**	**(*N*=34)**		**(*N*=8)**	**(*N*=14)**	
MDA [µM]	0.66 ± 0.31	0.73 ± 0.40	n.s.	0.66 ± 0.31	0.62 ± 0.28	n.s.
Protein damage	**(*N*=9)**	**(*N*=32)**	*P*	**(*N*=9)**	**(*N*=16)**	*P*
Carbonyl proteins [nmol/mg protein]	0.13 ± 0.12	0.12 ± 0.09	n.s.	0.13 ± 0.12	0.14 ± 0.18	n.s.
Inflammation	**(*N*=11)**	**(*N*=34)**	*P*	**(*N*=11)**	**(*N*=14)**	*P*
ChT [nmol/hr/mL]	33.18 ± 8.94	43.16 ± 17.28	0.018^{γ}	33.18 ± 8.94	43.46 ± 23.26	n.s.
	(*N*11)	**(*N*=34)**	*P*	**(*N*11)**	**(*N*=15)**	*P*
YKL-40 [ng/mL]	25.17 ± 11.67	22.86 ± 9.11	n.s.	25.17 ± 11.67	27.09 ± 13.54	n.s.
Antioxidant defence	**(*N*=10)**	**(*N*=33)**	*P*	**(*N*=10)**	**(*N*=15)**	*P*
GSH [µmol/g Hb]$^{\Phi}$	25.11 ± 12.29	32.04 ± 24.56	n.s.	25.11 ± 12.29	26.62 ± 11.69	n.s.
SH-protein groups [µmol/mg protein]	**(*N*=10)**	**(*N*=36)**	*P*	**(*N*=10)**	**(*N*=15)**	*P*
	9.05 ± 3.33	7.66 ± 1.65	n.s.	9.05 ± 3.33	7.76 ± 2.46	n.s.

Table 19. *Continuation*

	preCTX	postAnC	*P*	preCTX	postPTX	*P*
Lipid damage	**(*N*=17)**	**(*N*=105)**		**(*N*=17)**	**(*N*=34)**	
MDA [µM]	0.75 ± 0.40	0.67 ± 0.41	n.s.	0.75 ± 0.40	0.73 ± 0.40	n.s.
Protein damage	**(*N*=15)**	**(*N*=104)**	*P*	**(*N*=15)**	**(*N*=32)**	*P*
Carbonyl proteins [nmol/mg protein]	0.21 ± 0.20	0.14 ± 0.15	n.s.	0.21 ± 0.20	0.12 ± 0.09	n.s.
Inflammation	**(*N*=14)**	**(*N*=95)**	*P*	**(*N*=14)**	**(*N*=34)**	*P*
ChT [nmol/hr/mL]	52.17 ± 19.48	46.17 ± 24.91	n.s.	52.17 ± 19.48	43.16 ± 17.28	n.s.
	(*N*=15)	**(*N*=102)**	*P*	**(*N*=15)**	**(*N*=34)**	*P*
YKL-40 [ng/mL]	34.02 ± 20.51	26.27 ± 15.36	n.s.	34.02 ± 20.51	22.86 ± 9.11	0.047^{Ψ}
Antioxidant defence	**(*N*=12)**	**(*N*=91)**	*P*	**(*N*=12)**	**(*N*=33)**	*P*
GSH [µmol/g Hb]$^{\Phi}$	21.52 ± 9.06	32.10 ± 21.85	n.s.	21.52 ± 9.06	32.04 ± 24.56	n.s.
SH-protein groups [µmol/mg protein]	**(*N*=17)**	**(*N*=110)**	*P*	**(*N*=17)**	**(*N*=36)**	*P*
	8.06 ± 2.99	7.25 ± 2.65	n.s.	8.06 ± 2.99	7.66 ± 1.65	n.s.

Continue on next page

Table 19. *Continuation*

	preCTX	Labour	*P*	postAnC	postPTX	*P*
Lipid damage	**(*N*=17)**	**(*N*=14)**		**(*N*=105)**	**(*N*=34)**	
MDA [µM]	0.75 ± 0.40	0.62 ± 0.28	n.s.	0.67 ± 0.41	0.73 ± 0.40	n.s.
Protein damage	**(*N*=15)**	**(*N*=16)**	***P***	**(*N*=104)**	**(*N*=32)**	***P***
Carbonyl proteins [nmol/mg protein]	0.21 ± 0.20	0.14 ± 0.18	n.s.	0.14 ± 0.15	0.12 ± 0.09	n.s.
Inflammation	**(*N*=14)**	**(*N*=14)**	***P***	**(*N*=95)**	**(*N*=34)**	***P***
ChT [nmol/hr/mL]	52.17 ± 19.48	43.46 ± 23.26	n.s.	46.17 ± 24.91	43.16 ± 17.28	n.s.
	(*N*=15)	**(*N*=15)**	***P***	**(*N*=102)**	**(*N*=34)**	***P***
YKL-40 [ng/mL]	34.02 ± 20.51	27.09 ± 13.54	n.s.	26.27 ± 15.36	22.86 ± 9.11	n.s.
Antioxidant defence	**(*N*=12)**	**(*N*=15)**	***P***	**(*N*=91)**	**(*N*=33)**	***P***
GSH [µmol/g Hb]$^{\Phi}$	21.52 ± 9.06	26.62 ± 11.69	n.s.	32.10 ± 21.85	32.04 ± 24.56	n.s.
SH-protein groups [µmol/mg protein]	**(*N*=17)**	**(*N*=15)**	***P***	**(*N*=110)**	**(*N*=36)**	***P***
	8.06 ± 2.99	7.76 ± 2.46	n.s.	7.25 ± 2.65	7.66 ± 1.65	0.012^{Ψ}

Table 19. *Continuation*

	postAnC	Labour	*P*	postPTX	Labour	*P*
Lipid damage	**(*N*=105)**	**(*N*=14)**		**(*N*=34)**	**(*N*=14)**	
MDA [µM]	0.67 ± 0.41	0.62 ± 0.28	n.s.	0.73 ± 0.40	0.62 ± 0.28	n.s.
Protein damage	**(*N*=104)**	**(*N*=16)**	***P***	**(*N*=32)**	**(*N*=16)**	***P***
Carbonyl proteins [nmol/mg protein]	0.14 ± 0.15	0.14 ± 0.18	n.s.	0.12 ± 0.09	0.14 ± 0.18	n.s.
Inflammation	**(*N*=95)**	**(*N*=14)**	***P***	**(*N*=34)**	**(*N*=14)**	***P***
ChT [nmol/hr/mL]	46.17 ± 24.91	43.46 ± 23.26	n.s.	43.16 ± 17.28	43.46 ± 23.26	n.s.
	(*N*=102)	**(*N*=15)**	***P***	**(*N*=34)**	**(*N*=15)**	***P***
YKL-40 [ng/mL]	26.27 ± 15.36	27.09 ± 13.54	n.s.	22.86 ± 9.11	27.09 ± 13.54	n.s.
Antioxidant defence	**(*N*=91)**	**(*N*=15)**	***P***	**(*N*=33)**	**(*N*=15)**	***P***
GSH [µmol/g Hb]$^{\Phi}$	32.10 ± 21.85	26.62 ± 11.69	n.s.	32.04 ± 24.56	26.62 ± 11.69	n.s.
SH-protein groups [µmol/mg protein]	**(*N*=110)**	**(*N*=15)**	***P***	**(*N*=36)**	**(*N*=15)**	***P***
	7.25 ± 2.65	7.76 ± 2.46	n.s.	7.66 ± 1.65	7.76 ± 2.46	n.s.

Values are expressed as mean ± SD

$^{\Psi}$ Wilcoxon rank-sum test and $^{\gamma}$ Student's t test were used to assess differences between groups.

P value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.

A.2.1 Cluster analysis of significant biomarkers

Heatmap of statistically significant biomarkers, which visually exhibits the concentration changes among the groups studied is shown in **Figure 20**.

Decrease levels of inflammation **(A)** were observed in PABC patients after paclitaxel treatment compared to the beginning of chemotherapy treatment. On the other hand, the levels of antioxidant defence **(B)** were higher in PABC patients following paclitaxel treatment than after anthracyclines.

When comparing the effect of chemotherapy treatment along with pregnancy in PABC patients, we found differences with control-P group. Higher levels of inflammation **(C)** were observed before chemotherapy initiation and following paclitaxel treatment **(E)** in comparison with those measured in control-P group. Opposite, the levels of antioxidant defence were lower in PABC patients after treatment with anthracyclines than in control-P group **(D)**.

Despite perceiving essential differences among various comparative groups, further clustering visualization using principal component analysis (PCA) was not possible since the differences were not observed in more than one metabolite.

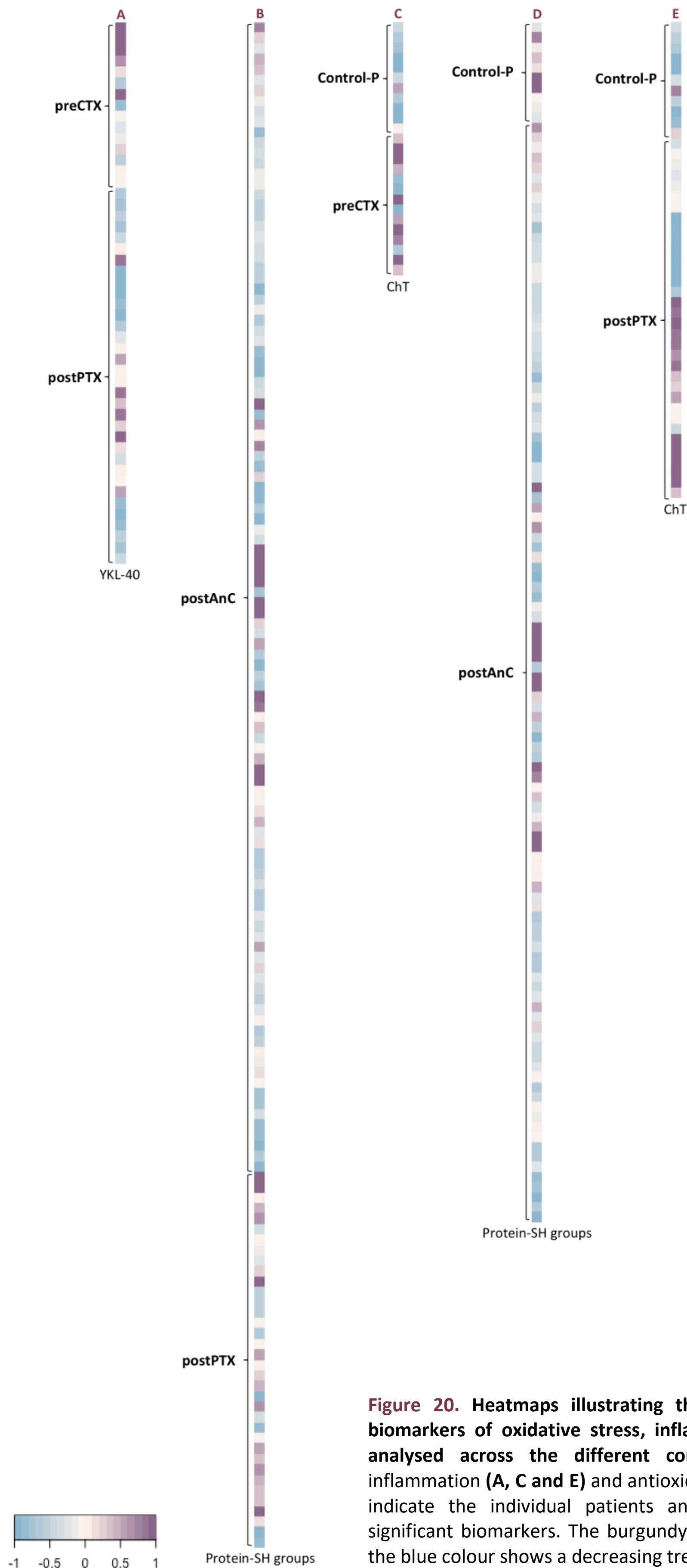


Figure 20. Heatmaps illustrating the clustering of all differentiating biomarkers of oxidative stress, inflammation and antioxidant defence analysed across the different comparative groups. Biomarkers of inflammation **(A, C and E)** and antioxidant defence **(B and D)**. The columns indicate the individual patients and the rows represent statistically significant biomarkers. The burgundy colour shows the trend of rise and the blue colour shows a decreasing trend.

A.2.2 Comparative study of biomarkers levels between tested groups

Oxidative damage to lipids

Results showed that MDA levels measured in plasma from PABC patients and healthy pregnant women were similar among all comparisons **(Figure 21)**.

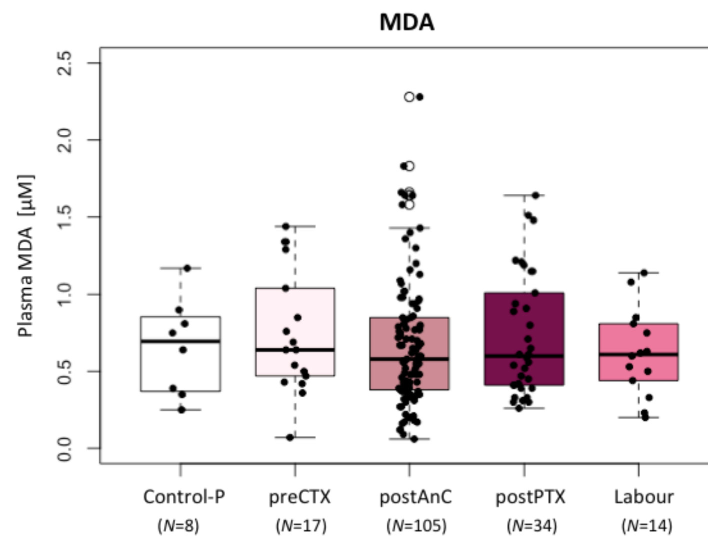


Figure 21. Boxplots of lipid peroxidation biomarker measured in plasma. MDA levels in healthy pregnant women (control-P), PABC patients prior chemotherapy (preCTX), following chemotherapy administration (anthracyclines or paclitaxel) and after labour. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.

P value of <0.05 was considered significant. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

Oxidative damage to proteins

The analysis of plasma concentrations of protein carbonyl shown no statistical differences among the comparative groups **(Figure 22)**.

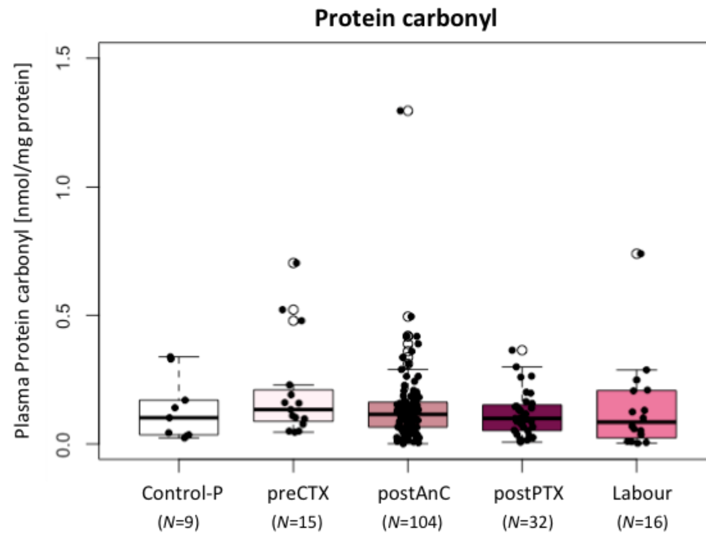


Figure 22. Boxplots of protein oxidation biomarker measured in plasma. Protein carbonyl levels in healthy pregnant women (control-P), PABC patients prior chemotherapy (preCTX), following chemotherapy administration (anthracyclines or paclitaxel) and after labour. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.

P value of <0.05 was considered significant. $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

Inflammatory mediators

Differences were detected in ChT plasma levels measured in PABC patients before chemotherapy treatment and control-P group reaching statistical significance (*P* value= 0.004). Likewise, ChT levels were statistically significantly higher following paclitaxel treatment (*P* value= 0.018) than in control-P group **(Figure 23.A)**.

As illustrated in **Figure 23.B**, PABC patients exhibited significantly increased plasma levels of YKL-40 before chemotherapy and respect to paclitaxel treatment (*P* value= 0.047).

Besides, it was appreciable an elevated trend of inflammation (especially ChT activity), in PABC patients in comparison with control-P group during the study. However, further experiments are needed to elucidate these observations.

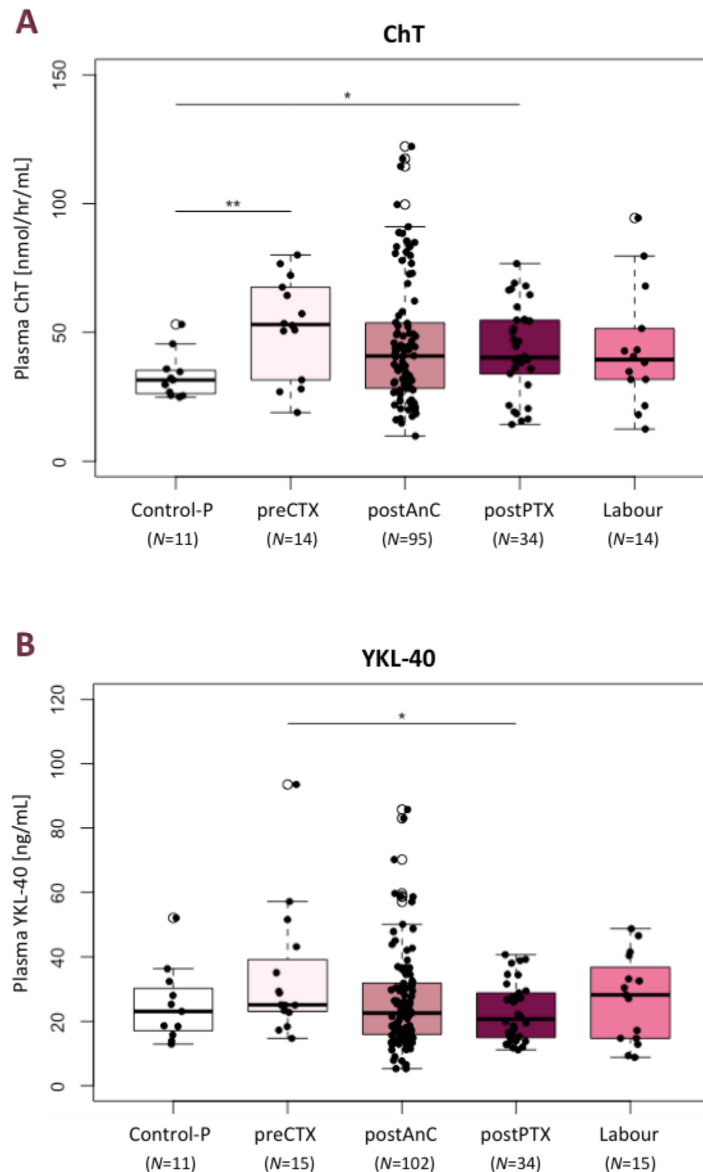


Figure 23. Boxplots of inflammatory biomarkers measured in plasma. Cht **(A)** and YKL-40 **(B)** levels in healthy pregnant women (control-P), PABC patients prior chemotherapy (preCTX), following chemotherapy administration (anthracyclines or paclitaxel) and after labour. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.

Student's t test **(A)** and wilcoxon rank-sum test **(B)** were used to assess differences between groups; a *P* value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.

Antioxidant defence

We observed that plasma levels of protein-SH groups were found statistically significant decreased in PABC patients after anthracyclines treatment respect to control-P group (*P* value= 0.010).

In addition, significant differences were observed in the levels of protein-SH groups between anthracyclines and paclitaxel treatments (*P* value= 0.012) (**Figure 24.A**).

Results showed no significant differences in GSH levels measured in RBC from all comparative groups **(Figure 24.B)**.

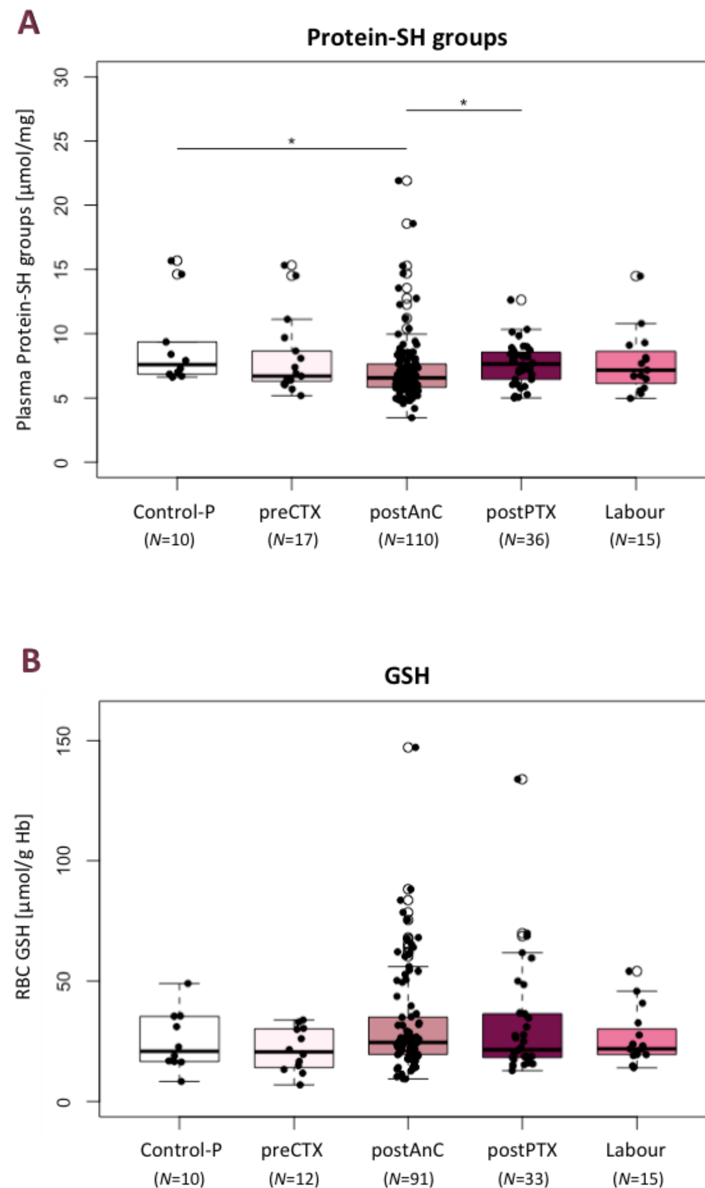


Figure 24. Boxplots of antioxidant defence biomarkers measured in plasma. Protein-SH groups **(A)** and GSH **(B)** levels in healthy pregnant women (control-P), PABC patients prior chemotherapy (preCTX), following chemotherapy administration (anthracyclines or paclitaxel) and after labour. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.
Wilcoxon rank-sum test was used to assess differences between groups;
P value of <0.05 was considered significant. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

A more extensive study that discloses the effect of anthracyclines and paclitaxel by cycles administered in PABC patients during pregnancy is available in **Suppl. table S5-S12**. Furthermore, the results are explained and visualized from **Supp.Figure S1** to **Supp.Figure S5.**

A.2.3 Multivariate and univariant regression analyses

Patient status and clinical characteristics

We did not find any association regarding patient status (disease-free or metastatic) with reproductive techniques (yes/no), hormone receptor (positive/negative), HER2/neu (positive/negative), parity (nulliparous/parous), gestational age at delivery (pre-term/full-term) and clinical stage (stage I+ stage II/stage III+ stage IV) using a multivariate logistic regression model.

Patient status and metabolites

Multivariate logistic regression analysis conducted on patient status (disease-free or metastatic) revealed non-significant association with biomarkers levels measured in PABC prior to chemotherapy and at labour.

Clinical stage and metabolites

Univariate logistic regression analysis for the association between metabolite levels measured before chemotherapy treatment in PABC patients and clinical stage showed non-significant results..

Metabolites and clinical characteristics

There was a significant positive association between reproductive techniques (yes/no) and protein carbonyl levels at labour according to multivariate linear regression analysis(*P* value=0.043; β coefficient=0.396). The remaining associations were not statistically significant **(Table 20)**.

Table 20. Multivariate linear regression analysis of the relation to clinical variables with the protein carbonyl levels measured in PABC patients at labour

Clinical characteristics	β	Std. Error	t-value	*P*
Intercept	0.22994	0.39342	0.584	0.575
Reproductive techniques	0.39621	0.16532	2.397	**0.0434**
Hormone receptor	-0.06597	0.11775	-0.56	0.5906
HER2/neu	0.06824	0.11845	0.576	0.5804
Surgery prior chemotherapy	0.10439	0.15121	0.69	0.5095
Parity	-0.40077	0.18172	-2.205	0.0585
Gestational age at delivery	-0.01159	0.22807	-0.051	0.9607
Clinical stage	0.27244	0.15027	1.813	0.1074

P value of <0.05 was considered significant. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.
Note: global *P* value= 0.289

Neonatal weight at birth and metabolites

Among all the metabolites, only protein carbonyl (*P* value=0.001; β coefficient = 1802.4) and YKL-40 (*P* value=0.033; β coefficient= -16.255) were found to be associated with neonatal weight at birth **(Table 21 and Table 22)**.

Table 21. Univariate lineal regression of the relation to protein carbonyl measured in PABC at labour and neonatal weight at birth

Metabolite	β	Std. Error	t-value	*P*
Intercept	2145.6	101.5	21.129	$3.73e^{-14}$
Protein carbonyl	1802.4	480.4	3.752	**0.00146**

P value of <0.05 was considered significant. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

Table 22. Univariate lineal regression of the relation to YKL-40 measured in PABC at labour and neonatal weight at birth

Metabolite	β	Std. Error	t-value	*P*
Intercept	2811.19	211.429	13.296	$2.06e^{-10}$
YKL-40	-16.255	6.997	-2.323	0.0328

P value of <0.05 was considered significant. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

Neonatal weight at birth and prematurity

Our results showed non- significant associations between birth weight and prematurity employing a univariate linear regression model.

Mode of delivery and metabolites

Univariate linear regression analysis for the association between mode of delivery (vaginal/caesarean) and all biomarkers measured in PBC patients at labour showed non-significant results.

A.2.4 Correlation analysis

Lipid damage

There were significant correlations between plasma MDA levels measured in PABC patients following anthracyclines with prior treatment (*R*=0.63, *P* value=0.017) and at labour(*R*=0.64, *P* value=0.013).

Protein damage

A significant positive correlation was detected between the plasma levels of protein carbonyl measured in PABC patients after anthracyclines and at labour (*R*=0.63, *P* value=0.012).

Inflammation

There were significant correlations between plasma ChT activity measured in PABC patients after anthracyclines with prior treatment (*R*=0.72, *P* value=0.006) and at labour(*R*=0.54, *P* value=0.047). In addition, there was a positive correlation between the plasma levels of ChT activity before chemotherapy and at labour (*R*=0.67, *P* value=0.013).

Significant positive correlations were observed between plasma YKL-40 activity measured in PABC patients following anthracyclines with before treatment (*R*=0.56, *P* value=0.039) and at labour(*R*=0.75, *P* value=0.001). In addition, there was a positive correlation between the plasma levels of ChT activity prior treatment and at labour (*R*=0.57, *P* value=0.033).

Antioxidant defence

There was a positive correlation between the RBC levels of GSH following anthracyclines and those measured at labour (*R*=0.56, *P* value=0.045).

However, non-significant correlation was noted between the plasma levels of protein thiol groups measured prior to chemotherapy, after anthracyclines and at labour.

STUDY I.B: *Accumulative effect of chemotherapy on the levels of oxidative stress, antioxidant defence and inflammation biomarkers in PABC patients*

The study was carried out using blood samples collected before chemotherapy treatment and at delivery from eleven out of seventeen PABC patients with a median gestational age at chemotherapy first dose of 23.3 weeks (mean = 23.35 weeks; range 15.6-30.3 weeks) and at birth of 36.53 weeks (mean = 36.5 weeks; range 34.4-38.5 weeks). The analysis of the remaining six PABC patients included initially in the study was not possible owing to sample missing. Besides, blood samples obtained before and after treatment with anthracyclines from ten non-PABC patients were also collected.

Blood was withdrawn immediately after delivery from ten out of sixteen pregnant controls included in the study (Control-L). Median age at birth of seven controls-L was 38.5 weeks (mean = 38.86 weeks; range 36.6-40.6 weeks) **(Figure 25)**. Samples from the remaining controls were not available.

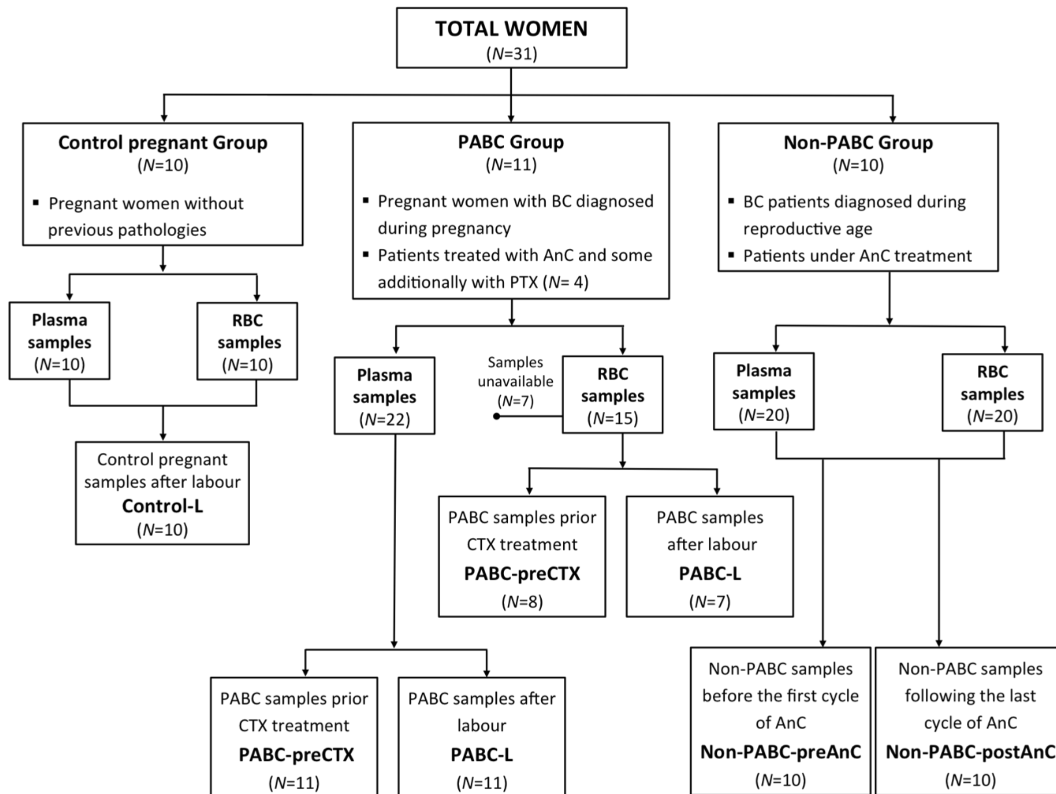


Figure 25. STUDY IB flow chart describing all participants of the study and the origin and type of the samples.

Various biomarkers of DNA damage, lipid damage, protein damage, inflammation and antioxidant defence were measured in the *Neonatal Research Group at the Health Research Institute Hospital La Fe* **(See Table 1)**.

Previous analysis, all values detected below LOQ were replaced by ½ LOQ.

B.1 Results of the statistical analyses of the study

Three different groups of participants, PABC patients, non-PABC patients and healthy pregnant women were evaluated **(see Figure 25)**. Blood samples obtained from PABC patients were classified into two comparative groups: before chemotherapy initiation (PABC-preCTX) and at birth (PABC-L). Besides, they were compared to blood samples from healthy pregnant women at labour (control-L).

As regards blood samples from non-PABC patients, they were also classified into two different comparative groups: prior (non-PABC-preAnC) and following (non-PABC-postAnC) anthracyclines treatment. Blood samples before treatment from non-PABC patients and PABC patients were additionally compared.

Shapiro-Wilk test was used to examine whether the biomarkers were normally distributed in all groups of study. After testing the normality, student t-test was employed once we accepted that the biomarkers measured in distinct test participants obey a Gaussian distribution whilst Wilcoxon rank-sum test was used when it was not.

However, following the same principle of normal distribution, student's t paired test (parametric test) and Wilcoxon signed-rank test (non-parametric test) were used when we compared the same participant at two different moments of the study (i.e., PABC and non-PABC patients).

The concentrations and ratios of the biomarkers analysed in all the comparative groups of the study IB, including the statistical test used are enlisted in **Table 23**, **Table 24** and **Table 25**.

Table 23. Plasma levels of DNA damage (ratios), protein damage (ratios), lipid damage (nM), inflammation (nM) and antioxidant defense (ratios) in PABC patients (PABC-preCTX and PABC-L) and healthy pregnant women at birth (Control-L).

	PABC-preCTX	PABC-L	*P*	PABC-L	Control-L	*P*
DNA damage	**(*N*=11)**	**(*N*=11)**		**(*N*=11)**	**(*N*=10)**	
8-OHdG/2dG	0.76 ± 0.08	0.87 ± 0.12	0.031^{λ}	0.87 ± 0.12	0.90 ± 0.16	n.s.
Protein damage	**(*N*=11)**	**(*N*=11)**	***P***	**(*N*=11)**	**(*N*=10)**	***P***
m-Tyr/Phe	0.78 ± 0.06	0.85 ± 0.15	n.s.	0.85 ± 0.15	1.00 ± 0.17	0.029^{Ψ}
o-Tyr/Phe	0.76 ± 0.09	0.95 ± 0.21	0.032^{δ}	0.95 ± 0.21	0.92 ± 0.16	n.s.
$3NO_2$-Tyr/p-Tyr	0.83 ± 0.10	0.85 ± 0.25	n.s.	0.85 ± 0.25	0.93 ± 0.22	n.s.
3Cl-Tyr/p-Tyr	<LOQ	<LOQ	–	<LOQ	<LOQ	–
Lipid damage	**(*N*=11)**	**(*N*=11)**	***P***	**(*N*=11)**	**(*N*=10)**	***P***
5-F_{2t}-IsoP + 5-epi-5-F_{2t}-IsoP	4.67 ± 3.99	5.52 ± 2.48	n.s.	5.52 ± 2.48	1.96± 1.78	0.031^{Ψ}
15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11β-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$ –IsoP	0.65 ± 0.22	0.68 ± 0.14	n.s.	0.68 ± 0.14	0.54 ± 0.05	$3.72e^{-03\Psi}$
15-F_{2t}-IsoP	1.00 ± 0.40	0.93 ± 0.23	n.s.	0.93 ± 0.23	1.04 ± 0.31	n.s.
15-*epi*-15-F_{2t}-IsoP	1.01 ± 0.46	0.86 ± 0.28	n.s.	0.86 ± 0.28	0.86 ± 0.32	n.s.
4-F_{4t}-NeuroP + 4-*epi*-4-F_{4t}-NeuroP	3.34 ± 1.85	3.71 ± 2.10	n.s.	3.71 ± 2.10	2.98 ± 0.98	n.s.
10-*epi*-10-F_{4t}-NeuroP	1.77 ± 0.52	1.70 ± 0.57	n.s.	1.70 ± 0.57	1.54 ± 0.23	n.s.
10-F_{4t}-NeuroP	1.02 ± 0.28	1.10 ± 0.33	n.s.	1.10 ± 0.33	0.77 ± 0.31	0.031^{Ψ}
14*(RS)*-14-F_{4t}-NeuroP	1.54 ± 1.73	2.50 ± 1.94	n.s.	2.50 ± 1.94	2.03 ± 0.45	n.s.
4*(RS)*-ST-Δ^5-8-Neuro	–	–	–	–	–	–
17*(RS)*-F_{2t}-dihomo-IsoP + 17-*epi*-17-F_{2t}-dihomo-IsoP	0.72 ± 0.68	0.66 ± 0.74	n.s.	0.66 ± 0.74	1.08 ± 1.12	n.s.
ent-7*(RS)*-F_{2t}-dihomo-IsoP	1.67 ± 1.06	0.65 ± 1.07	n.s.	0.65 ± 1.07	0.58 ± 0.65	n.s.
17*(RS)*-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF	3.34 ± 2.14	2.75 ± 2.14	n.s.	2.75 ± 2.14	3.43 ± 1.80	n.s.
7*(RS)*-ST- Δ^{18}-11-dihomo-IsoF	5.76 ± 2.02	6.58 ± 1.86	n.s.	6.58 ± 1.86	4.60 ± 2.38	n.s.

Continue on next page

Table 23. *Continuation*

Inflammation	PABC-preCTX (*N*=11)	PABC-L (*N*=11)	*P*	PABC-L (*N*=11)	Control-L (*N*=10)	*P*
1a,1b-dihomo $PGF_{2\alpha}$	4.50 ± 4.39	3.56 ± 3.54	n.s.	3.56 ± 3.54	4.92 ± 5.36	n.s.
11ß-$PGF_{2\alpha}$	0.46 ± 0.25	0.42 ± 0.32	n.s.	0.42 ± 0.32	0.40 ± 0.18	n.s.
6-keto-$PGF_{1\alpha}$	0.49 ± 0.27	0.51 ± 0.31	n.s.	0.51 ± 0.31	0.58 ± 0.36	n.s.
$PGF_{2\alpha}$	5.79 ± 1.49	6.04 ± 1.04	n.s.	6.04 ± 1.04	3.48 ± 2.49	0.026^{Ψ}
GSA	<LOQ	<LOQ	–	<LOQ	<LOQ	–
Antioxidant defence	(*N*=11)	(*N*=11)	*P*	(*N*=11)	(*N*=10)	*P*
GSH/GSSG	1.20 ± 0.26	1.07 ± 0.12	n.s.	1.07 ± 0.12	0.93 ± 0.09	$4.79e^{-03\gamma}$
CYS/CYSS	–	–	–	–	–	–

Values are expressed as mean ± SD

$^{\Psi}$ Wilcoxon rank-sum test, $^{\gamma}$ Student's t test, $^{\delta}$ Wilcoxon signed-rank test and $^{\lambda}$ Student's t paired test were used to assess differences between groups.

P value of ≤ 0.05 was considered significant.

Note: Values below LOQ were replaced by ½ LOQ. Ratio values were normalized between 1 and 2 employing Min-Max scaling technique.

Table 24. Plasma levels of DNA damage (ratios), protein damage (ratios), lipid damage (nM), inflammation (nM) and antioxidant defense (ratios) in PABC patients (PABC-preCTX and PABC-L) and non-PABC patients (non-PABC-preAnC and non-PABC-postAnC).

	PABC-preCTX	Non-PABC-preAnC	*P*	Non-PABC-preAnC	Non-PABC-postAnC	*P*
DNA damage	(*N*=11)	(*N*=10)		(*N*=10)	(*N*=10)	
8-OHdG/2dG	0.76 ± 0.08	0.97 ± 0.15	1.52e-$^{03\Psi}$	0.97 ± 0.15	1.23 ± 0.38	0.029$^{\lambda}$
Protein damage	(*N*=11)	(*N*=10)	*P*	(*N*=10)	(*N*=10)	*P*
m-Tyr/Phe	0.78 ± 0.06	1.04 ± 0.25	7.88e$^{-04\Psi}$	1.04 ± 0.25	0.84 ± 0.20	0.027$^{\delta}$
o-Tyr/Phe	0.76 ± 0.09	0.88 ± 0.16	n.s.	0.88 ± 0.16	0.85 ± 0.15	n.s.
3NO_2-Tyr/p-Tyr	0.83 ± 0.10	0.82 ± 0.09	n.s.	0.82 ± 0.09	0.84 ± 0.11	n.s.
3Cl-Tyr/p-Tyr	<LOQ	<LOQ	–	<LOQ	<LOQ	–
Lipid damage	(*N*=11)	(*N*=10)	*P*	(*N*=10)	(*N*=10)	*P*
5-F_{2t}-IsoP + 5-epi-5-F_{2t}-IsoP	4.67 ± 3.99	1.68 ± 0.69	3.86e$^{-03\Psi}$	1.68 ± 0.69	1.86± 1.02	n.s.
15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11β-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$ -IsoP	0.65 ± 0.22	0.50± 0.21	n.s.	0.50± 0.21	0.55 ± 0.18	n.s.
15-F_{2t}-IsoP	1.00 ± 0.40	1.41 ± 0.68	n.s.	1.41 ± 0.68	1.24 ± 0.73	n.s.
15-*epi*-15-F_{2t}-IsoP	1.01 ± 0.46	1.67 ± 1.00	n.s.	1.67 ± 1.00	1.36 ± 0.70	n.s.
4-F_{4t}-NeuroP + 4-*epi*-4-F_{4t}-NeuroP	3.34 ± 1.85	8.74 ± 5.07	1.35e$^{-03\Psi}$	8.74 ± 5.07	6.93 ± 3.49	n.s.
10-*epi*-10-F_{4t}-NeuroP	1.77 ± 0.52	2.48± 0.98	0.010$^{\Psi}$	2.48± 0.98	2.42 ± 0.80	n.s.
10-F_{4t}-NeuroP	1.02 ± 0.28	0.83 ± 0.16	n.s.	0.83 ± 0.16	0.78 ± 0.30	n.s.
14*(RS)*-14-F_{4t}-NeuroP	1.54 ± 1.73	2.18 ± 1.30	n.s.	2.18 ± 1.30	2.10 ± 1.15	n.s.
4*(RS)*-ST-Δ^{5}-8-NeuroF	–	–	–	–	–	–
17*(RS)*-F_{2t}-dihomo-IsoP + 17-*epi*-17-F_{2t}-dihomo-IsoP	0.72 ± 0.68	1.48 ± 0.74	n.s.	1.48 ± 0.74	1.82 ± 1.72	n.s.
ent-7*(RS)*-F_{2t}-dihomo-IsoP	1.67 ± 1.06	2.01 ± 0.74	n.s.	2.01 ± 0.74	2.07 ± 1.10	n.s.
17*(RS)*-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF	3.34 ± 2.14	4.66 ± 1.47	n.s.	4.66 ± 1.47	4.87 ± 2.47	n.s.
7*(RS)*-ST- Δ^{18}-11-dihomo-IsoF	5.76 ± 2.02	<LOQ	–	<LOQ	<LOQ	–

Continue on next page

Table 24. *Continuation*

Inflammation	PABC-preCTX (*N*=11)	Non-PABC-preAnC (*N*=10)	*P*	Non-PABC-preAnC (*N*=10)	Non-PABC-postAnC (*N*=10)	*P*
1a,1b-dihomo $PGF_{2\alpha}$	4.50 ± 4.39	8.31 ± 2.99	0.016$^{\Psi}$	8.31 ± 2.99	7.91 ± 2.58	n.s.
11ß-$PGF_{2\alpha}$	0.46 ± 0.25	0.35 ± 0.19	n.s.	0.35 ± 0.19	0.45 ± 0.53	n.s.
6-keto-$PGF_{1\alpha}$	0.49 ± 0.27	0.22 ± 0.14	n.s.	1.32 ± 0.25	1.16 ± 0.37	n.s.
$PGF_{2\alpha}$	5.79 ± 1.49	2.93 ± 0.92	5.65e$^{-05\gamma}$	2.93 ± 0.92	2.66 ± 1.31	n.s.
GSA	<LOQ	<LOQ	–	<LOQ	<LOQ	–
Antioxidant defence	(*N*=11)	(*N*=10)	*P*	(*N*=10)	(*N*=10)	*P*
GSH/GSSG	1.20 ± 0.26	0.88 ± 0.06	5.67e$^{-06\Psi}$	0.88 ± 0.06	0.90 ± 0.08	n.s.
CYS/CYSS	–	–	–	–	–	–

Values are expressed as mean ± SD

$^{\Psi}$ Wilcoxon rank-sum test, $^{\gamma}$ Student's t test, $^{\delta}$ Wilcoxon signed-rank test and $^{\lambda}$ Student's t paired test were used to assess differences between groups.

P value of ≤ 0.05 was considered significant.

Note: Values below LOQ were replaced by ½ LOQ. Ratio values were normalized between 1 and 2 employing Min-Max scaling technique.

Table 25. RBC levels (ratios) of antioxidant defence in PABC patients (PABC-preCTX and PABC-L), healthy pregnant women at birth (Control-L) and non-PABC patients (non-PABC-preAnC and non-PABC-postAnC).

Antioxidant defence	PABC-preCTX (*N*=6)	PABC-L (*N*=6)	*P*	PABC-L (*N*=7)	Control-L (*N*=10)	*P*
GSH/GSSG[b]	1.19± 0.20	1.31 ± 0.13	n.s.	1.24 ± 0.23	1.11 ± 0.14	n.s.
	PABC-preCTX (*N*=8)	**Non-PABC-preAnC (*N*=10)**	***P***	**Non-PABC-preAnC (*N*=10)**	**Non-PABC-postAnC (*N*=10)**	***P***
GSH/GSSG[c]	1.12 ± 0.22	1.20 ± 0.26	n.s.	1.20 ± 0.10	1.27 ± 0.24	n.s.

Values are expressed as mean ± SD

[b]Data are available from six patients with PABC-preCTX and PABC-L samples and from seven patients with PABC-L samples measured in RBCs

[c]Data are available from eight patients with PABC-preCTX samples measured in RBCs

$^{\Psi}$ Wilcoxon rank-sum test, $^{\gamma}$ Student's t test, $^{\delta}$ Wilcoxon signed-rank test and $^{\lambda}$ Student's t paired test were used to assess differences between groups

P value of ≤ 0.05 was considered significant

Note: Values below LOQ were replaced by ½ LOQ. Ratio values were normalized between 1 and 2 employing Min-Max scaling technique.

Suppl. Table S13 and **Suppl**. **Table S14** summarised the main descriptors of the distribution of concentrations of the metabolites measured in the first and second LC-MS/MS method. From the 26 parameters quantified employing both methods, 20 were detected in the studied samples. All metabolites were measured in plasma, except for GSH and GSSG that were also analysed in RBC.

B.2.1 Cluster analysis of significant biomarkers

A heatmap of differential biomarkers of oxidative stress, inflammation and antioxidant defence, which visualises the concentration changes among the groups studied, is presented in **Figure 26**. Increase of DNA **(A)** and protein **(B)** damage was observed between PABC-preCTX group and PABC-L group. The levels of protein damage biomarker **(C)** were decreased in the plasma of PABC-L group compared to the levels in control-L, but the levels of lipid peroxidation **(D)**, inflammation **(E)** and antioxidant defence **(F)** biomarkers were elevated. When comparing PABC-preCTX group with the non-PABC-preAnC group, biomarkers of oxidative damage to DNA **(C)**, proteins **(H)**, lipids **(I)** and inflammation **(J)** were decreased in PABC-preCTX group. Opposite, three biomarkers of lipid damage **(I)**, inflammation **(J)** and antioxidant defence **(K)** were increased in PABC-preCTX. The levels of DNA damage **(L)** biomarker were increased during anthracyclines in non-PABC group, although the levels of protein damage **(M)** were the opposite.

As shown in **Figure 27**, significant differences among groups were additionally evaluated by principal component analysis (PCA). PABC patients **(A)** and non-PABC **(B)** patients exhibited different metabolic profile prior to and after chemotherapy treatment. Score plot from patients included in PABC-L group suggested a distinct metabolic profile from control-L group **(C)**. PABC-preCTX group showed discrepancies in the metabolic profile in comparison with non-PABC-preAnC **(D)**.

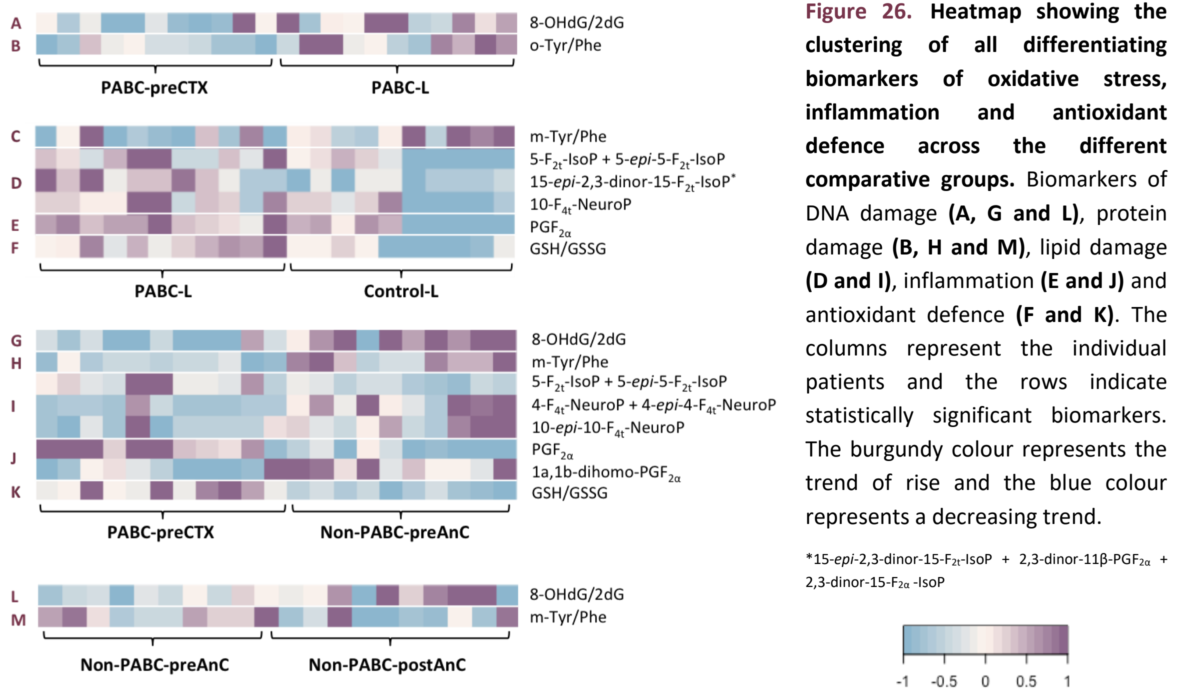


Figure 26. Heatmap showing the clustering of all differentiating biomarkers of oxidative stress, inflammation and antioxidant defence across the different comparative groups. Biomarkers of DNA damage **(A, G and L)**, protein damage **(B, H and M)**, lipid damage **(D and I)**, inflammation **(E and J)** and antioxidant defence **(F and K)**. The columns represent the individual patients and the rows indicate statistically significant biomarkers. The burgundy colour represents the trend of rise and the blue colour represents a decreasing trend.

*15-*epi*-2,3-dinor-$15\text{-}F_{2t}$-IsoP + 2,3-dinor-11β-$PGF_{2\alpha}$ + 2,3-dinor-$15\text{-}F_{2\alpha}$-IsoP

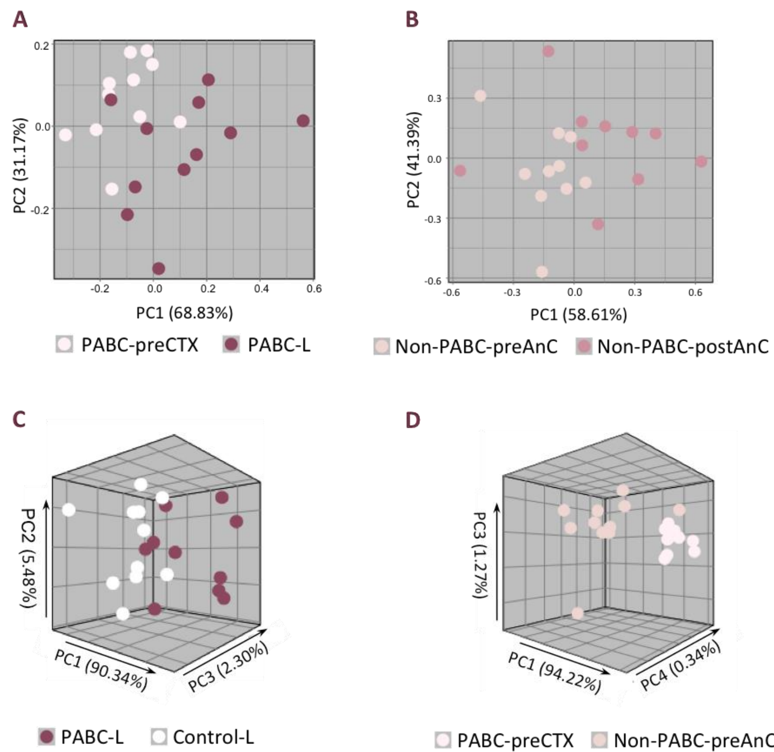


Figure 27. Plots of principal component analysis (PCA) were used to explain the statistically significant metabolic differences among groups. (A) Score plot of plasma samples obtained from PABC-preCTX group and PABC-L group. PC1, and PC2 account for 99.93% of the data's variance. **(B)** Score plot of plasma samples obtained from non-PABC-preAnc group and non-PABC-postAnC. PC1, and PC2 account for 100% of the data's variance. **(C)** Score plot of plasma samples obtained from PABC-L group and control-L group. PC1, PC2 and PC3 account for 98.48% of the data's variance. **(D)** Score plot of plasma samples obtained from PABC-preCTX group and non-PABC-preAnC. PC1, PC3 and PC4 account for 95.83% of the data's variance. Coloured circles represent the samples of each group.

Abbreviation. PC1, principal component 1; PC2, principal component 2; PC3, principal component 3; PC4, principal component 4.

B.2.2 Comparative study of biomarkers levels among tested groups

Oxidative damage to DNA

Results showed that 8-OHdG/2dG ratio measured in plasma was significantly elevated in PABC patients at labour in comparison to prior chemotherapy initiation ((*P* value= 0.031). However, the levels of 8OH-dG/2dG ratio from PABC-L patients did not differ from those detected in control-L women (**Figure 28.A**).

Likewise, 8-OHdG/2dG plasma levels were also significantly incremented during anthracyclines treatment in non-PABC patient (*P* value= 0.029). However, comparing PABC and non-PABC patients before chemotherapy treatment, the levels of 8-OHdG/2dG plasma ratio were statistically significantly higher in non-PABC-preAnC (*P* value= $1.52e^{-03}$) (**Figure 28.B**).

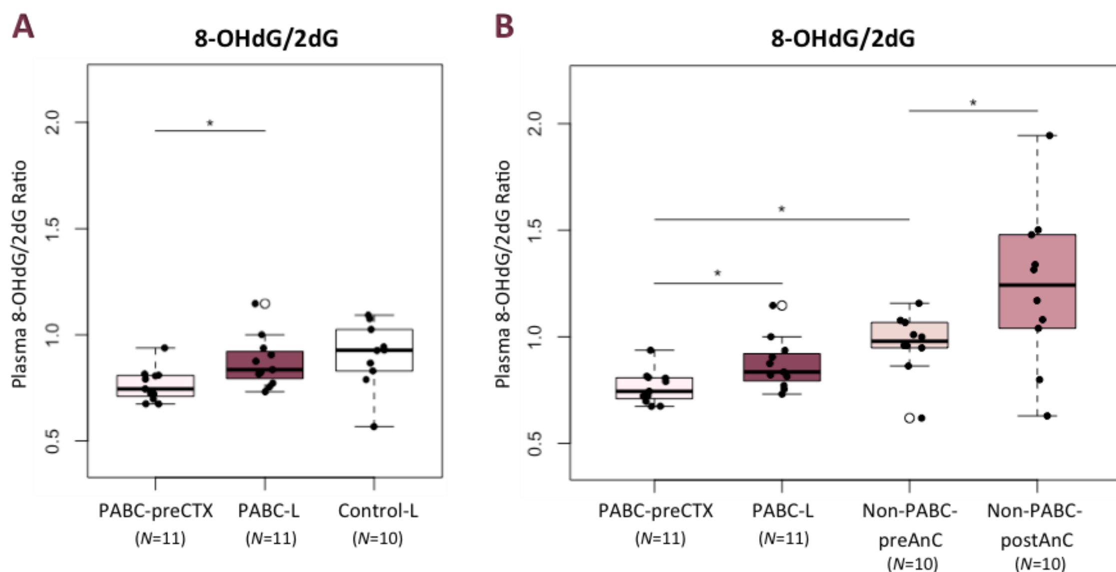


Figure 28. Boxplots of DNA damage biomarker measured in plasma. (A) Effect of chemotherapy treatment during pregnancy. **(B)** Comparative effect of breast cancer occurrence in PABC and non-PABC patients and the repercussion of anthracycline-based regimens administered in non-PABC patients. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.

Student's t paired test **(A** and **B)** and Wilcoxon rank-sum test **(B, PABC-preCTX vs non-PABC-preAnC)** were used to assess differences between groups;

P value of <0.05 was considered significant. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

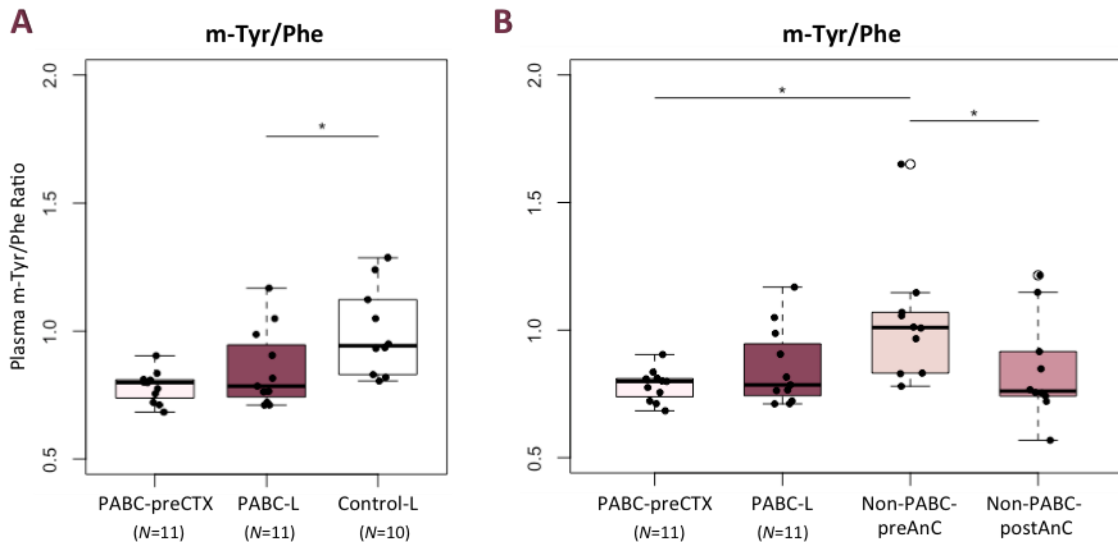
Note: Values below LOQ were replaced by ½ LOQ. Ratio values were normalized between 1 and 2 employing Min-Max scaling technique.

Oxidative damage to proteins

We observed differences in m-Tyr/Phe plasma levels measured in labour between PABC-L and control-L group reaching statistical significance (*P* value= 0.029) **(Figure 29.A)**. Moreover, they were remarkably elevated in non-PABC-preAnC in comparison with non-PABC-postAnC and PABC-preCTX patients, (*P* value= 0.027, *P* value= $7.88e^{-04}$, respectively) **(Figure 29.B)**. However, non-significant differences were detected in m-Tyr/ Phe plasma levels between PABC-preCTX and PABC-L groups.

On the other hand, o-Tyr/Phe plasma levels were notably incremented during chemotherapy treatment as the concentrations detected in PABC-preCTX group were lower than those from PABC-L group (*P* value= 0.032) **(Figure 29.C)**. Nevertheless, o-Tyr/Phe levels were similar among the remaining comparative groups (**Figure 29.C and D)**.

Besides, the levels of $3NO_2$-Tyr/p-Tyr were not statistically different among all comparisons **(Figure 29.E and F)** while the levels of 3Cl-Tyr/p-Tyr were detected below LOQ.



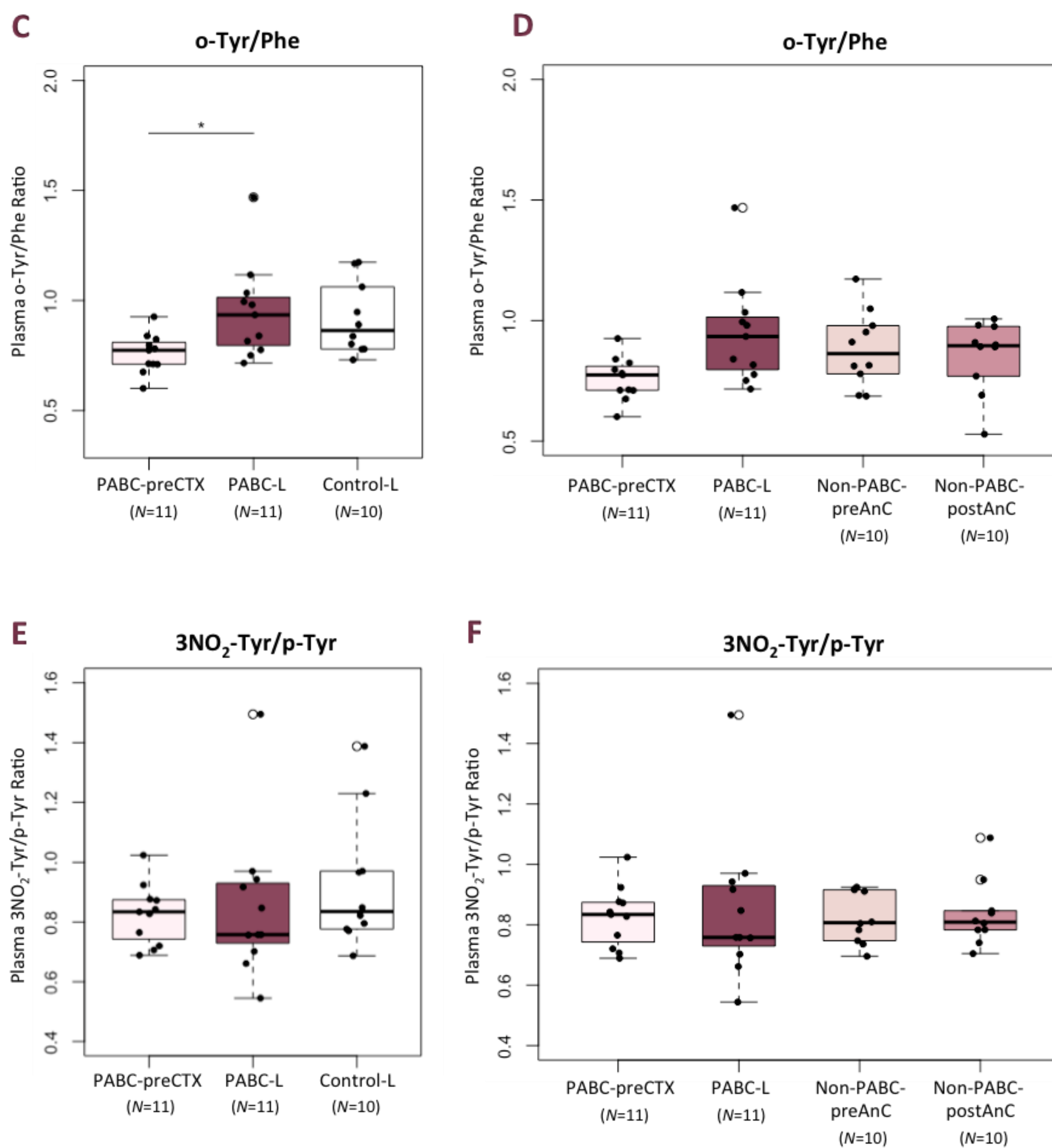


Figure 29. Boxplots of protein damage biomarkers measured in plasma. (A,C and **E)** Effect of chemotherapy treatment during pregnancy. **(B,D** and **F)** Comparative effect of breast cancer occurrence in PABC and non-PABC patients and the repercussion of anthracycline-based regimens administered in non-PABC patients. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.

Wilcoxon rank-sum test **(A and B)**, Wilcoxon signed-rank test **(C)** Student's t paired test **(B, non-PABC-preAnC vs non-PABC-postAnC)** were used to assess differences between groups; *P* value of <0.05 was considered significant. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

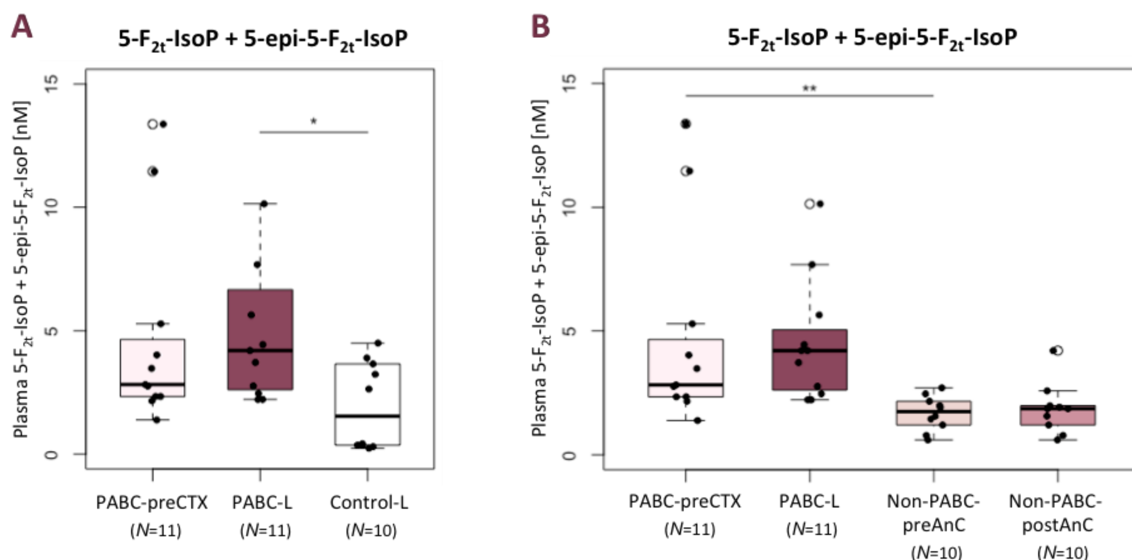
Note: Values below LOQ were replaced by ½ LOQ. Ratio values were normalized between 1 and 2 employing Min-Max scaling technique.

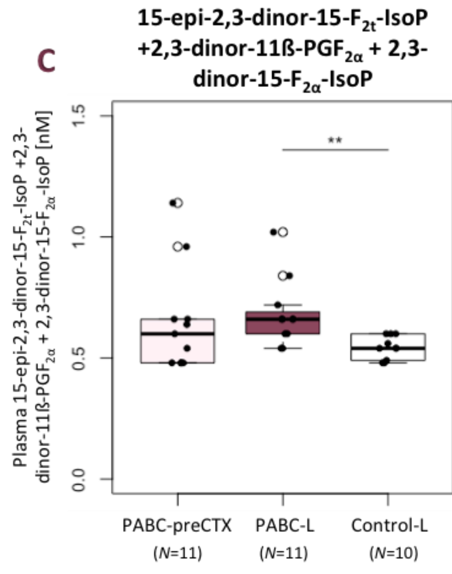
Oxidative damage to lipids

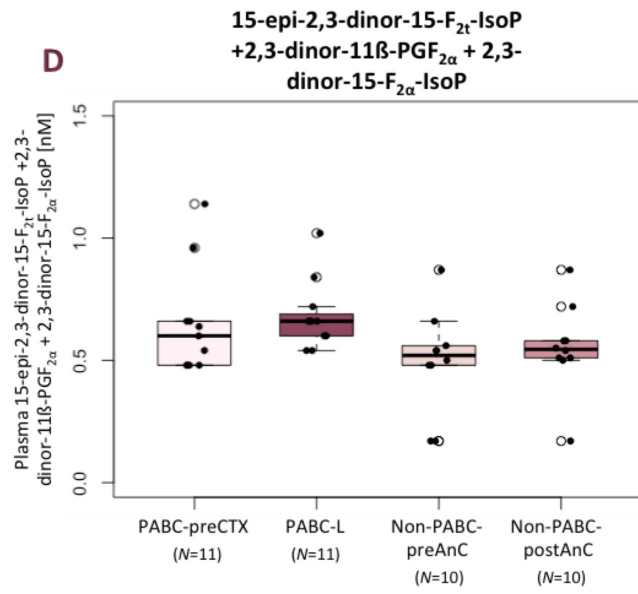
As shown in **Figure 30.A** and **30.C**, some isoprostane compounds levels (i.e., 5-F_2t-IsoP + 5-epi-5-F_{2t}-IsoP and 15-epi-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11ß-PGF2α + 2,3-dinor-15-$F_{2\alpha}$-IsoP) were statistically elevated in PABC-L group respect to control-L group (*P* value= 0.031, *P* value= 3.72e^{-03}, respectively). In addition, 5-F2t-IsoP + 5-epi-5-F2t-IsoP plasma levels were found statistically significant lower in non-PABC than in PABC patients prior to chemotherapy initiation (*P* value= 3.86e^{-03}) **(Figure 30.B)**. However, the levels of these isoprostanes were similar among the remaining comparisons **(Figure 30.A-D)**.

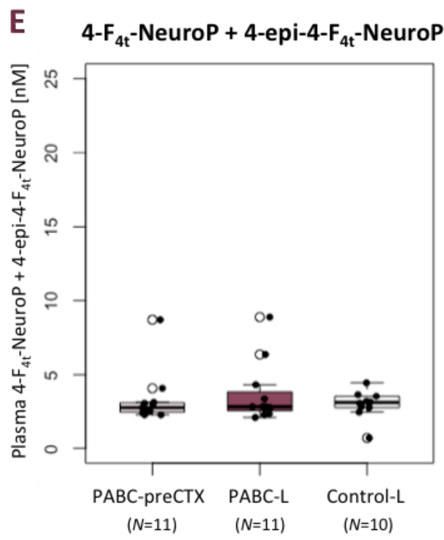
Plasma biomarkers of lipid peroxidation from the neuroprostanes family, 4-F_{4t}-NeuroP + 4-epi-4-F_{4t}-NeuroP and 10-epi-10-F_{4t}-NeuroP, were especially significantly decreased in PABC-preCTX patients in comparison with non-PABC-preAnC patients group (*P* value= 1.35^{e-03}, *P* value= 0.030, respectively) **(Figure 30.F** and **30.H)**. Conversely, only 10-F_{4t}-NeuroP plasma levels were detected significantly elevated in PABC-L patients respect to control-L (*P* value= 0.031) **(Figure 30.I)**. Nevertheless, we observed non-significant differences in the levels of these NeuroPs among the rest of the comparative groups (**Figure 30.E-J)**.

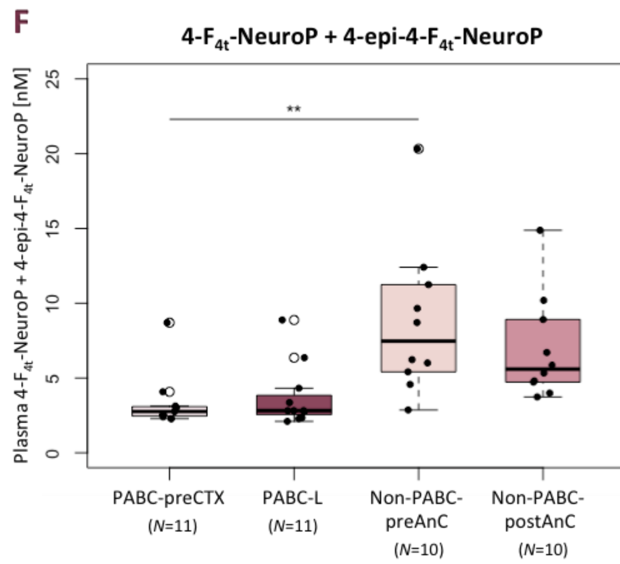
The remaining compounds analysed did not show statistically significant differences among all comparisons **(see Suppl. Figure S6)**. Exceptionally, 4*(RS)*-ST-Δ^5-8-NeuroF compound was not detected.

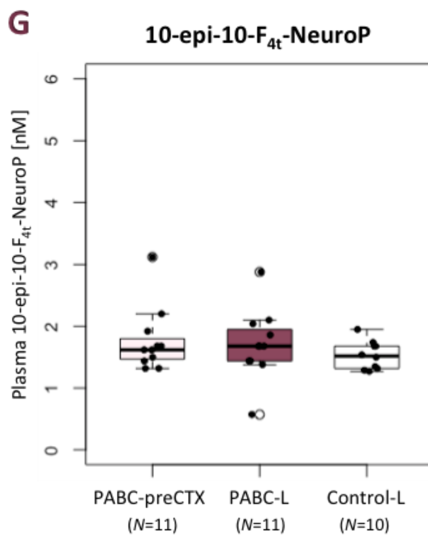


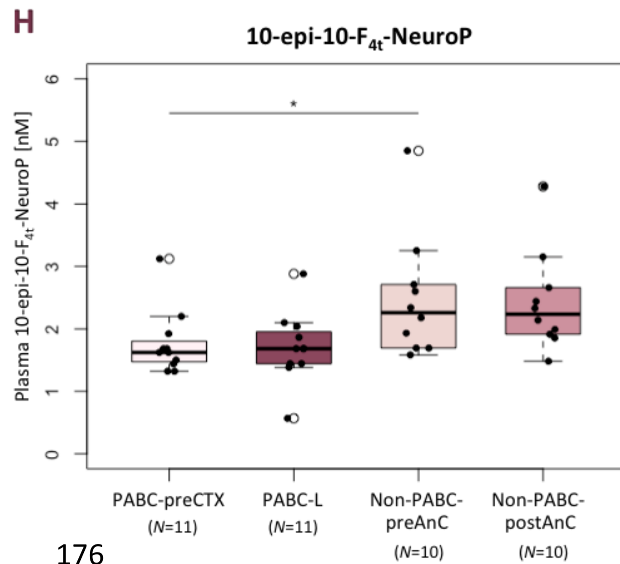

C
15-epi-2,3-dinor-15-F_{2t}-IsoP +2,3-dinor-11ß-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$-IsoP
Plasma 15-epi-2,3-dinor-15-F_{2t}-IsoP +2,3-dinor-11ß-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$-IsoP [nM]
**
0.0
0.5
1.0
1.5
PABC-preCTX
(N=11)
PABC-L
(N=11)
Control-L
(N=10)


D
15-epi-2,3-dinor-15-F_{2t}-IsoP +2,3-dinor-11ß-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$-IsoP
Plasma 15-epi-2,3-dinor-15-F_{2t}-IsoP +2,3-dinor-11ß-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$-IsoP [nM]
0.0
0.5
1.0
1.5
PABC-preCTX
(N=11)
PABC-L
(N=11)
Non-PABC-preAnC
(N=10)
Non-PABC-postAnC
(N=10)


E
4-F_{4t}-NeuroP + 4-epi-4-F_{4t}-NeuroP
Plasma 4-F_{4t}-NeuroP + 4-epi-4-F_{4t}-NeuroP [nM]
0
5
10
15
20
25
PABC-preCTX
(N=11)
PABC-L
(N=11)
Control-L
(N=10)


F
4-F_{4t}-NeuroP + 4-epi-4-F_{4t}-NeuroP
Plasma 4-F_{4t}-NeuroP + 4-epi-4-F_{4t}-NeuroP [nM]
**
0
5
10
15
20
25
PABC-preCTX
(N=11)
PABC-L
(N=11)
Non-PABC-preAnC
(N=10)
Non-PABC-postAnC
(N=10)


G
10-epi-10-F_{4t}-NeuroP
Plasma 10-epi-10-F_{4t}-NeuroP [nM]
0
1
2
3
4
5
6
PABC-preCTX
(N=11)
PABC-L
(N=11)
Control-L
(N=10)


H
10-epi-10-F_{4t}-NeuroP
Plasma 10-epi-10-F_{4t}-NeuroP [nM]
*
0
1
2
3
4
5
6
PABC-preCTX
(N=11)
PABC-L
(N=11)
Non-PABC-preAnC
(N=10)
Non-PABC-postAnC
(N=10)

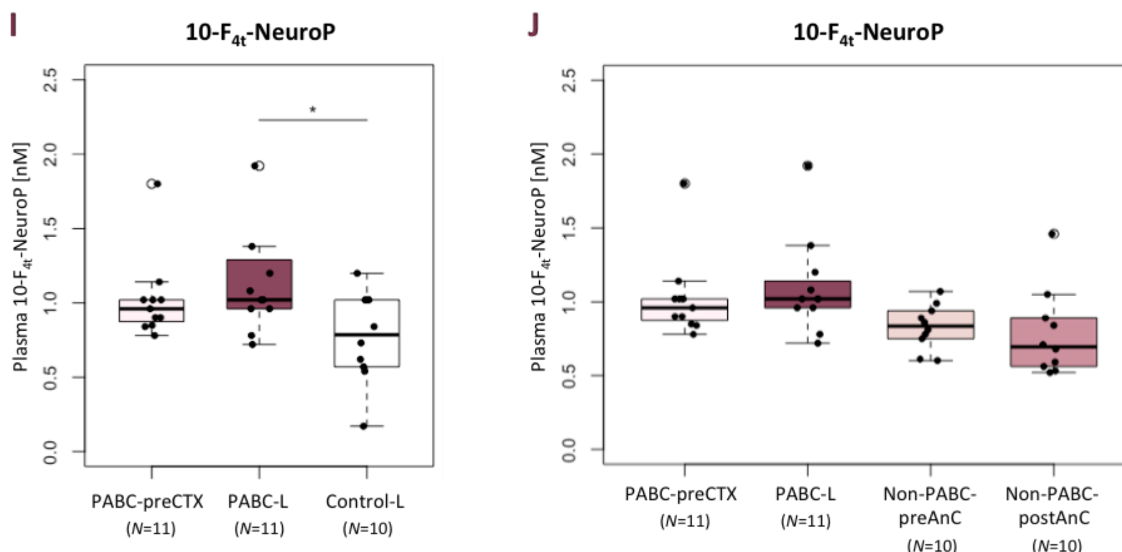


Figure 30. Boxplots of lipid damage biomarker measured in plasma. (A, C, E, G and I) Effect of chemotherapy treatment during pregnancy. **(B, D, F and J)** Comparative effect of breast cancer occurrence in PABC and non-PABC patients and the repercussion of anthracycline-based regimens administered in non-PABC patients. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers. Wilcoxon rank-sum test was used to assess differences between groups; a *P* value of <0.05 was considered significant. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.
Note: Values below LOQ were replaced by ½ LOQ.

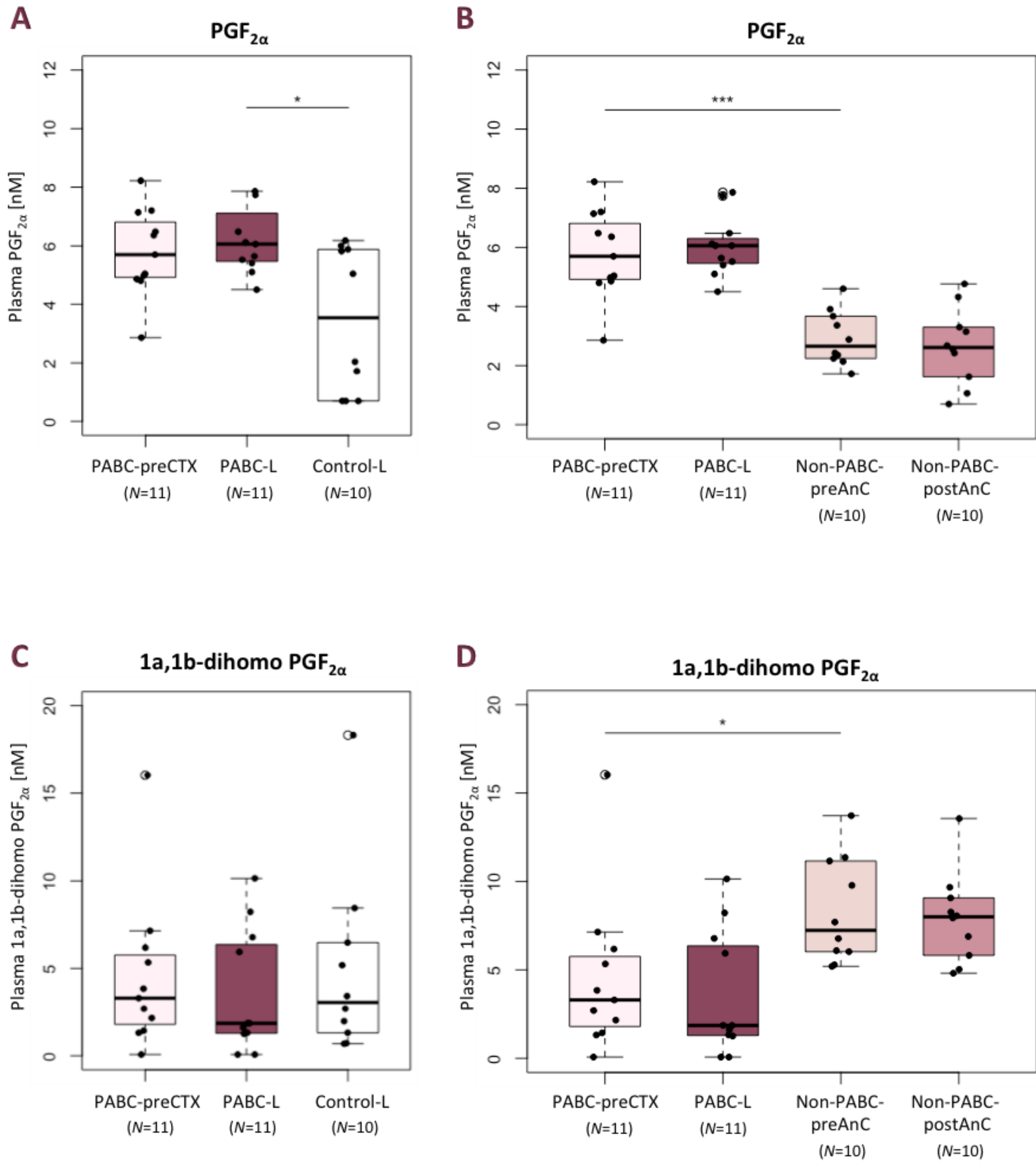
Inflammatory mediators

Significant differences in prostaglandins levels were appreciable among some groups**.** **Figure 31.A** illustrates an increase of $PGF_{2\alpha}$ plasma levels detected in PABC-L group compared to control-L group (*P* value= 0.026). Additionally, $PGF_{2\alpha}$ plasma levels were highly incremented in PABC patients respect to non-PABC previous chemotherapy initiation (*P* value= $5.65e^{-05}$) **(Figure 31.B)**. These differences were not observed in the rest of the groups.

Conversely, plasma concentrations of 1a,1b-dihomo-$PGF_{2\alpha}$ were detected significantly more decreased in PABC-preCTX patients than in non-PABC-preAnC (*P* value= 0.016) **(Figure 31.D)**. However, the evaluation of this prostaglandin in both PABC and non-PABC groups following chemotherapy showed similar levels.

In addition, we observed non-significant differences among the levels of 1a,1b-dihomo-$PGF_{2\alpha}$ following chemotherapy and between PABC-L and control-L groups at birth **(Figure 31.C)**.

Excepting GSA, which was detected below LOQ, we found no statistically significant differences among all the comparison in the remaining biomarkers analysed (**Figure 31.F-I**).



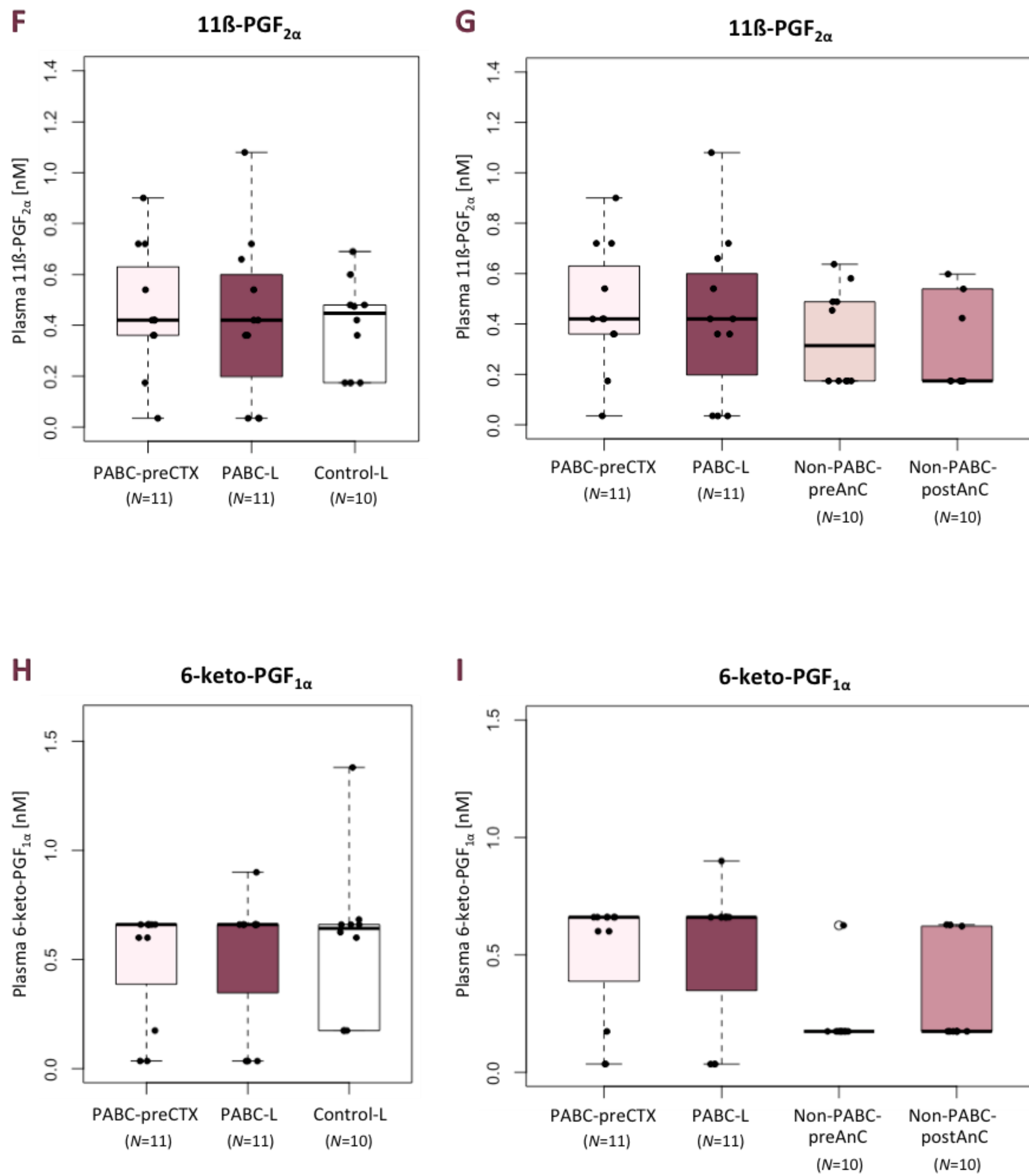


Figure 31. Boxplots of inflammation biomarker measured in plasma. (A, C, E, F and **H)** Effect of chemotherapy treatment during pregnancy. **(B, D, G** and **I)** Comparative effect of breast cancer occurrence in PABC and non-PABC patients and the repercussion of anthracycline-based regimens administered in non-PABC patients. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.

Wilcoxon rank-sum test **(A** and **D)**, Student's t test **(B),** were used to assess differences between groups; *P* value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.

Note: Values below LOQ were replaced by ½ LOQ.

Antioxidant defence

The analysis of antioxidant defence biomarker showed some discrepancies among the groups studied. The plasma levels of GSH/GSSG ratio were statistically significantly higher in patients from PABC-L in comparison with those from control-L (*P* value= $4.79e^{-03}$), although they did not differ from those measured previous chemotherapy administration **(Figure 32.A)**.

Moreover, GSH/GSSG plasma levels were significantly increased in PABC-preCTX patients respect to non-PABC-preAnC (*P* value= $5.67e^{-06}$) **(Figure 32.B)**. However, the comparison between non-PABC groups revealed no significant differences in the levels of GSH/GSSG measured.

Regarding the analysis of GSH/GSSG in RBCs, we did not observe significant differences among all the comparisons (**Figure 33.A**-**B)**.

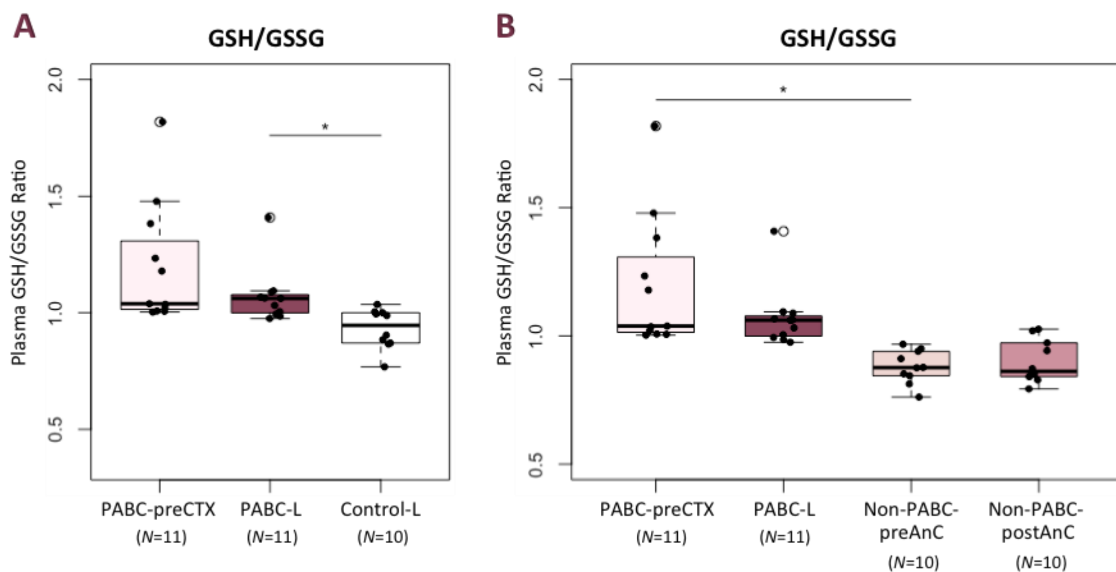


Figure 32. Boxplots of antioxidant defence biomarker measured in plasma. (A) Effect of chemotherapy treatment during pregnancy. **(B)** Comparative effect of breast cancer occurrence in PABC and non-PABC patients and the repercussion of anthracycline-based regimens administered in non-PABC patients. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.

Student's t test **(A)** and Wilcoxon rank-sum test **(B)** were used to assess differences between groups; a *P* value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.

Note: Values below LOQ were replaced by ½ LOQ.

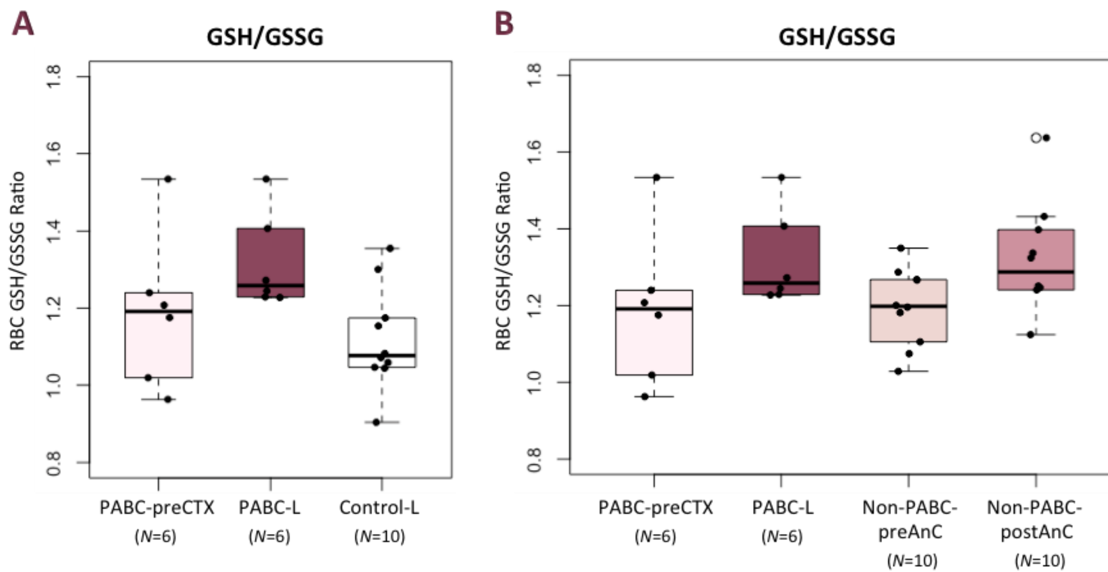


Figure 33. Boxplots of antioxidant defence biomarker measured in RBC. (A) Effect of chemotherapy treatment during pregnancy. **(B)** Comparative effect of breast cancer occurrence in PABC and non-PABC patients and the repercussion of anthracycline-based regimens administered in non-PABC patients. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.

P value of <0.05 was considered significant. $*P \le 0.05$; $**P \le 0.01$; $***P \le 0.001$.

Note: Values below LOQ were replaced by ½ LOQ.

B.2.3 Multivariate and univariate regression analyses

Patient status and metabolites

Univariate logistic regression analysis showed non-significant association of the biomarkers levels measured in PABC prior chemotherapy and at labour with patient status (Disease-free or metastatic)

Metabolites and clinical characteristics

There was a significant negative association between surgery prior chemotherapy (yes/no) and the plasma levels of m-Tyr/Phe ratio before chemotherapy treatment according to a univariate linear regression analysis (*P* value=0.043; β=-0.131) **(Table 26)**. Opposite, we found a positive association with the plasma levels of 5-F_{2t}-IsoP + 5-*epi*-5-F_{2t}-IsoP measured prior chemotherapy (*P* value=0.049; β=5.73) **(Table 27)**.

Table 26. Univariate lineal regression of the relation to clinical characteristics and m-Tyr/Phe plasma ratio measured in PABC patients before chemotherapy

Clinical characteristics	β	Std. Error	t-value	*P*
Intercept	-18.54	7.524	-2.464	0.090
Reproductive techniques	-4.95	2.465	-2.008	0.138
Hormone receptor	9.57	4.994	1.916	0.151
Her2	-4.47	1.788	-2.5	0.088
Surgery prior chemotherapy	5.73	1.788	3.204	**0.049**
Parity	15.24	6.786	2.246	0.110
Gestational age at delivery	-1.41	4.629	-0.305	0.781
Clinical stage	0.72	2.039	0.353	0.747

P value of <0.05 was considered significant. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.
Note: global *P* value= 0.227

Table 27. Univariate lineal regression of the relation to clinical characteristics and 5-F_{2t}-IsoP + 5-*epi*-5-F_{2t}-IsoP plasma levels measured in PABC patients before chemotherapy

Clinical characteristics	β	Std. Error	t-value	*P*
Intercept	1.15001	0.16333	7.041	0.006
Reproductive techniques	0.03747	0.05351	0.7	0.534
Hormone receptor	-0.13384	0.10843	-1.234	0.304
HER2/neu	-0.08941	0.03882	-2.303	0.104
Surgery prior chemotherapy	-0.13124	0.03882	-3.381	**0.043**
Parity	-0.25232	0.14732	-1.713	0.185
Gestational age at delivery	0.14768	0.10049	1.47	0.238
Clinical stage	-0.00118	0.04426	-0.027	0.980

P value of <0.05 was considered significant. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.
Note: global *P* value= 0.095

Furthermore, we observed a significant positive association between parity (nulliparous/parous) (*P* value=0.035; β=0.035) and clinical stage (stage I+ stage II/stage III+ stage IV) (*P* value=0.035; β=0.010) with the plasma levels of GSH/GSSG ratio measured prior chemotherapy treatment **(Table 28)**.

Table 28. Univariate lineal regression of the relation to clinical characteristics and the plasma levels GSH/GSSG ratio measured in PABC patients before chemotherapy

Clinical characteristics	β	Std. Error	t-value	*P*
Intercept	0.02728	0.19101	0.143	0.896
Reproductive techniques	-0.12838	0.06258	-2.051	0.133
Hormone receptor	0.10497	0.1268	0.828	0.469
HER2/neu	0.08852	0.0454	1.95	0.146
Surgery prior chemotherapy	0.05472	0.0454	1.205	0.3145
Parity	0.6295	0.17229	3.654	**0.0354**
Gestational age at delivery	-0.06885	0.11752	-0.586	0.5991
Clinical stage	0.30038	0.05177	5.803	**0.0102**

P value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.
Note: global *P* value= 0.006

Significant positive associations between surgery prior chemotherapy (yes/no), gestational age at delivery (pre-term/full-term) and clinical stage (stage I+ stage II/stage III+ stage IV) with the plasma levels of 17(*RS*)-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF was detected in PABC patients before chemotherapy (*P* value=0.040; β=2.64, *P* value=0.021; β=8.65, and *P* value=0.025; β=3.60 respectively). Contrary to these findings, we observed significant negative associations between hormone receptor (positive/negative) HER2/neu (positive/negative) and parity (nulliparous/parous) (*P* value=0.043; β=-7.12, *P* value=0.045; β=-2.52, *P* value=0.044; β=-9.71) with the plasma levels 17(*RS*)-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF measured in PABC patients prior chemotherapy **(Table 29)**.

Table 29. Univariate lineal regression of the relation to clinical characteristics and the plasma levels of 17(*RS*)-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF measured in PABC patients before chemotherapy.

Clinical characteristics	β	Std. Error	t-value	*P*
Intercept	7.9408	3.1963	2.484	0.089
Reproductive techniques	1.7614	1.0472	1.682	0.191
Hormone receptor	-7.1225	2.1218	-3.357	**0.043**
HER2/neu	-2.5193	0.7597	-3.316	**0.045**
Surgery prior chemotherapy	2.6393	0.7597	3.474	**0.040**
Parity	-9.7115	2.883	-3.369	**0.044**
Gestational age at delivery	8.6546	1.9665	4.401	**0.021**
Clinical stage	3.6014	0.8662	4.158	**0.025**

P value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.
Note: global *P* value= 0.049

There was a significant negative association between parity (nulliparous/parous) (*P* value=0.044; β=-5.64) and 15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$-IsoP **(Table 30)**.

Table 30. Univariate lineal regression of the relation to clinical characteristics and the plasma levels of 15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$ -IsoP measured in PABC patients before chemotherapy.

Clinical characteristics	β	Std. Error	t-value	*P*
Intercept	6.6431	1.8811	3.531	0.0386
Reproductive techniques	1.1859	0.6163	1.924	0.15
Hormone receptor	-3.5561	1.2488	-2.848	0.0652
Her2	-0.1832	0.4471	-0.41	0.7095
Surgery prior chemotherapy	-1.0549	0.4471	-2.359	0.0995
Parity	-5.6437	1.6967	-3.326	**0.0448**
Gestational age at delivery	3.1503	1.1574	2.722	0.0724
Clinical stage	0.7564	0.5098	1.484	0.2345

P value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.
Note: global *P* value= 0.258

Neonatal weight at birth and metabolites

Univariate lineal regression conducted on each birth weight revealed a negative significant association with m-Tyr/Phe levels measured in PABC patients at labour **(Table 31)**.

Table 31. Univariate lineal regression of the relation to neonatal birth weight and the plasma levels of m-Tyr/Phe ratio measured in PABC patients at labour

Clinical characteristics	β	Std. Error	t-value	*P*
Intercept	3827.4	497.6	7.691	$9.48e^{-06}$
m-Tyr/Phe	-1455.4	562	-2.59	**0.0251**

P value of <0.05 was considered significant. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

Mode of delivery and metabolites

Our study did not observed an association between the mode of delivery (vaginal and caesarean) and biomarkers levels detected in PABC patients at labour.

Clinical stage and metabolites

Univariate logistic regression analysis for the association between metabolite levels measured before chemotherapy treatment in PABC patients and clinical stage showed non-significant results.

B.2.5 Correlation analyses

DNA damage

There were non-significant correlations between plasma 8-OHdG/2dG levels measured in PABC patients prior treatment and at labour.

Protein damage

There were non-significant correlations between plasma m-Tyr/Phe, o-Tyr/Phe and $3NO_2$-Tyr/Phe levels measured in PABC patients prior treatment and at labour.

Lipid damage

There were non-significant correlations between plasma levels of isoprostanes, isofurans, neuroprostanes, neurofurans, dihomo-Isoprostanes and dihomo-Isofurans in PABC patients prior treatment and at labour.

Inflammation

There were non-significant correlations between plasma levels of prostaglandins in PABC patients prior treatment and at labour.

Antioxidant defence

There were non-significant correlations between plasma levels of GSH/GSSG ratio in PABC patients prior treatment and at labour. We also did not found a correlation between RBC levels of GSH/GSSG ratio before treatment and at labour.

STUDY II: *Oxidative stress, inflammation and antioxidant defence biomarkers measured in cord blood and urine samples from neonates exposed to chemotherapy in utero*

Blood samples were obtained from thirteen neonates, who were exposed to chemotherapy *in utero* (PABC-N) and fourteen neonates from healthy gestation (control-N) **(Figure 34)**. Every neonate included in PABC-N group was born after exposure to varying numbers of cycles of anthracyclines *in utero*. Four neonates were exposed to two cycles of anthracyclines, one neonate was exposed to three cycles, seven neonates were exposed to four cycles, and one neonate was exposed to five cycles. Some neonates received additional exposure to paclitaxel *in utero* (*N*=4). Two neonates were exposed to four cycles of paclitaxel, and the remaining two were exposed to eight and ten, cycles respectively.

Blood was withdrawn from the umbilical cord shortly after birth. All neonates from PABC-N group and five neonates from control-N group were born at the *Vall d´Hebron University Hospital*. The remaining neonates were born at the *Son Espases University Hospital.*

Additionally, urine samples were collected from five out of thirteen neonates exposed to chemotherapy *in utero* included in the study. Urine samples from the remaining neonates were missing. Regarding the control-N group, urine samples were collected from six neonates delivered at the *La Fe and Polytechnic University Hospital.* All samples were acquired within 24h of life.

The exposure anthracycline-based regimen *in utero* was as follow: one neonate was exposed to two cycles of anthracyclines, three neonates were exposed to four cycles, and one neonate was exposed to five cycles. Furthermore, three of them were exposed to paclitaxel following anthracyclines treatment during their intrauterine development. Specifically, they were exposed to four, eight and ten cycles of paclitaxel, respectively.

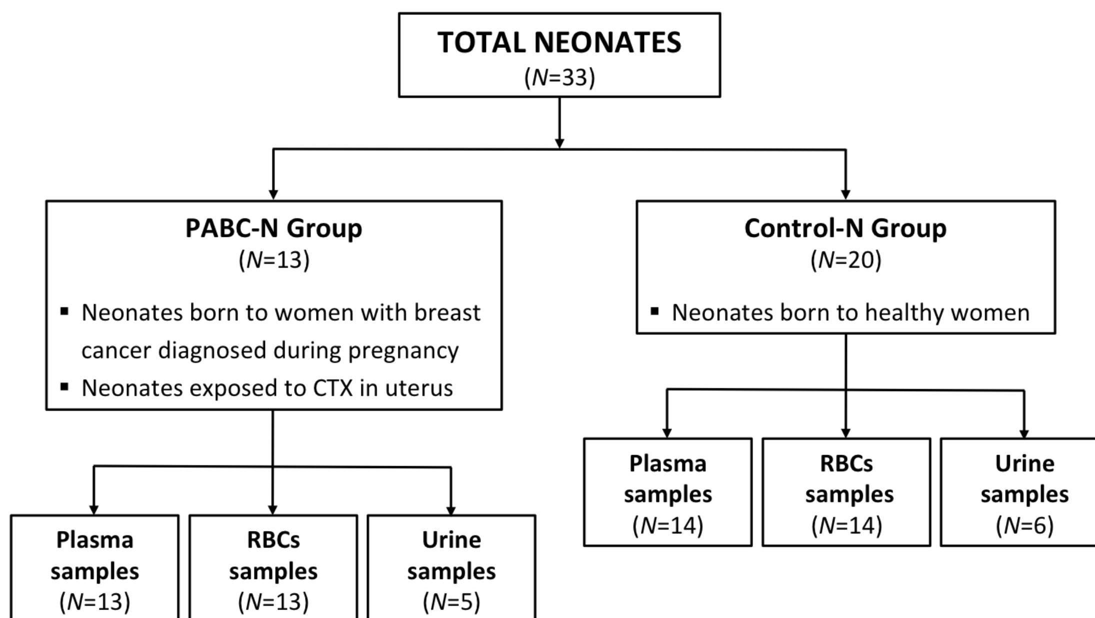


Figure 34. STUDY II flow chart describing all participants of the study and type of the samples.

2.1 Neonatal characteristics of the study populations

The clinical information of the thirty-eight neonates included are listed in **Table 32** and **Table 33**. All neonates included in the case group were born after exposure to varying numbers of anthracycline-based regimens *in utero* (100%). Additionally, 30.77% and 60% of neonates with blood and urine analysed respectively, were exposed to paclitaxel following anthracyclines treatment. In total, the mean chemotherapy cycles that were exposed was 5.36 cycles (range 4-14 cycles) in neonates with blood analysed and 8.20 cycles (4-14 cycles) in those with urine analysed.

Neonates with blood analysed were exposed to a mean number of anthracyclines cycles of 3.38 (range 2-5 cycles), whereas the neonates with urine analysed were 3.80 (range 2-5 cycles). Among those neonates that additionally were exposed to paclitaxel, the mean number cycles were 6.50 (range 4-10 cycles) and 7.33 (range 4-10 cycles) in neonates with blood and urine analysed, respectively.

Regarding the clinical data from neonates with blood analysed, the gestational age was significantly reduced in neonates exposed to chemotherapy in utero as compared to control-L group (*P* value= $4.06e^{-06}$). Therefore, ten out of thirteen PABC-N neonates were classified as premature (76.92%) whereas only one out of fourteen controls were born pre-term (7.14%). Moreover, neonates under chemotherapy exposure *in utero* had statistically significant lower weight than those not exposed (*P* value= $7.87e^{-05}$). The mean birth weight was 2560.38 g (range 1995-3350 g) with a mean weight percentile of 47.23 (range 4-98; data of one neonate was not available) in PABC-N group. The mean birth weight was 3187.86 g (range 2250-3630 g) with a mean weight percentile of 56.71 (range 2-97) in neonates from control-N group.

On the other hand, the mean gestational age at delivery did not differ statistically among urine groups, and the proportion of prematurity was similar. Specifically, the mean gestational age at delivery in the urine group of PABC-N was 36.82 weeks (range 34.4-38.5 weeks) with two neonates born prematurely (40%) and 37.10 weeks (range 36.2-38.1 weeks) with also two pre-term neonates (33.33%) in the group of controls. Furthermore, the mean weight at birth in PABC-group was 2673 g (range 2370-3250) with a weight percentile of 29.20 (range 7-55) As a consequence of their weight at birth, one neonate of PABC-N group (10%) and three of control-N group (50%) were diagnosed with low birth weight.

Despite the differences detailed previously, the neonatal Apgar scores of 1 min and 5 min were within the normal range in all neonates included in the study.

Table 32 . Clinical information of neonates exposed to chemotherapy *in utero* (PABC-N) and neonates born to healthy pregnant women (control-N) included in cord blood group

	PABC-N *(Cord blood)*		**Control-N *(Cord blood)***	
CHARACTERISTIC	*N*=13	%	*N*=14	%
Chemotherapy				
Anthracyclines	13	100%	-	-
Cycles	3.38 ± 1.04	(2-5)	-	-
Paclitaxel	4	30.77%	-	-
Cycles	6.50 ± 2.60	(4-10)	-	-
Total cycles	5.38 ± 3.62	(14)	-	-
GA at delivery (wk)	36.09 ± 1.17***	(34.4-38.5)	39.09 ± 1.45	(36.6-41.3)
Preterm	10	76.92%	1	7.14%
Term	3	23.08%	13	92.86%
Birth weight (g)	2560.38 ± 398.84	(1995-3350)	3328.57 ± 447.96	(2250-4160)
Birth weight (p)	47.23 ± 31.17	(4-98)	56.71 ± 29.76	(2-97)
Low birth weight	1	7.69%	0	-
1 min Apgar	8.76 ± 0.44	(8-9)	8.86 ± 0.86	(6-10)
5 min Apgar	9.62 ± 0.77	(8-10)	10 ± 0	100%

Student's t test were used to assess differences between groups; a *P* value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.

Table 33. Clinical information of neonates exposed to chemotherapy *in utero* (PABC-N) and neonates born to healthy pregnant women (control-N) included in urine group

	PABC-N (*Urine*)		**Control-N (*Urine*)**	
CHARACTERISTIC	*N*=5	%	*N*=6	%
Chemotherapy				
Anthracyclines	5	100%	-	-
Cycles	3.80 ± 1.10	(2-5)	-	-
Paclitaxel	3	60%	-	-
Cycles	4.40 ± 4.56	(4-10)	-	-
Total cycles	8.20 ± 4.04	(4-14)	-	-
GA at delivery (wk)	36.82 ± 1.56	(34.4-38.5)	37.10 ± 0.70	(36.2-38.1)
Preterm	2	40%	2	33.33%
Term	3	60%	4	66.67%
Birth weight (g)	[143]3.00 ± 344.99	(2370-3250)	2605.00 ± 582.02	(1940-3440)
Birth weight (p)	29.20 ± 22.45	(7-55)	30.5 ± 41.89	(2-93)
Low birth weight	1	20%	3	50%
1 min Apgar	9 .00 ± 0	(9)	9.00 ± 0.63	(8-10)
5 min Apgar	10.10 ± 0	(10)	9.83 ± 0.41	(9-10)

2.2 Results of the statistical analysis of the study

The main objective of this study was to compare the levels of different biomarkers in neonates born to those PABC women included in STUDY I and consequently prenatally exposed to chemotherapy (PABC-N) with neonates born to healthy pregnant women (control-L) **(see Figure 34)**. This comparison was made by analysing the neonatal cord blood collected at birth and urine obtained within the first 24h after birth.
Additionally, cord blood from neonates exposed to chemotherapy (PABC-N) was compared with the corresponding maternal blood (PABC-L) at delivery.

Regarding the statistical tests employed in the present study, first, we determined whether the biomarkers followed a normal distribution in all groups by using Shapiro-Wilk test. Second, following the test of normally, we employed the student t-test or Wilcoxon rank-sum test if we accepted that the biomarkers analysed went by normal distribution or the opposite respectively.
On the other hand, student's t paired test (normal distribution accepted) and Wilcoxon signed-rank test (normal distribution not accepted) were used when we compared groups that could influence each other (i.e., PABC-N and PABC-L)

The concentrations and ratios of the biomarkers measured in all comparative groups included in the study II detailing the statistical test employed are summarized from **Table 34** to **Table 37**.

Table 34. Plasma and urine levels of DNA damage (ratios), protein damage (ratios), lipid damage (nM), inflammation (nM) and antioxidant defense (ratios) in neonates exposed to chemotherapy *in utero* (PABC-N) and neonates born to healthy women at birth (Control-N).

	PLASMA			**URINE**		
	PABC-N	Control-N	*P*	PABC-N	Control-N	*P*
DNA damage	(*N*=13)	(*N*=14)		(*N*=5)	(*N*=6)	
8-OHdG/2dG	0.77 ± 0.14	0.76 ± 0.19	n.s.	1.11 ± 0.26	0.95 ± 0.12	n.s.
Protein damage	(*N*=13)	(*N*=14)	*P*	(*N*=5)	(*N*=6)	*P*
m-Tyr/Phe	0.80 ± 0.11	0.74 ± 0.12	n.s.	<LOQ	<LOQ	-
o-Tyr/Phe	0.88 ± 0.12	0.93 ±0.14	n.s.	<LOQ	<LOQ	-
3NO_2-Tyr/p-Tyr	0.66 ± 0.05	0.75 ± 0.14	0.038^{γ}	<LOQ	<LOQ	-
3Cl-Tyr/p-Tyr	<LOQ	<LOQ	-	2.94 ± 1.78	2.94 ± 1.78	-
Lipid damage	(*N*=13)	(*N*=14)	*P*	(*N*=5)	(*N*=6)	*P*
5-F_{2t}-IsoP + 5-*epi*-5-F_{2t}-IsoP	4.43 ± 1.46	2.94 ± 1.78	0.025^{γ}	3.06 ± 2.45	8.09 ± 5.75	n.s
15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$ -IsoP	0.66 ± 0.10	0.65 ± 0.11	n.s.	19.39 ± 20.11	49.21 ± 33.34	n.s
15-F_{2t}-IsoP	0.88 ± 0.35	1.62 ± 1.02	$8.05e^{-03\Psi}$	1.92 ± 0.55	6.64 ± 3.52	0.021^{γ}
15-*epi*-15-F_{2t}-IsoP	1.68 ± 0.54	1.95 ± 1.04	n.s.	6.03 ± 3.96	13.82 ± 11.75	n.s
15-E_{2t}-IsoP	-	-	-	4.10 ± 0.82	8.86 ± 8.00	n.s
15-keto-15-F_{2t}-IsoP	-	-	-	8.79 ± 4.29	8.36 ± 7.37	n.s
15-keto-15-E_{2t}-IsoP	-	-	-	3.79 ± 1.84	6.06 ± 3.36	n.s
4-F_{4t}-NeuroP + 4-*epi*-4-F_{4t}-NeuroP	3.00 ± 0.58	7.41 ± 9.67	0.012^{Ψ}	5.24 ± 2.30	9.22 ± 4.49	n.s.
10-*epi*-10-F_{4t}-NeuroP	1.58 ± 0.11	1.94 ± 0.99	n.s.	1.65 ± 0.93	2.67 ± 1.74	n.s
10-F_{4t}-NeuroP	1.04 ± 0.12	0.88 ± 0.34	n.s.	1.32 ± 0.89	2.30 ± 1.53	n.s
14*(RS)*-14-F_{4t}-NeuroP	1.62 ± 1.97	2.46 ± 1.75	n.s.	4.47 ± 1.51	7.31 ± 1.41	0.012^{γ}
4*(RS)*-ST-Δ^5-8-NeuroF	-	-	-	-	-	-

Continue on next page

Table 34. *Continuation*

	PLASMA			URINE		
	PABC-N	Control-N	*P*	PABC-N	Control-N	*P*
Lipid damage	(*N*=13)	(*N*=14)		(*N*=5)	(*N*=6)	
17*(RS)*-F_{2t}-dihomo-IsoP + 17-*epi*-17-F_{2t}-dihomo-IsoP	1.64 ± 1.44	2.69 ± 1.68	0.025$^{\Psi}$	5.46 ± 4.26	10.64 ± 7.29	n.s
ent-7*(RS)*-F_{2t}-dihomo-IsoP	2.05 ± 0.63	2.13 ± 2.24	n.s.	2.85 ± 2.84	3.92 ± 3.68	n.s
17*(RS)*-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF	2.05 ± 2.23	6.14 ± 5.78	1.79e$^{-03\Psi}$	20.10 ± 4.75	21.06 ± 12.04	n.s
7*(RS)*-ST- Δ^{18}-11-dihomo-IsoF	3.48 ± 2.33	3.30 ± 1.99	n.s.	40.11 ± 42.62	16.58 ± 12.69	n.s
Inflammation	(*N*=13)	(*N*=14)	*P*	(*N*=5)	(*N*=6)	*P*
1a,1b-dihomo $PGF_{2\alpha}$	8.68 ± 5.51	13.8 ± 11.55	n.s.	15.33 ± 14.16	18.49 ± 11.37	n.s
11ß-$PGF_{2\alpha}$	0.45 ± 0.20	0.64 ± 0.37	n.s.	1.85 ± 0.78	6.11 ± 3.49	0.029$^{\gamma}$
6-keto-$PGF_{1\alpha}$	1.22 ± 1.61	2.15 ± 2.74	n.s.	2.60 ± 0.70	8.95 ± 9.66	n.s.
$PGF_{2\alpha}$	6.45 ± 1.10	5.56 ± 3.05	n.s.	9.51 ± 4.84	27.31 ± 17.03	n.s.
PGE_2	-	-	-	6.58 ± 4.45	6.91 ± 4.62	n.s.
GSA	<LOQ	<LOQ	-	559.73 ± 164.72	272.02 ± 130.68	0.016$^{\gamma}$
Antioxidant defence	(*N*=13)	(*N*=14)	*P*	(*N*=5)	(*N*=6)	*P*
GSH/GSSG	1.02 ± 0.03	1.02 ± 0.20	n.s.	<LOQ	<LOQ	-
CYS/CYSS	-	-	-	<LOQ	<LOQ	-

Values are expressed as mean ± SD. *P* value of ≤ 0.05 was considered significant.
$^{\Psi}$ Wilcoxon rank-sum test, $^{\gamma}$ Student's t test,
Note: Values below LOQ were replaced by ½ LOQ. Ratio values were normalized between 1 and 2 employing Min-Max scaling technique.

Table 35. RBCs levels of antioxidant defense (ratio) in neonates exposed to chemotherapy *in utero* (PABC-N) and neonates born to healthy women at birth (Control-N).

	RBC		
	PABC-N	Control-N	*P*
Antioxidant defence	(*N*=13)	(*N*=14)	
GSH/GSSG	1.44 ± 0.22	1.17 ± 0.20	3.99e$^{-03\Psi}$

Values are expressed as mean ± SD. *P* value of ≤ 0.05 was considered significant.
$^{\Psi}$ Wilcoxon rank-sum test, $^{\gamma}$ Student's t test,
Note: Values below LOQ were replaced by ½ LOQ. Ratio values were normalized between 1 and 2 employing Min-Max scaling technique.

Table 36. Plasma levels of DNA damage (ratios), protein damage (ratios), lipid damage (nM), inflammation (nM) and antioxidant defense (ratios) in neonates exposed to chemotherapy *in utero* (PABC-N) and the corresponding mothers at birth (PABC-L).

	PABC-N	PABC-L	*P*
DNA damage	(*N*=13)	(*N*=11)	
8-OHdG/2dG	0.77 ± 0.14	0.87 ± 0.12	0.042$^{\gamma}$
Protein damage	(*N*=13)	(*N*=11)	*P*
m-Tyr/Phe	0.80 ± 0.11	0.85 ± 0.15	n.s
o-Tyr/Phe	0.88 ± 0.12	0.95 ± 0.21	n.s
$3NO_2$-Tyr/p-Tyr	0.66 ± 0.05	0.85 ± 0.25	0.039$^{\Psi}$
3Cl-Tyr/p-Tyr	<LOQ	<LOQ	-
Lipid damage	(*N*=13)	(*N*=11)	*P*
5-F_{2t}-IsoP + 5-*epi*-5-F_{2t}-IsoP	4.43 ± 1.46	5.52 ± 2.48	n.s
15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$ -IsoP	0.66 ± 0.10	0.68 ± 0.14	n.s
15-F_{2t}-IsoP	0.88 ± 0.35	0.93 ± 0.23	n.s
15-*epi*-15-F_{2t}-IsoP	1.68 ± 0.54	0.86 ± 0.28	0.002$^{\gamma}$
4-F_{4t}-NeuroP + 4-*epi*-4-F_{4t}-NeuroP	3.00 ± 0.58	3.71 ± 2.10	n.s
10-*epi*-10-F_{4t}-NeuroP	1.58 ± 0.11	1.70 ± 0.57	n.s
10-F_{4t}-NeuroP	1.04 ± 0.12	1.10 ± 0.33	n.s
14*(RS)*-14-F_{4t}-NeuroP	1.62 ± 1.97	2.50 ± 1.94	-
4*(RS)*-ST-Δ^5-8-NeuroF	-	–	-
17*(RS)*-F_{2t}-dihomo-IsoP + 17-*epi*-17-F_{2t}-dihomo-IsoP	1.64 ± 1.44	0.66 ± 0.74	n.s
ent-7*(RS)*-F_{2t}-dihomo-IsoP	2.05 ± 0.63	0.65 ± 1.07	0.010$^{\Psi}$
17*(RS)*-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF	2.05 ± 2.23	2.75 ± 2.14	n.s
7*(RS)*-ST- Δ^{18}-11-dihomo-IsoF	3.48 ± 2.33	6.58 ± 1.86	0.005$^{\gamma}$

Continue on next page

Table 36. *Continuation*

Inflammation	PABC-N (*N*=13)	PABC-L (*N*=11)	*P*
1a,1b-dihomo $PGF_{2\alpha}$	8.68 ± 5.51	3.56 ± 3.54	0.046$^{\gamma}$
11ß-$PGF_{2\alpha}$	0.45 ± 0.20	0.42 ± 0.32	n.s
6-keto-$PGF_{1\alpha}$	1.22 ± 1.61	0.51 ± 0.31	n.s
$PGF_{2\alpha}$	6.45 ± 1.10	6.04 ± 1.04	n.s
GSA	<LOQ	<LOQ	-
Antioxidant defence	(*N*=13)	(*N*=11)	*P*
GSH/GSSG	1.02 ± 0.03	1.07 ± 0.12	n.s.
CYS/CYSS	-	-	-

Values are expressed as mean ± SD. *P* value of ≤ 0.05 was considered significant.
$^{\Psi}$ Wilcoxon rank-sum test, $^{\gamma}$ Student's t test,
Note: Values below LOQ were replaced by ½ LOQ. Ratio values were normalized between 1 and 2 employing Min-Max scaling technique.

Table 37. RBC levels of antioxidant defense (ratio) in neonates exposed to chemotherapy *in utero* (PABC-N) and the corresponding mothers at birth (PABC-L).

Antioxidant defence	PABC-N (*N*=8)	PABC-L (*N*=8)	*P*
GSH/GSSG	1.21 ± 0.20	1.40 ± 0.13	0.039$^{\Psi}$

Values are expressed as mean ± SD. *P* value of ≤ 0.05 was considered significant.
$^{\Psi}$ Wilcoxon rank-sum test, $^{\gamma}$ Student's t test,
Note: Values below LOQ were replaced by ½ LOQ. Ratio values were normalized between 1 and 2 employing Min-Max scaling technique.

The main descriptors of the distribution of concentrations of each meatabolite measured in plasma and urine employing both LC-MS/MS methods are summarised from **Suppl. table S15** to **S18**. From the 55 parameters quantified employing both methods, 46 were detected in the studied samples. All metabolites were measured in plasma and urine except for GSH and GSSG that were also analysed in RBC.

2.2.1 Cluster analysis of significant biomarkers

Heatmaps of significant biomarkers of oxidative stress, inflammation and antioxidant defence, which illustrate the concentration changes between PABC-N and control-N groups measured in neonatal cord blood (plasma and RBCs) and urine samples, is showed in **Figure 35**.

Higher levels of protein damage **(A)** measured in plasma were detected in control-N group than PABC-N. Likewise, five out of six biomarkers of lipid peroxidation measured in plasma were found elevated in control-N group, whereas the remaining one was increased in PABC-L group **(B)**. Levels of antioxidant defence biomarker **(C)** analysed in RBCs, were more elevated in PABC-N group than control-N group.

When comparing both groups in urinary samples, two biomarkers of oxidative damage to lipids **(D)** and one biomarker of inflammation **(F)** were decreased in PABC-N group. Opposite, the remaining inflammatory mediator was increased in PABC-N group.

Additional, **Figure 36** illustrated the heatmaps of those biomarkers of oxidative stress, inflammation and antioxidant defence that were statistically significant between PABC-L and PABC-N groups detected in neonatal cord blood (plasma and RBCs) and maternal plasma at birth, respectively.

Elevated plasma levels of DNA **(A)** and protein **(B)** were observed in PABC-L group as compared to PABC-N. Opposite results were found in plasma levels of lipid peroxidation **(C)** as two out of biomarkers were increased in PABC-N group respect to PABC-L whereas the remaining one was decreased.

Lastly, RBC levels of antioxidant defence **(D)** were higher in PABC-N group than PABC-L group.

Differential metabolic profiles analysed in blood and samples between PABC-N and control-L groups were further evaluated by PCA **(Figure 37)**. Score plot from neonates included in PABC-N exhibited distinct metabolic profile either in blood **(A)** or urine **(B)** samples when was compared to control-L group. Moreover, **Figure 39** shows a PCA evaluating the differential metabolic profile detected in plasma and RBC samples between PABC-L and PABC-N groups.

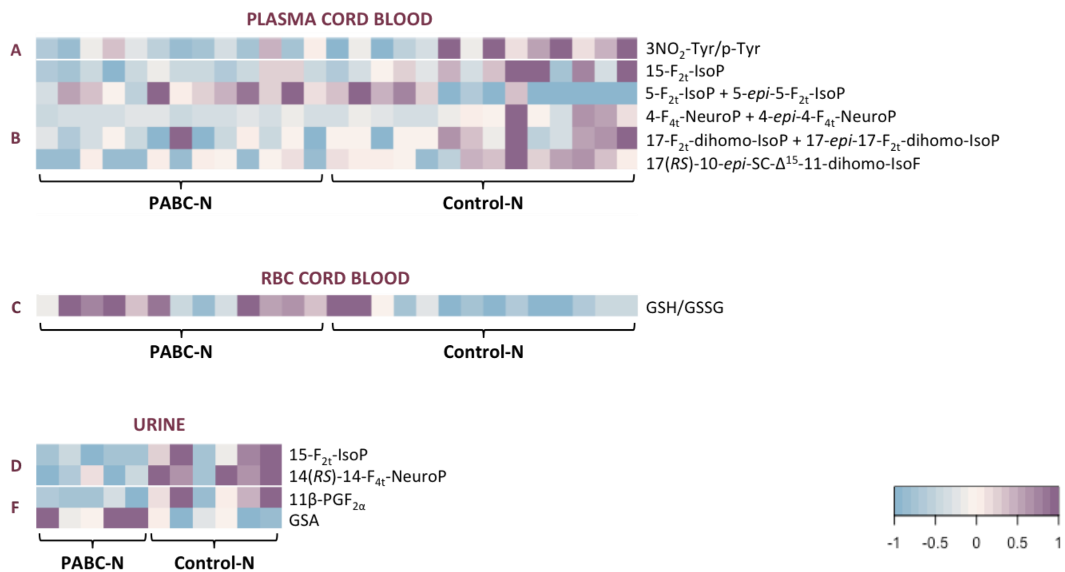


Figure 35.Heatmaps showing the clustering of all differentiating biomarkers of oxidative stress inflammation and antioxidant defence analysed in blood and urine across the different comparative groups. The columns represent the individual neonates and the rows indicate statistically significant biomarkers. The burgundy colour represents the trend of rise and the blue colour represents a decreasing trend. Biomarker of protein oxidation (**A**) and lipid peroxidation **(B)** were statistically significant in plasma whereas the biomarker of antioxidant defence **(C)** was in RBCs. In addition, biomarkers of lipid peroxidation **(D)** and inflammation **(E)** were statistically significant in urine.

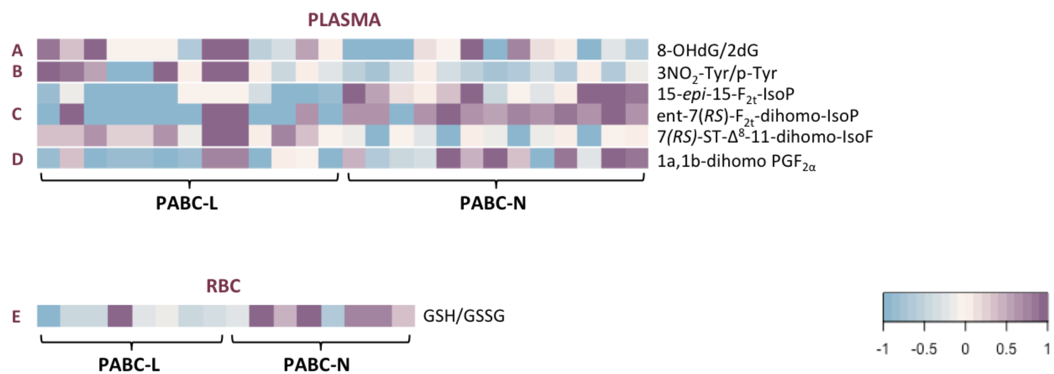


Figure 36. Heatmaps showing the clustering of all differentiating biomarkers of oxidative stress and antioxidant defence measured in blood between PABC-L and PABC-N groups . The columns represent the individual subjects and the rows indicate statistically significant biomarkers. The burgundy colour represents the trend of rise and the blue colour represents a decreasing trend. Biomarkers of DNA oxidation (**A**), protein nitration (**B**), lipid peroxidation **(C)** and inflammation **(D)** were statistically significant in plasma while antioxidant defence biomarker **(E)** was in RBCs.

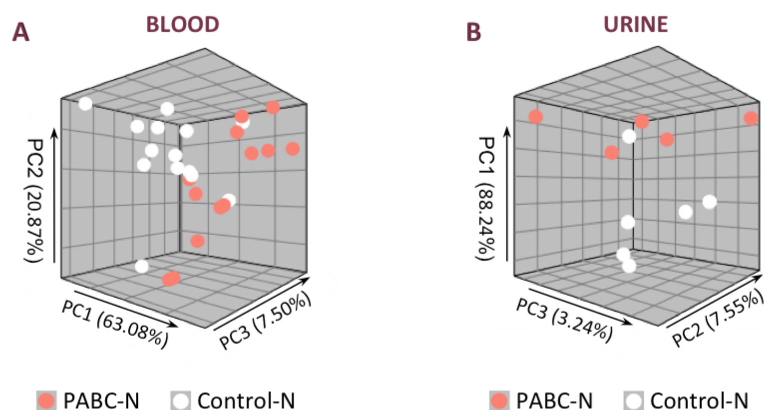


Figure. 37 Plots of principal component analysis (PCA) were used to explain the statistically significant metabolic differences between PABC-N and control-N groups. (A) Score plot of blood samples obtained from PABC-N group and control-N group. PC1, PC3 and PC2 account for 91.45% of the data's variance. **(B)** Score plot of urine samples obtained from obtained from PABC-N group and control-N group. PC3, PC2 and PC1 account for 99.03% of the data's variance.
Abbreviation. PC1, principal component 1; PC2, principal component 2; PC3, principal component 3; PC4, principal component 4.

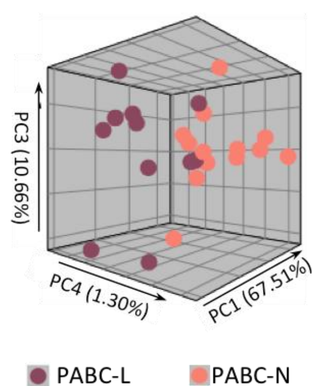


Figure. 38 Plot of principal component analysis (PCA) were used to explain the statistically significant metabolic difference between PABC-L and PABC-N groups. Score plot of plasma samples obtained from PABC-L group and PABC-N group. PC4, PC1 and PC3 account for 79.46% of the data's variance.
Abbreviation. PC1, principal component 1; PC3, principal component 3; PC4, principal component 4.

2.2.2. Comparative study of biomarkers levels between tested groups

Oxidative damage to DNA

The evaluation of 8-OHdG/2dG ratio showed no significant differences between neonates from PABC-N group and control-N group either un plasma or urinary samples **(Figure 39.A** and **Figure 40)**. Nevertheless, the ratio of 8-OHdG/2dG was significantly increased in maternal plasma in comparison with plasma cord blood (*P* value= 0.042) **(Figure 39.B)**.

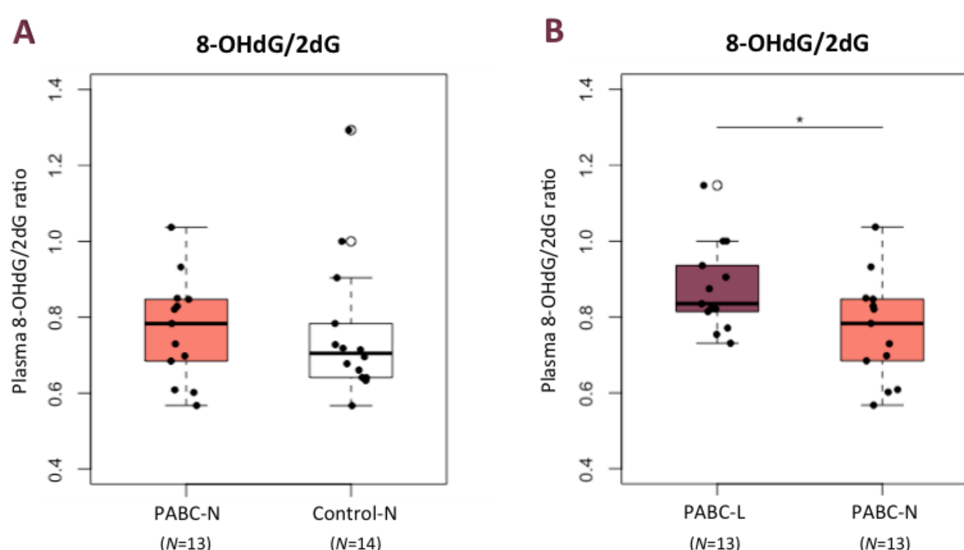


Figure 39. Boxplot of DNA defence biomarker measured in plasma. (A) Effect of chemotherapy exposure in *utero* **(B)** Maternal and cord blood oxidative stress at birth following chemotherapy. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.

Student's t test was used to assess differences between groups; *P* value of <0.05 was considered significant. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

Note: Values below LOQ were replaced by ½ LOQ. Ratio values were normalized between 1 and 2 employing Min-Max scaling technique.

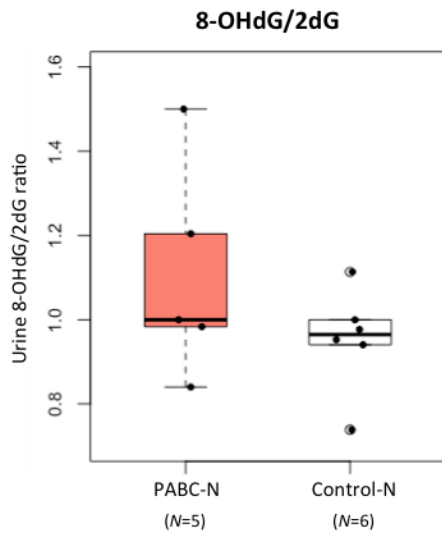
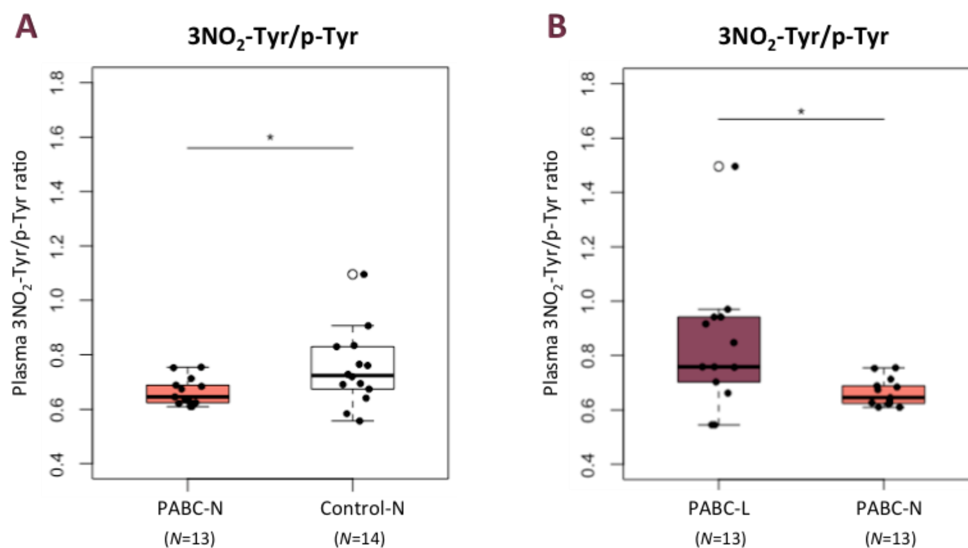


Figure 40. Boxplot of the effect of chemotherapy *in utero* on urinary DNA defence biomarker. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.
P value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.
Note: Values below LOQ were replaced by ½ LOQ. Ratio values were normalized between 1 and 2 employing Min-Max scaling technique.

Oxidative damage to proteins

Results showed lower plasma levels of $3NO_2$-Tyr/p-Tyr ratio in PABC-N group than in control-N group (*P* value= 0.038) **(Figure 41.A)**. However, plasma levels of m-Tyr/Phe and o-Tyr/Phe were not statistically different between those groups **(Figure 41.C** and **E)**. On the other hand plasma maternal showed increased levels of $3NO_2$-Tyr/p-Tyr at birth in comparison with plasma cord blood (*P* value= 0.039) **(Figure 41.B)**. These results were not observed in m-Tyr/Phe and o-Tyr/Phe ratios **(Figure 41.D** and **F).**

Regarding the urinary analysis, the levels of all tyrosine isomers (o-Tyr/Phe, m-Tyr/Phe, $3NO_2$-Tyr/p-Tyr and 3Cl-Tyr/p-Tyr) measured in the study were below LOQ.



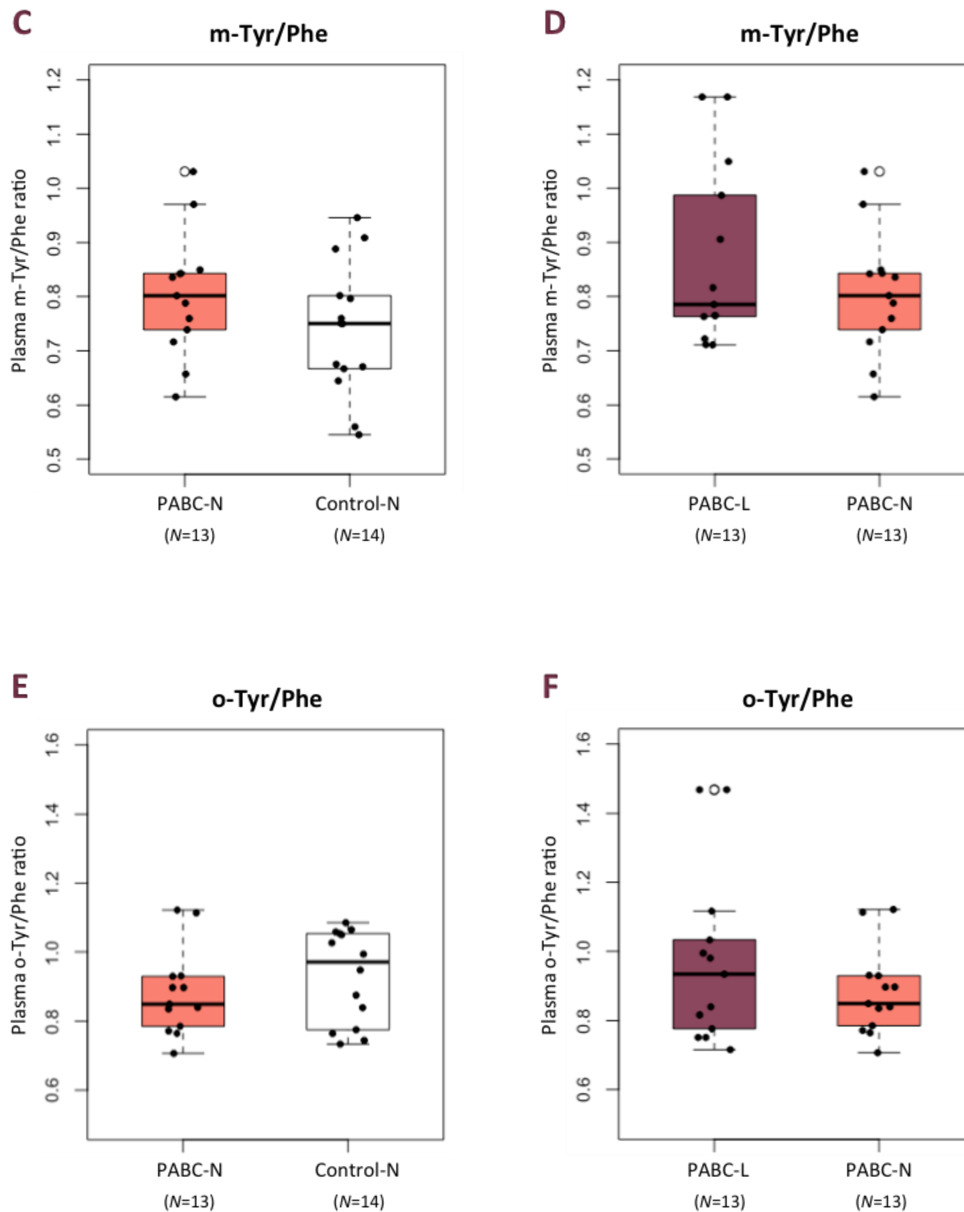


Figure 41. Boxplot of protein nitration and oxidation biomarkers measured in plasma. (A, C and E) Effect of chemotherapy exposure in *utero.* **(B, D and F)** Maternal and cord blood oxidative stress at birth following chemotherapy. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.
Student's t test **(A)** and Wilcoxon rank-sum test **(B)** were used to assess differences between groups;
P value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.

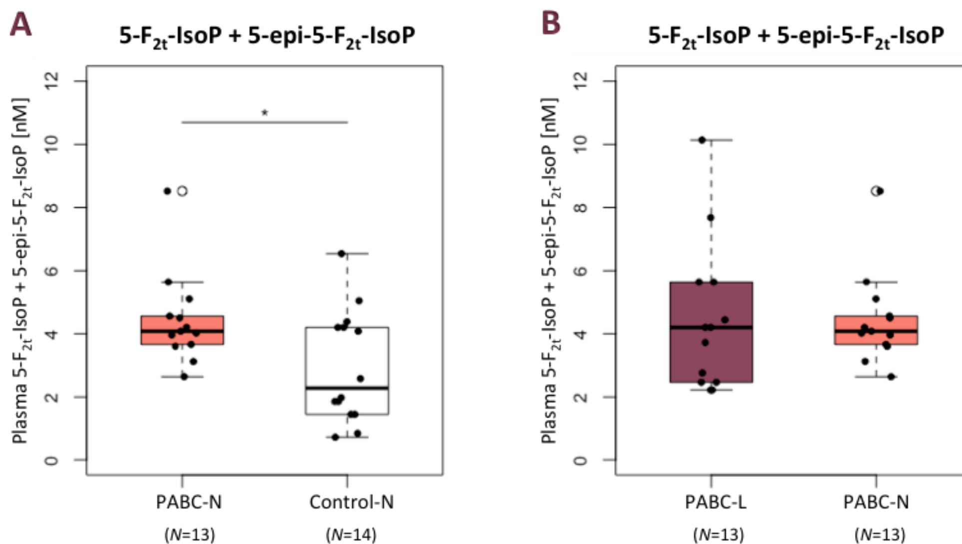
Note: Values below LOQ were replaced by ½ LOQ. Ratio values were normalized between 1 and 2 employing Min-Max scaling technique.

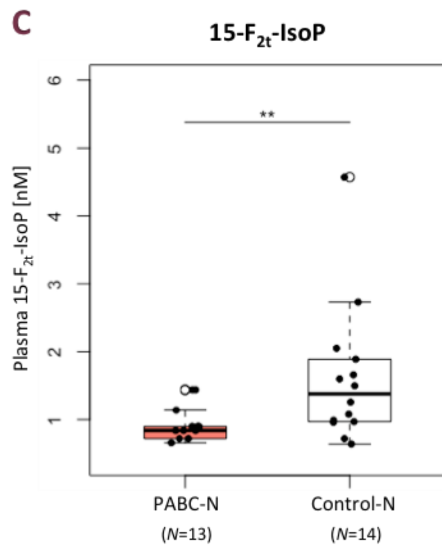
Oxidative damage to lipids

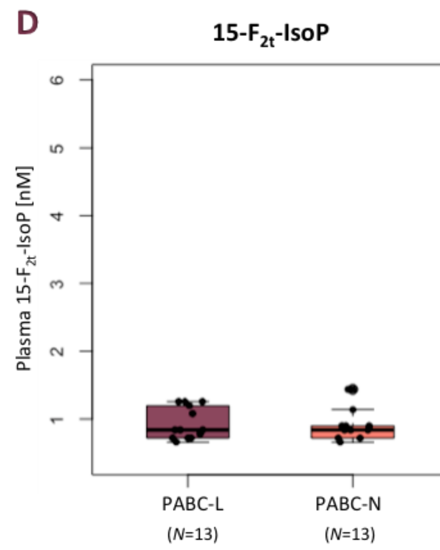
We observed differentiating levels of some particular isoprostanes and neuroprostens biomarkers between neonates exposed and not exposed to chemotherapy *in utero*. An increase and decrease of plasma F_{2t}-IsoP + 5-epi-5-F_{2t}-IsoP and 15-F_{2t}-IsoP levels respectively were observed in PABC-N group respect to control-N group (*P* value= 0.025 and *P* value= 0.008 respectively) **(Figure 42.A** and **Figure C).** In addition, 4-epi-4-F_{4t}-NeuroP plasma levels were detected statistically significant augmented in control-N group (*P* value= 0.012) **(Figure 42.G).** As we illustrated in **Figure 42.I** and **42.N**, significant differences of two specifics dihomo-IsoP and dihomo-IsoF biomarkers were appreciable between groups. 17(RS)-F_{2t}-dihomo-IsoP+17-epi-17-F_{2t}-dihomo-IsoP (*P* value= 0.025) and especially 17(RS)-10-epi-SC-Δ^{15}-11-dihomo-IsoF (*P* value= 0.002) plasma levels were significant decreased in neonates exposed to chemotherapy *in utero*.

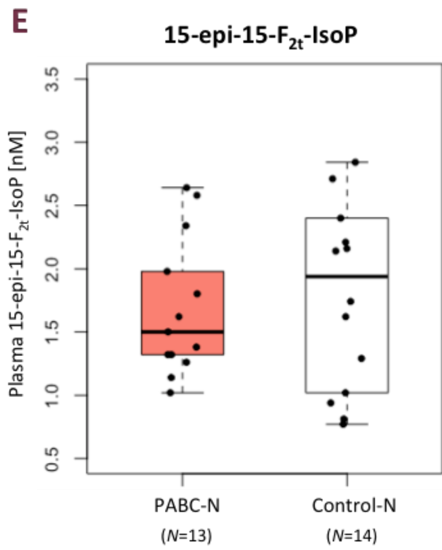
The remaining compounds analysed in neonatal plasma were not statically significant despite most of them showed a slightly tendency of increasing levels in control-N group **(see Suppl. Figure S7)**. 4(RS)-ST Δ5-8-NeuroF compound was directly not detected.

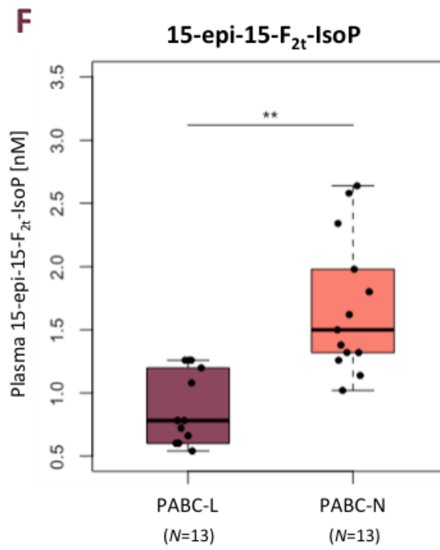
On the other hand, plasma levels of 15-epi-15-F_{2t}-IsoP, ent-7*(RS)*-F_{2t}-dihomo-IsoP and 7*(RS)*-ST- Δ^{18}-11-dihomo-IsoF were incremented in neonates exposed to chemotherapy as compared to the mothers at birth **(Figure 42.F, 42.L** and **42.P)**. See **Suppl. Figure S7** to visualize the compounds that were not statisticallt significant.

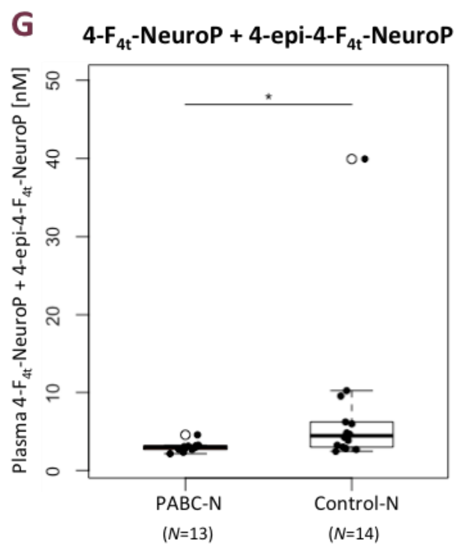


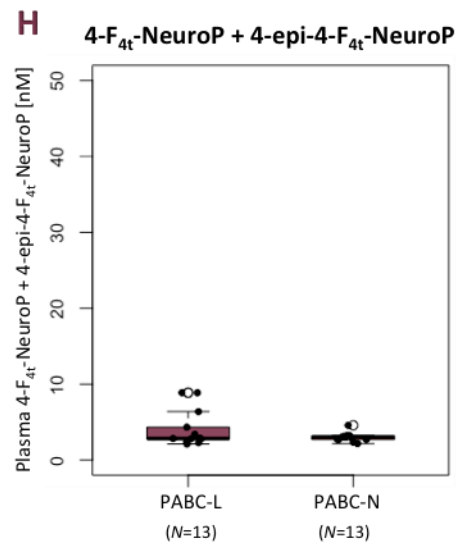

C
15-F_{2t}-IsoP
Plasma 15-F_{2t}-IsoP [nM]
**
PABC-N
(N=13)
Control-N
(N=14)

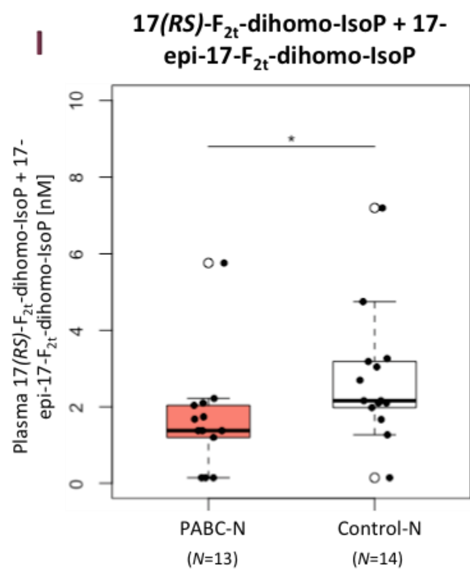

D
15-F_{2t}-IsoP
Plasma 15-F_{2t}-IsoP [nM]
PABC-L
(N=13)
PABC-N
(N=13)

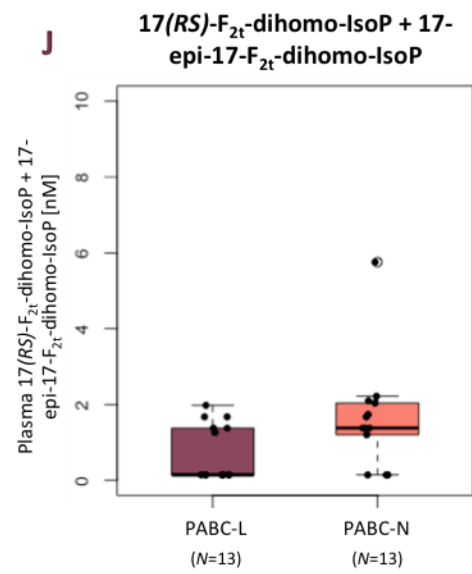

E
15-epi-15-F_{2t}-IsoP
Plasma 15-epi-15-F_{2t}-IsoP [nM]
PABC-N
(N=13)
Control-N
(N=14)

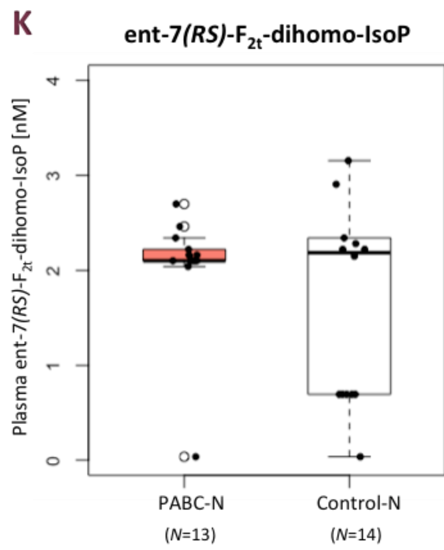

F
15-epi-15-F_{2t}-IsoP
Plasma 15-epi-15-F_{2t}-IsoP [nM]
**
PABC-L
(N=13)
PABC-N
(N=13)

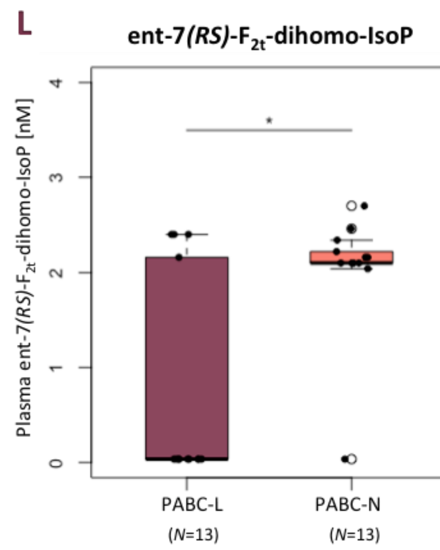

G
4-F_{4t}-NeuroP + 4-epi-4-F_{4t}-NeuroP
Plasma 4-F_{4t}-NeuroP + 4-epi-4-F_{4t}-NeuroP [nM]
*
PABC-N
(N=13)
Control-N
(N=14)

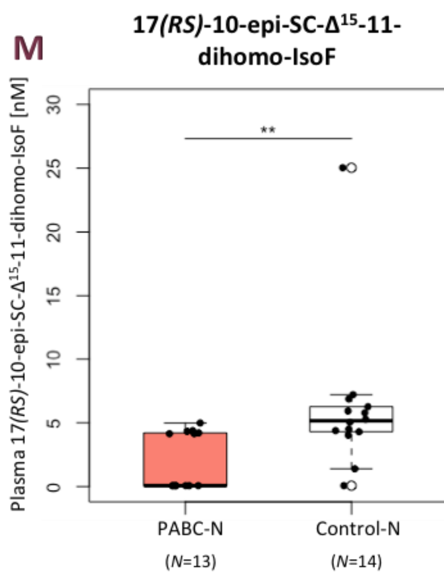

H
4-F_{4t}-NeuroP + 4-epi-4-F_{4t}-NeuroP
Plasma 4-F_{4t}-NeuroP + 4-epi-4-F_{4t}-NeuroP [nM]
PABC-L
(N=13)
PABC-N
(N=13)

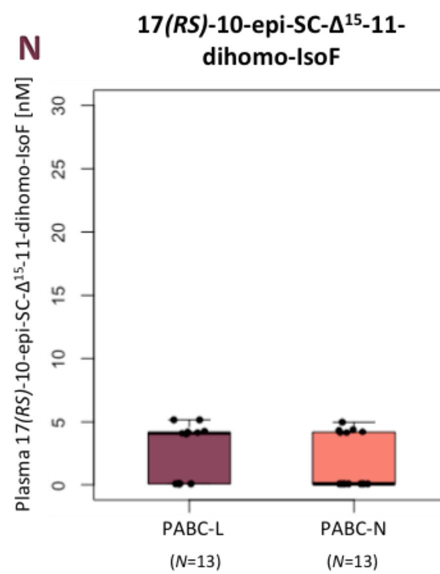

I
17(RS)-F2t-dihomo-IsoP + 17-epi-17-F2t-dihomo-IsoP
Plasma 17(RS)-F2t-dihomo-IsoP + 17-epi-17-F2t-dihomo-IsoP [nM]
*
PABC-N
(N=13)
Control-N
(N=14)


J
17(RS)-F2t-dihomo-IsoP + 17-epi-17-F2t-dihomo-IsoP
Plasma 17(RS)-F2t-dihomo-IsoP + 17-epi-17-F2t-dihomo-IsoP [nM]
PABC-L
(N=13)
PABC-N
(N=13)


K
ent-7(RS)-F2t-dihomo-IsoP
Plasma ent-7(RS)-F2t-dihomo-IsoP [nM]
PABC-N
(N=13)
Control-N
(N=14)


L
ent-7(RS)-F2t-dihomo-IsoP
Plasma ent-7(RS)-F2t-dihomo-IsoP [nM]
*
PABC-L
(N=13)
PABC-N
(N=13)


M
17(RS)-10-epi-SC-Δ15-11-dihomo-IsoF
Plasma 17(RS)-10-epi-SC-Δ15-11-dihomo-IsoF [nM]
**
PABC-N
(N=13)
Control-N
(N=14)


N
17(RS)-10-epi-SC-Δ15-11-dihomo-IsoF
Plasma 17(RS)-10-epi-SC-Δ15-11-dihomo-IsoF [nM]
PABC-L
(N=13)
PABC-N
(N=13)

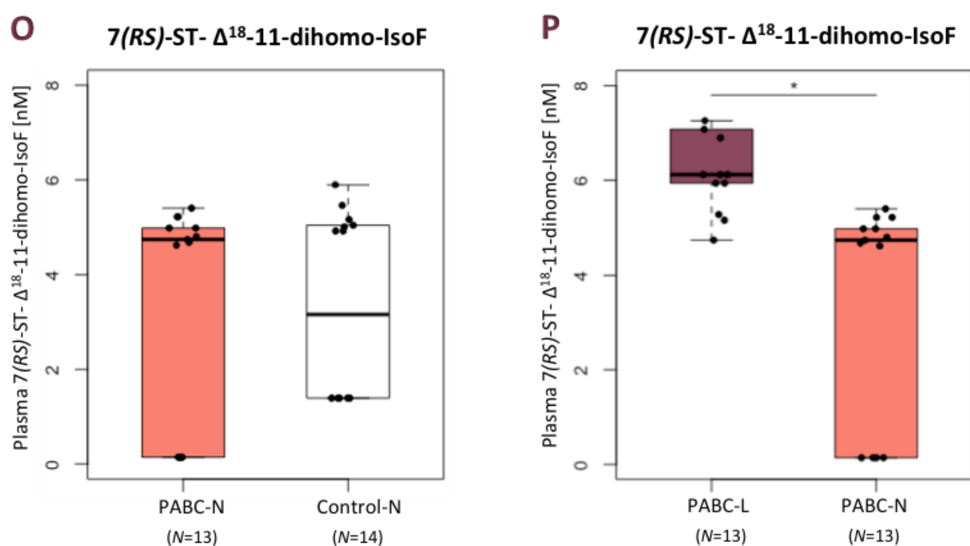


Figure 42. Boxplot of lipid peroxidation biomarkers measured in plasma. (A, C, E, G, I, K, M and **O)** Effect of chemotherapy exposure in *utero.* **(B, D, F, H, J, L, N** and **P)** Maternal and cord blood oxidative stress at birth following chemotherapy. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.
Student's t test **(A, F, I, M** and **P)** and Wilcoxon rank-sum test **(C, G** and **L)** were used to assess differences between groups;
P value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.
Note: Values below LOQ were replaced by ½ LOQ. Ratio values were normalized between 1 and 2 employing Min-Max scaling technique.

Regarding urinary samples, levels of 15-F2t-IsoP compound were detected significantly more elevated in control-N group than in PABC-N group (*P* value= 0.021) **(Figure 43.A)**. Similarly, a decrease of 14(RS)-14-F4t-NeuroP levels was found in PABC-N group compared to the control-N group (*P* value= 0.012) **(Figure 43.B)**.

Like in plasma, we observed a suggestive but non-significant trend of higher levels in control-N in most of the remaining compounds **(see Suppl. Figure S8).** 4*(RS)*-ST-Δ^5-8-NeuroF compound was not detected.

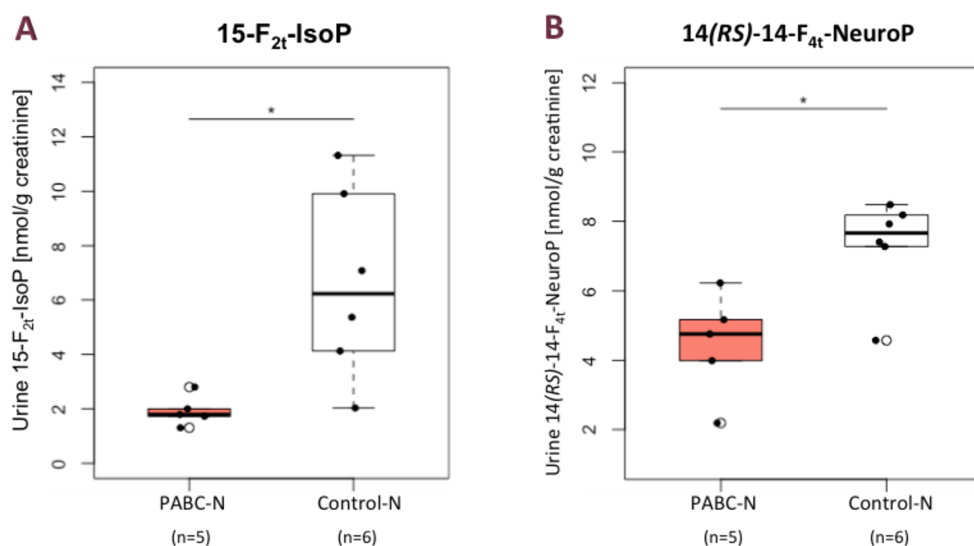
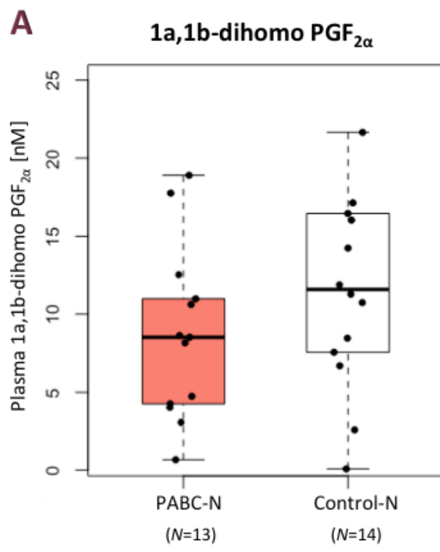


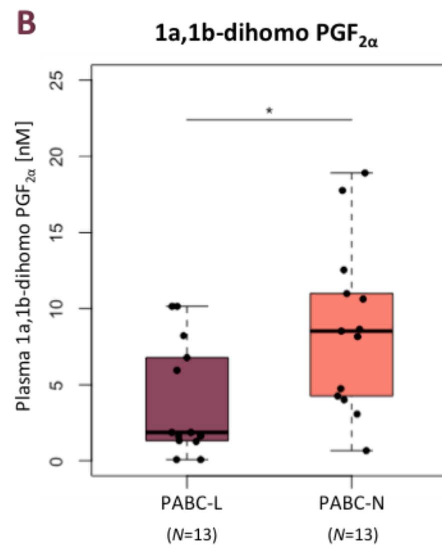
Figure 43. Boxplot of the effect of chemotherapy *in utero* on urinary lipid peroxidation biomarkers. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.
Student's t test was used to assess differences between groups; a *P* value of <0.05 was considered significant. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$. **Note:** values below LOQ were replaced by ½ LOQ.

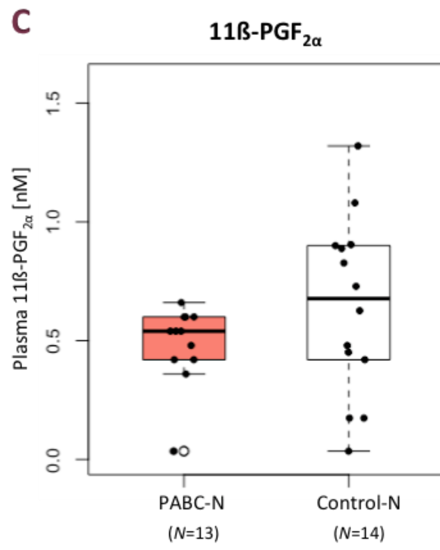
Inflammatory mediators

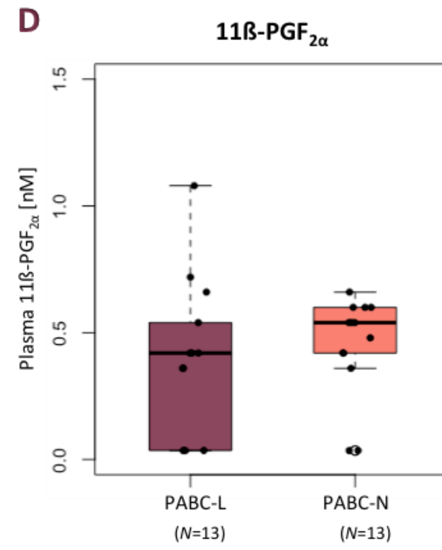
There were no significant differences in plasma levels of prostaglandins measured in neonates prenatally exposed to chemotherapy and those born to healthy pregnant women **(Figure 44.A-G)**. Alternatively, we found significant differences in the levels of 1a,1b-dihomo $PGF_{2\alpha}$ between maternal plasma and plasma cord blood at birth (*P* value= 0.046) **(Figure 44.B).** These differences were not observed among the other compounds **(Figure 44.D, 44.F** and **44.H)**.

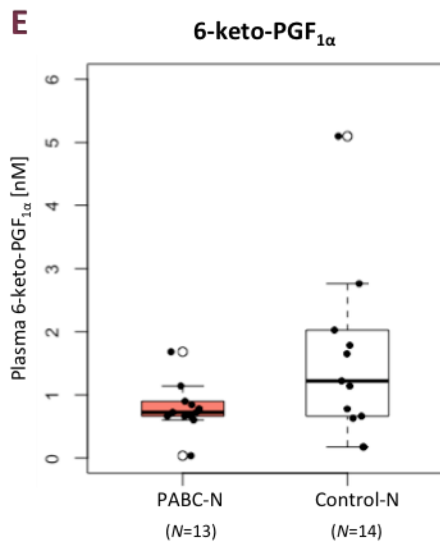
On the other hand, plasma levels of GSA were detected below LOQ.

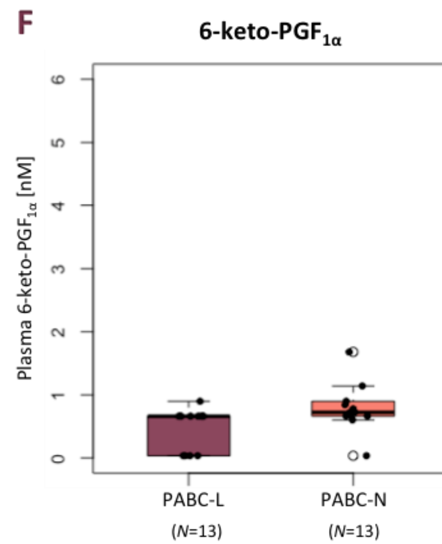

A
1a,1b-dihomo PGF2α
Plasma 1a,1b-dihomo PGF2α [nM]
0
5
10
15
20
25
PABC-N
(N=13)
Control-N
(N=14)


B
1a,1b-dihomo PGF2α
Plasma 1a,1b-dihomo PGF2α [nM]
0
5
10
15
20
25
*
PABC-L
(N=13)
PABC-N
(N=13)


C
11ß-PGF2α
Plasma 11ß-PGF2α [nM]
0.0
0.5
1.0
1.5
PABC-N
(N=13)
Control-N
(N=14)


D
11ß-PGF2α
Plasma 11ß-PGF2α [nM]
0.0
0.5
1.0
1.5
PABC-L
(N=13)
PABC-N
(N=13)


E
6-keto-PGF1α
Plasma 6-keto-PGF1α [nM]
0
1
2
3
4
5
6
PABC-N
(N=13)
Control-N
(N=14)


F
6-keto-PGF1α
Plasma 6-keto-PGF1α [nM]
0
1
2
3
4
5
6
PABC-L
(N=13)
PABC-N
(N=13)

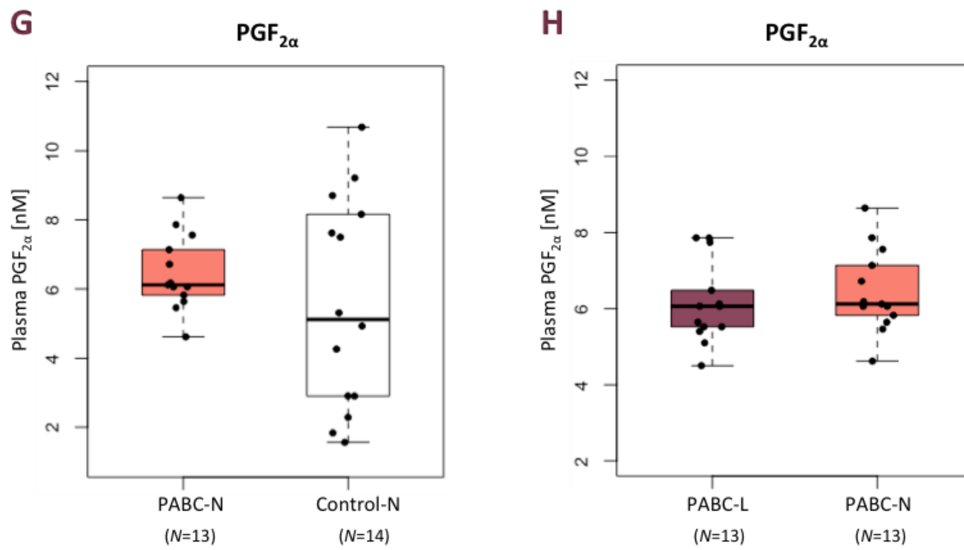
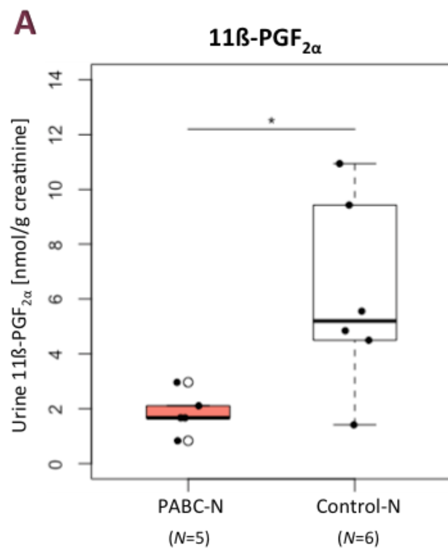
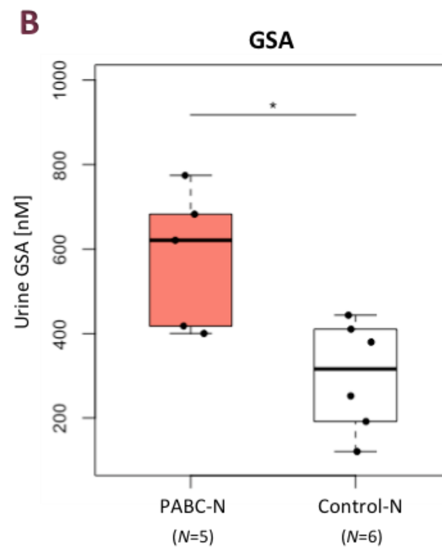


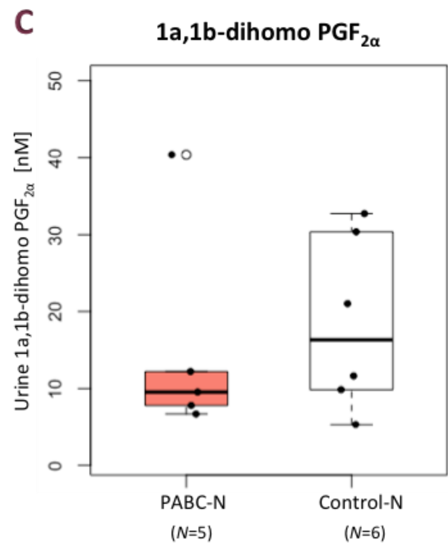
Figure 44. Boxplot of inflammatory biomarkers measured in plasma. (A, C, E and G) Effect of chemotherapy exposure in *utero.* **(B, D, F and H)** Maternal and cord blood oxidative stress at birth following chemotherapy. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.
Student's t test was used to assess differences between groups;
P value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.
Note: Values below LOQ were replaced by ½ LOQ. Ratio values were normalized between 1 and 2 employing Min-Max scaling technique.

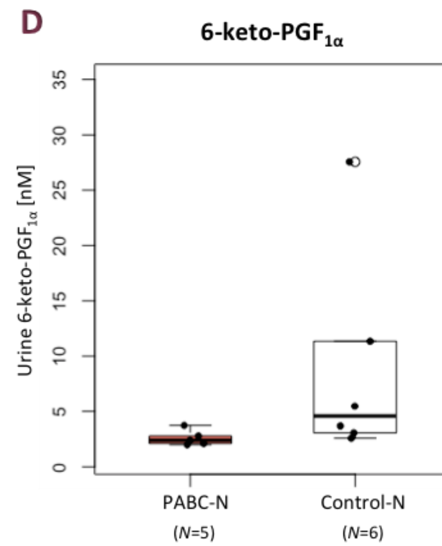
We observed significantly decreased levels of 11ß-$PGF_{2\alpha}$, a prostaglandin compound, in neonates exposed to chemotherapy *in utero* as compared to the controls (*P* value= 0.029) **(Figure 45.A)**. Although the remaining compounds did not show any statistical differences between the groups of study, the levels measured in control-N were slightly incremented in nearly all of them **(Figure 45.C, 45.D, 45.E** and **45.F)**.

As we showed in **Figure 45.B**, opposite results we found in urine levels of GSA as they were detected significantly higher in neonates from PABC-N group than control-N group (*P* value= 0.016).


A
11ß-PGF2α
Urine 11ß-PGF2α [nmol/g creatinine]
*
PABC-N
(N=5)
Control-N
(N=6)


B
GSA
Urine GSA [nM]
*
PABC-N
(N=5)
Control-N
(N=6)


C
1a,1b-dihomo PGF2α
Urine 1a,1b-dihomo PGF2α [nM]
PABC-N
(N=5)
Control-N
(N=6)


D
6-keto-PGF1α
Urine 6-keto-PGF1α [nM]
PABC-N
(N=5)
Control-N
(N=6)

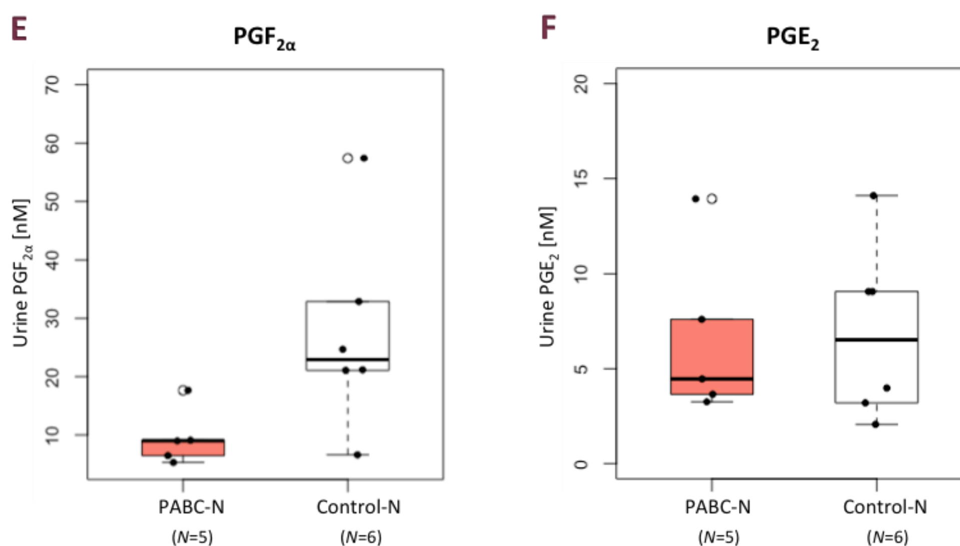


Figure 45. Boxplot of the effect of chemotherapy *in utero* on urinary inflammatory biomarkers. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.
Student's t test was used to assess differences between groups;
P value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001. **Note:** values below LOQ were replaced by ½ LOQ.

Antioxidant defence

We observed non-significant differences among plasma levels of GSH/GSSG ratio from neonates exposed to chemotherapy *in utero* and control group **(Figure 46.A)** or in comparison with plasma maternal **(Figure 46.B)**.

Opposite, the levels of GSH/GSSG ratio were significantly increased in RBCs belonging to neonates from PABC-N group as compared to those from control-N group (*P* value= $3.99e^{-03}$) **(Figure 47.A)**. In addition, the levels of GSH/GSSG ratio was also incremented in neonates exposed to chemotherapy in utero as compared to the mothers (*P* value= 0.039) **(Figure 47.B)**.

On the other hand, plasma and urinary levels of Cys/CySS ratio were not detected in all groups of study.

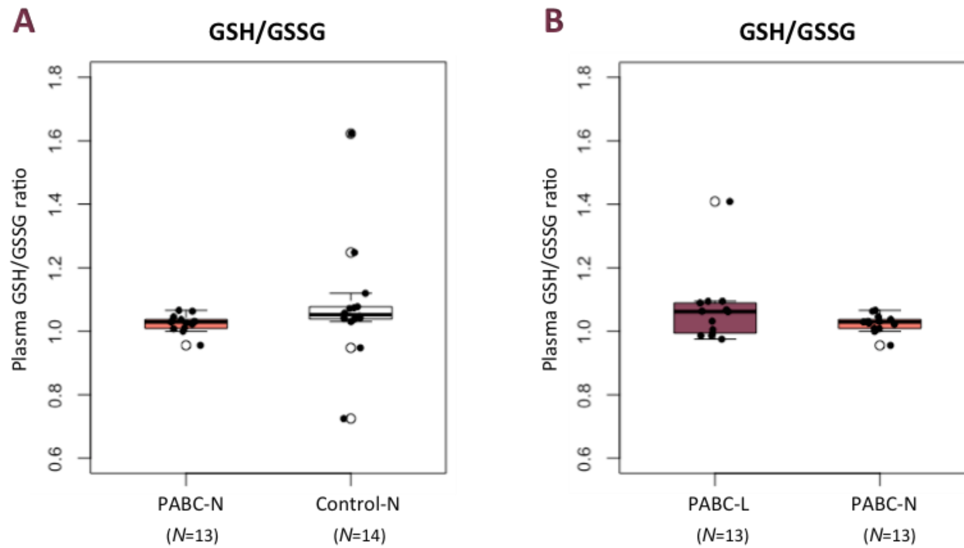


Figure 46. Boxplot of antioxidant defence biomarker measured in plasma. (A) Effect of chemotherapy exposure in *utero.* **(B)** Maternal and cord blood oxidative stress at birth following chemotherapy. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.

P value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.

Note: Values below LOQ were replaced by ½ LOQ. Ratio values were normalized between 1 and 2 employing Min-Max scaling technique.

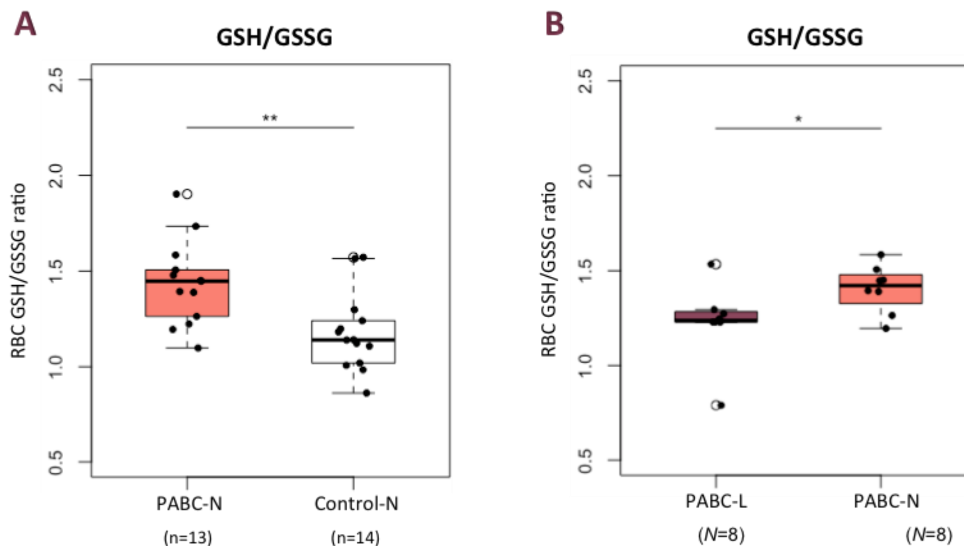


Figure 47. Boxplot of antioxidant defence biomarker measured in RBC. (A) Effect of chemotherapy exposure in *utero.* **(B)** Maternal and cord blood oxidative stress at birth following chemotherapy. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.

Wilcoxon rank-sum test was used to assess differences between groups; a *P* value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.

Note: Values below LOQ were replaced by ½ LOQ. Ratio values were normalized between 1 and 2 employing Min-Max scaling technique.

2.2.3 Multivariate and univariate regression analyses

Neonatal weight at birth and prematurity

There was a positive association between the weight at birth and the RBC levels of GSH/GSSG ratio (*P* value= 0.034; β= 1060.6) measured in cord blood from neonates exposed to chemotherapy *in utero* **(Table 38)**.

Table 38. Univariate lineal regression of the relation to neonatal birth weight and the RBC levels of GSH/GSSG ratio measured neonates exposed to chemotherapy *in utero*

Clinical characteristics	β	Std. Error	t-value	*P*
Intercept	1037.5	637.9	1.626	0.1321
GSH/GSSH	1060.6	439.5	2.413	**0.0344**

P value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.

Mode of delivery and metabolites

We did not observed an association between the mode of delivery (vaginal/caesarean) and the levels of all metabolites analysed in plasma, RBC and urine samples from neonates exposed to chemotherapy in perinatal period employing an univariate lineal regression model.

2.2.4 Correlation analyses

We studied the correlation between the biomarkers of oxidative stress and inflammation with the levels of GSH/GSSG to study the homeostasis imbalance. There were some negative significant correlation between GSH/GSSG ratio measured in plasma cord blood from neonates with intrauterine exposure to chemotherapy **(Table 39).**

Table 39. Correlations observed between biomarkers analysed in plasma cord blood with GSH/GSSG ratio from neonates exposed to chemotherapy *in utero*

	$3NO_2$-Tyr/p-Tyr	6-keto-$PGF_{1\alpha}$	1a,1b-dihomo $PGF_{2\alpha}$
GSH/GSSG	*R* =-0.66)	*R* =-0.72	*R* =-0.70
	(*P*=0.014)	(*P*=0.006	*P*=0.008

P value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.

We additionally studied the correlation between the levels of oxidative stress, inflammation and antioxidant defence biomarkers measured in neonates exposed to chemotherapy in utero and their corresponding PABC mothers:

Lipid damage

There were negative significant correlations between levels of 15-*epi*-15-F_{2t}-IsoP and 2,3-dinor-15-F_{2t}-IsoP (*R*=-0.55, *P* value=0.049 and *R*=-0.68, *P* value=0.011 respectively) measured in plasma cord blood from neonates exposed to chemotherapy in utero and maternal plasma.

VII. DISCUSSION

DISCUSSION STUDY I

I.A REDOX STATUS AND INFLAMMATION PROFILE IN PABC PATIENTS TREATED WITH ANTHRACYCLINES AND PACLITAXEL DURING PREGNANCY.

The current study is the first to evaluate the effect of two specific treatments, anthracyclines and paclitaxel in PABC patients on the production of lipid and protein damage, inflammatory mediators and antioxidant defence during pregnancy.

The vulnerability to lipid peroxidation and protein oxidation was not associated with the administration of anthracyclines and paclitaxel in PABC patients. Oppositely, PABC patients exhibited a moderately elevated inflammatory profile in comparison with healthy pregnant women, which it was then affected by the administration of chemotherapy during pregnancy. Besides, our study strongly demonstrated a differential impact on the levels of inflammation (i.e., YKL-40) and antioxidant defence (i.e., protein thiol) depending on the treatment with anthracyclines and paclitaxel.

Oxidative stress is considered an imbalance between elevated exposure to ROS and a deficiency antioxidant defence system. The consequences of an overproduction of ROS are critical since it may cause direct damage to DNA, lipids and proteins[268143]. Thus, oxidative stress is a very prominent feature of a great variety of diseases, including cancer or endothelial dysfunction. Unfortunately, despite the efforts of the scientific community to elucidate the mechanisms behind this association, they have not been fully discovered yet.

The current study was undertaken to gain further insight into the role of oxidative stress and inflammation and pregnancy complications in PABC patients undergoing chemotherapy treatment; to this end, and to better characterise oxidative stress and inflammatory status, the study was approached from several angles (before chemotherapy, after anthracyclines and paclitaxel treatment and at labour) and conducted in PABC patients and healthy pregnant women.

In recent years many authors have highlighted the crucial role of lipid peroxidation and protein oxidation on the pathogenesis of several diseases, including cancer and placental insufficiency[196,163,90]

Lipid peroxidation is a chain reaction that involves the oxidative conversion of fatty acids to primary compounds, finally resulting in a loss of cell membrane viability[147]. The findings of our study showed that plasma levels of MDA, a well-known biomarker of secondary end products of lipid peroxidation, did not significantly increase in PABC patients compared with those of healthy pregnant women. Bhattacharjee *et al.* reported increased levels of MDA in breast cancer patients as compared to the healthy controls[269]. Similar observations were found in the studies conducted by Portakal *et al.* and Hristozov *et al.* where MDA levels were elevated in patients with breast cancer suggesting augmentation of cancer-induced ROS generation[270,271].

The study performed by Taherkhani *et al.* and colleagues evaluated the effect of chemotherapy treatment on the lipid peroxidation of breast cancer patients using MDA as a biomarker. The authors found that MDA levels increased following three cycles of anthracyclines[272].

In another study performed by Hewala *et al.* and co-workers, however, a non-significant association between patients with breast cancer following six cycles of chemotherapy and control group was observed[273]. Our results were inconsistent with the first study but similar to the second since non-significant differences were observed between PABC patients before chemotherapy and after both treatments. However, we found a correlation between the levels of MDA following anthracyclines with those prior to chemotherapy initation and at labour.

Moreover, high levels of MDA were associated with different pregnancy complications. D'Souza *et al.* found elevated plasma levels of MDA in women with PE as compared to healthy pregnant women[274]. Along this line, our results did not significantly differ from PABC patients and healthy pregnant women, which would explain the appearances of the relatively low frequency of pregnancy complications included in the study.

Therefore, we speculate that a very efficient antioxidant protective mechanism against chemotherapy-induced lipid peroxidation reflects the normal pregnancy development in most of PABC patients.

Proteins are usually the main target of ROS direct reactions or secondary by-products of oxidative stress as they are the primary component of human cells. Damage proteins are involved in several intracellular pathways triggering different disorders and pathologies. Protein carbonyl groups are generated by different sources including direct oxidation of amino acid residues, oxidative cleavage of proteins or reacting with MDA[172–174]. Considering this fact, we measured the protein carbonylation content to characterise the oxidative damage to proteins. Nevertheless, little data have been published regarding the levels of protein carbonylation content in breast cancer or generated by chemotherapy treatment[275]. In the study performed by Aryal *et al*. showed elevating levels of selective protein carbonylation in breast cancer tissue compared to adjacent healthy epithelial tissue[276]. In another study conducted by El-azem *et al.* and co-workers, they evaluated the levels of protein carbonyl group following six weeks of paclitaxel in Sprague-Dawley rats[277]. The authors showed that the levels were higher after exposure to chemotherapy than in the control group.

On the contrary, Söylemez *et al.* found similar serum levels of protein carbonyl group in breast patients after three cycles of chemotherapy in comparison with healthy controls. However, they observed a statistically significant difference between serum protein carbonyl group levels of breast cancer patients before and after chemotherapy and between healthy controls and breast cancer before chemotherapy[278]. Our results are inconsistent with these studies as we failed to find evidence that circulating protein carbonylation content are significantly affected by the administration of chemotherapy during pregnancy in plasma of PABC patients. However, we found a correlation between the levels of protein carbonyl measured afer anthracyclines and at labour.

Furthermore, oxidative damage to protein does not seem to be related to carcinogenesis because no differences were found in PABC patients as compared to healthy pregnant control. Therefore, these results may also explain the low frequency of pregnancy complications observed in our study, as various authors have reported significant increases in protein carbonylation content in pregnancy disorders[244,279].

Oxidative stress and inflammation are tightly linked with one another, especially in pathological diseases such as cancer. Inflammation uses the immune cells to promote the secretion of various cytokines with the purpose of recruiting more immune cells to the site of oxidative stress. Reflexively, the ROS produced by the immune cells at the place of inflammation induces oxidative stress and tissue damage[280].

Considering that inflammation is constituted by the action of different inflammatory cells and enzymes rather than a single product, two indicators were used in this study to evaluate the inflammatory status; therefore we quantified both plasma activity of ChT and YKL-40. These enzymes are usually generated from neutrophils and macrophages and then secreted in response to various inflammatory stimuli[281,282]. YKL-40 has been more characterised in cancer, as some authors also demonstrated that tumours cells might increase its activity to promote carcinogenesis[283,284].

In general terms, the levels of inflammation decreased during chemotherapy treatment but were still elevated in comparison with healthy pregnant control. However, these observations were not statistically significant among all comparisons. In a recent study conducted by Thein *et al.* the serum Cht activity was higher in breast cancer patients than healthy controls[285]. Our data support this observation as we found not only significantly elevated levels of plasma ChT activity in PABC patients before chemotherapy but also following paclitaxel in comparison with healthy pregnant controls.

YKL-40 has been recently also defined as a risk cancer predictor; therefore, it was essential to describe its activity in PABC patients[286]. However, YKL-40 plasma levels were decreased in PABC patients a consequence of chemotherapy administration. This observation was, in particular, statistically significant following paclitaxel. These results are in agreement with a previous study conducted by Coskun *et al* [287]. The author and associates observed non-significant decreased serum levels of YLK-40 in advanced breast cancer patients before and after three cycles of anthracyclines.

Although we suggest that cancer may be partially responsible for the inflammatory status that surrounds PABC patients, the role of these inflammatory mediators in breast cancer is barely understood. In previous studies, the involvement of macrophages in the

processes of implantation and placentation in normal and pathological pregnancy has been discussed[288,289]. Whereas elevated Cht enzyme activity was linked to an augmentation of macrophage activation in PE women, increased serum levels of YKL-40 were associated with the onset of gestational diabetes mellitus[290,291]. Contrary to the above studies, although we described an elevated activity of ChT and YKL-40 (significantly evident on ChT levels), most of PABC patients progressed as expected without apparent complications related to inflammation. Despite these results, it is interesting to point out that in both enzymes, ChT and YKL-40 the levels measured in all groups were correlated among each other.

The present study investigated two different antioxidant defence systems in PABC, showing a great capacity to counteract the effect of chemotherapy-induced oxidative stress. The levels of plasma thiol groups, a powerful extracellular antioxidant, were decreased in PABC following anthracyclines as compared with the control group. However, the levels were restored after treatment with paclitaxel. Eryilmaz *et al.* described statistically significantly lower and higher native thiol and serum disulfide levels, respectively, in breast cancer patients[292]. These findings suggested an alteration of the thiol-disulfide homeostasis that may help to the pathogenesis of breast cancer. In another study conducted by kedzierska et al., the levels of plasma protein thiol groups were distinctly decreased in patients with invasive breast cancer[293]. On the other hand, based on the hypothetical role of the antioxidant protection against chemotherapy-induced toxicity, *Topuz et al.* and colleges found an impaired thiol/disulfide homeostasis in breast cancer patients following anthracyclines treatment[294]. Although our study did not measure the disulfide levels as the above studies did, we also speculate that plasma protein-SH groups were reduced in order to attempt to prevent the deleterious effects of free radicals during chemotherapy treatment.

Reduced GSH is the most abundant and crucial low-molecular-weight water-soluble antioxidant in human cells. GSH intervenes in different metabolic processes principally to maintain the redox homeostasis through its role of thiol-disulfide exchange and antioxidant protection[295]. Nowadays, reports of many authors point out the disruption of

the GSH synthesis or changes in its content are features of initiation and progression of malignant tumours. Kasapović et al. and Yeh et al., showed reduced RBC levels of GSH in breast cancer patients in comparison with the control group[296,297]. On the other hand, Amin *et al.* suggested that oxidative stress is involved In the pathogenesis of breast cancer and chemotherapy aggravates this oxidative stress leading to tissue damage and toxicities[218]. The authors observed reduced levels of GSH following two cycles of anthracyclines while MDA levels were incremented. These findings are consistent with those reported by Gomes *et al*. where GSH levels were progressively decreased after four cycles of chemotherapy compared with healthy women[298]. However, we observed non-significant changes regarding GSH content during the whole study. Therefore, our findings differ from those explained previously in which GSH was reduced in breast cancer patients following chemotherapy treatment and respect to control group. Even so, we found a correlation between the levels measured before anthracyclines and those measured at labour.

Besides, these results also shed light on the reduced frequency of pregnancy complications associated with oxidative stress since an imbalance of antioxidant homeostasis may be related to in some pregnancy disorders[299,300]. Llurba *et al*. and Ahmad *et al*. described in two independent studies reduced GSH levels, pregnant women, with PE[244,301].

Strength and limitations of the study

The main limitation of this assay was the small sample size. PABC is a relatively unusual event with very low incidence and even when appears, some patients decide to terminate the pregnancy. Besides, we could not measure these biomarkers in non-pregnant breast cancer patients before and after chemotherapy treatment. In the absence of these population groups, it was impossible for us to fully understand the role of chemotherapy-induced oxidative stress in pregnancy and the neonates. Finally, PABC patients received a different number of chemotherapy cycles during pregnancy, and even some of them were not treated with paclitaxel. The main reason for this limitation resides in the complexity of a standardised number of cycles during pregnancy since all PABC patients had different gestational age at the moment of the diagnosis. Thus the length of the treatment varies

among patients, and it was principally designed according to their own needs. Unfortunately, this also could influence on the statistical results.

A significant strength of the study is the novel evaluation of these well-recognised biomarkers in four periods, before, during and after treatment with two of the most common chemotherapy agents and at labour, to provide a more comprehensive assessment of the role of oxidative stress and inflammation in cancer during pregnancy. Few authors have described the consequences of anthracyclines and paclitaxel administration on oxidative stress and inflammation in breast cancer patients. However, to date, we are the first study to evaluate both chemotherapy agents on a PABC population in a prospective manner. On the other hand, the fact that the population-based study design made sure to incorporate cases and controls from the same source population could also be accepted as another strength of the study.

In the future, it would be interesting to assess a relationship between oxidative stress and inflammation with all different stages of cancer in PABC patients during treatment with anthracyclines and paclitaxel. It is also necessary to perform these analyses taking into account other factors that may trigger the overproduction of oxidative stress in pregnancy such as obesity, nutrition and smoking. Furthermore, approaches from other angles (e.g., placental tissue examination and DNA/RNA studies) are required to validate the impact of anthracyclines and paclitaxel on placental insufficiency.

Conclusions

In general terms, no apparent changes in lipid peroxidation, protein damage and inflammation biomarkers were observed in PABC patients in comparison with healthy pregnant women. These results may indicate that tumour cells were not taken advantage to an imbalance of the redox homeostasis to promote the progression of the disease at least disrupting the physiological pathways of these biomarkers.

A noteworthy finding of this study was the different impact of anthracyclines and paclitaxel treatment on the levels of plasma YKL-40 activity and protein thiol in PABC patients. According to YKL-40 plasma levels, we speculate that paclitaxel treatment was

much efficient, reducing inflammation in PABC patients.

Furthermore, proteins that possess a carbon-bonded sulfhydryl were very susceptible to be oxidised to counterbalance the oxidative stress induced by anthracyclines. However, the levels were slightly restored after paclitaxel, which leads us to suggest that paclitaxel is less cytotoxic than anthracyclines.

Regarding the oxidative stress and inflammation induced by anthracyclines and paclitaxel and the onset of pregnancy complications, the results of this study reported may lead us to suggest that there is no apparent influence of overproduction of oxidative stress and maternal and perinatal disorders. Thus, the use of chemotherapy agents during pregnancy may not influence on the overproduction of oxidative stress. Furthermore, these observations are tightly connected to the efficient production of antioxidant defence systems that remove circulating oxidatively modified compounds in PABC patients.

I.B REDOX STATUS AND INFLAMMATION PROFILE IN PABC PATIENTS BEFORE TREATMENT WITH CHEMOTHERAPY AND AT LABOUR.

This study provides evidence of a disturbance of the generation of oxidative stress, inflammatory mediators and antioxidant defence in women with PABC undergoing chemotherapy treatment by comparing the levels before treatment and at labour. The susceptibility to DNA and protein damage was strongly associated with chemotherapy administration regardless of pregnancy or pregnancy condition. Besides, labour samples from PABC patients genuinely demonstrated a significant augmentation of some biomarkers of oxidative stress, inflammation and antioxidant defence than samples from healthy pregnancies. Likewise, our study sincerely found a differential impact of tumour effect on the redox state and inflammation between pregnant and non-pregnant patients before chemotherapy treatment.

Persistent oxidative stress through the overproduction of ROS may cause direct damage to DNA. In particular, we used 8-OHdG biomarker and its oxidised form, 2dG to evaluate DNA oxidative injury because guanine has a low one-electron reduction potential as compared to other nucleosides in DNA[302]. In the present study, we appreciated an

augmentation of 8-OHdG/2dG ratio during chemotherapy treatment in both; pregnant and non-pregnant breast cancer patients. These observations agreed with previous studies where 8-OHdG was increased in cancer patients undergoing chemotherapy treatment. Atukeren *et al.* reported elevated levels of 8-OHdG in breast cancer patients after two cycles of chemotherapy[303]. Also, Crohns, *et al.* detailed an increase in 8OH-dG levels following chemotherapy in patients with lung cancer[304]. Our report, however, is the first to successfully describe elevated levels of oxidative damage to DNA prior chemotherapy initiation in non-pregnant breast cancer women than in PABC patients. In fact, these results are quite impressive since reduced 8-OHdG levels may be associated with poor survival in breast cancer patients.

The study conducted by Nour Eldin *et al.* detailed that 8-OHdG levels were gradually decreased in advanced cancer stages comparing to the early stages in breast cancer patients[305]. Another recent study by Rashed et al. also showed a relationship between reduced 8-OHdG levels and increasing cancer severity[306]. These findings could be entirely explained by the capacity of malignant cells to take advantage of multiple antioxidant mechanisms to avoid excessive overproduction of ROS. In doing so, the formation of oxidative stress is reduced, allowing the tumour cells to escape from apoptosis and promoting cancer progression and metastasis. Interestingly, PABC patients also exhibited a potent antioxidant reducing systems during pregnancy that would reinforce this mechanism.

Based on this belief, we hypothesise that PABC patients suffer from a more aggressive cancer environment than non-PABC patients and that pregnancy may be responsible for tumour cells developing the necessary mechanisms to do so.

It is well known that DNA stability and integrity is necessary for the correct functioning of the living cells[180]. Therefore, oxidative DNA modifications are crucial to initiate the neoplastic process and transform normal cells into malignant ones[177,178]. In recent studies, it was shown that breast cancer patients had elevated levels of 8-OHdG compared to healthy women[307–310]. Our results are inconsistent with these studies since we observed decreased plasma 8-OHdG/2dG ratio in PABC patients in comparison to

healthy pregnant women at birth. However, these differences may be as a result of increased DNA damage during pregnancy as we did not measure the 8-OHdG/2dG ratio in healthy pregnant women.

On the other hand, plasma and urinary 8-OHdG analyses in women with complicated and uncomplicated pregnancies have so far provided strong supporting evidence of how high 8-OHdG levels impact negatively on pregnancy outcomes[311]. Nevertheless, contradictory to these findings, our study found similar plasma levels of 8-OHdG/2dG ratio in PABC-L and control-L groups. The absence of DNA damage at birth may support that chemotherapy-induced oxidative stress is not responsible for pregnancy complications.

Oxidative damage to proteins induces the generation of protein-protein cross-linked derivatives or amino acids side-chains oxidation that eventually results in loss of physiological functions[162]. The role of ROS-induced protein damage along the development and progression in cancer has been evaluated in previous works. However, only a very few numbers of clinical studies have assessed the role of oxidatively modified derivatives of phenylalanine such as m-Tyr and o-Tyr (tyrosine isoforms) in normal and pathological processes [161,165].

In the present study, we found increasing plasma levels of o-Tyr/Phe ratio following chemotherapy during pregnancy in PABC patients. Interestingly, while this increasing plasma level was not significantly observed in m-Tyr/Phe ratio in PABC patients during chemotherapy, non-PABC patients showed significant decreasing levels of plasma m-Tyr/Phe ratio following anthracycline therapy. Based on these findings, we could speculate that pregnancy status may be responsible for the increased levels of m- Tyr/Phe ratio measured in PABC patients following chemotherapy. Despite this hypothesis, plasma m-Tyr/Phe ratio was detected at low concentrations in PABC patients at labour compared to healthy pregnant women at childbirth. Consequently, the lack of protein damage may reinforce the theory that there is a need for high levels of oxidative damage to trigger the onset of pregnancy complications but that chemotherapy is not inducing it.

Interestingly, the results of this study reflect reduced levels of m-Tyr/Phe ratio in PABC patients before being treated with chemotherapy. Ruggiero *et al.* suggested that m-Tyr and –to a less extent– o-Tyr intervene as inhibitors of secondary tumour growth in the so-called concomitant tumour resistance[312]. This phenomenon states that selected primary tumours can restrain the growth of a secondary tumour and metastasis[313,314] In line with this research, the workgroup of Gueron *et al.* recently proposed m-Tyr as a novel approach for therapeutic purposes exerting its anti-tumour activity[315]. Bearing the observations mentioned above in mind, we suggest that PABC patients may show a significant predisposition to spread the tumour to different body parts, at least before being treated with chemotherapy. Thus, a delay in breast cancer diagnosis and treatment in pregnant women could be more dangerous than in non-PABC patients.

Regarding the potential implications of ROS-induced lipid peroxidation in the pathology of a wide variety of human diseases (e.g., cancer) and normal physiological conditions (e.g., pregnancy), isoprostanes have been the widest biomarkers used to assess oxidative stress status in vivo in clinical research. Although the production of isoprostanes increases in direct proportion to the level of oxidative stress does not necessarily imply a causative link role for the diseases. Alternatively, neuroprostanes also appear to be important biomarkers of lipid damage, especially for neuronal oxidant injury. Besides, they are involved in biological activities including cardiac protection, anti-inflammatory response, and breast cancer cell inhibition[316].

Many studies have demonstrated an association between elevated levels of isoprostanes with breast cancer risk, breast cancer survival or normal pregnancy. Whereas, Rossner *et al.* found higher levels of F_2-IsoPs in breast cancer subjects than healthy women, Ishihara *et al.* reported significantly higher levels of 15F_{2t}-IsoP in healthy pregnant women as compared to the non-pregnancy[317,318].

To the best of our knowledge, this is the first longitudinal report that describes the effect of chemotherapy in the generation of various isoprostanes and neuroprostanes metabolites including 5-F_{2t}-IsoPs, 15-F_{2t}-IsoPs and F_{4t}-NeuroPs series in pregnant and non-pregnant women with breast cancer. While we showed significantly increased 5-F_{2t}-IsoP + 5-*epi*-5-F_{2t}-IsoP levels in PABC patients before chemotherapy as compared to non-PABC-

prior anthracyclines, the levels of 10-*epi*-10-F_{4t}-NeuroP and 4-*epi*-4-F_{4t}-NeuroP were instead reduced. The difference between isoprostanes and neuroprostanes compounds could possible reside whether breast cancer appears or not during pregnancy. Therefore, although PABC patients may be more susceptible to the peroxidation of arachidonic acid than docosahexaenoic acid than non-PABC patients, does not permit us to conclude that lipid peroxidation might be a pathogenically relevant process causally contributing to worsening breast cancer prognosis.

Concerning the effect of chemotherapy treatment on lipid damage, PABC patients exhibited a suggestive non-significant tendency of high lipid peroxidation production. However, these observations were not reported in non-PABC undergoing anthracyclines. As detailed in this thesis, lipid peroxidation alters the integrity of cell membranes leading to a loss of function and structure. Many studies have provided evidence that lipid peroxidation may play a role in placental insufficiency. The study conducted by Barden *et al.* showed for the first time an association between elevated levels of plasma isofurans and neuroprostanes with PE[319]. Other study conducted by Harsem *et al.* found similar results by analysing F_{2t}-IsoPs in plasma from PE women[320].
Contrary to the above publications, a salient finding of our study was that despite PABC patients showed significantly increased plasma levels of F_{2t}-IsoP and F_{4t}-NeuroP metabolites (i.e., 5-F_{2t}-IsoP + 5-*epi*-5-F_{2t}-IsoP, 15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11ß-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$-IsoP and 10-*epi*-10-F_{4t}-NeuroP) as compared to healthy pregnant women at labour, did not increment the pregnancy disorders. It should be borne in mind that, pregnancy is a condition that continuously generates lipid peroxidation reaching the highest values toward the end of the pregnancy. Consequently, it seems plausible that the observed findings in PABC patients represent the levels generated by the coexistence of cancer and its corresponding chemotherapy treatment with pregnancy.

As previously mentioned, inflammation and oxidative stress show to be reciprocally linked and very involved in the pathogenesis of several diseases, including cancer and pregnancy[188]. To further delineate the role of inflammation in PABC patients, the

inflammatory status was evaluated through the quantification of plasma prostaglandins. Prostaglandins are traditional eicosanoids formed enzymatically with several biological functions described, although they are mainly known for their role on the inflammatory response modulation[194]. According to various studies, elevated levels of prostaglandins have been reported to appear early in breast cancer, in tumour spread and host immune response evasion[321]. In the current study, PABC patients before chemotherapy showed higher plasma levels of $PGF_{2\alpha}$ than non-PABC patients before anthracyclines.
Furthermore, increased plasma levels of $PGF_{2\alpha}$ were found in PABC patients at labour in comparison with control-P group. This is also in keeping with data from various experimental studies, which suggested that increased plasma levels of $PGF_{2\alpha}$ are necessary for various normal biological processes of pregnancy, especially in parturition. Ishihara et al. found significantly higher plasma levels of $PGF_{2\alpha}$ during normal gestation than in non-pregnancy[229]. The results of this study suggest that although cancer may partly increase the levels of $PGF_{2\alpha}$ in breast cancer patients, pregnancy is the main responsible for the differences observed between PABC and non-PABC. In the light of the facts, we assumed that $PGF_{2\alpha}$ was contributing in the preparation of the utero or the onset of labour[322,323].

Relatively little information is published regarding 1a,1b-dihomo-$PGF_{2\alpha}$ implication, either in breast cancer or pregnancy[324]. However, our study reported opposite results in comparison with $PGF_{2\alpha}$ since plasma levels of 1a,1b-dihomo-$PGF_{2\alpha}$ was significantly lower in PABC patients before chemotherapy than in the non-PABC patients before anthracyclines. Considering these findings, we speculate PABC patients exhibit a low inflammatory profile, perhaps induced by the administration of anti-inflammatory drugs such as glucocorticoids to prevent chemotherapy side effects.

GSH is the major thiol antioxidant, and redox buffer of the human cell and GSSG is its oxidised form, which is accumulated inside the cells[144]. The disruption of GSH metabolism is closely related to several pathological and physiological conditions. Kędzierska et al., reported lower levels of plasma GSH/GSSG ratio in breast cancer patients following anthracyclines treatment as compared to healthy subjects[325]. Contrary

to these findings, the current study showed no changes in antioxidant defence following treatment either in PABC or non-PABC patients.

In our study, however, PABC patients exhibited a highly efficient GSH/GSSG ratio during the study in comparison with non-PABC patients. This observation was especially statistically significant before chemotherapy treatment in both groups. This is in keeping with the hypothesis proposed above regarding the ability of tumour cells to enhance the activity of antioxidant defence and thus skip apoptosis and promote carcinogenesis[153,155,197]. The effect of GSH on oxidative stress and carcinogenesis is highly complicated because of the dual role that GSH plays. Although it is critical in several points of carcinogenesis including detoxification and cell survival, elevated levels of GSH protect the tumour cells by conferring them resistance to apoptosis and chemotherapeutic agents[326,327]. Therefore, it seems plausible that the observed findings represent an adaptation of tumour to proliferate by inducing reduced oxidative damage and increased antioxidant the activity. Even though we should not discard the possibility of the elevated GSH/GSSG ratio being caused by the need to battle an overexpression of ROS without inducing significant consequences in tumour progression.

Pregnancy is a physiological condition accompanied by elevated oxygen requirement and high-energy demand[328]. Therefore, an antioxidant defence system capable of counteracting elevated levels of oxidative stress is necessary to avoid pregnancy complications. In this study, the levels of plasma GSH/GSSG ratio were significantly higher in PABC patients than in healthy pregnant patients in labour reflecting an apparent increase in antioxidant activity in response to elevated levels of oxidising species.

Further approaches of these biomarkers in different states of breast cancer with a larger group of patients are required to validate the hypothesis of an aggressive tumour microenvironment in PABC patients.
Although this assay helps shed lights on the safety of chemotherapy in pregnancy, more specific studies measuring these metabolites in different periods of anthracyclines and paclitaxel treatment are needed to keep ensuring that the onset of pregnancy complications are related not to their administration.

Besides, the replication of this study employing placental tissue may be an interesting approach to validate our findings further.

Strength and limitations

This study has some significant limitations. First, it was a prospective analysis with a small population size. Therefore, further evaluations must be done, including more patients in each group. Second, we did not discriminate between periods of treatments. Consequently, the study the effect of anthracyclines and paclitaxel on these biomarkers separately would be interesting. Third, our study lacked samples from healthy women during pregnancy. Since various biomarkers were significantly different in control-P group compared to PABC-L group, it may be of interest to compare these metabolites with gestational-age-matched PABC patients prior to chemotherapy initiation. In doing so, it would provide a more comprehensive assessment of the role of oxidative stress generation during chemotherapy.

The principal strengths of this study were the evaluation of multiple biomarkers of oxidative stress (i.e., DNA, protein and lipid damage) inflammation and antioxidant defence, employing two validated methods and the detection of these metabolites in PABC patients, non-PABC patients and healthy pregnant women[258,261,262,264,267,329].

Conclusions

In summary, the findings of this extensive study show that long-prolonged exposition to chemotherapeutic agents promotes DNA susceptibility to oxidation and consequently, the 8-OHdG/2dG ratio was found raised in PABC and non-PABC patients after treatment. However, PABC exhibited explicitly lower levels of 8-OHdG/2dG and m-Tyr/Phe ratios than non-PABC patients, which may play a role in, breast cancer severity. Simultaneously we observed that the levels of GSH/GSSG ratio measured in PABC patients might favour tumour progression since cancer cells are recognised for promoting antioxidant defence to avoid death cell. Consequently, the determination of these biomarkers could be of interest for further investigations regarding aggressiveness.

Opposite of what was expected, the present study successfully found evidences of low DNA and protein damage in PABC patients at labour suggesting that 8-OHdG/2dG ratio and tyrosine isomers (m-Tyr/Phe and o-Tyr/Phe ratios) may be useful indicators for pregnancy complications. Contradictory results were found in PABC patients regarding lipid peroxidation and inflammation as we expected that increasing or decreasing levels of those biomarkers would make more likely to develop obstetric complications. Thus the simplest explanation of our results is that the levels of isoprostanes and neuroprostanes, despite being significant elevated in PABC patients, they were not enough to increase the risk of pregnancy complications or as $PGF_{2\alpha}$ that may be was necessary to the onset of parturition.

On the contrary, chemotherapy administration was not associated with increased levels of inflammatory mediators in PABC and non-PABC patients. We suggest that the use of glucocorticoids before chemotherapy may restrain the augmentation of inflammation during treatment.

Moreover, PABC exhibited explicitly lower levels of 8-OHdG/2dG and m-Tyr/Phe ratios, which are reported to play a role in breast cancer severity. Simultaneously we observed that the levels of GSH/GSSG ratio measured in PABC patients might favour tumour progression, since cancer cells are recognised for promoting antioxidant defence to avoid death cell. Consequently, the determination of these biomarkers could be used as a prognostic factor for cancer aggressiveness.

Lastly, the findings of this exhaustive study suggest that chemotherapy-induced oxidative stress and inflammation does not clearly influence of the onset of maternal and perinatal complications by overproducing ROS levels. However, in the hypothetical case that chemotherapy in fact induces the overproduction of ROS, PABC patients showed an efficient production of antioxidant defence systems that could balance the redox state.

DISCUSSION STUDY II

To our knowledge, this study presents the first evidence of low levels of protein and lipid damage and high levels of antioxidant defence in plasma and urine samples of neonates exposed to chemotherapy in utero for maternal breast cancer compared to neonates from healthy gestation. Furthermore, we also observed discrepancies in the inflammatory profile of the neonates with intrauterine exposure to chemotherapy. Regarding the relationship between the oxidative state of the mother and the neonate at birth, we found significant differences among some oxidative biomarkers and antioxidant defence in neonates exposed to chemotherapy as compared to the mothers. However, the only correlations that we found were on the levels of 15-*epi*-15-F_{2t}-IsoP and 15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$-IsoP between both groups.

Chemotherapy exposure during prenatally period is not contraindicated after the first trimester of pregnancy despite the possibility of inducing an alteration of the redox homeostasis and inflammation. Oxidative stress and inflammation have been postulated as potentially contributors to multiple perinatal complications[77,85] Therefore, the onset of those neonatal disorders can appear more frequently undergoing chemotherapy treatment. Yet, chemotherapy is still commonly used in pregnancy despite the lack of general consensus regarding its role on ROS overproduction and the consequences for the neonate. The root of the controversy might lay in the fact that the evidences for its safety administration has been mostly described in observational studies with contradictory results and the lack of comparing published data to establish the real effect of oxidative stress and inflammation on perinatal development[92,97,136].

DNA damage, a central feature of oxidant stress and a source of genomic instability very advantageous to tumour progression and other disorders, was evaluated in the study[153,330]. Our results showed non-significant changes in plasma and urine 8-OHdG/2dG ratio, from neonates exposed to chemotherapy *in utero* as compared to those born to healthy mothers. Our findings are inconsistent with a recent study in placental tissue. Verheecke *et al.* and associates suggested that the placenta suffers from an increase of

oxidative DNA damage following chemotherapy exposure that might impact negatively on foetal development[331]. If we take this study into consideration, we could speculate that chemotherapy indeed may induce DNA damage in placental tissue but it is not reflected on the cord blood circulation and urine.

On the other hand, the levels of cord blood 8-OHdG/2dG ratio were lower than the corresponding maternal blood levels showing that the foetus may not be affected by maternal oxidative damage to DNA.

Several amino acid residues can experience oxidative modifications, including the oxidation, nitration and chlorination of aromatic amino acids (i.e., Phe and p-Tyr). The present study showed significant differences regarding tyrosine nitration (3-NO_2-Tyr/p-Tyr) but not tyrosine oxidation (m-Tyr/Phe and o-Tyr/Phe) and chlorination (3-Cl-Tyr/p-Tyr). In pregnancy, elevated levels of 3-NO_2-Tyr/p-Tyr have been associated with PE and IUGR[121,332] .However, comparing the results of the present study with those studies that found these associations, we showed that lower plasma levels of 3-NO_2-Tyr/p-Tyr are observed in the absence of perinatal complications. Therefore, we speculate that reduced protein damage may help to avoid the onset of perinatal complications.

Interestingly, the levels of 3-NO_2-Tyr/p-Tyr ratio were also lower in cord blood plasma of neonates with exposure to chemotherapy *in utero* than in maternal plasma. These findings may suggest that maternal oxidative stress regarding protein damage, do not affect on neonates with intrauterine expose to chemotherapy at birth.

With regard to lipid peroxidation, the hallmark of oxidative stress, various non-enzymatic prostaglandin-like compounds were employed in this study to characterise the oxidative damage to lipids in neonates with intrauterine exposure to chemotherapy. In general terms, no signs of lipid damage were observed as a result of chemotherapy expose during perinatal period. If we focus on the most specific and reliable biomarker of lipid peroxidation, isoprostanes, we found decreased levels of 15-F_{2t}-IsoP in plasma and urine while increased levels of 5-F_{2t}-IsoP + 5-*epi*-5-F_{2t}-IsoP in neonates exposed to chemotherapy *in utero* in comparison with those born to healthy mothers at birth. Furthermore, other non-enzymatic prostaglandin-like compounds belonging to the family

of neuroprostanes, dihomo-IsoPs and dihomo-IsoF exhibited the significantly differences as 15-F_{2t}-IsoP. In particular, plasma levels of 4-*epi*-4-F_{4t}-NeuroP, urine levels of 14RS-14-F_{4t}- 14RS-14-F_{4t}-NeuroP, in addition to the lesser known 17-F_{2t}-dihomo-IsoP+17-*epi*-17-F_{2t}-dihomo-IsoP and 17(RS)-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF compounds were found significantly elevated in neonates from healthy pregnancies respect to those exposed to chemotherapy *in utero*.

Concerning the variations of lipid peroxidation levels between cord blood and maternal blood, we found significantly higher levels of 15-*epi*-15F_{2t}-IsoP ent-7*(RS)*-F_{2t}-dihomo-IsoP and 7*(RS)*-ST-Δ^{8}-11-dihomo-IsoF in cord blood plasma from neonates prenatally exposed to chemotherapy than the corresponding maternal plasma levels. Opposite, 7*(RS)*-ST-Δ^{8}-11-dihomo-IsoF was increased in maternal plasma as compared to the neonates. However, we only observed a correlation between both groups on the levels of 15-*epi*-15-F_{2t}-IsoP and 15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$-IsoP compounds.

We suggest that, the absence of DNA, protein and lipid damage observed in neonates exposed prenatally to chemotherapy may indicate a protective role of the placenta against oxidative stress. A further explanation for these results would lay in the development of an efficient activation of different antioxidant mechanisms.

Perinatal inflammation was evaluated through the quantification of prostaglandins, a well-recognised inflammatory biomarker and GSA, a specific biomarker for HOCl both in plasma and urine from neonates exposed to chemotherapy *in utero*. However, we found contradictory results between both biomarkers. While urinary levels of GSA were increased in neonates exposed to chemotherapy *in utero* in comparison with those born to healthy pregnant women, urinary levels of 11β-$PGF_{2\alpha}$ were instead decreased. These results suggest that additional maternal medication such as glucocorticoids may reduce specifically affect the prostaglandins but not GSA[113,114,333].

GSA is a biomarker of HOCl generation produced as consequence of the activation of neutrophils in inflammatory processes[334]. Previous studies have showed a pro-

inflammatory environment in preterm neonates[258,335]. Given that nearly all of neonates included in this study were born prematurely, we suggest that this condition promotes the activation of different inflammatory pathways including neutrophils stimulation. However, little literature exists regarding the role of GSA in pregnancy complications.

In this perspective, two reasonable interpretations could explain these results. First, some studies have observed decreased circulating levels of antioxidants in neonates at birth[336,337]. Robles *et al.* showed a diminution of the antioxidant defence proportional with gestational age in neonates born to normal pregnancy reflecting the substantial oxidative stress generation at birth[338]. For that reason, is comprehensible to suggest that chemotherapy may generated an initial ROS overproduction promptly followed by induction of prenatal antioxidant defences to reinforce resistance against oxidative damage to DNA, protein and lipids and as well as inflammatory mediators. Second, glucocorticoids (e.g., dexamethasone) are well recognised for decreasing pro-inflammatory mediators and modify the concentration of free circulating isoprostanes and prostaglandins by exerting depletion of phospholipase enzyme[339].

Although glucocorticoids can cross the placental barrier, they are routinely prescribed following each cycle of chemotherapy to reduce nausea and vomiting. Thus they may indirectly balance the redox state in favour of the antioxidant defence mechanisms by decreasing lipid peroxidation. In addition, this theory may also demonstrate why the cord plasma and urinary levels of prostaglandins were reduced while the urinary levels of GSA were increased in neonates exposed to chemotherapy during perinatal period.

Consequently, the exposure to chemotherapy *in utero* or its combination with glucocorticoids may induce, in part, an increase neonatal availability of antioxidant defence which would explain the lack of DNA, protein and lipid damage and well as high levels of prostaglandins compounds. Nevertheless, the hypothetical mechanism responsible for this is still not elucidated and further research is required.

Concentrations of prostaglandins are usually more elevated in neonates than in their mothers leading to believe that placenta is an important source of prostaglandins in foetal circulation at birth. In our study, we observed significantly differences on the levels

of 1a,1b-dihomo $PGF_{2\alpha}$ maternal plasma and cord blood plasma. However, we did not find any correlation.

Concerning the antioxidant defence systems, we used GSH, which is considered the most important scavengers of ROS and its ratio with GSSG. This study found that neonates with exposure to chemotherapy during the perinatal period exhibited higher levels of GSH/GSSG ratio in RBCs than those born to healthy pregnant women. Based on this observation, we may speculate that GSH/GSSG homeostasis was altered in neonates exposed to chemotherapy treatment leading to enhance the production of GSH levels to resist against oxidative stress. Nevertheless, a further possible explanation of this result would lie in a significant influence of the maternal antioxidant defence on the neonatal since increased levels of cord blood RBCs GSH/GSSG ratio were observed in neonates exposed to chemotherapy *in utero* as compared to maternal RBCs levels. However, a correlation regarding the of GSH/GSSG between both groups were not found.

Strength and limitations

It is also important to note certain limitations of our study. First, because of the rarity of being exposed to chemotherapy during perinatal period, we conducted this study with a small size population. Second, we were not able to accomplish the obtaining of urine samples from all neonates born to PABC patients under chemotherapy included in the study. This issue may be attributed to the lack of coordination, since not all medical staff was aware of the project. Therefore, the significant results achieved from analysing urine samples should be interpreted with caution. Third, all neonates received a different number of chemotherapy cycles *in utero*. Even more, not all of them were also exposed to paclitaxel following anthracylines. Fourth, the origins of the blood and urine samples included in the control group are different, thus they do not belong to the same participant. This may partially explain why the results differed between both fluids. Fifth, blood and urine samples were obtained from neonates born at three different hospitals. Despite following the same protocols established for the corresponding study, there is a risk of bias from the possibility of collecting and processing the samples differently. Sixth, the doses of glucocorticoids varied among patients. Therefore is highly complicated to fully establish their effect on the placenta and foetal development through oxidative

stress and inflammation.

Lastly, due to the lack of previous similar studies regarding oxidative stress during perinatal period, we were unable to compare our results with others.

However, this study also has several strengths. To our knowledge this is the first study that aims to assess an association between intrauterine exposure to chemotherapy and neonatal complication by analysing the redox state and inflammatory profile in plasma, RBCs and urine fluids in neonates exposed to chemotherapy *in utero*.

Second, we were also the first to study a correlation between maternal and neonatal oxidative stress and inflammation. Lastly, opposite to other publications, we evaluated a great number of biomarkers of DNA, protein and lipid damage, inflammation and antioxidant defence with different validated methods.

The current study may stimulate further investigations aimed to better characterisation of the chemotherapy-induced oxidative stress in pregnancy with larger population and different insight. For instance, it would highly interesting to fully examine the placental tissue as we were not able to fully discard the possibility of the existence of a oxidative damage. Moreover, future exploration of these neonates would be important to obtain more objective data of the safety of chemotherapy during pregnancy and long-time side effects.

Conclusions

In summary, intrauterine exposure to chemotherapy is significantly associated with elevated antioxidant defence and reduced oxidative damage to DNA, proteins and lipid. Regarding inflammation, contradictory data was found as GSA was elevated while 11β-$PGF_{2\alpha}$ was decreased in neonates exposed to chemotherapy in perinatal period. This discrepancy may be caused by the negative effect of glucocorticoids on the levels of prostaglandins. Nonetheless, these results are highly valuable because reinforce the security of the chemotherapy administration during the second and third trimester dismissing any relationship with foetal growth impairment.

Moreover, neonates exposed to chemotherapy *in utero* whose cord blood were analysed, were born weighing much less than those born to healthy pregnant women. However, we

assume that their low weight at birth was more due to their prematurity than for being exposed to chemotherapy in the perinatal period.

Likewise, we suggest that prematurity is due to maternal treatment rather than the effect of chemotherapy exposure in perinatal period. Even though, it is worth noting the importance of a multidisciplinary team that continuously assures the foetal and maternal well being while undergoing chemotherapy in pregnancy. At least three weeks between a cycle of chemotherapy and labour initiation are recommended. Once the pulmonary maturation is done, the neonate can be born safely and to be exposed to another dose of chemotherapy through the placenta is unnecessary.

FINAL CONSIDERATIONS

The studies presented in this thesis are the first to evaluate a significant number of biomarkers of oxidative stress, inflammation and antioxidant defence in PABC patients alongside their offspring employing different biochemical techniques and well-validated methods. Moreover, along with the different studies, we compared cases-population (PABC patients or neonates exposed to chemotherapy *in utero*) with their corresponding controls (healthy pregnant women; non-PABC patients or neonates born to healthy mothers) giving strength to the studies. Therefore, we gain new insight into a metabolic profile that could lead us to understand some of the pathological pathways that could lead to the onset of some pregnancy complications.

Nevertheless, we showed that despite PABC patients might exhibit significantly increased levels of some biomarkers of oxidative stress and inflammation, the antioxidant defence mechanisms of these women were highly efficient en both studies; therefore we speculate that their antioxidant defence capacity was able to counteract the adverse effects of oxidative stress. Moreover, according to the results of the study I.A, we also suggest that anthracyclines and paclitaxel may affect biochemically different on the overproduction of ROS.

Furthermore, we could demonstrate that PABC patients had different metabolic profile than non-PABC patients. However, the natural progression of pregnancy may be responsible for some of these differences.
Lastly, even though the administration of chemotherapy in pregnancy may result controversial because of the fear that the well-being of the foetus is compromised, those neonates did not show perinatal complications secondary to chemotherapy administration during pregnancy.

The knowledge generated by these studies regarding oxidative stress and inflammatory status in PABC patients and their offspring could motivate further studies with larger sample size, that validate our results and that assess whether there are mechanisms that justify for cancer prognosis in this patients.

VIII. CONCLUSIONS

CONCLUSIONS

1. Our data showed that PABC patients exhibited similar levels of oxidative stress and antioxidant defence markers as compared to pregnant controls before the initiation of chemotherapy treatment.

2. Before treatment, PABC patients exhibited significantly higher levels of ChT as compared to healthy pregnant women. Levels of ChT in PABC patients were reduced through treatment.

3. After chemotherapy treatment, the activity of YKL-40 was significantly reduced following paclitaxel treatment, whereas protein-SH groups were especially decreased after anthracyclines.

4 Chemotherapy treatment during pregnancy resulted in elevated levels of DNA damage (i.e., 8-OHdG/2dG ratio) and protein damage (i.e., o-Tyr/Phe ratio) levels, as compared to baseline (before treatment) and at labour.

5 PABC patients showed reduced levels of protein damage (i.e., m-Tyr/Phe) as compared to healthy pregnant women at labour. Additionally, levels of isoprostanes and neuroprostanes (i.e., 5-F_{2t}-IsoP+5-*epi*-5-F_{2t}-IsoP, 15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$ -IsoP and 10-F_{4t}-NeuroP), inflammation ($PGF_{2\alpha}$) and antioxidant defence biomarkers (i.e., GSH/GSSG ratio) were significantly higher compared to controls.

6 non-PABC patients showed elevated levels of DNA damage (i.e., 8-OHdG/2dG ratio), protein damage (i.e., m-Tyr/Phe ratio) and neuroprostanes (i.e., 4-F_{4t}-NeuroP + 4-*epi*-4-F_{4t}-NeuroP and 10-*epi*-10-F_{4t}-NeuroP) biomarkers as well as reduced levels of inflammation ($PGF_{2\alpha}$) and isoprostane compound (i.e., 5-F_{2t}-IsoP + 5-*epi*-5-F_{2t}-IsoP) in comparison with PABC patients.

7 Neonates exposed to chemotherapy *in utero* demonstrated an efficient antioxidant defence system to battle oxidative stress induced by chemotherapy treatment. Neonates exhibited reduced levels of lipid peroxidation and inflammation markers compared to neonates born to healthy women.

8 Overall, the administration of chemotherapy during pregnancy is significantly associated with the disruption of redox homeostasis, although it seems to be efficiently counterbalanced by the action of the antioxidant defence system.

9 Adverse effects on maternal and neonatal outcomes reported in the study secondary to chemotherapy treatment might not be caused by an increase of oxidative and inflammatory pathways, or if increased, they were counteracted by the redox system.

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X. APPENDIX

SUPPLEMENTARY MATERIAL

1. List of supplementary tables

Supplementary table S1. Description of all biomarkers of oxidative stress, inflammation and antioxidant defence analysed in study IA.

Oxidative damage to Lipids	*Modification*	*Effects*
MDA	Polyunsaturated fatty acids peroxidation	Disruption of cellular homeostasis in several pathologic processes
Oxidative damage to Proteins	*Modification*	*Effects*
Protein carbonyl	Direct oxidation of amino acids or via the binding of aldehydes produced from lipid peroxidation processes	Disruption of cellular homeostasis in several pathologic processes
Inflammatory mediators	*Modification*	*Effects*
ChT	Activated by macrophages	Inflammatory response
YKL-40	Secreted by different cells such macrophages, neutrophils and some types of malignant cells	Inflammatory response, angiogenesis, cell proliferation and survival
Antioxidant defence	*Modification*	*Effects*
GSH	Redox regulation	Antioxidant defence systems and metabolic processes
SH-protein groups	Redox regulation	

Supplementary table S2. Description of all biomarkers of oxidative stress, inflammation and antioxidant defence analysed in study IB and II.

Oxidative damage to Proteins		*Modification*	*Effects*
o-Tyr/Phe		Tyrosine Hydroxylation	Disruption of cellular homeostasis in several pathologic processes
m-Tyr/Phe		Tyrosine Hydroxylation	Disruption of cellular homeostasis in several pathologic processes
$3NO_2$-Tyr/p-Tyr		Tyrosine Nitration	Inflammatory response and apoptosis
3Cl-Tyr/p-Tyr		Tyrosine Chlorination	Inflammatory response
Oxidative damage to DNA		*Modification*	*Effects*
8-OHdG/2dG		Hydroxylation DNA nucleotides	Transversion mutations especially during DNA replication (GC --> TA)
Oxidative damage to Lipids		*Modification*	*Effects*
Neuroprostanes	**4-F_{4t}-NeuroP + 4-*epi*-4-F_{4t}-NeuroP** **10-*epi*-10-F_{4t}-NeuroP** **10-F_{4t}-NeuroP** **14(*RS*)-14-F_{4t}-NeuroP**	Docosahexaenoic acid peroxidation	Neuronal oxidative damage
Neurofurans	**4(*RS*)-ST-Δ^5-8-NeuroF**		
Isoprostanes	**5-F_{2t}-IsoP + 5-*epi*-5-F_{2t}-IsoP** **15-F_{2t}-IsoP** **15-E_{2t}-IsoP$^{\varpi}$** **15-*epi*-15-F_{2t}-IsoP** **15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11ß-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$-IsoP** **15-keto-15-F2t-IsoP$^{\varpi}$** **15-keto-15-E2t-IsoP$^{\varpi}$**	Arachidonic acid peroxidation	Adverse effects of oxidant injury mediator

Continue on next page

Supplementary table S2. *Continuation*

Oxidative damage to Lipids		*Modification*	*Effects*
dihomo-IsoPs	**17-F_{2t}-dihomo-IsoP + 17-*epi*-17-F_{2t}-dihomo-IsoP**	Adrenic acid peroxidation	Neuronal oxidative damage
	ent-7(*RS*)-F_{2t}-dihomo-IsoP		
dihomo-IsoFs	**17(*RS*)-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF**		
	7(*RS*)-ST- Δ^{8}-11-dihomo-IsoF		
Inflammatory mediators		*Modification*	*Effects*
Prostaglandins	**1a,1b-dihomo $PGF_{2\alpha}$**	Arachidonic acid peroxidation mediated by enzymes	Inflammatory response
	11ß-$PGF_{2\alpha}$		
	6-keto-$PGF_{1\alpha}$		
	$PGF_{2\alpha}$		
	PGE_2[ϖ]		
GSA		GSH oxidation mediated by hypochlorous acid (HOCL)	
Antioxidants		*Modification*	*Effects*
GSH/GSSG		Redox status	Antioxidant defence systems and metabolic processes
CYS/CYSS			

[ϖ] Metabolites only detected in urine because in plasma are not stable during the KOH hydrolysis

Supplementary table S3. Treatment cycles administered to PABC patients

	CYCLES OF ANTHRACYCLINES						CYCLES OF PACLITAXEL									
Patients	AnC$_{C1}$	AnC$_{C2}$	AnC $_{C3}$	AnC $_{C4}$	AnC$_{C5}$	AnC$_{C6}$	PTX$_{C1}$	PTX$_{C2}$	PTX$_{C3}$	PTX$_{C4}$	PTX$_{C5}$	PTX$_{C6}$	PTX$_{C7}$	PTX$_{C8}$	PTX$_{C9}$	PTX$_{C10}$
PABC-1	✓	✓	✓													
PABC-2	✓	✓	✓	✓			✓	✓	✓	✓	✓					
PABC-3	✓	✓	✓	✓												
PABC-4	✓	✓	✓	✓	✓	✓	✓	✓								
PABC-5	✓	✓														
PABC-6	✓	✓	✓	✓			✓	✓	✓	✓	✓	✓	✓	✓		
PABC-7	✓	✓	✓	✓			✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
PABC-8	✓	✓	✓													
PABC-9	✓	✓	✓	✓			✓	✓	✓	✓						
PABC-10	✓	✓	✓	✓												
PABC-11	✓	✓	✓	✓												
PABC-12	✓	✓														
PABC-13	✓	✓														
PABC-14	✓	✓	✓	✓												
PABC-15	✓	✓					✓	✓	✓	✓	✓	✓	✓	✓		
PABC-16	✓	✓	✓	✓	✓											
PABC-17	✓	✓	✓	✗			✗	✗	✗	✓	✗					

Supplementary table S4. Analysis of control-P group expressed as mean.

	Control-P
Oxidative damage to Lipids	**(*N*=8)**
MDA	0.66 ± 0.31
Oxidative damage to Proteins	**(*N*=9)**
Carbonyl proteins	0.13 ± 0.12
Inflammatory mediators	**(*N*=10)**
ChT	25.11 ± 12.29
	(*N*=10)
YKL-40	9.05 ± 3.33
Antioxidant defence	**(*N*=10)**
GSH	33.18 ± 8.94
	(*N*=11)
SH-protein groups	25.17 ± 11.67

The median GA was 29.6 weeks (mean = 29 weeks; range 23.5-33.3 weeks)

Supplementary table S5. Comparative levels of protein damage, lipid damage, inflammation and antioxidant defence in PABC patients during treatment with anthracyclines (PreAnC vs PostAnC) and paclitaxel (PrePTX vs PostPTX) and pregnancy (PreCTX vs Labour).

	PreAnC	PostAnC	*P*	PrePTX	PostPTX	*P*	PreCTX	Labour	*P*
Oxidative damage to Lipids	(*N*=15)	(*N*=15)		(*N*=6)	(*N*=6)		(*N*=14)	(*N*=14)	
MDA	0.69 ± 0.39	0.59 ± 0.24	n.s.	0.69 ± 0.33	0.86 ± 0.38	0.031[δ]	0.68 ± 0.37	0.62 ± 0.28	n.s.
Oxidative damage to Proteins	(*N*=13)	(*N*=13)	*P*	(*N*=5)	(*N*=5)	*P*	(*N*=14)	(*N*=14)	*P*
Carbonyl proteins	0.19 ± 0.20	0.12 ± 0.08	n.s.	0.09 ± 0.06	0.08 ± 0.04	n.s.	0.21 ± 0.21	0.14 ± 0.19	n.s.
Inflammatory mediators	(*N*=14)	(*N*=14)	*P*	(*N*=6)	(*N*=6)	*P*	(*N*=14)	(*N*=14)	*P*
ChT	50.94 ± 19.20	51.28 ± 22.92	n.s.	57.76 ± 21.98	34.57 ± 9.65	0.031[λ]	52.17 ± 19.48	43.46 ± 23.26	n.s.
	(*N*=15)	(*N*=15)	*P*	(*N*=6)	(*N*=6)	*P*	(*N*=15)	(*N*=15)	*P*
YKL-40	33.66 ± 20.73	26.00 ± 11.73	0.026[δ]	25.85 ± 8.18	29.67 ± 10.13	n.s.	34.02 ± 20.51	27.09 ± 13.54	n.s.
Antioxidant defence	(*N*=8)	(*N*=8)	*P*	(*N*=5)	(*N*=5)	*P*	(*N*=11)	(*N*=11)	*P*
GSH	20.87 ± 9.51	39.14 ± 21.32	0.046[λ]	28.01 ± 21.70	18.93 ± 5.31	n.s.	21.52 ± 9.50	27.44 ± 13.35	n.s.
	(*N*=15)	(*N*=15)	*P*	(*N*=6)	(*N*=6)	*P*	(*N*=15)	(*N*=15)	*P*
SH-protein groups	8.26 ± 3.12	6.67 ± 1.27	0.030[λ]	6.10 ± 1.20	8.59 ± 1.46	0.038[λ]	7.85 ± 3.77	7.76 ± 2.46	n.s.

Supplementary table S6. Levels of protein damage, lipid damage, inflammation and antioxidant defence in PABC patients in each cycle of anthracyclines in comparison with before chemotherapy treatment.

	PreCTX	AnC_{C1}	*P*	PreCTX	AnC_{C2}	*P*	PreCTX	AnC_{C3}	*P*
Oxidative damage to Lipids	**(*N*=16)**	**(*N*=16)**		**(*N*=16)**	**(*N*=16)**		**(*N*=12)**	**(*N*=12)**	
MDA	0.72 ± 0.39	0.64 ± 0.41	n.s.	0.72 ± 0.39	0.58 ± 0.25	0.034^{λ}	0.66 ± 0.40	0.71 ± 0.42	n.s.
Oxidative damage to Proteins	**(*N*=15)**	**(*N*=15)**	*P*	**(*N*=15)**	**(*N*=15)**	*P*	**(*N*=11)**	**(*N*=11)**	*P*
Carbonyl proteins	0.21 ± 0.20	0.14 ± 0.11	n.s.	0.21 ± 0.20	0.14 ± 0.11	n.s.	0.24 ± 0.22	0.11 ± 0.04	n.s.
Inflammatory mediators	**(*N*=14)**	**(*N*=14)**	*P*	**(*N*=14)**	**(*N*=14)**	*P*	**(*N*=11)**	**(*N*=11)**	*P*
ChT	52.17 ± 19.48	62.35 ± 25.74	n.s.	52.17 ± 19.48	56.66 ± 27.90	n.s.	52.81 ± 20.43	56.80 ± 30.36	n.s.
	(*N*=15)	**(*N*=15)**	*P*	**(*N*=15)**	**(*N*=15)**	*P*	**(*N*=11)**	**(*N*=11)**	*P*
YKL-40	34.02 ± 20.51	32.53 ± 23.93	n.s.	34.02 ± 20.51	24.93 ± 12.00	0.030^{δ}	37.16 ± 23.25	28.82 ± 14.03	n.s.
Antioxidant defence	**(*N*=11)**	**(*N*=11)**	*P*	**(*N*=8)**	**(*N*=8)**	*P*	**(*N*=7)**	**(*N*=7)**	*P*
GSH	20.48 ± 8.70	32.91 ± 23.88	0.019^{δ}	23.08 ± 8.98	36.98 ± 18.48	n.s.	23.65 ± 9.40	35.28 ± 23.89	0.011^{λ}
	(*N*=17)	**(*N*=17)**	*P*	**(*N*=17)**	**(*N*=17)**	*P*	**(*N*=12)**	**(*N*=12)**	*P*
SH-protein groups	8.07 ± 2.98	8.46 ± 4.71	n.s.	8.07 ± 2.98	6.93 ± 1.80	n.s.	8.68 ± 3.33	6.94 ± 1.39	n.s.

Supplementary table S6. *Continuation*

Oxidative damage to Lipids	PreCTX (*N*=9)	AnC_{C4} (*N*=9)	*P*
MDA	0.61 ± 0.39	0.59 ± 0.30	n.s.
Oxidative damage to Proteins	(*N*=9)	(*N*=9)	*P*
Carbonyl proteins	0.22 ± 0.23	0.18 ± 0.23	n.s.
Inflammatory mediators	(*N*=9)	(*N*=9)	*P*
ChT	56.83 ± 18.96	57.12 ± 20.47	n.s.
	(*N*=9)	(*N*=9)	*P*
YKL-40	33.39 ± 14.13	26.30 ± 12.57	0.039^{δ}
Antioxidant defence	(*N*=7)	(*N*=7)	*P*
GSH	25.06 ± 9.71	26.59 ± 13.95	n.s.
	(*N*=9)	(*N*=9)	*P*
SH-protein groups	8.92 ± 3.62	6.46 ± 1.30	n.s.

Values are expressed as mean ± SD

$^{\Psi}$ Wilcoxon rank-sum test, $^{\gamma}$ Student's t test, $^{\delta}$ Wilcoxon signed-rank test and $^{\lambda}$ Student's t paired test were used to assess differences between groups.

P value of ≤ 0.05 was considered significant.

Supplementary table S7. Levels of protein damage, lipid damage, inflammation and antioxidant defence in PABC patients in each cycle of paclitaxel in comparison with before chemotherapy treatment.

	PreCTX	PTX_{C1}		PreCTX	PTX_{C2}		PreCTX	PTX_{C3}	
Oxidative damage to Lipids	**(*N*=6)**	**(*N*=6)**	***P***	**(*N*=5)**	**(*N*=5)**	***P***	**(*N*=4)**	**(*N*=4)**	***P***
MDA	0.55 ± 0.42	0.65 ± 0.35	n.s.	0.40 ± 0.22	0.65 ± 0.23	n.s.	0.49 ± 0.14	0.51 ± 0.22	n.s.
Oxidative damage to Proteins	**(*N*=5)**	**(*N*=5)**	***P***	**(*N*=4)**	**(*N*=4)**	***P***	**(*N*=4)**	**(*N*=4)**	***P***
Carbonyl proteins	0.08 ± 0.03	0.08 ± 0.05	n.s.	0.09 ± 0.03	0.12 ± 0.13	n.s.	0.09 ± 0.03	0.06 ± 0.06	n.s.
Inflammatory mediators	**(*N*=6)**	**(*N*=6)**	***P***	**(*N*=5)**	**(*N*=5)**	***P***	**(*N*=4)**	**(*N*=4)**	***P***
ChT	57.65 ± 17.89	45.10 ± 16.37	n.s.	56.32 ± 19.67	39.09 ± 14.52	0.008^{λ}	57.25 ± 22.59	41.18 ± 19.55	n.s.
	(*N*=6)	**(*N*=6)**	***P***	**(*N*=5)**	**(*N*=5)**	***P***	**(*N*=4)**	**(*N*=4)**	***P***
YKL-40	33.80 ± 14.55	22.84 ± 11.51	n.s.	35.56 ± 15.54	22.51 ± 7.89	n.s.	35.67 ± 17.94	20.89 ± 7.29	n.s.
Antioxidant defence	**(*N*=4)**	**(*N*=4)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***	**(*N*=2)**	**(*N*=2)**	***P***
GSH	19.09 ± 10.80	56.69 ± 55.93	n.s.	21.00 ± 12.36	39.96 ± 27.01	n.s.	28.05 ± 2.76	28.80 ± 22.48	n.s.
	(*N*=6)	**(*N*=6)**	***P***	**(*N*=5)**	**(*N*=5)**	***P***	**(*N*=4)**	**(*N*=4)**	***P***
SH-protein groups	7.69 ± 1.35	7.44 ± 1.92	n.s.	7.61 ± 1.49	8.10 ± 1.38	n.s.	7.79 ± 1.66	8.20 ± 0.65	n.s.

Supplementary table S7. *Continuation*

	PreCTX	PTX_{C4}	*P*	PreCTX	PTX_{C5}	*P*	PreCTX	PTX_{C6}	*P*
Oxidative damage to Lipids	**(*N*=5)**	**(*N*=5)**		**(*N*=3)**	**(*N*=3)**		**(*N*=3)**	**(*N*=3)**	
MDA	0.65 ± 0.38	0.65 ± 0.35	n.s.	0.64 ± 0.44	0.76 ± 0.36	n.s.	0.73 ± 0.49	0.85 ± 0.69	n.s.
Oxidative damage to Proteins	**(*N*=3)**	**(*N*=3)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***
Carbonyl proteins	0.08 ± 0.03	0.10 ± 0.07	n.s.	0.08 ± 0.05	0.10 ± 0.04	n.s.	0.21 ± 0.24	0.15 ± 0.12	n.s.
Inflammatory mediators	**(*N*=5)**	**(*N*=5)**	***P***	**(*N*=4)**	**(*N*=4)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***
ChT	58.66 ± 19.81	42.57 ± 17.78	n.s.	59.97 ± 22.63	44.27 ± 22.19	n.s.	55.92 ± 25.88	47.45 ± 31.36	n.s.
	(*N*=5)	**(*N*=5)**	***P***	**(*N*=4)**	**(*N*=4)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***
YKL-40	33.54 ± 16.25	22.87 ± 12.29	n.s.	35.64 ± 17.96	19.28 ± 8.95	n.s.	33.14 ± 21.13	24.59 ± 12.35	n.s.
Antioxidant defence	**(*N*=3)**	**(*N*=3)**	***P***	–	–	***P***	**(*N*=2)**	**(*N*=2)**	***P***
GSH	23.14 ± 8.72	27.55 ± 18.20	n.s.	–	–	–	21.67 ± 11.79	19.76 ± 7.45	n.s.
	(*N*=5)	**(*N*=5)**	***P***	**(*N*=4)**	**(*N*=4)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***
SH-protein groups	7.85 ± 1.45	9.06 ± 2.02	n.s.	7.39 ± 1.17	7.79 ± 1.23	n.s.	6.96 ± 0.98	7.56 ± 1.31	n.s.

Supplementary table S7. *Continuation*

	PreCTX	PTX_{C7}	*P*	PreCTX	PTX_{C8}	*P*
Oxidative damage to Lipids	**(*N*=3)**	**(*N*=3)**		**(*N*=2)**	**(*N*=2)**	
MDA	0.73 ± 0.49	0.67 ± 0.43	n.s.	0.73 ± 0.49	1.21 ± 0.51	n.s.
Oxidative damage to Proteins	**(*N*=3)**	**(*N*=3)**	***P***	**(*N*=2)**	**(*N*=2)**	***P***
Carbonyl proteins	0.21 ± 0.24	0.13 ± 0.07	n.s.	0.07 ± 0.03	0.09 ± 0.02	n.s.
Inflammatory mediators	**(*N*=3)**	**(*N*=3)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***
ChT	55.92 ± 25.88	46.56 ± 27.73	n.s.	55.92 ± 25.88	37.22 ± 16.56	n.s.
	(*N*=3)	**(*N*=3)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***
YKL-40	33.14 ± 21.13	22.26 ± 7.14	n.s.	33.14 ± 21.13	29.10 ± 10.70	n.s.
Antioxidant defence	–	–	***P***	–	–	***P***
GSH	–	–	–	–	–	–
	(*N*=3)	**(*N*=3)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***
SH-protein groups	6.96 ± 0.98	7.03 ± 2.72	n.s.	6.96 ± 0.98	9.19 ± 1.54	n.s.

Values are expressed as mean ± SD

$^{\Psi}$ Wilcoxon rank-sum test, $^{\gamma}$ Student's t test, $^{\delta}$ Wilcoxon signed-rank test and $^{\lambda}$ Student's t paired test were used to assess differences between groups.

P value of ≤ 0.05 was considered significant.

Supplementary table S8. Comparative levels of protein damage, lipid damage, inflammation and antioxidant defence in PABC patients during each cycle of anthracyclines.

	AnC$_{C1}$	AnC$_{C2}$	*P*	AnC$_{C1}$	AnC$_{C3}$	*P*	AnC$_{C1}$	AnC$_{C4}$	*P*
Oxidative damage to Lipids	**(*N*=16)**	**(*N*=16)**		**(*N*=12)**	**(*N*=12)**		**(*N*=9)**	**(*N*=9)**	
MDA	0.64 ± 0.41	0.58 ± 0.25	n.s.	0.60 ± 0.44	0.71 ± 0.42	n.s.	0.60 ± 0.49	0.59 ± 0.30	n.s.
Oxidative damage to Proteins	**(*N*=15)**	**(*N*=15)**	*P*	**(*N*=11)**	**(*N*=11)**	*P*	**(*N*=9)**	**(*N*=9)**	*P*
Carbonyl proteins	0.14 ± 0.11	0.14 ± 0.11	n.s.	0.12 ± 0.06	0.11 ± 0.04	n.s.	0.11 ± 0.06	0.18 ± 0.23	n.s.
Inflammatory mediators	**(*N*=15)**	**(*N*=15)**	*P*	**(*N*=11)**	**(*N*=11)**	*P*	**(*N*=9)**	**(*N*=9)**	*P*
ChT	62.35 ± 23.74	56.66 ± 27.90	n.s.	61.27 ± 19.41	56.80 ± 30.36	n.s.	62.01 ± 20.36	57.12 ± 20.47	n.s.
	(*N*=16)	**(*N*=16)**	*P*	**(*N*=12)**	**(*N*=12)**	*P*	**(*N*=9)**	**(*N*=9)**	*P*
YKL-40	31.51 ± 23.48	24.96 ± 11.60	n.s.	34.96 ± 26.18	27.82 ± 13.82	n.s.	34.16 ± 23.42	26.30 ± 12.57	n.s.
Antioxidant defence	**(*N*=10)**	**(*N*=10)**	*P*	**(*N*=8)**	**(*N*=8)**	*P*	**(*N*=7)**	**(*N*=7)**	*P*
GSH	32.93 ± 24.88	28.80 ± 13.86	n.s.	30.62 ± 22.47	48.25 ± 27.17	0.027^{λ}	30.43 ± 24.40	31.52 ± 17.52	n.s.
	(*N*=17)	**(*N*=17)**	*P*	**(*N*=12)**	**(*N*=12)**	*P*	**(*N*=8)**	**(*N*=8)**	*P*
SH-protein groups	8.46 ± 4.71	6.93 ± 1.80	n.s.	9.40 ± 5.34	6.94 ± 1.39	n.s.	8.81 ± 4.33	6.13 ± 0.88	n.s.

Supplementary table S8. *Continuation*

	AnC$_{C2}$	AnC$_{C3}$	*P*	AnC$_{C2}$	AnC$_{C4}$	*P*	AnC$_{C3}$	AnC$_{C4}$	*P*
Oxidative damage to Lipids	(*N*=12)	(*N*=12)		(*N*=9)	(*N*=9)		(*N*=9)	(*N*=9)	
MDA	0.57 ± 0.27	0.71 ± 0.42	n.s.	0.53 ± 0.30	0.59 ± 0.29	n.s.	0.63 ± 0.35	0.59 ± 0.29	n.s.
Oxidative damage to Proteins	(*N*=12)	(*N*=12)	*P*	(*N*=9)	(*N*=9)	*P*	(*N*=9)	(*N*=9)	*P*
Carbonyl proteins	0.14 ± 0.11	0.11 ± 0.04	n.s.	0.15 ± 0.12	0.18 ± 0.23	n.s.	0.12 ± 0.05	0.18 ± 0.23	n.s.
Inflammatory mediators	(*N*=11)	(*N*=11)	*P*	(*N*=9)	(*N*=9)	*P*	(*N*=9)	(*N*=9)	*P*
ChT	61.49 ± 27.16	56.80 ± 30.36	n.s.	65.31 ± 28.56	57.12 ± 20.47	n.s.	62.85 ± 30.17	57.12 ± 20.47	n.s.
	(*N*=12)	(*N*=12)	*P*	(*N*=9)	(*N*=9)	*P*	(*N*=9)	(*N*=9)	*P*
YKL-40	26.34 ± 11.19	27.82 ± 13.82	n.s.	25.32 ± 10.52	26.30 ± 12.57	n.s.	29.63 ± 15.02	26.30 ± 12.57	n.s.
Antioxidant defence	(*N*=7)	(*N*=7)	*P*	(*N*=6)	(*N*=6)	*P*	(*N*=7)	(*N*=7)	*P*
GSH	24.21 ± 9.63	45.49 ± 27.79	0.049^{λ}	30.08 ± 19.63	29.08 ± 17.92	n.s.	38.49 ± 26.24	31.05 ± 17.86	n.s.
	(*N*=12)	(*N*=12)	*P*	(*N*=8)	(*N*=8)	*P*	(*N*=8)	(*N*=8)	*P*
SH-protein groups	7.24 ± 1.73	6.94 ± 1.39	n.s.	6.61 ± 1.35	6.13 ± 0.88	n.s.	7.03 ± 1.58	6.13 ± 0.88	n.s.

Supplementary table S8. *Continuation*

	AnC$_{C4}$	AnC$_{C5}$	*P*
Oxidative damage to Lipids	–	–	
MDA	–	–	–
Oxidative damage to Proteins	–	–	*P*
Carbonyl proteins	–	–	–
Inflammatory mediators	**(*N*=2)**	**(*N*=2)**	*P*
ChT	52.65 ± 13.36	44.29 ± 13.45	n.s.
	–	–	*P*
YKL-40	–	–	–
Antioxidant defence	–	–	*P*
GSH	–	–	–
	(*N*=2)	**(*N*=2)**	*P*
SH-protein groups	6.63 ± 1.01	5.16 ± 0.25	n.s.

Values are expressed as mean ± SD

$^{\Psi}$ Wilcoxon rank-sum test, $^{\gamma}$ Student's t test, $^{\delta}$ Wilcoxon signed-rank test and $^{\lambda}$ Student's t paired test were used to assess differences between groups.

P value of ≤ 0.05 was considered significant.

Supplementary table S9. Comparative levels of protein damage, lipid damage, inflammation and antioxidant defence in PABC patients during each cycle of paclitaxel.

	PTX_{C1}	PTX_{C2}	*P*	PTX_{C1}	PTX_{C3}	*P*	PTX_{C1}	PTX_{C4}	*P*
Oxidative damage to Lipids	**(*N*=5)**	**(*N*=5)**		**(*N*=4)**	**(*N*=4)**		**(*N*=5)**	**(*N*=5)**	
MDA	0.54 ± 0.23	0.65 ± 0.23	n.s.	0.57 ± 0.25	0.51 ± 0.22	n.s.	0.70 ± 0.37	0.74 ± 0.31	n.s.
Oxidative damage to Proteins	**(*N*=4)**	**(*N*=4)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=4)**	**(*N*=4)**	*P*
Carbonyl proteins	0.07 ± 0.06	0.12 ± 0.13	n.s.	0.10 ± 0.05	0.08 ± 0.07	n.s.	0.10 ± 0.04	0.12 ± 0.05	n.s.
Inflammatory mediators	**(*N*=5)**	**(*N*=5)**	*P*	**(*N*=4)**	**(*N*=4)**	*P*	**(*N*=5)**	**(*N*=5)**	*P*
ChT	41.21 ± 14.88	39.09 ± 14.52	n.s.	41.57 ± 17.16	41.18 ± 19.55	n.s.	46.16 ± 18.07	42.57 ± 17.78	n.s.
	(*N*=5)	**(*N*=5)**	*P*	**(*N*=4)**	**(*N*=4)**	*P*	**(*N*=5)**	**(*N*=5)**	*P*
YKL-40	24.61 ± 11.91	22.51 ± 7.89	n.s.	20.97 ± 10.03	20.89 ± 7.29	n.s.	19.57 ± 9.23	22.87 ± 12.29	n.s.
Antioxidant defence	**(*N*=4)**	**(*N*=4)**	*P*	**(*N*=4)**	**(*N*=4)**	*P*	**(*N*=5)**	**(*N*=5)**	*P*
GSH	60.33 ± 52.50	36.22 ± 23.29	n.s.	60.83 ± 51.97	31.75 ± 25.47	n.s.	51.23 ± 49.86	23.93 ± 13.79	n.s.
	(*N*=5)	**(*N*=5)**	*P*	**(*N*=4)**	**(*N*=4)**	*P*	**(*N*=5)**	**(*N*=5)**	*P*
SH-protein groups	7.12 ± 1.97	8.10 ± 1.38	n.s.	7.14 ± 2.27	8.20 ± 0.65	n.s.	7.52 ± 2.14	9.06 ± 2.02	n.s.

Supplementary table S9. *Continuation*

	PTX_{C1}	PTX_{C5}	*P*	PTX_{C1}	PTX_{C6}	*P*	PTX_{C1}	PTX_{C7}	*P*
Oxidative damage to Lipids	**(*N*=4)**	**(*N*=4)**		**(*N*=3)**	**(*N*=3)**		**(*N*=3)**	**(*N*=3)**	
MDA	0.76 ± 0.39	0.76 ± 0.36	n.s.	0.85 ± 0.42	0.85 ± 0.69	n.s.	0.85 ± 0.42	0.67 ± 0.43	n.s.
Oxidative damage to Proteins	**(*N*=3)**	**(*N*=3)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***
Carbonyl proteins	0.08 ± 0.04	0.10 ± 0.04	n.s.	0.08 ± 0.04	0.15 ± 0.12	n.s.	0.08 ± 0.04	0.13 ± 0.07	n.s.
Inflammatory mediators	**(*N*=4)**	**(*N*=4)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***
ChT	45.16 ± 20.70	44.27 ± 22.19	n.s.	48.28 ± 24.17	47.45 ± 31.36	n.s.	48.28 ± 24.17	46.56 ± 27.73	n.s.
	(*N*=4)	**(*N*=4)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***
YKL-40	19.07 ± 10.59	19.28 ± 8.95	n.s.	19.90 ± 12.81	24.59 ± 12.35	n.s.	19.90 ± 12.81	22.26 ± 7.14	n.s.
Antioxidant defence	**(*N*=3)**	**(*N*=3)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***	**(*N*=2)**	**(*N*=2)**	***P***
GSH	20.18 ± 7.28	21.19 ± 5.65	n.s.	55.69 ± 67.85	20.20 ± 5.69	n.s.	16.58 ± 5.27	16.98 ± 2.43	n.s.
	(*N*=4)	**(*N*=4)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***	**(*N*=3)**	**(*N*=3)**	***P***
SH-protein groups	8.08 ± 2.01	7.79 ± 1.23	n.s.	7.32 ± 1.63	7.56 ± 1.31	n.s.	7.32 ± 1.63	7.03 ± 2.72	n.s.

Supplementary table S9. *Continuation*

	PTX_{C1}	PTX_{C8}	*P*	PTX_{C2}	PTX_{C3}	*P*	PTX_{C2}	PTX_{C4}	*P*
Oxidative damage to Lipids	**(*N*=3)**	**(*N*=3)**		**(*N*=4)**	**(*N*=4)**		**(*N*=4)**	**(*N*=4)**	
MDA	0.85 ± 0.42	1.21 ± 0.50	n.s.	0.66 ± 0.26	0.51 ± 0.22	0.009^{λ}	0.66 ± 0.26	0.62 ± 0.20	n.s.
Oxidative damage to Proteins	**(*N*=2)**	**(*N*=2)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*
Carbonyl proteins	0.11 ± 0.01	0.07 ± 0.10	n.s.	0.16 ± 0.13	0.08 ± 0.07	n.s.	0.16 ± 0.13	0.10 ± 0.03	n.s.
Inflammatory mediators	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=4)**	**(*N*=4)**	*P*	**(*N*=4)**	**(*N*=4)**	*P*
ChT	48.28 ± 24.17	37.22 ± 16.56	n.s.	40.94 ± 16.08	41.18 ± 19.55	n.s.	40.94 ± 16.08	36.62 ± 13.63	n.s.
	(*N*=3)	**(*N*=3)**	*P*	**(*N*=4)**	**(*N*=4)**	*P*	**(*N*=4)**	**(*N*=4)**	*P*
YKL-40	19.90 ± 12.81	29.10 ± 10.70	n.s.	20.53 ± 7.54	20.89 ± 7.29	n.s.	20.53 ± 7.54	25.38 ± 12.63	n.s.
Antioxidant defence	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*
GSH	55.69 ± 67.85	23.47 ± 11.77	n.s.	43.33 ± 22.60	36.10 ± 29.32	n.s.	43.33 ± 22.60	28.58 ± 17.25	n.s.
	(*N*=3)	**(*N*=3)**	*P*	**(*N*=4)**	**(*N*=4)**	*P*	**(*N*=4)**	**(*N*=4)**	*P*
SH-protein groups	7.32 ± 1.63	9.19 ± 1.54	n.s.	7.79 ± 1.38	8.20 ± 0.65	n.s.	7.79 ± 1.38	9.22 ± 2.30	n.s.

Supplementary table S9. *Continuation*

	PTX$_{C2}$	PTX$_{C5}$	*P*	PTX$_{C2}$	PTX$_{C6}$	*P*	PTX$_{C2}$	PTX$_{C7}$	*P*
Oxidative damage to Lipids	**(*N*=3)**	**(*N*=3)**		**(*N*=2)**	**(*N*=2)**		**(*N*=2)**	**(*N*=2)**	
MDA	0.71 ± 0.30	0.62 ± 0.30	0.021$^{\lambda}$	0.71 ± 0.43	0.46 ± 0.18	n.s.	0.71 ± 0.43	0.43 ± 0.18	n.s.
Oxidative damage to Proteins	**(*N*=2)**	**(*N*=2)**	***P***	–	–	***P***	–	–	***P***
Carbonyl proteins	0.08 ± 0.02	0.11 ± 0.06	n.s.	–	–	–	–	–	–
Inflammatory mediators	**(*N*=3)**	**(*N*=3)**	***P***	**(*N*=2)**	**(*N*=2)**	***P***	**(*N*=2)**	**(*N*=2)**	***P***
ChT	37.87 ± 18.19	36.37 ± 19.07	n.s.	47.55 ± 9.97	32.85 ± 26.23	n.s.	47.55 ± 9.97	35.30 ± 27.86	n.s.
	(*N*=3)	**(*N*=3)**	***P***	**(*N*=2)**	**(*N*=2)**	***P***	**(*N*=2)**	**(*N*=2)**	***P***
YKL-40	18.28 ± 7.39	21.10 ± 10.00	n.s.	19.72 ± 9.84	27.61 ± 15.83	n.s.	19.72 ± 9.84	25.79 ± 5.21	n.s.
Antioxidant defence	–	–	***P***	–	–	***P***	–	–	***P***
GSH	–	–	–	–	–	–	–	–	–
	(*N*=3)	**(*N*=3)**	***P***	**(*N*=2)**	**(*N*=2)**	***P***	**(*N*=2)**	**(*N*=2)**	***P***
SH-protein groups	7.97 ± 1.63	7.64 ± 1.46	n.s.	7.04 ± 0.32	7.22 ± 1.65	n.s.	7.04 ± 0.32	5.48 ± 0.60	n.s.

Supplementary table S9. *Continuation*

	PTX$_{C2}$	PTX$_{C8}$	*P*	PTX$_{C3}$	PTX$_{C4}$	*P*	PTX$_{C3}$	PTX$_{C5}$	*P*
Oxidative damage to Lipids	(*N*=2)	(*N*=2)		(*N*=4)	(*N*=4)		(*N*=3)	(*N*=3)	
MDA	0.71 ± 0.43	1.05 ± 0.61	n.s.	0.51 ± 0.22	0.62 ± 0.20	0.036$^{\lambda}$	0.55 ± 0.25	0.62 ± 0.30	n.s.
Oxidative damage to Proteins	–	–	*P*	(*N*=4)	(*N*=4)	*P*	(*N*=2)	(*N*=2)	*P*
Carbonyl proteins	–	–	–	0.06 ± 0.06	0.08 ± 0.05	n.s.	0.04 ± 0.01	0.11 ± 0.06	n.s.
Inflammatory mediators	(*N*=2)	(*N*=2)	*P*	(*N*=4)	(*N*=4)	*P*	(*N*=3)	(*N*=3)	*P*
ChT	47.55 ± 9.97	32.25 ± 20.01	n.s.	41.18 ± 19.55	36.62 ± 13.63	n.s.	41.70 ± 21.91	36.37 ± 19.07	n.s.
	(*N*=2)	(*N*=2)	*P*	(*N*=4)	(*N*=4)	*P*	(*N*=3)	(*N*=3)	*P*
YKL-40	19.72 ± 9.84	33.87 ± 9.61	n.s.	20.89 ± 7.29	25.38 ± 12.63	n.s.	19.17 ± 7.87	21.10 ± 10.01	n.s.
Antioxidant defence	–	–	*P*	(*N*=3)	(*N*=3)	*P*	(*N*=2)	(*N*=2)	*P*
GSH	–	–	–	36.10 ± 29.32	28.58 ± 17.25	n.s.	20.55 ± 2.62	23.05 ± 6.58	n.s.
	(*N*=2)	(*N*=2)	*P*	(*N*=4)	(*N*=4)	*P*	(*N*=3)	(*N*=3)	*P*
SH-protein groups	7.04 ± 0.32	9.92 ± 1.23	n.s.	8.20 ± 0.65	9.22 ± 2.30	n.s.	8.01 ± 0.66	7.64 ± 1.46	n.s.

Supplementary table S9. *Continuation*

	PTX_{C3} (*N*=2)	PTX_{C6} (*N*=2)	*P*	PTX_{C3} (*N*=2)	PTX_{C7} (*N*=2)	*P*	PTX_{C3} (*N*=2)	PTX_{C8} (*N*=2)	*P*
Oxidative damage to Lipids									
MDA	0.55 ± 0.35	0.46 ± 0.18	n.s.	0.55 ± 0.35	0.43 ± 0.18	n.s.	0.55 ± 0.35	1.05 ± 0.61	n.s.
Oxidative damage to Proteins	–	–	*P*	–	–	*P*	–	–	*P*
Carbonyl proteins	–	–	–	–	–	n.s.	–	–	–
Inflammatory mediators	(*N*=2)	(*N*=2)	*P*	(*N*=2)	(*N*=2)	*P*	(*N*=2)	(*N*=2)	*P*
ChT	43.00 ± 33.66	32.85 ± 26.23	n.s.	43.00 ± 33.66	35.30 ± 27.86	n.s.	43.00 ± 33.66	32.25 ± 20.01	n.s.
	(*N*=2)	(*N*=2)	*P*	(*N*=2)	(*N*=2)	*P*	(*N*=2)	(*N*=2)	*P*
YKL-40	19.82 ± 11.02	27.61 ± 15.83	n.s.	19.82 ± 11.02	25.79 ± 5.21	n.s.	19.82 ± 11.02	33.87 ± 9.61	n.s.
Antioxidant defence	–	–	*P*	–	–	*P*	–	–	*P*
GSH	–	–	–	–	–	–	–	–	–
	(*N*=2)	(*N*=2)	*P*	(*N*=2)	(*N*=2)	*P*	(*N*=2)	(*N*=2)	*P*
SH-protein groups	8.33 ± 0.54	7.22 ± 1.65	n.s.	8.33 ± 0.54	5.48 ± 0.60	n.s.	8.33 ± 0.54	9.92 ± 1.23	n.s.

Supplementary table S9. *Continuation*

	PTX_{C4}	PTX_{C5}	*P*	PTX_{C4}	PTX_{C6}	*P*	PTX_{C4}	PTX_{C7}	*P*
Oxidative damage to Lipids	**(*N*=4)**	**(*N*=4)**		**(*N*=3)**	**(*N*=3)**		**(*N*=3)**	**(*N*=3)**	
MDA	0.62 ± 0.20	0.76 ± 0.36	n.s.	0.82 ± 0.41	0.85 ± 0.69	n.s.	0.82 ± 0.41	0.67 ± 0.43	n.s.
Oxidative damage to Proteins	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*
Carbonyl proteins	0.12 ± 0.07	0.10 ± 0.04	n.s.	0.10 ± 0.09	0.15 ± 0.12	n.s.	0.10 ± 0.09	0.13 ± 0.07	n.s.
Inflammatory mediators	**(*N*=4)**	**(*N*=4)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*
ChT	44.74 ± 19.75	44.27 ± 22.19	n.s.	47.59 ± 23.16	47.45 ± 31.36	n.s.	47.59 ± 23.16	46.56 ± 27.73	n.s.
	(*N*=4)	**(*N*=4)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*
YKL-40	19.98 ± 12.06	19.28 ± 8.95	n.s.	21.65 ± 14.19	24.59 ± 12.35	n.s.	21.65 ± 14.19	22.26 ± 7.14	n.s.
Antioxidant defence	**(*N*=2)**	**(*N*=2)**	*P*	**(*N*=2)**	**(*N*=2)**	*P*	–	–	*P*
GSH	17.25 ± 2.19	22.59 ± 7.23	n.s.	18.33 ± 0.18	22.63 ± 5.41	n.s.	–	–	–
	(*N*=4)	**(*N*=4)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*
SH-protein groups	9.26 ± 2.28	7.79 ± 1.23	n.s.	9.53 ± 2.71	7.56 ± 1.31	n.s.	9.53 ± 2.71	7.03 ± 2.72	n.s.

Supplementary table S9. *Continuation*

	PTX_{C4}	PTX_{C8}	*P*	PTX_{C5}	PTX_{C6}	*P*	PTX_{C5}	PTX_{C7}	*P*
Oxidative damage to Lipids	**(*N*=3)**	**(*N*=3)**		**(*N*=3)**	**(*N*=3)**		**(*N*=3)**	**(*N*=3)**	
MDA	0.82 ± 0.41	1.21 ± 0.51	n.s.	0.79 ± 0.43	0.85 ± 0.69	n.s.	0.79 ± 0.43	0.67 ± 0.43	n.s.
Oxidative damage to Proteins	**(*N*=2)**	**(*N*=2)**	*P*	**(*N*=2)**	**(*N*=2)**	*P*	**(*N*=2)**	**(*N*=2)**	*P*
Carbonyl proteins	0.14 ± 0.08	0.09 ± 0.02	n.s.	0.12 ± 0.04	0.21 ± 0.07	n.s.	0.12 ± 0.04	0.17 ± 0.04	n.s.
Inflammatory mediators	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*
ChT	47.59 ± 23.16	37.22 ± 16.56	n.s.	46.26 ± 26.74	47.45 ± 31.36	n.s.	46.26 ± 26.74	46.56 ± 27.73	n.s.
	(*N*=3)	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*
YKL-40	21.65 ± 14.19	29.10 ± 10.70	n.s.	19.06 ± 10.95	24.59 ± 12.35	n.s.	19.06 ± 10.95	22.26 ± 7.14	n.s.
Antioxidant defence	**(*N*=2)**	**(*N*=2)**	*P*	**(*N*=2)**	**(*N*=2)**	*P*	**(*N*=2)**	**(*N*=2)**	*P*
GSH	18.33 ± 0.18	27.95 ± 12.52	n.s.	17.94 ± 0.65	17.07 ± 2.45	n.s.	17.94 ± 0.65	16.98 ± 2.43	n.s.
	(*N*=3)	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*
SH-protein groups	9.53 ± 2.71	9.19 ± 1.54	n.s.	7.41 ± 1.18	7.56 ± 1.31	n.s.	7.41 ± 1.18	7.03 ± 2.71	n.s.

Supplementary table S9. *Continuation*

	PTX$_{C5}$	PTX$_{C8}$	*P*	PTX$_{C6}$	PTX$_{C7}$	*P*	PTX$_{C6}$	PTX$_{C8}$	*P*
Oxidative damage to Lipids	**(*N*=3)**	**(*N*=3)**		**(*N*=3)**	**(*N*=3)**		**(*N*=3)**	**(*N*=3)**	
MDA	0.79 ± 0.43	1.21 ± 0.51	0.036^{λ}	0.85 ± 0.69	0.67 ± 0.43	n.s.	0.85 ± 0.69	1.21 ± 0.51	n.s.
Oxidative damage to Proteins	**(*N*=2)**	**(*N*=2)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=2)**	**(*N*=2)**	*P*
Carbonyl proteins	0.12 ± 0.04	0.09 ± 0.02	n.s.	0.15 ± 0.12	0.13 ± 0.07	n.s.	0.21 ± 0.07	0.09 ± 0.02	n.s.
Inflammatory mediators	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*
ChT	46.26 ± 26.74	37.22 ± 16.56	n.s.	47.45 ± 31.36	46.56 ± 27.73	n.s.	47.45 ± 31.36	37.22 ± 16.56	n.s.
	(*N*=3)	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*
YKL-40	19.06 ± 10.95	29.10 ± 10.70	n.s.	24.59 ± 12.35	22.26 ± 7.14	n.s.	24.59 ± 12.35	29.10 ± 10.70	n.s.
Antioxidant defence	**(*N*=2)**	**(*N*=2)**	*P*	**(*N*=2)**	**(*N*=2)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*
GSH	17.94 ± 0.65	16.98 ± 3.24	n.s.	17.07 ± 2.45	16.98 ± 2.43	n.s.	20.20 ± 5.69	23.47± 11.77	n.s.
	(*N*=3)	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*	**(*N*=3)**	**(*N*=3)**	*P*
SH-protein groups	7.41 ± 1.18	9.19 ± 1.54	n.s.	7.56 ± 1.31	7.03 ± 2.72	n.s.	7.56 ± 1.31	9.19 ± 1.54	n.s.

Supplementary table S9. *Continuation*

	PTX_{C7}	PTX_{C8}	*P*
Oxidative damage to Lipids	**(*N*=3)**	**(*N*=3)**	
MDA	0.67 ± 0.43	1.21 ± 0.51	n.s.
Oxidative damage to Proteins	**(*N*=2)**	**(*N*=2)**	*P*
Carbonyl proteins	0.17 ± 0.04	0.09 ± 0.02	n.s.
Inflammatory mediators	**(*N*=3)**	**(*N*=3)**	*P*
ChT	46.56 ± 27.73	37.22 ± 16.56	n.s.
	(*N*=3)	**(*N*=3)**	*P*
YKL-40	22.26 ± 7.14	29.10 ± 10.70	n.s.
Antioxidant defence	**(*N*=2)**	**(*N*=2)**	*P*
GSH	16.98 ± 2.43	16.81 ± 3.24	n.s.
	(*N*=3)	**(*N*=3)**	*P*
SH-protein groups	7.03 ± 2.72	9.19 ± 1.54	n.s.

Values are expressed as mean ± SD

$^{\Psi}$ Wilcoxon rank-sum test, $^{\gamma}$ Student's t test, $^{\delta}$ Wilcoxon signed-rank test and $^{\lambda}$ Student's t paired test were used to assess differences between groups.

P value of ≤ 0.05 was considered significant.

Supplementary table S10. Levels of protein damage, lipid damage, inflammation and antioxidant defence in PABC patients prior chemotherapy administration, before and after treatment with anthracyclines and paclitaxel and in labour compared to control group.

	Controls	PreCTX	*P*	Controls	PreAnC	*P*	Controls	PostAnC	*P*
Oxidative damage to Lipids	**(*N*=8)**	**(*N*=8)**		**(*N*=8)**	**(*N*=12)**		**(*N*=8)**	**(*N*=9)**	
MDA	0.66 ± 0.31	0.75 ± 0.40	n.s.	0.66 ± 0.31	0.77 ± 0.42	n.s.	0.66 ± 0.31	0.59 ± 0.24	n.s.
Oxidative damage to Proteins	**(*N*=9)**	**(*N*=15)**	*P*	**(*N*=9)**	**(*N*=15)**	*P*	**(*N*=9)**	**(*N*=15)**	*P*
Carbonyl proteins	0.14 ± 0.12	0.21 ± 0.20	n.s.	0.14 ± 0.12	0.21 ± 0.20	n.s.	0.14 ± 0.12	0.11 ± 0.08	n.s.
Inflammatory mediators	**(*N*=11)**	**(*N*=14)**	*P*	**(*N*=11)**	**(*N*=14)**	*P*	**(*N*=11)**	**(*N*=14)**	*P*
ChT	33.18 ± 8.94	52.17 ± 19.48	0.004$^{\gamma}$	33.18 ± 8.94	50.94 ± 19.20	0.006$^{\gamma}$	33.18 ± 8.94	51.28 ± 22.92	0.041$^{\gamma}$
	(*N*=11)	**(*N*=15)**	*P*	**(*N*=11)**	**(*N*=15)**	*P*	**(*N*=11)**	**(*N*=15)**	*P*
YKL-40	25.17 ± 11.67	34.02 ± 20.51	n.s.	25.17 ± 11.67	33.66 ± 20.73	n.s.	25.17 ± 11.67	26.00 ± 11.73	n.s.
Antioxidant defence	**(*N*=10)**	**(*N*=12)**	*P*	**(*N*=10)**	**(*N*=12)**	*P*	**(*N*=10)**	**(*N*=14)**	*P*
GSH	25.11 ± 12.29	21.52 ± 9.06	n.s.	25.11 ± 12.29	20.94 ± 9.28	n.s.	25.11 ± 12.29	32.61 ± 17.77	n.s.
	(*N*=10)	**(*N*=17)**	*P*	**(*N*=10)**	**(*N*=17)**	*P*	**(*N*=10)**	**(*N*=15)**	*P*
SH-protein groups	9.05 ± 3.33	8.06 ± 2.99	n.s.	9.05 ± 3.33	8.04 ± 2.99	n.s.	9.05 ± 3.33	6.67 ± 1.27	0.005$^{\Psi}$

Supplementary table S10. *Continuation*

	Controls	PrePTX		Controls	PostPTX		Controls	Labour	
Oxidative damage to Lipids	**(*N*=8)**	**(*N*=9)**	***P***	**(*N*=8)**	**(*N*=6)**	***P***	**(*N*=8)**	**(*N*=9)**	***P***
MDA	0.66 ± 0.31	0.69 ± 0.33	n.s.	0.66 ± 0.31	0.86 ± 0.38	n.s.	0.66 ± 0.31	0.62 ± 0.28	n.s.
Oxidative damage to Proteins	**(*N*=9)**	**(*N*=5)**	***P***	**(*N*=9)**	**(*N*=6)**	***P***	**(*N*=9)**	**(*N*=16)**	***P***
Carbonyl proteins	0.14 ± 0.12	0.09 ± 0.06	n.s.	0.14 ± 0.12	0.06 ± 0.05	n.s.	0.14 ± 0.12	0.14 ± 0.18	n.s.
Inflammatory mediators	**(*N*=11)**	**(*N*=6)**	***P***	**(*N*=11)**	**(*N*=6)**	***P***	**(*N*=11)**	**(*N*=14)**	***P***
ChT	33.18 ± 8.94	57.76 ± 21.98	0.039$^{\gamma}$	33.18 ± 8.94	34.57 ± 9.65	n.s.	33.18 ± 8.94	43.46 ± 23.26	n.s.
	(*N*=11)	**(*N*=6)**	***P***	**(*N*=11)**	**(*N*=6)**	***P***	**(*N*=11)**	**(*N*=15)**	***P***
YKL-40	25.17 ± 11.67	25.85 ± 8.18	n.s.	25.17 ± 11.67	29.67 ± 10.13	n.s.	25.17 ± 11.67	27.09 ± 13.54	n.s.
Antioxidant defence	**(*N*=10)**	**(*N*=5)**	***P***	**(*N*=10)**	**(*N*=6)**	***P***	**(*N*=10)**	**(*N*=15)**	***P***
GSH	25.11 ± 12.29	28.01 ± 21.70	n.s.	25.11 ± 12.29	19.03 ± 4.76	0.022$^{\gamma}$	25.11 ± 12.29	26.62 ± 11.69	n.s.
	(*N*=10)	**(*N*=6)**	***P***	**(*N*=10)**	**(*N*=6)**	***P***	**(*N*=10)**	**(*N*=15)**	***P***
SH-protein groups	9.05 ± 3.33	6.10 ± 1.20	0.007$^{\Psi}$	9.05 ± 3.33	8.59 ± 1.46	n.s.	9.05 ± 3.33	7.76 ± 2.46	n.s.

Values are expressed as mean ± SD

$^{\Psi}$ Wilcoxon rank-sum test, $^{\gamma}$ Student's t test, $^{\delta}$ Wilcoxon signed-rank test and $^{\lambda}$ Student's t paired test were used to assess differences between groups.

P value of ≤ 0.05 was considered significant.

Supplementary table S11. Levels of protein damage, lipid damage, inflammation and antioxidant defence in PABC patients in each cycle of anthracyclines-based regimens in comparison with control group.

	Controls	AnC_{C1}	*P*	Controls	AnC_{C2}	*P*	Controls	AnC_{C3}	*P*
Oxidative damage to Lipids	**(*N*=8)**	**(*N*=16)**		**(*N*=8)**	**(*N*=16)**		**(*N*=8)**	**(*N*=12)**	
MDA	0.66 ± 0.31	0.64 ± 0.41	n.s.	0.66 ± 0.31	0.58 ± 0.25	n.s.	0.66 ± 0.31	0.71 ± 0.42	n.s.
Oxidative damage to Proteins	**(*N*=9)**	**(*N*=15)**	***P***	**(*N*=9)**	**(*N*=17)**	***P***	**(*N*=9)**	**(*N*=12)**	***P***
Carbonyl proteins	0.14 ± 0.12	0.14 ± 0.11	n.s.	0.14 ± 0.12	0.13 ± 0.10	n.s.	0.14 ± 0.12	0.11 ± 0.04	n.s.
Inflammatory mediators	**(*N*=11)**	**(*N*=14)**	***P***	**(*N*=11)**	**(*N*=14)**	***P***	**(*N*=11)**	**(*N*=11)**	***P***
ChT	33.18 ± 8.94	62.35 ± 25.74	0.001[γ]	33.18 ± 8.94	56.66 ± 27.90	0.009[γ]	33.18 ± 8.94	56.80 ± 30.36	0.034[γ]
	(*N*=11)	**(*N*=16)**	***P***	**(*N*=16)**	**(*N*=15)**	***P***	**(*N*=12)**	**(*N*=11)**	***P***
YKL-40	25.17 ± 11.67	31.51 ± 23.48	n.s.	25.17 ± 11.67	24.96 ± 11.60	n.s.	25.17 ± 11.67	27.82 ± 13.82	n.s.
Antioxidant defence	**(*N*=10)**	**(*N*=14)**	***P***	**(*N*=10)**	**(*N*=13)**	***P***	**(*N*=10)**	**(*N*=10)**	***P***
GSH	25.11 ± 12.29	29.85 ± 21.81	n.s.	25.11 ± 12.29	30.07 ± 16.95	n.s.	25.11 ± 12.29	44.08 ± 25.65	n.s.
	(*N*=10)	**(*N*=17)**	***P***	**(*N*=10)**	**(*N*=17)**	***P***	**(*N*=10)**	**(*N*=12)**	***P***
SH-protein groups	9.05 ± 3.33	8.46 ± 4.71	n.s.	9.05 ± 3.33	6.94 ± 1.80	n.s.	9.05 ± 3.33	6.94 ± 1.39	n.s.

Supplementary table S11. *Continuation*

	Controls	AnC_{C4}	*P*
Oxidative damage to Lipids	**(*N*=8)**	**(*N*=9)**	
MDA	0.66 ± 0.31	0.59 ± 0.30	n.s.
Oxidative damage to Proteins	**(*N*=9)**	**(*N*=9)**	***P***
Carbonyl proteins	0.14 ± 0.12	0.18 ± 0.23	n.s.
Inflammatory mediators	**(*N*=11)**	**(*N*=9)**	***P***
ChT	33.18 ± 8.94	57.12 ± 20.47	n.s.
	(*N*=11)	**(*N*=9)**	***P***
YKL-40	25.17 ± 11.67	26.30 ± 12.57	n.s.
Antioxidant defence	**(*N*=10)**	**(*N*=9)**	***P***
GSH	25.11 ± 12.29	28.87 ± 16.07	n.s.
	(*N*=10)	**(*N*=9)**	***P***
SH-protein groups	9.05 ± 3.33	6.46 ± 1.30	0.006^{Ψ}

Values are expressed as mean ± SD

$^{\Psi}$ Wilcoxon rank-sum test, $^{\gamma}$ Student's t test, $^{\delta}$ Wilcoxon signed-rank test and $^{\lambda}$ Student's t paired test were used to assess differences between groups.

P value of ≤ 0.05 was considered significant.

Supplementary table S12. Levels of protein damage, lipid damage, inflammation and antioxidant defence in PABC patients in each cycle of paclitaxel compared to control group

	Controls	PTX_{C1}	*P*	Controls	PTX_{C2}	*P*	Controls	PTX_{C3}	*P*
Oxidative damage to Lipids	(*N*=8)	(*N*=6)		(*N*=8)	(*N*=5)		(*N*=8)	(*N*=4)	
MDA	0.66 ± 0.31	0.65 ± 0.35	n.s.	0.66 ± 0.31	0.65 ± 0.23	n.s.	0.66 ± 0.31	0.51 ± 0.22	n.s.
Oxidative damage to Proteins	(*N*=9)	(*N*=5)	*P*	(*N*=9)	(*N*=4)	*P*	(*N*=9)	(*N*=4)	*P*
Carbonyl proteins	0.14 ± 0.12	0.08 ± 0.05	n.s.	0.14 ± 0.12	0.12 ± 0.13	n.s.	0.14 ± 0.12	0.06 ± 0.06	n.s.
Inflammatory mediators	(*N*=11)	(*N*=6)	*P*	(*N*=11)	(*N*=5)	*P*	(*N*=11)	(*N*=4)	*P*
ChT	33.18 ± 8.94	45.10 ± 16.37	n.s.	33.18 ± 8.94	39.09 ± 14.52	n.s.	33.18 ± 8.94	41.18 ± 19.55	n.s.
	(*N*=11)	(*N*=6)	*P*	(*N*=16)	(*N*=5)	*P*	(*N*=12)	(*N*=4)	*P*
YKL-40	25.17 ± 11.67	22.84 ± 11.51	n.s.	25.17 ± 11.67	22.51 ± 7.89	n.s.	25.17 ± 11.67	20.89 ± 7.29	n.s.
Antioxidant defence	(*N*=10)	(*N*=6)	*P*	(*N*=10)	(*N*=4)	*P*	(*N*=10)	(*N*=4)	*P*
GSH	25.11 ± 12.29	45.74 ± 46.58	n.s.	25.11 ± 12.29	36.22 ± 23.29	n.s.	25.11 ± 12.29	31.75 ± 25.47	n.s.
	(*N*=10)	(*N*=6)	*P*	(*N*=10)	(*N*=4)	*P*	(*N*=10)	(*N*=5)	*P*
SH-protein groups	9.05 ± 3.33	7.44 ± 1.92	n.s.	9.05 ± 3.33	8.10 ± 1.38	n.s.	9.05 ± 3.33	8.20 ± 0.65	n.s.

Supplementary table S12. *Continuation*

	Controls	PTX_{C4}	*P*	Controls	PTX_{C5}	*P*	Controls	PTX_{C6}	*P*
Oxidative damage to Lipids	**(*N*=8)**	**(*N*=5)**		**(*N*=8)**	**(*N*=4)**		**(*N*=8)**	**(*N*=3)**	
MDA	0.66 ± 0.31	0.74 ± 0.31	n.s.	0.66 ± 0.31	0.76 ± 0.36	n.s.	0.66 ± 0.31	0.85 ± 0.69	n.s.
Oxidative damage to Proteins	**(*N*=9)**	**(*N*=5)**	*P*	**(*N*=9)**	**(*N*=3)**	*P*	**(*N*=9)**	**(*N*=3)**	*P*
Carbonyl proteins	0.14 ± 0.12	0.10 ± 0.07	n.s.	0.14 ± 0.12	0.10 ± 0.04	n.s.	0.14 ± 0.12	0.15 ± 0.12	n.s.
Inflammatory mediators	**(*N*=11)**	**(*N*=5)**	*P*	**(*N*=11)**	**(*N*=4)**	*P*	**(*N*=11)**	**(*N*=3)**	*P*
ChT	33.18 ± 8.94	42.57 ± 17.78	n.s.	33.18 ± 8.94	44.27 ± 22.19	n.s.	33.18 ± 8.94	47.45 ± 31.36	n.s.
	(*N*=11)	**(*N*=5)**	*P*	**(*N*=11)**	**(*N*=4)**	*P*	**(*N*=16)**	**(*N*=3)**	*P*
YKL-40	25.17 ± 11.67	22.87 ± 12.29	n.s.	25.17 ± 11.67	19.28 ± 8.95	n.s.	25.17 ± 11.67	24.59 ± 12.35	n.s.
Antioxidant defence	**(*N*=10)**	**(*N*=5)**	*P*	**(*N*=10)**	**(*N*=3)**	*P*	**(*N*=10)**	**(*N*=3)**	*P*
GSH	25.11 ± 12.29	23.93 ± 13.79	n.s.	25.11 ± 12.29	21.19 ± 5.65	n.s.	25.11 ± 12.29	20.20 ± 5.69	n.s.
	(*N*=10)	**(*N*=5)**	*P*	**(*N*=10)**	**(*N*=4)**	*P*	**(*N*=10)**	**(*N*=3)**	*P*
SH-protein groups	9.05 ± 3.33	9.05 ± 2.02	n.s.	9.05 ± 3.33	7.79 ± 1.23	n.s.	9.05 ± 3.33	7.56 ± 1.31	n.s.

Supplementary table S12. *Continuation*

	Controls	PTX_{C7}	*P*	Controls	PTX_{C8}	*P*
Oxidative damage to Lipids	(*N*=8)	(*N*=3)		(*N*=8)	(*N*=3)	
MDA	0.66 ± 0.31	0.67 ± 0.43	n.s.	0.66 ± 0.31	1.21 ± 0.51	n.s.
Oxidative damage to Proteins	(*N*=9)	(*N*=2)	*P*	–	–	*P*
Carbonyl proteins	0.14 ± 0.12	0.13 ± 0.07	n.s.	–	–	–
Inflammatory mediators	(*N*=11)	(*N*=3)	*P*	(*N*=11)	(*N*=3)	*P*
ChT	33.18 ± 8.94	46.56 ± 27.73	n.s.	33.18 ± 8.94	37.22 ± 16.56	n.s.
	(*N*=12)	(*N*=3)	*P*	(*N*=11)	(*N*=3)	*P*
YKL-40	25.17 ± 11.67	22.26 ± 7.14	n.s.	25.17 ± 11.67	29.10 ± 10.70	n.s.
Antioxidant defence	–	–	*P*	(*N*=10)	(*N*=3)	*P*
GSH	–	–	–	25.11 ± 12.29	23.47 ± 11.77	n.s.
	(*N*=10)	(*N*=3)	*P*	(*N*=10)	(*N*=3)	*P*
SH-protein groups	9.05 ± 3.33	7.03 ± 2.72	n.s.	9.05 ± 3.33	9.19 ± 1.54	n.s.

Values are expressed as mean ± SD

$^{\Psi}$ Wilcoxon rank-sum test, $^{\gamma}$ Student's t test, $^{\delta}$ Wilcoxon signed-rank test and $^{\lambda}$ Student's t paired test were used to assess differences between groups.

P value of ≤ 0.05 was considered significant.

Supplementary table S13. Main descriptors of the distribution of ratios of the DNA damage, protein damage and antioxidant defence biomarkers in plasma samples measured by the first method.

ANALYTE	RANGE	MEDIAN	IQR	MEAN ± SD	>LOQ (%)
8-OHdG/2dG	0.57-1.94	0.93	0.28	0.94 ± 0.24	100
o-Tyr/Phe	0.53-1.47	0.84	0.18	0.87 ± 0.16	100
m-Tyr/Phe	0.57-1.65	0.82	0.26	0.91 ± 0.21	94
3Cl/p-Tyr	0.63-1.35	0.80	0.14	1.04 ± 0.17	38
3NO_2-Tyr/p-Tyr	0.54-1.76	0.83	0.16	0.87 ± 0.20	96
GSA	–	–	–	–	–
GSH/GSSG	0.64-1.82	1.00	0.16	0.99 ± 0.18	93
CYS/CYSS	–	–	–	–	–

Metabolites concentrations detected above the LOQ in ≥ 60% of samples, were accepted

Supplementary table S14. Main descriptors of the distribution of concentration [nM] of the lipid peroxidation biomarkers in plasma samples measured by the second method

ANALYTE	RANGE	MEDIAN	IQR	MEAN ± SD	>LOQ (%)
5-F_{2t}-IsoP + 5-*epi*-5-F_{2t}-IsoP	0.24-296.34	2.61	2.48	8.00 ± 35.69	100
15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11_-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$ -IsoP	0.48-1.14	0.58	0.12	0.61 ± 0.14	96
15-F_{2t}-IsoP	0.64-12.54	1.03	0.58	1.45 ± 1.80	97
15-*epi*-15-F_{2t}-IsoP	0.54-15.84	0.96	0.7	1.51 ± 2.11	99
4-F_{4t}-NeuroP + 4-epi-4-F_{4t}-NeuroP	2.1-59.05	3.7	3.9	6.76 ± 0.69	97
10-*epi*-10-F_{4t}-NeuroP	1.27-16.13	1.74	0.71	2.40 ± 2.49	99
10-F_{4t}-NeuroP	0.52-9.06	0.94	0.3	1.16 ± 1.23	99
14(RS)-14-F_{4t}-NeuroP	3.54-21.78	3.72	0.5	4.88 ± 3.77	44
4(RS)-ST-Δ^5-8-NeuroF	–	–	–	–	–
17*(RS)*-F_{2t}-dihomo-IsoP + 17-*epi*-17-F_{2t}-dihomo-IsoP	1.04-8.82	1.65	1.18	2.26 ± 1.64	59
ent-7(RS)-F_{2t}-dihomo-IsoP	2.04-10.98	2.17	0.55	2.81 ± 1.75	57
17(RS)-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF	4.02-15.36	4.50	1.03	5.22 ± 2.07	75
7*(RS)*-ST- Δ^{18}-11-dihomo-IsoF	4.74-52.08	6.03	1.71	7.61 ± 7.22	62
1a,1b-dihomo $PGF_{2\alpha}$	0.60-57.85	6.03	5.67	8.14 ± 10.11	93
11ß-$PGF_{2\alpha}$	0.36-1.88	0.48	0.18	0.55 ± 0.25	68
6-keto-$PGF_{1\alpha}$	0.60-1.38	0.66	0.03	0.68 ± 0.13	52
$PGF_{2\alpha}$	1.06-63.3	5.04	3.31	5.87 ± 7.64	94

Metabolites concentrations detected above the LOQ in ≥ 60% of samples, were accepted

Supplementary table S15. Main descriptors of the distribution of ratios of the DNA damage, protein damage and antioxidant defense biomarkers in plasma and RBCs samples measured by the first method.

ANALYTE	RANGE		MEDIAN		IQR		MEAN ± SD		>LOQ (%)	
	PLASMA	RBC	PLASMA	RBC	PLASMA	RBC	PLASMA	RBC	PLASMA	RBC
8-OHdG/2dG	0.57-1.29	–	0.72	–	0.19	–	0.76± 0.16	–	100	–
o-Tyr/Phe	0.71-1.47	–	0.90	–	0.26	–	0.91 ± 0.13	–	100	–
m-Tyr/Phe	0.55-1.03	–	0.76	–	0.17	–	0.77± 0.12	–	100	–
3Cl/p-Tyr	0.70-1.10	–	0.83	–	0.09	–	0.85± 0.11	–	33	–
3NO$_2$-Tyr/p-Tyr	0.56-1.10	–	0.69	–	0.12	–	0.71± 0.11	–	100	–
GSA	–	–	–	–	–	–	–	–	–	–
GSH/GSSG	0.72-1.62	0.86-1.90	1.04	1.24	0.04	0.33	1.06 ± 0.15	1.28 ± 0.25	81	100
CyS/CySS	–	–	–	–	–	–	–	–	–	–

Metabolites concentrations detected above the LOQ in ≥ 60% of samples, were accepted

Supplementary Table S16. Main descriptors of the distribution of DNA damage and antioxidant defense biomarkers and concentration [nM] of the inflammatory mediator in urine samples measured by the first method.

ANALYTE	RANGE	MEDIAN	IQR	MEAN ± SD	>LOQ (%)
8-OHdG/2dG	0.74-1.6	0.98	0.11	1.01 ± 0.20	91
o-Tyr/Phe	–	–	–	–	–
m-Tyr/Phe	–	–	–	–	–
3Cl/p-Tyr	–	–	–	–	–
$3NO_2$-Tyr/p-Tyr	–	–	–	–	–
GSA	1.00-2.00	1.44	0.33	1.44-0.31	100
GSH/GSSG	–	–	–	–	–
CyS/CySS	0.66-2.00	0.97	0.41	0.96 ± 0.39	100

Metabolites concentrations detected above the LOQ in ≥ 60% of samples, were accepted

Supplementary table S17. Main descriptors of the distribution of concentration [nM] of the lipid peroxidation biomarkers in plasma samples measured by the second method.

ANALYTE	RANGE	MEDIAN	IQR (25-75)	MEAN ± SD	>LOQ (%)
5-F_{2t}-IsoP + 5-*epi*-5-F_{2t}-IsoP	0.72-8.52	4.02	2.16	3.66 ± 1.77	100.
15-*epi*-2,3-dinor-15-F_{2t}-IsoP + 2,3-dinor-11_-PG$F_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$ –IsoP	0.49-0.84	0.66	0.16	0.66 ± 0.10	100
15-F_{2t}-IsoP	0.64-4.57	0.98	0.645	1.31 ± 0.83	99
15-epi-15-F_{2t}-IsoP	0.77-4.62	1.62	1.00	1.82 ± 0.33	100
4-F_{4t}-NeuroP + 4-*epi*-4-F_{4t}-NeuroP	2.16-39.93	3.18	1.84	5.28 ± 7.21	100
10-*epi*-10-F_{4t}-NeuroP	1.30-5.23	1.62	0.24	1.77 ± 0.72	100
10-F_{4t}-NeuroP	0.46-1.55	1.02	0.26	0.96 ± 0.27	100
14(RS)-14-F_{4t}-NeuroP	3.60-5.43	4.02	0.78	4.15 ± 0.57	41
4(RS)-ST-Δ^{5}-8-NeuroF	–	–	–	–	–
17*(RS)*-F_{2t}-dihomo-IsoP + 17-*epi*-17-F_{2t}-dihomo-IsoP	1.02-7.20	2.1	1.20	2.54 ± 1.50	85
ent-7(RS)-F_{2t}-dihomo-IsoP	2.04-9.10	2.22	0.27	2.65 ± 1.55	74
17(RS)-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF	4.02-25.03	4.74	1.58	6.15 ± 4.81	67
7*(RS)*-ST- Δ^{18}-11-dihomo-IsoF	4.62-5.89	4.99	0.33	5.07 ± 0.33	60
1a,1b-dihomo PG$F_{2\alpha}$	0.66-48.41	10.68	8.68	11.77 ± 9.26	96
11ß-PG$F_{2\alpha}$	0.36-1.32	0.6	0.32	0.65 ± 0.24	81
6-keto-PG$F_{1\alpha}$	0.60-98.34	1.02	1.14	5.94 ± 19.82	89
PG$F_{2\alpha}$	1.57-10.68	6.06	2.81	5.99 ± 2.33	100

Metabolites concentrations detected above the LOQ in ≥ 60% of samples, were accepted

Supplementary table S18. Main descriptors of the distribution of concentration [nM] of the lipid peroxidation biomarkers in urine samples measured by the second method.

ANALYTE	RANGE	MEDIAN	IQR (25-75)	MEAN ± SD	>LOQ (%)
5-F$_{2t}$-IsoP + 5-*epi*-5-F$_{2t}$-IsoP	1.31-17.79	3.962	6.42	5.81 ± 5.08	100
15-*epi*-2,3-dinor-15-F$_{2t}$-IsoP + 2,3-dinor-11_-PGF$_{2\alpha}$ + 2,3-dinor-15-F$_{2\alpha}$ -IsoP	6.81-100.9	28.42	28.81	35.65 ± 30.98	100
15-F$_{2t}$-IsoP	1.72-11.32	2.80	4.33	4.50 ± 3.52	100
15-*epi*-15-F$_{2t}$-IsoP	2.88-37.02	7.47	6.24	10.28 ± 9.58	100
15-E$_{2t}$-IsoP	3.13-24.72	4.44	2.52	6.70 ± 6.20	100
15-keto-15-F$_{2t}$-IsoP	2.56-21.99	7.47	5.64	8.56 ± 5.88	100
15-keto-15-E$_{2t}$-IsoP	0.73-11.11	5.09	3.64	5.03 ± 2.90	100
4-F$_{4t}$-NeuroP + 4-*epi*-4-F$_{4t}$-NeuroP	3.30-17.08	7.44	4.65	7.41 ± 4.06	100
10-*epi*-10-F$_{4t}$-NeuroP	0.42-4.79	1.37	1.87	2.20 ± 1.47	100
10-F$_{4t}$-NeuroP	0.34-4.15	1.28	1.94	1.86 ± 1.32	100
14(RS)-14-F$_{4t}$-NeuroP	2.19-8.48	6.23	3.00	6.02 ± 2.03	100
4(RS)-ST-Δ^{5}-8-NeuroF	115.789-234.098	197.22	38.20	189.25 ± 45.86	45
17*(RS)*-F$_{2t}$-dihomo-IsoP + 17-epi-17-F$_{2t}$-dihomo-IsoP	3.77-20.49	8.47	6.13	10.02 ± 5.72	82
ent-7(RS)-F$_{2t}$-dihomo-IsoP	0.66-9.99	3.22	4.68	4.33 ± 3.33	73
17(RS)-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF	4.58-89.07	19.05	17.36	34.15 ± 31.52	82
7*(RS)*-ST- Δ^{18}-11-dihomo-IsoF	1.45-110.54	19.46	23.91	27.28 ± 30.96	100
1a,1b-dihomo PGF$_{2\alpha}$	5.29-40.38	11.64	17.00	17.05 ± 12.14	100
11ß-PGF$_{2\alpha}$	1.41-10.94	3.73	3.60	4.51 ± 3.35	91
6-keto-PGF$_{1\alpha}$	1.99-27.55	2.78	1.28	3.09 ± 1.09	73
PGF$_{2\alpha}$	5.32-57.44	17.66	15.13	19.22 ± 15.52	100
PGE$_{2}$	2.06-135.65	4.46	10.56	28.07 ± 48.89	100

Metabolites concentrations detected above the LOQ in ≥ 60% of samples, were accepted

2. Results of the statistical analyses of the supplementary study

Blood samples were obtained from PABC patients in different stages of the treatment to compare their own accumulative effect on oxidative stress, inflammation and antioxidant defence status as a consequence of chemotherapy administration. The comparative PABC groups were: before chemotherapy (preCTX), before anthracyclines (preAnC), after X cycles of anthracyclines (AnC_{xc}), after anthracyclines (postAnC), after X cycles of paclitaxel (PTX_{xc}), after paclitaxel (postPTX) and at birth (labour). Besides, they were compared to blood samples from healthy pregnant women (controls).

Shapiro-Wilk test was used to examine whether the biomarkers were normally distributed in all groups of study. After testing the normality, student's t paired test (parametric test) and Wilcoxon signed-rank test (non-parametric test) were used to compare PABC patients at different moments of the study. Following the same premise of normal distribution, student t-test was employed once it was accepted whilst Wilcoxon rank-sum test was used when it was not. These tests were used when we compared PABC patient with control group.

The concentrations of the biomarkers analysed in all the comparative groups of the study, including the statistical test employed are enlisted from **Suppl. table S5** to **Suppl. table S12**.

Cluster analysis of significant biomarkers

Heatmap of statistically significant biomarkers of oxidative stress, inflammation and antioxidant defence, which show the concentration changes among the groups studied, is illustrated in **Suppl. Figure S1**.

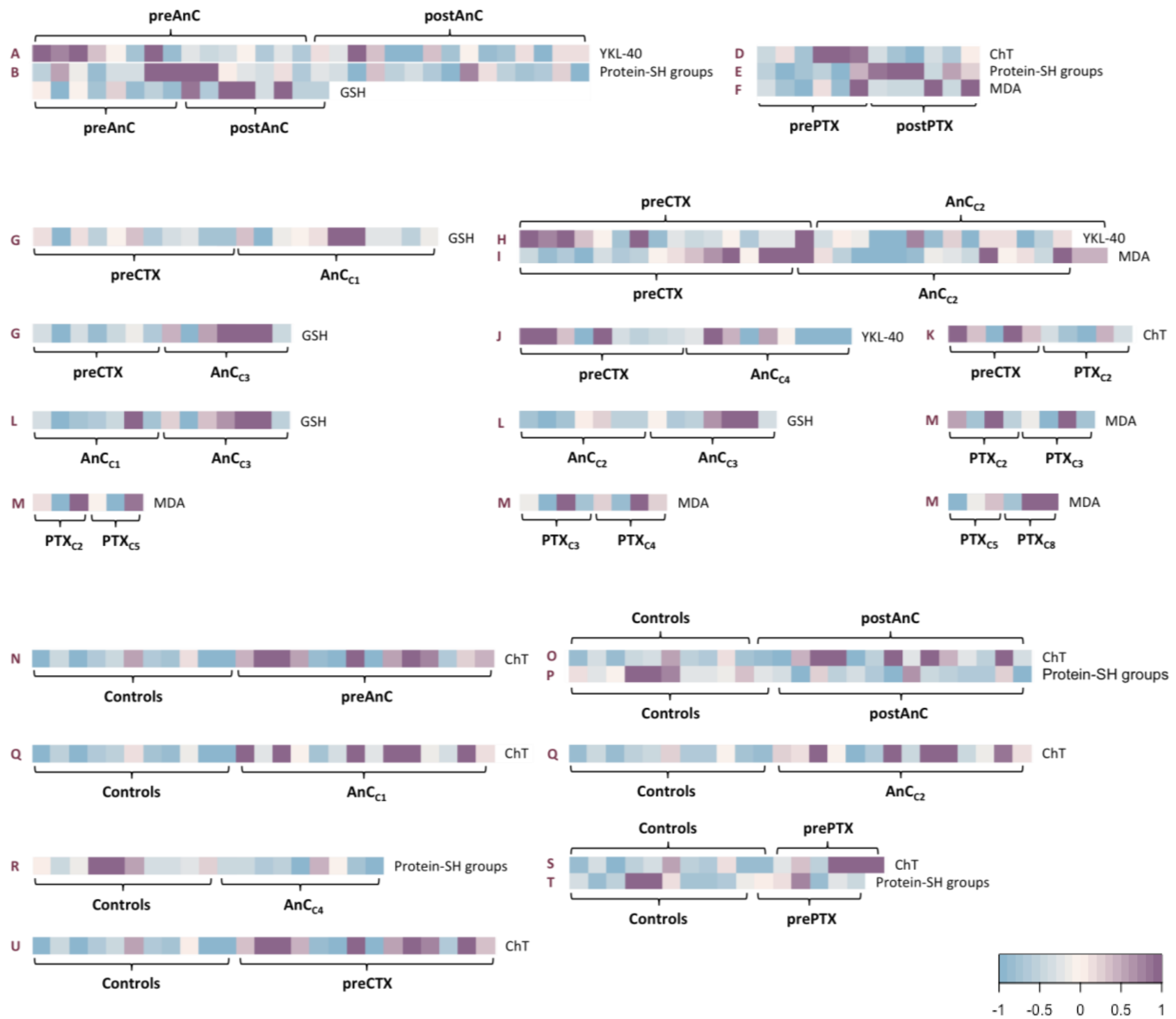
Decrease levels of inflammatory mediators **(A)** were observed after the administration of anthracycline-based regimens in PABC. Likewise, the levels of one biomarker of antioxidant defence analysed **(B)** were also found decreased whereas the remaining biomarker showed increase levels after treatment with anthracyclines **(C).** On the other hand**,** the levels of inflammatory mediator **(D)** were decreased in plasma following

paclitaxel although the levels of antioxidant defence **(E)** and lipid peroxidation **(F)** biomarkers were the opposite.

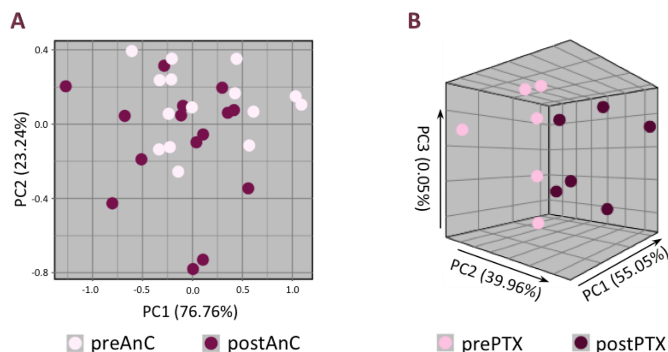
In fact, the levels of antioxidant defence **(G)** were increased in certain cycles of anthracyclines compared to the beginning of chemotherapy treatment although they were decreased in biomarkers of inflammation **(H)** and lipid peroxidation **(I)**. Additionally, a different inflammatory mediator was also found decreased in a specific cycle of paclitaxel respect to chemotherapy first administration **(J)**.

When comparing the effect of chemotherapy treatment along pregnancy in PABC patients, we found differences among cycles of anthracyclines and paclitaxel administered. Higher levels of antioxidant defence biomarker were observed among particular cycles of anthracyclines **(K)**. Opposite, the levels of lipid peroxidation were elevated among different cycles of paclitaxel despite being firstly decreased during the beginning of paclitaxel treatment **(L** and **M r**espectively**)**.
Raised levels of inflammation were detected before **(N)** and after **(O)** anthracyclines treatment compared to control group. Moreover, the biomarker of inflammation was more elevated in PABC patients before paclitaxel treatment than control group **(Q)**. However, the comparison between the levels of antioxidant defence measured in PABC following anthracyclines therapy and control group showed opposite results **(P)**.
Increase of inflammatory biomarker in different cycles of anthracyclines **(S)** administration was observed in PABC patients in comparison with control group. Nevertheless, the levels of antioxidant defence biomarker were decreased in certain cycles **(T)** of anthracyclines respect to control group resulting to low levels prior to paclitaxel initiation **(R)**.
Decrease levels of inflammatory mediator were also detected in control group compared to PABC patients before starting chemotherapy treatment **(U)**.

Significant differences observed in those comparative groups with enough data available to be further evaluated by PCA were illustrated in **Suppl. Figure S2**.
PABC patients exhibited dissimilar metabolic profile prior the first cycle of anthracyclines **(A)** or paclitaxel **(B)** in comparison with the end of both therapies.



Supplementary Figure S1. **Heatmaps illustrating the clustering of all differentiating biomarkers of oxidative stress, inflammation and antioxidant defence analysed across the different comparative groups.** Biomarkers of inflammation (**A, D, H, J, K, N, O, Q, S and U**), antioxidant defence **(B, C, E, G, L, P, R and T)** and lipid peroxidation **(F, I and M)**. The columns indicate the individual patients and the rows represent statistically significant biomarkers. The burgundy colour shows the trend of rise and the blue colour shows a decreasing trend.



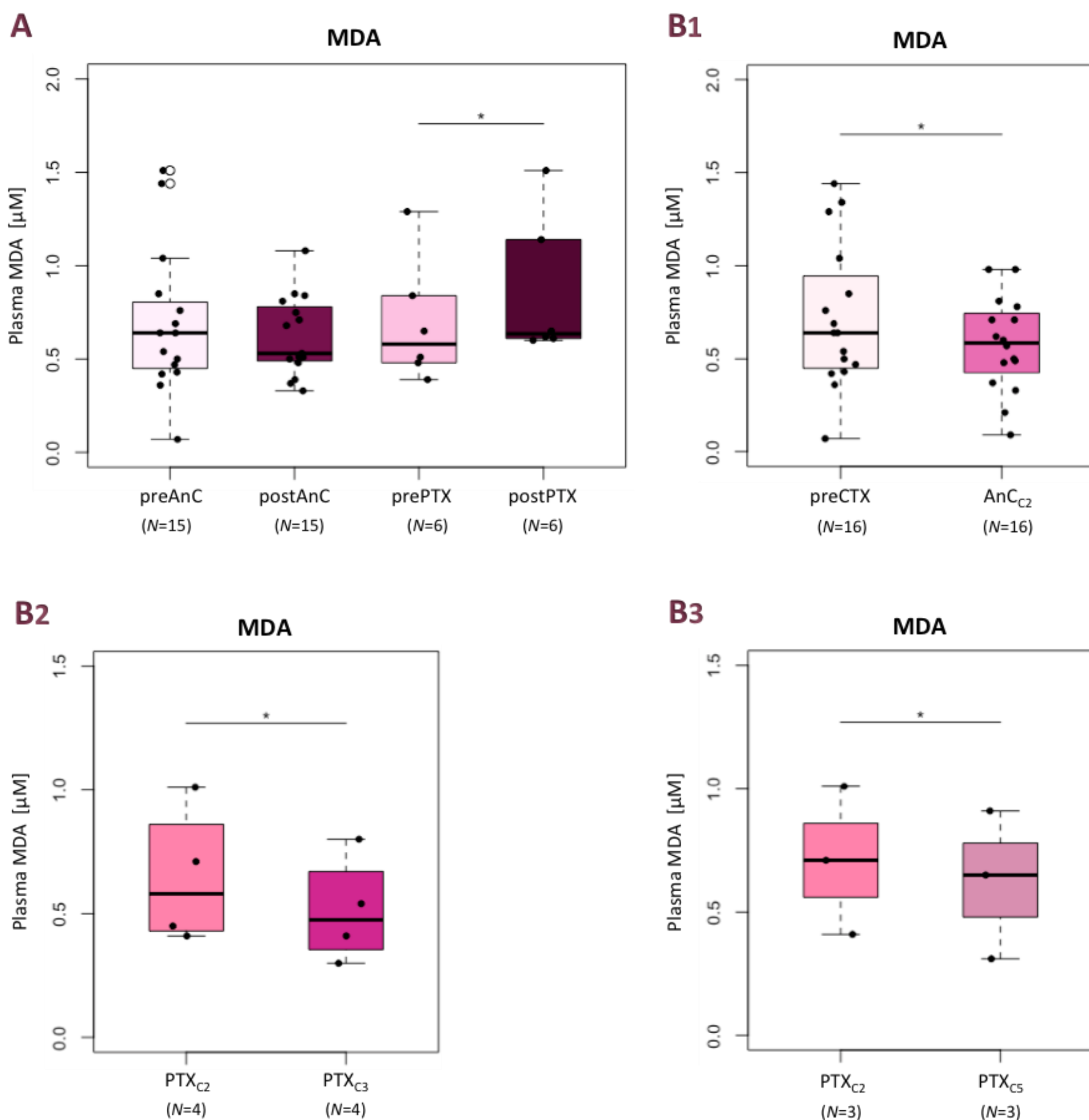
Supplementary Figure S2. **Plots of principal component analysis (PCA) were used to explain the statistically significant metabolic differences among groups. (A)** Score plot of plasma samples obtained from prePTX group and postPTX group. PC1, PC2 and PC3 account for 95.06% of the data's variance. **(B)** Score plot of plasma samples obtained from preAnC group and postAnC. PC1, and PC2 account for 100% of the data's variance. Coloured circles represent the samples of each group.

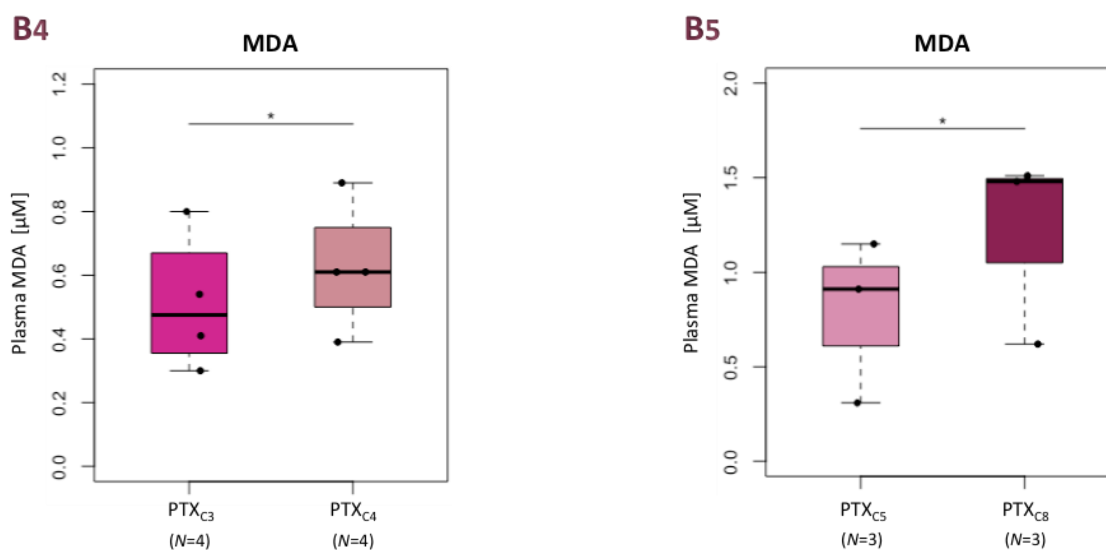
Abbreviation. PC1, principal component 1; PC2, principal component 2 and PC3, principal component 3.

Comparative study of biomarkers levels between tested groups

Oxidative damage to lipids

Results showed that MDA levels measured in plasma were significantly incremented during paclitaxel treatment in PABC patients (*P* value= 0.031) but were not during anthracyclines treatment **(Suppl. Figure S3.A)**. However, as shown in **Suppl. Figure S3.B1**, MDA levels measured in cycle two of anthracyclines were reduced compared to prior chemotherapy initiation (*P* value= 0.034). Likewise, MDA levels detected in cycle three and five of paclitaxel were decreased respect to cycle two of paclitaxel (*P* value= 0.009; *P* value= 0.021) (**Suppl. fig S3.B; Suppl. Figure S3.B3)**. Nevertheless, the levels of MDA were incremented as PTX treatment went on as illustrated in **Suppl. Figure S3.B4** and **Suppl. Figure S3.B5** (*P* value= 0.036 and *P* value= 0.036, respectively).





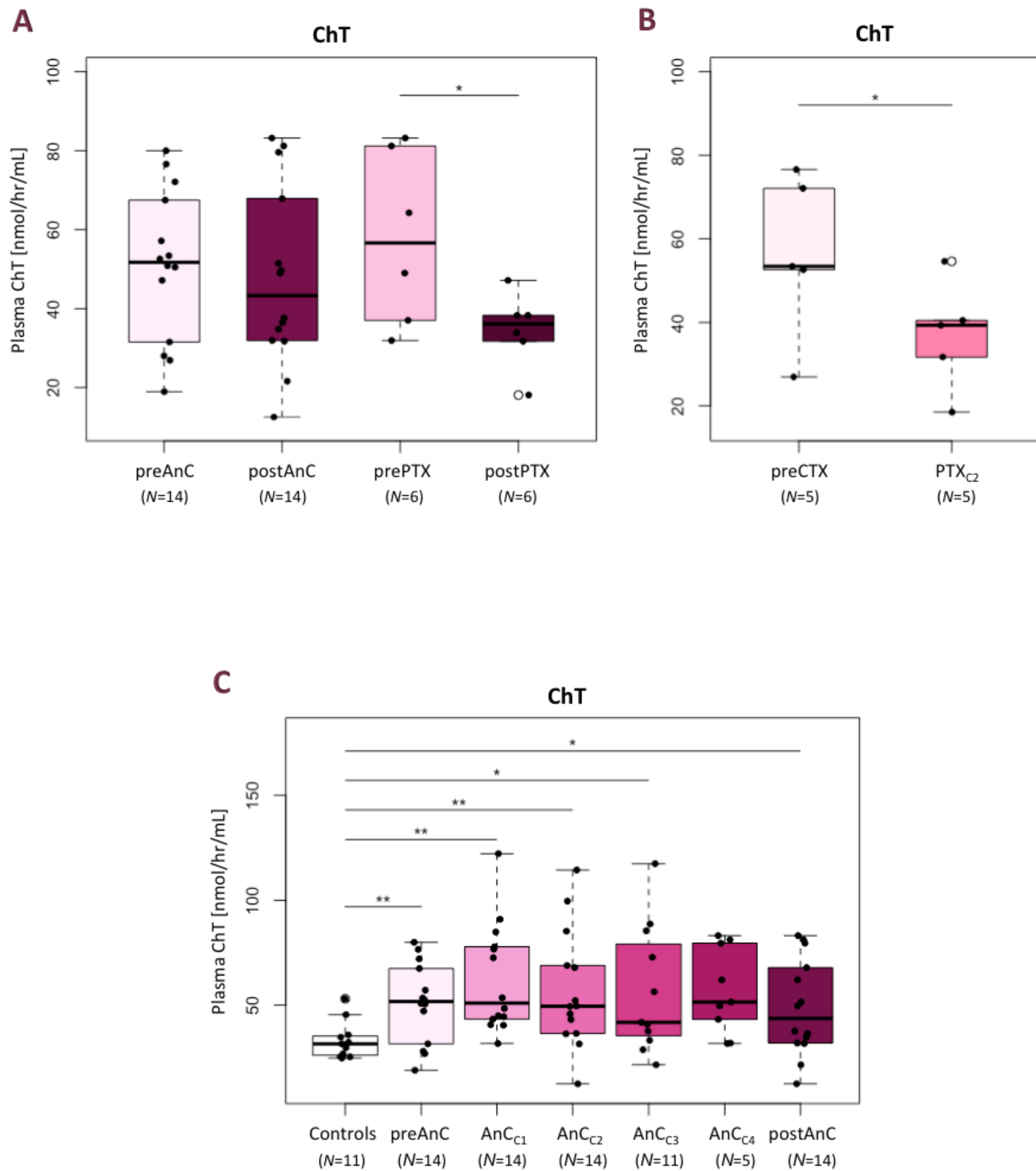
Supplementary Figure S3. Boxplots of lipid damage biomarker measured in plasma. (A) Effect of anthracyclines (AnC) and paclitaxel (PTX) treatment during pregnancy. **(B)** Effect of chemotherapy administration by cycle of chemotherapy. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.
Wilcoxon signed-rank test **(A)** and Student's t paired test **(B)** were used to assess differences between groups; a *P* value of <0.05 was considered significant.
$^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.
Note: Values below LOQ were replaced by ½ LOQ.

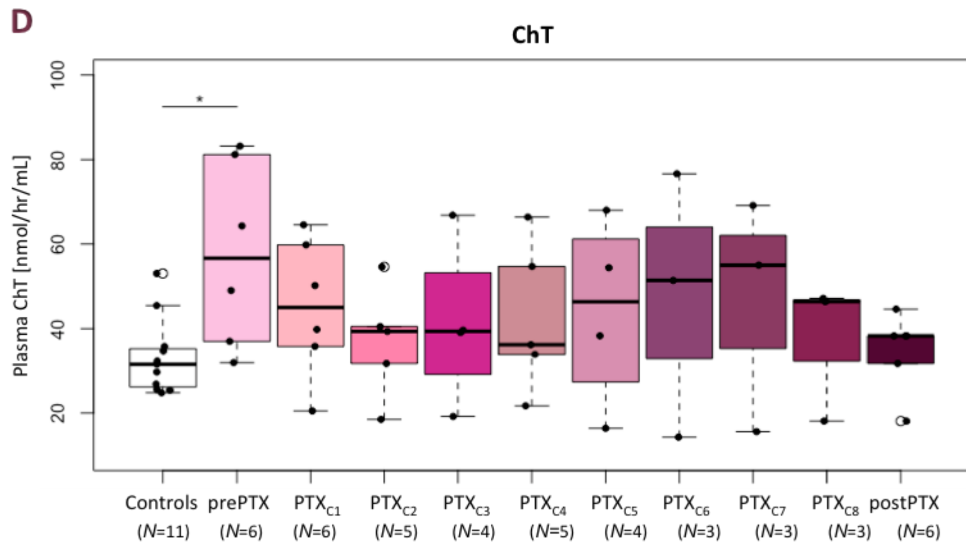
Inflammatory mediators

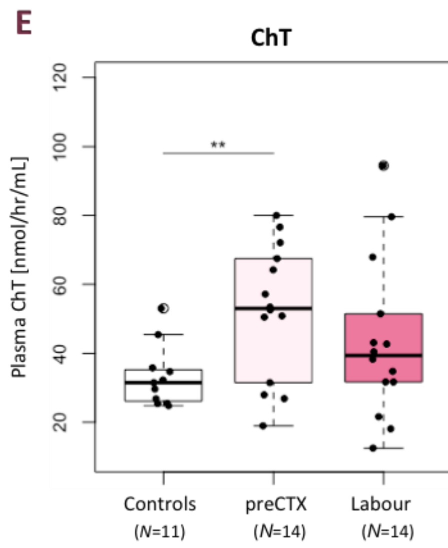
Differences were seen in ChT plasma levels measured in PABC patients before and after paclitaxel treatment reaching statistical significance (*P* value= 0.030) **(Suppl. Figure S4.A)**. In fact, they were notably decreased in cycle two of paclitaxel in comparison with those levels from prior chemotherapy treatment (*P* value= 0.02) **(Suppl. Figure S4.B)**.

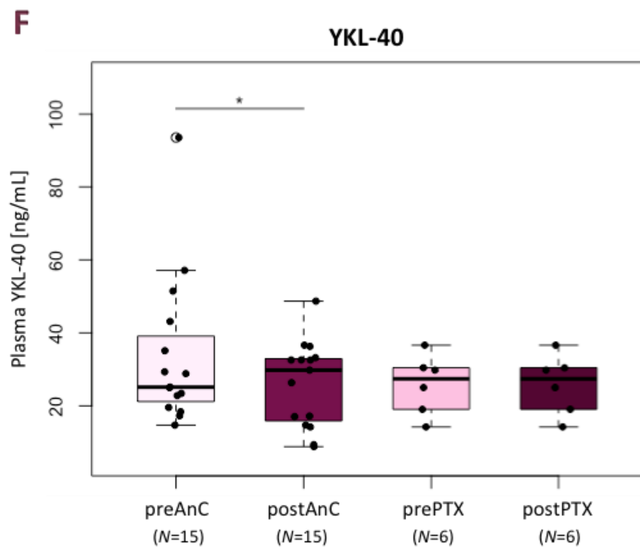
However, significant differences of ChT levels measured in control group and PABC patients undergoing CTX treatment were appreciable among some cycles of anthracyclines and PTX**.** As depicted by **Suppl. Figure S4.C** and **Suppl. Figure S4.D** ChT levels were statistically significantly higher in preAnC group (*P* value= 0.006), cycle one of anthracyclines (*P* value= 0.001), cycle two of anthracyclines (*P* value= 0.009), cycle three of anthracyclines (*P* value= 0.034), postAnC group (*P* value= 0.001) and prePTX group (*P* value= 0.039) than in control group. Likewise, PABC patients showed elevated levels of ChT prior CTX treatment in comparison with control group but not at birth (*P* value= 0.041) **(Suppl. Figure S4.E)**.

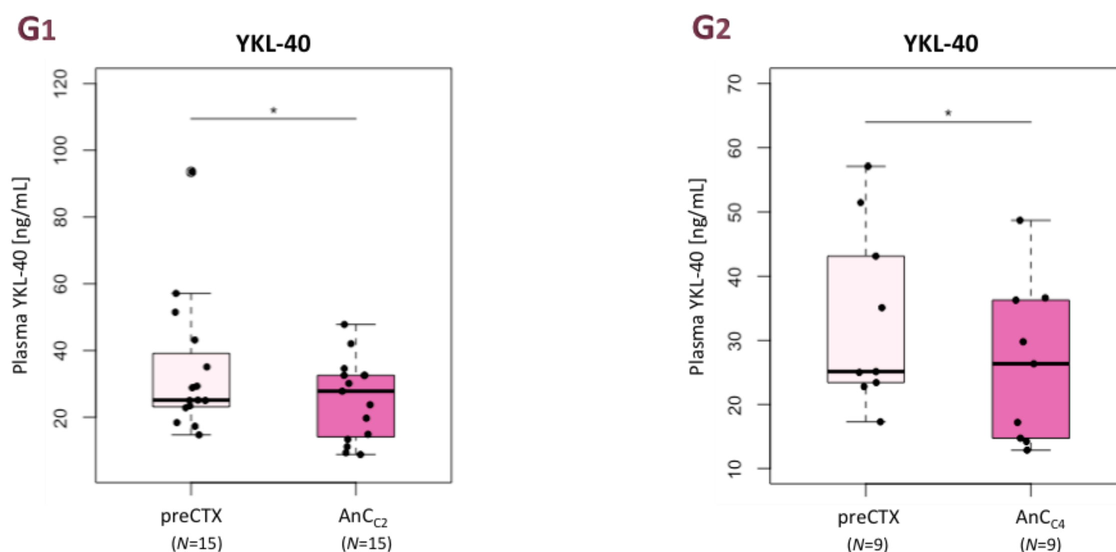
On the other hand, YKL-40 plasma levels were incremented following anthracyclines treatment (*P* value= 0.026) **(Suppl. Figure S4.F). Suppl. Figure S4.G1** and **Suppl. Figure S4.G2** illustrate an increase of YKL-40 levels measured in cycle two and four of AnC compared to CTX initiation (*P* value= 0.030 and *P* value= 0.039 respectively).




D
ChT
Plasma ChT [nmol/hr/mL]
Controls (N=11)
prePTX (N=6)
PTXC1 (N=6)
PTXC2 (N=5)
PTXC3 (N=4)
PTXC4 (N=5)
PTXC5 (N=4)
PTXC6 (N=3)
PTXC7 (N=3)
PTXC8 (N=3)
postPTX (N=6)


E
ChT
Plasma ChT [nmol/hr/mL]
Controls (N=11)
preCTX (N=14)
Labour (N=14)


F
YKL-40
Plasma YKL-40 [ng/mL]
preAnC (N=15)
postAnC (N=15)
prePTX (N=6)
postPTX (N=6)



Supplementary Figure S4. Boxplots of inflammatory biomarkers measured in plasma. (A and F) Effect of anthracyclines (AnC) and paclitaxel (PTX) treatment during pregnancy **(B and G).** Effect of chemotherapy administration by cycle of chemotherapy **(C).**Comparative levels of each cycle of anthracyclines (AnC) administered in PABC patients with control group. **(D)** Comparative levels of each cycle of paclitaxel (PTX) administered in PABC patients with control group. **(E)** Effect of chemotherapy treatment before and in labour. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.
Student's t paired test **(A and B),** Wilcoxon signed-rank test **(F and G),** and Student's test **(C, D and E)** were used to assess differences between groups; *P* value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.
Note: Values below LOQ were replaced by ½ LOQ.

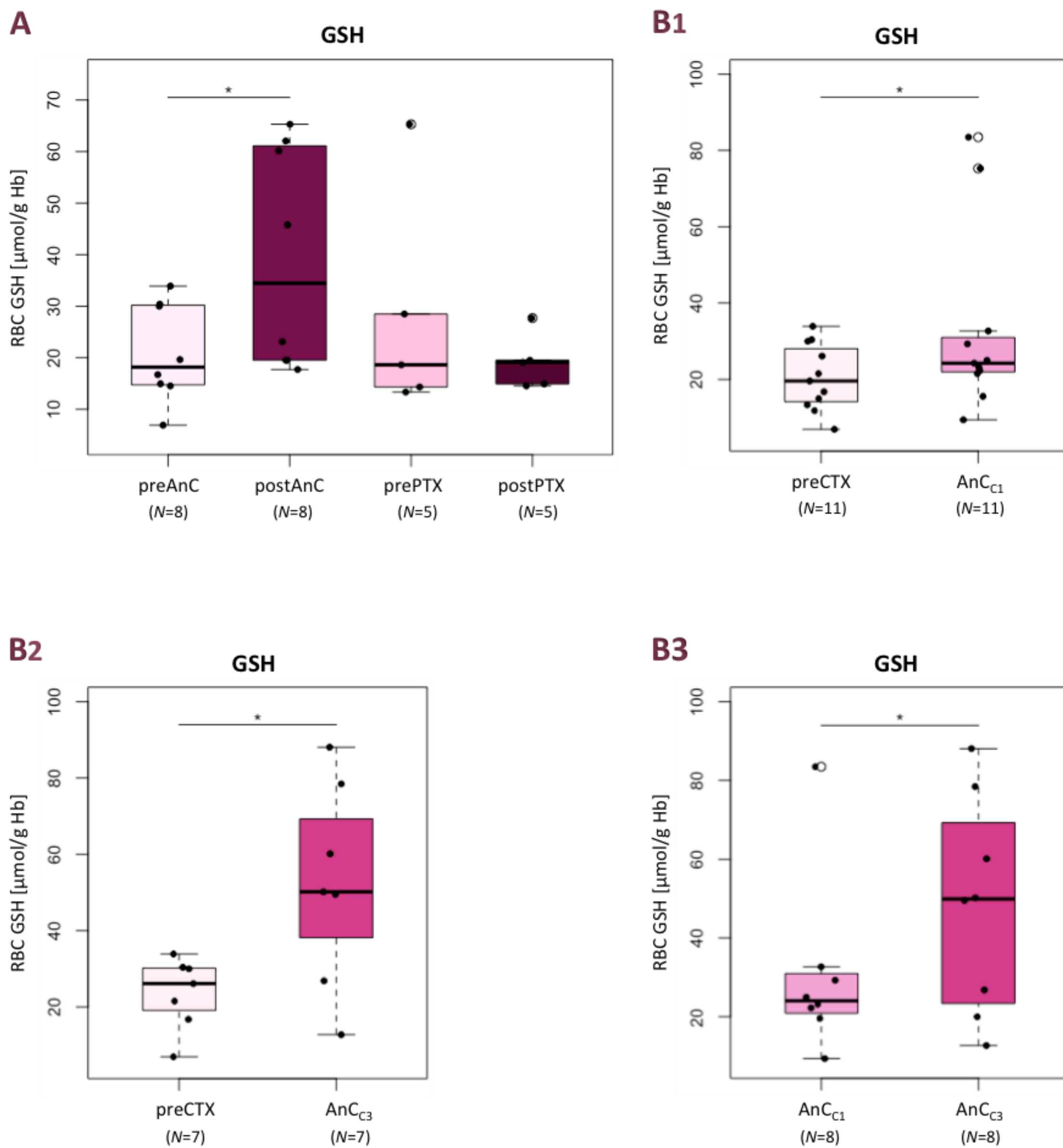
Antioxidant defence

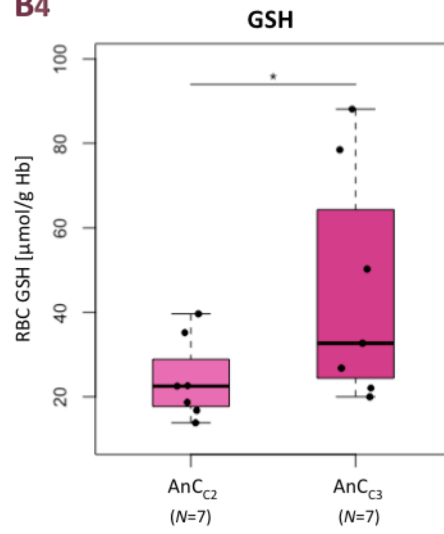
As shown in **Suppl. Figure S5.A**, GSH RBC levels were found statistically significant elevated after anthracyclines treatment respect before anthracyclines initiation (*P* value= 0.046). Precisely, we detected increase levels of GSH in cycle one of anthracyclines (*P* value= 0.019) and cycle two of anthracyclines (*P* value= 0.011) in comparison with those measured before chemotherapy initiation **(Suppl. Figure S5.B1 and Suppl. Figure S5.B2)**. Likewise, differentiating levels of GSH between cycle three of anthracyclines compared to cycle one (*P* value= 0.027) and two of anthracyclines (*P* value= 0.049) were observed in PABC patients **(Suppl. Figure S5.B3** and **Suppl. Figure S5.B4)**.

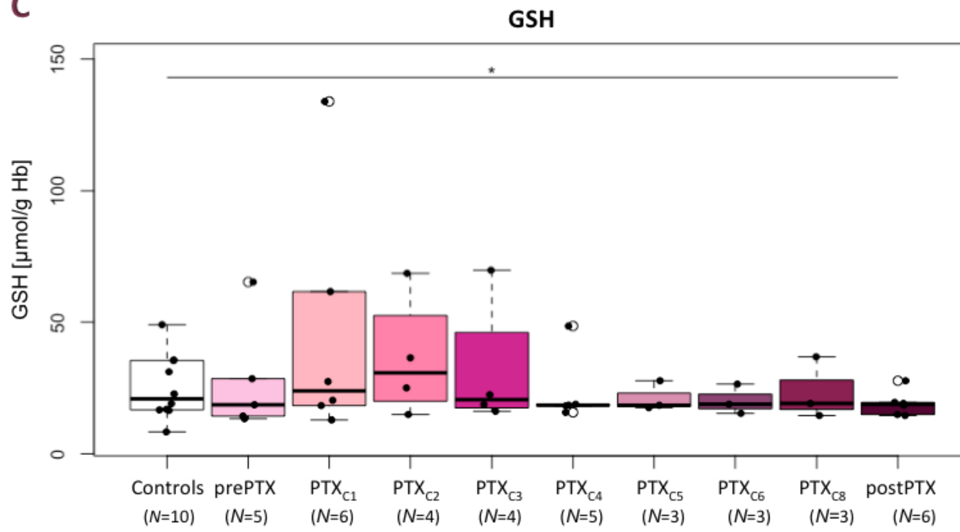
As for the effect of paclitaxel in PABC patients **(Suppl. Figure S5.C)**, we found decrease levels of GSH after paclitaxel treatment in comparison with control group (*P* value= 0.022).

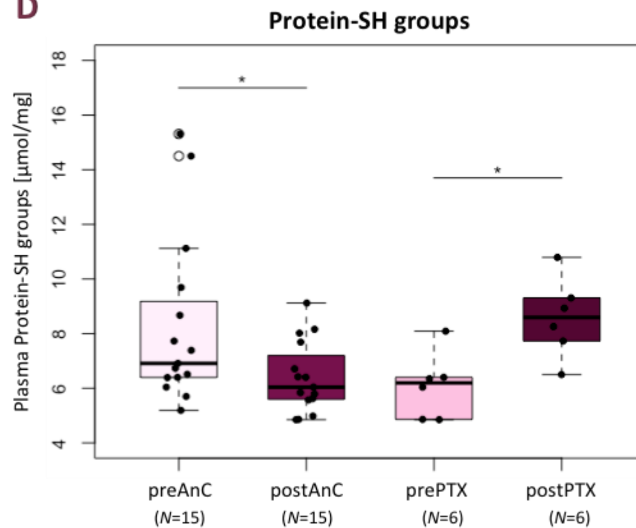
In addition, the levels of protein-SH groups measured in plasma were decreased after anthracyclines treatment while they were incremented following paclitaxel treatment (*P* value= 0.030 and *P* value= 0.038 respectively) **(Suppl. Figure S5.D).**

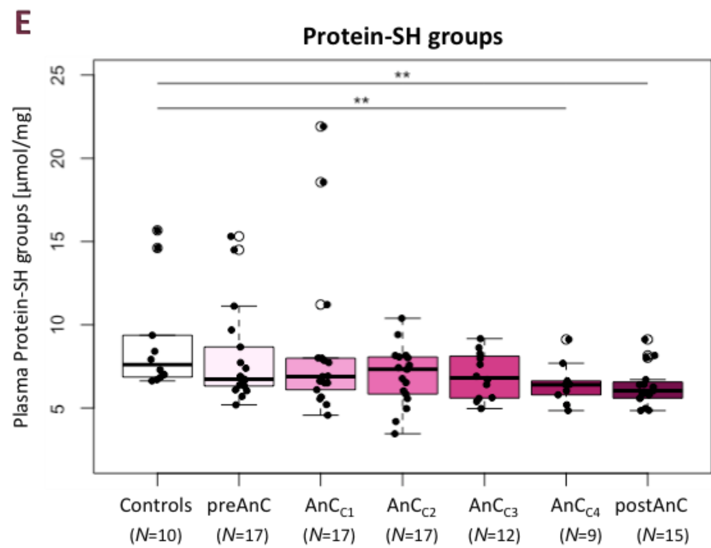
Futhermore, the levels of protein-SH groups were especially significantly decreased in cycle nine of anthracyclines (*P* value= 0.005) and postAnC group (*P* value= 0.005) in comparison with control group **Suppl. Figure S5.E.** Nevertheless, the levels of protein-SH groups were stabilized as the treatment with paclitaxel went on despite they were reduced before its initiation (*P* value= 0.007) **(Suppl. Figure S5.F).**

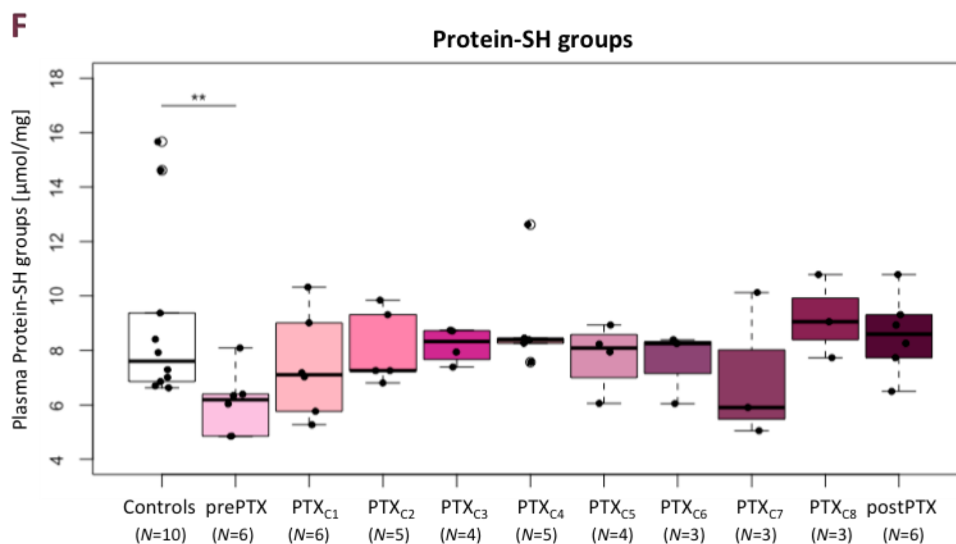



B4
GSH
RBC GSH [µmol/g Hb]
AnC$_{C2}$
(N=7)
AnC$_{C3}$
(N=7)
*


C
GSH
GSH [µmol/g Hb]
Controls (N=10)
prePTX (N=5)
PTX$_{C1}$ (N=6)
PTX$_{C2}$ (N=4)
PTX$_{C3}$ (N=4)
PTX$_{C4}$ (N=5)
PTX$_{C5}$ (N=3)
PTX$_{C6}$ (N=3)
PTX$_{C8}$ (N=3)
postPTX (N=6)
*


D
Protein-SH groups
Plasma Protein-SH groups [µmol/mg]
preAnC (N=15)
postAnC (N=15)
prePTX (N=6)
postPTX (N=6)
*
*

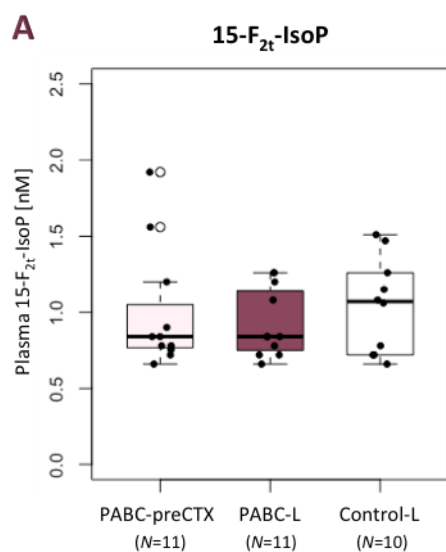


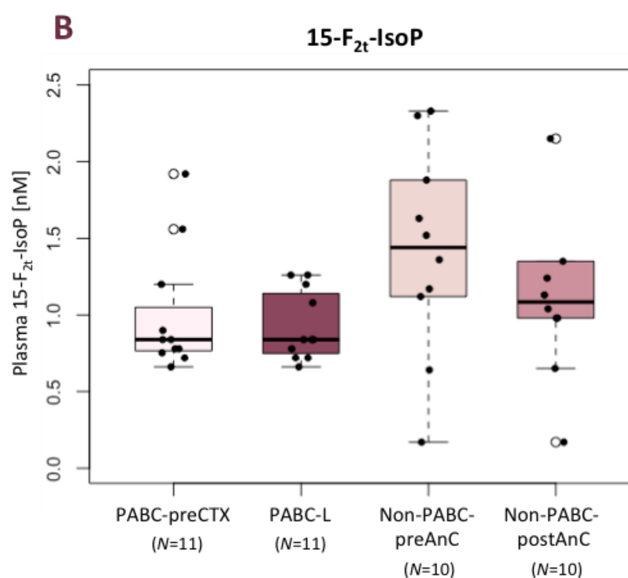


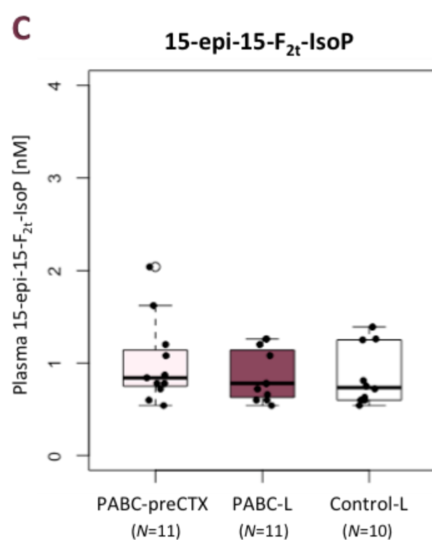
Supplementary Figure S5. Boxplots of antioxidant defence biomarkers measured in plasma and RBC. (A and C) Effect of anthracyclines (AnC) and paclitaxel (PTX) treatment during pregnancy. **(B)** Effect of chemotherapy administration by cycle of chemotherapy. **(D)** Comparative levels of each cycle of anthracyclines (AnC) administered in PABC patients with control group. **(E)** Comparative levels of each cycle of paclitaxel (PTX) administered in PABC patients with control group. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.

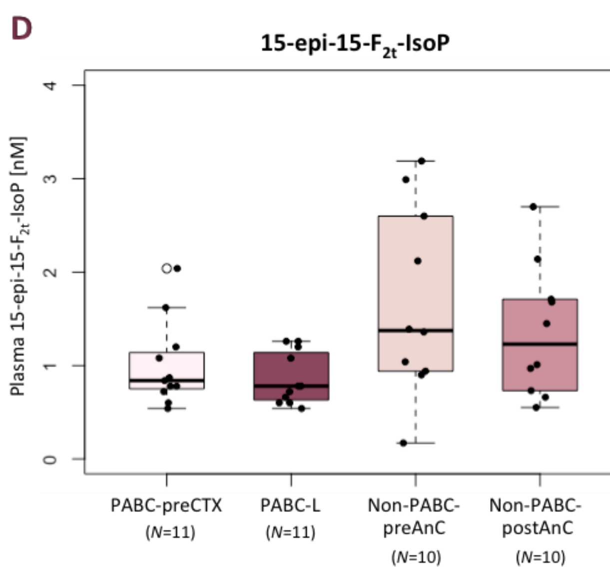
Student's t paired test **(A, B2, B3, B4 and D),** Wilcoxon signed-rank test **(B1),** Student's t test **(C)** and Wilcoxon rank-sum test **(E and F)** were used to assess differences between groups; *P* value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.

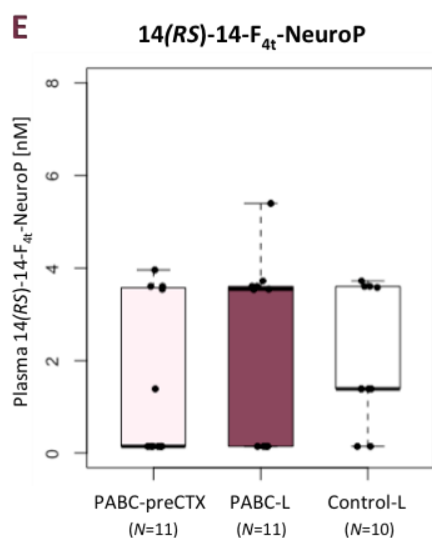
Note: Values below LOQ were replaced by ½ LOQ.

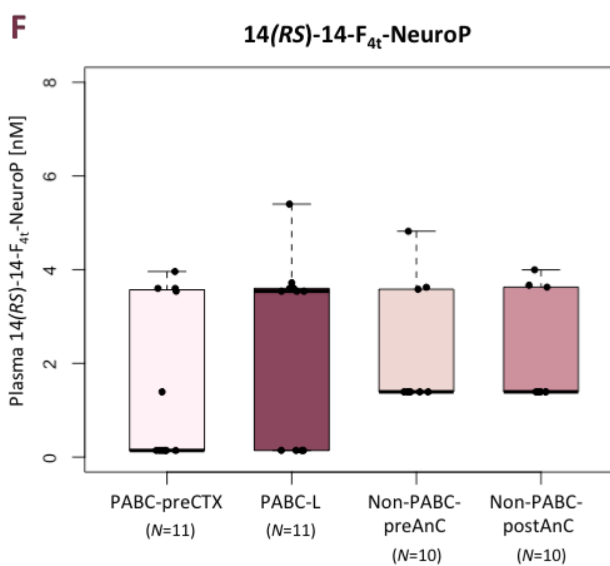

A
15-F2t-IsoP
Plasma 15-F2t-IsoP [nM]
PABC-preCTX (N=11)
PABC-L (N=11)
Control-L (N=10)

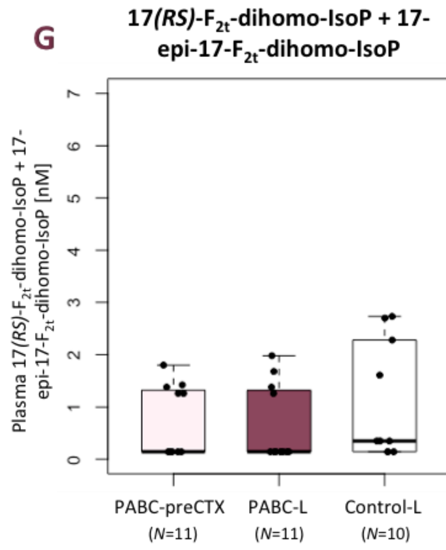

B
15-F2t-IsoP
Plasma 15-F2t-IsoP [nM]
PABC-preCTX (N=11)
PABC-L (N=11)
Non-PABC-preAnC (N=10)
Non-PABC-postAnC (N=10)

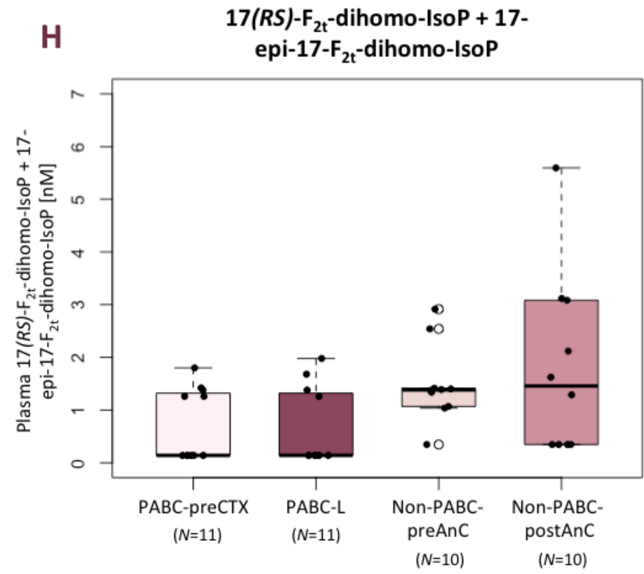

C
15-epi-15-F2t-IsoP
Plasma 15-epi-15-F2t-IsoP [nM]
PABC-preCTX (N=11)
PABC-L (N=11)
Control-L (N=10)

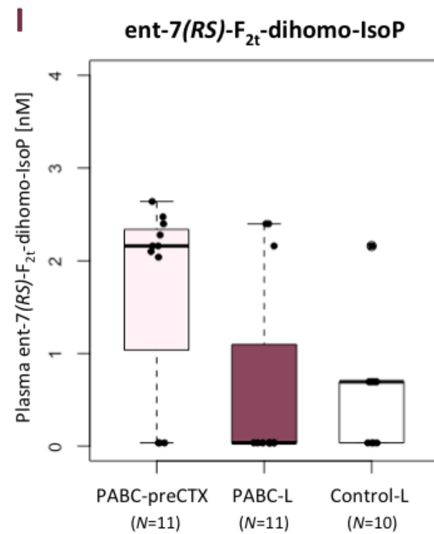

D
15-epi-15-F2t-IsoP
Plasma 15-epi-15-F2t-IsoP [nM]
PABC-preCTX (N=11)
PABC-L (N=11)
Non-PABC-preAnC (N=10)
Non-PABC-postAnC (N=10)

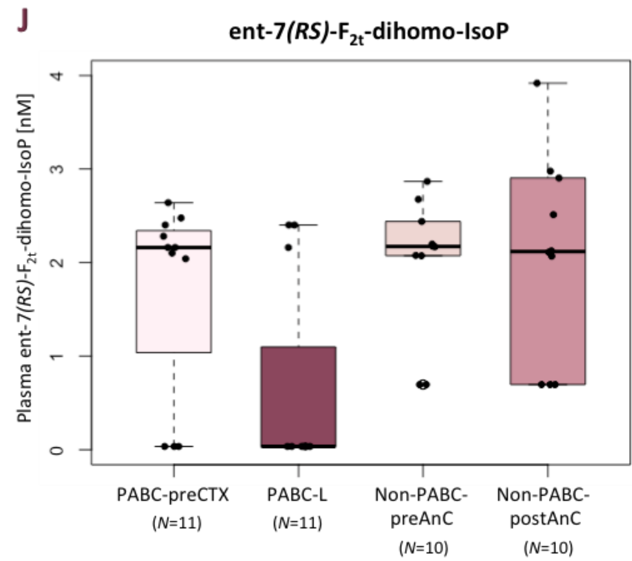

E
14(RS)-14-F4t-NeuroP
Plasma 14(RS)-14-F4t-NeuroP [nM]
PABC-preCTX (N=11)
PABC-L (N=11)
Control-L (N=10)

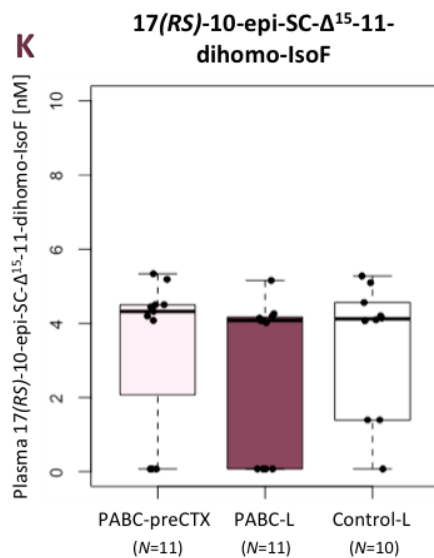

F
14(RS)-14-F4t-NeuroP
Plasma 14(RS)-14-F4t-NeuroP [nM]
PABC-preCTX (N=11)
PABC-L (N=11)
Non-PABC-preAnC (N=10)
Non-PABC-postAnC (N=10)

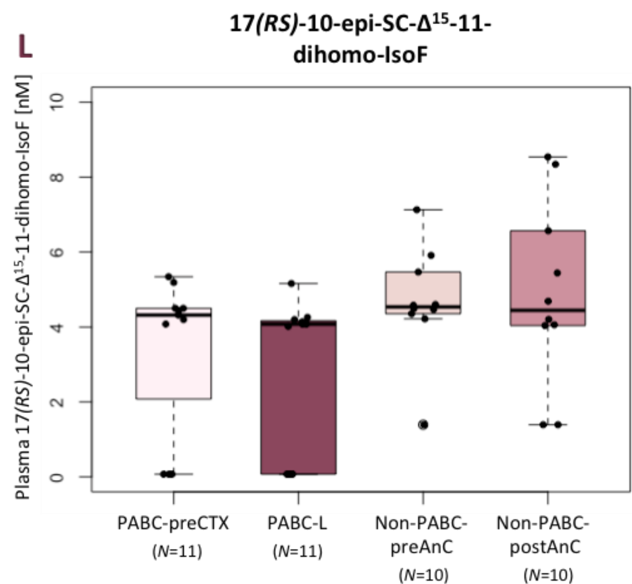

G
17(RS)-F2t-dihomo-IsoP + 17-epi-17-F2t-dihomo-IsoP
Plasma 17(RS)-F2t-dihomo-IsoP + 17-epi-17-F2t-dihomo-IsoP [nM]
PABC-preCTX (N=11)
PABC-L (N=11)
Control-L (N=10)

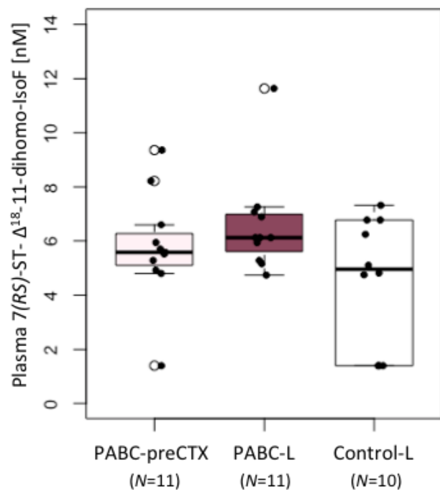

H
17(RS)-F2t-dihomo-IsoP + 17-epi-17-F2t-dihomo-IsoP
Plasma 17(RS)-F2t-dihomo-IsoP + 17-epi-17-F2t-dihomo-IsoP [nM]
PABC-preCTX (N=11)
PABC-L (N=11)
Non-PABC-preAnC (N=10)
Non-PABC-postAnC (N=10)


I
ent-7(RS)-F2t-dihomo-IsoP
Plasma ent-7(RS)-F2t-dihomo-IsoP [nM]
PABC-preCTX (N=11)
PABC-L (N=11)
Control-L (N=10)


J
ent-7(RS)-F2t-dihomo-IsoP
Plasma ent-7(RS)-F2t-dihomo-IsoP [nM]
PABC-preCTX (N=11)
PABC-L (N=11)
Non-PABC-preAnC (N=10)
Non-PABC-postAnC (N=10)


K
17(RS)-10-epi-SC-Δ15-11-dihomo-IsoF
Plasma 17(RS)-10-epi-SC-Δ15-11-dihomo-IsoF [nM]
PABC-preCTX (N=11)
PABC-L (N=11)
Control-L (N=10)

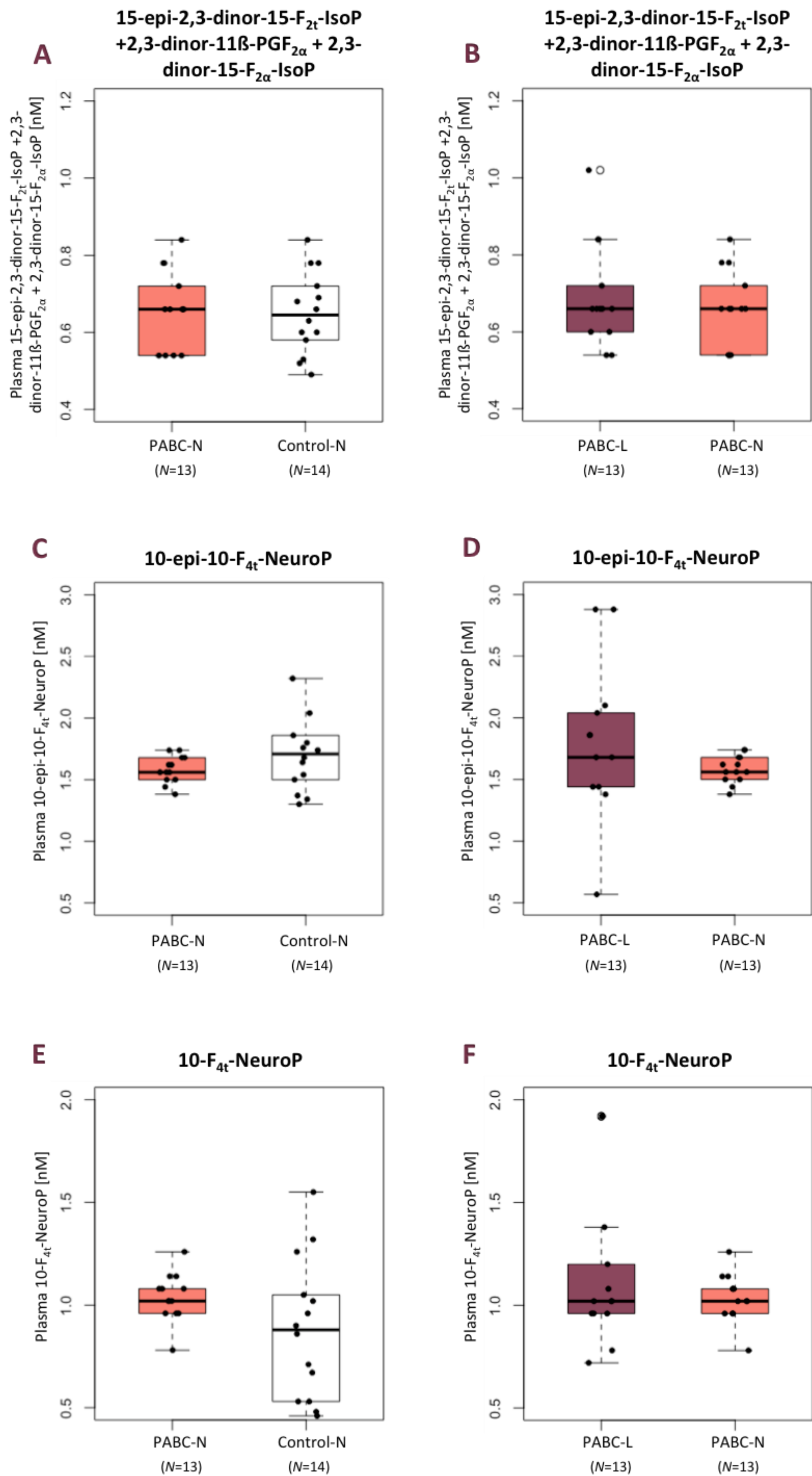

L
17(RS)-10-epi-SC-Δ15-11-dihomo-IsoF
Plasma 17(RS)-10-epi-SC-Δ15-11-dihomo-IsoF [nM]
PABC-preCTX (N=11)
PABC-L (N=11)
Non-PABC-preAnC (N=10)
Non-PABC-postAnC (N=10)

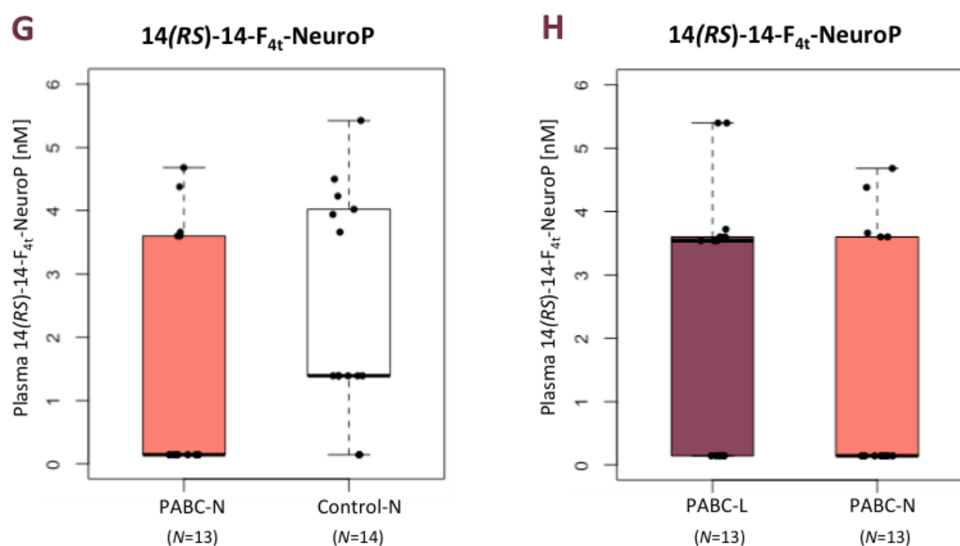


Supplementary Figure S6. Boxplots of lipid damage biomarker measured in plasma. (A, C, E, G, I, K, and **M).** Effect of chemotherapy treatment during pregnancy. **(B, D, F , H, J** and **L)** Comparative effect of breast cancer occurrence in PABC and non-PABC patients and the repercussion of anthracycline-based regimens administered in non-PABC patients. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.

P value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.

Note: Values below LOQ were replaced by ½ LOQ.

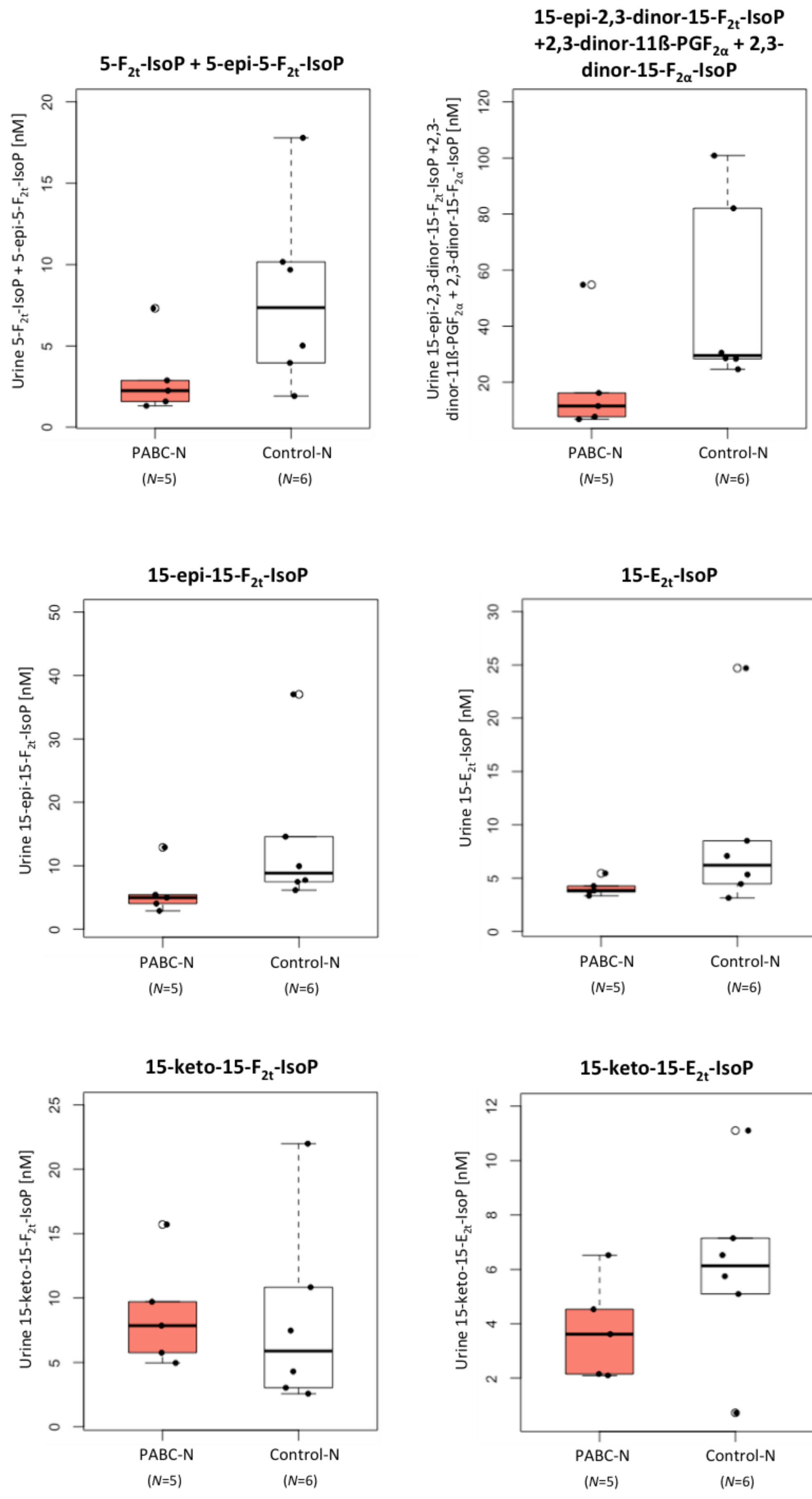

A
15-epi-2,3-dinor-15-F2t-IsoP +2,3-dinor-11ß-PGF2α + 2,3-dinor-15-F2α-IsoP
Plasma 15-epi-2,3-dinor-15-F2t-IsoP +2,3-dinor-11ß-PGF2α + 2,3-dinor-15-F2α-IsoP [nM]
PABC-N
(N=13)
Control-N
(N=14)
B
15-epi-2,3-dinor-15-F2t-IsoP +2,3-dinor-11ß-PGF2α + 2,3-dinor-15-F2α-IsoP
PABC-L
(N=13)
PABC-N
(N=13)
C
10-epi-10-F4t-NeuroP
Plasma 10-epi-10-F4t-NeuroP [nM]
PABC-N
(N=13)
Control-N
(N=14)
D
10-epi-10-F4t-NeuroP
PABC-L
(N=13)
PABC-N
(N=13)
E
10-F4t-NeuroP
Plasma 10-F4t-NeuroP [nM]
PABC-N
(N=13)
Control-N
(N=14)
F
10-F4t-NeuroP
PABC-L
(N=13)
PABC-N
(N=13)



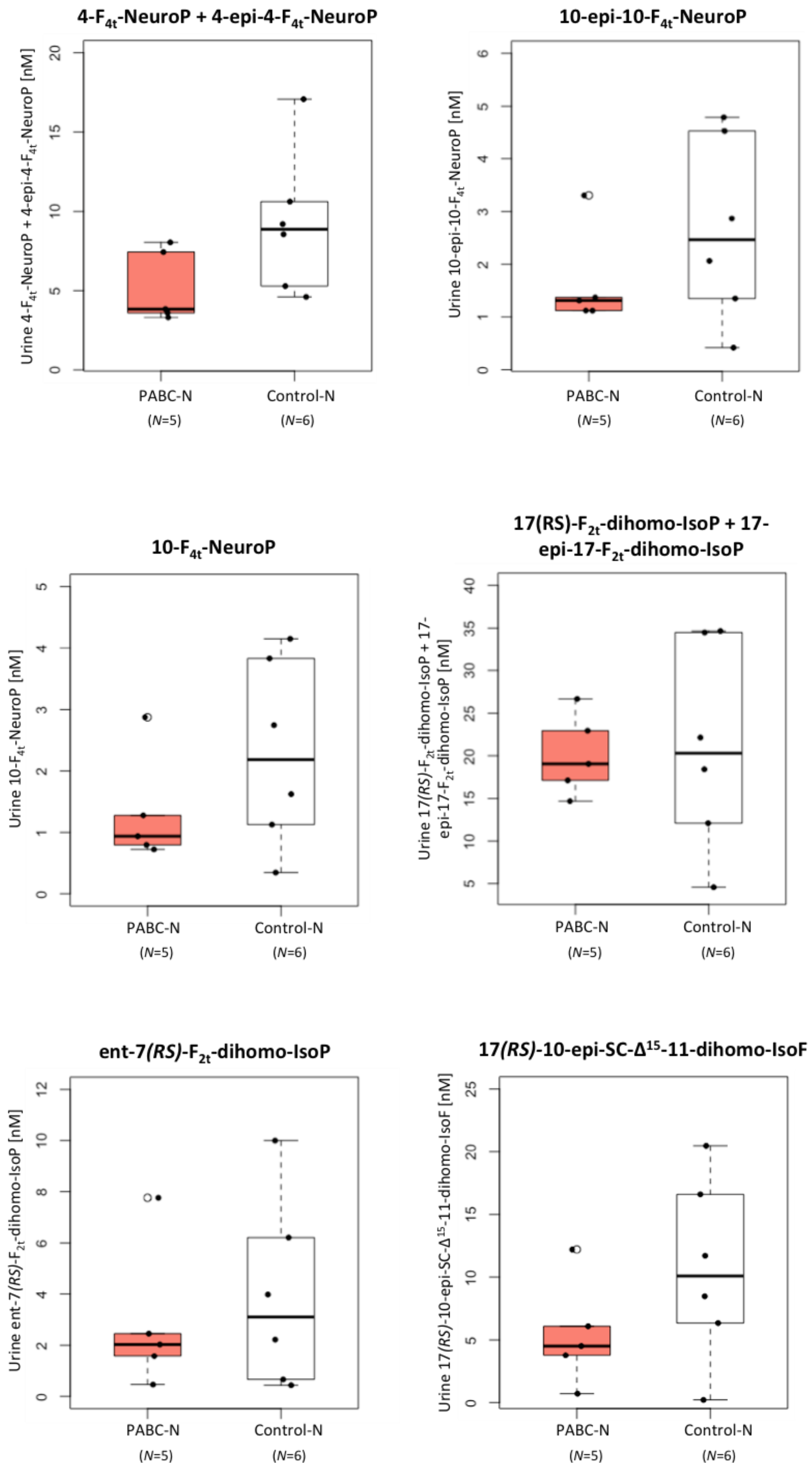
Supplementary Figure S7. Boxplots of lipid damage biomarker measured in plasma. (A, C, E, G, I, K, and **M).** Effect of chemotherapy treatment during pregnancy. **(B, D, F , H, J** and **L)** Comparative effect of breast cancer occurrence in PABC and non-PABC patients and the repercussion of anthracycline-based regimens administered in non-PABC patients. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.

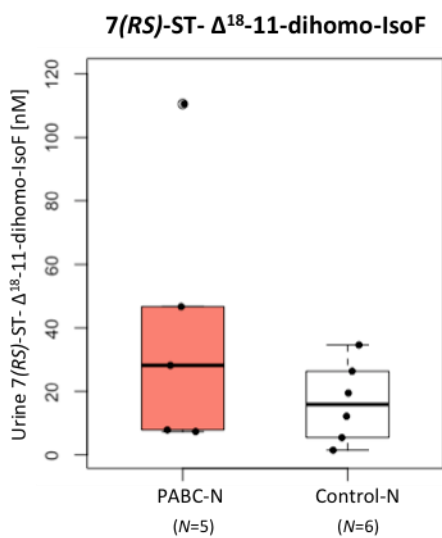
P value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.

Note: Values below LOQ were replaced by ½ LOQ.



5-F_{2t}-IsoP + 5-epi-5-F_{2t}-IsoP
Urine 5-F_{2t}-IsoP + 5-epi-5-F_{2t}-IsoP [nM]
15-epi-2,3-dinor-15-F_{2t}-IsoP +2,3-dinor-11ß-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$-IsoP
Urine 15-epi-2,3-dinor-15-F_{2t}-IsoP +2,3-dinor-11ß-$PGF_{2\alpha}$ + 2,3-dinor-15-$F_{2\alpha}$-IsoP [nM]
15-epi-15-F_{2t}-IsoP
Urine 15-epi-15-F_{2t}-IsoP [nM]
15-E_{2t}-IsoP
Urine 15-E_{2t}-IsoP [nM]
15-keto-15-F_{2t}-IsoP
Urine 15-keto-15-F_{2t}-IsoP [nM]
15-keto-15-E_{2t}-IsoP
Urine 15-keto-15-E_{2t}-IsoP [nM]
PABC-N
(N=5)
Control-N
(N=6)


4-F_{4t}-NeuroP + 4-epi-4-F_{4t}-NeuroP
Urine 4-F_{4t}-NeuroP + 4-epi-4-F_{4t}-NeuroP [nM]
10-epi-10-F_{4t}-NeuroP
Urine 10-epi-10-F_{4t}-NeuroP [nM]
10-F_{4t}-NeuroP
Urine 10-F_{4t}-NeuroP [nM]
17(RS)-F_{2t}-dihomo-IsoP + 17-epi-17-F_{2t}-dihomo-IsoP
Urine 17(RS)-F_{2t}-dihomo-IsoP + 17-epi-17-F_{2t}-dihomo-IsoP [nM]
ent-7(RS)-F_{2t}-dihomo-IsoP
Urine ent-7(RS)-F_{2t}-dihomo-IsoP [nM]
17(RS)-10-epi-SC-Δ^{15}-11-dihomo-IsoF
Urine 17(RS)-10-epi-SC-Δ^{15}-11-dihomo-IsoF [nM]
PABC-N
(N=5)
Control-N
(N=6)



Supplementary Figure S8. Boxplot of the effect of chemotherapy *in utero* on urinary lipid peroxidation biomarkers.. Boxes indicate the 1st and the 3rd quartiles; the median is shown as a black line; whiskers mark indicate the highest and lowest value; black circles represent the value of the samples and unfilled circles are outliers.

P value of <0.05 was considered significant. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤0.001.

Note: Values below LOQ were replaced by ½ LOQ.