A PILOT STUDY: BISPHENOL A AND BISPHENOL A-GLUCURONIDE AND THEIR ASSOCIATION WITH SEX STEROID HORMONES AND 25 HYDROXY VITAMIN D AMONG MOTHER AND CHILD PAIRS

Dr Verena Gounden
983170122
Department of Medical Biochemistry

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (Medical Biochemistry) in the School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal

Supervisors
Professor Anil Amichund Chuturgoon
Professor Rajen Naidoo

Date of Submission 25 August 2021

DECLARATION

I, Dr Verena Gounden, declare as follows:

1. That the work described in this thesis has not been submitted to UKZN or other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

2. That my contribution to the project was as follows:
I was involved in all stages of the study. The original hypothesis was mine. I developed the LC-MS/MS method for the measurement of BPA and BPA-glucuronide and performed part of the sample analysis. I validated and was involved in sample analysis for steroid hormone analysis on the LC-MS/MS methodology. I reviewed all the chromatograms for LC-MS/MS analysis. I optimised and performed all DNA methylation studies. I captured all data and analysed all data as well as carried out all statistical analyses

3. That the contributions of others to the project were as follows:
Professors A. Chuturgoon and R. Naidoo supervised the study, were involved in the supervision of conceptualization of the study components and advisory role in write up. Mr Z. Warasally and Mr T. Magwai assisted with validation of LC-MS/MS methodologies and sample analysis.

4. This thesis does not contain other persons' data, pictures, graphs, or other information, unless specifically, acknowledged as being sourced from other persons.

5. This thesis does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
a) their words have been re-written but the general information sourced has been referenced to the authors;
b) where their exact words have been used, their writing has been placed within quotation marks, and referenced.

6. Where I have reproduced a publication of which I am an author, co-author, or editor, I have indicated in detail which part of the publication was written by myself alone and have fully referenced such publications.

7. This thesis does not contain text, graphics, or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the thesis and reference section.

The research described in this study was carried out in the Disciplines of Chemical Pathology and Medical Biochemistry, School of Laboratory Medicine and Medical Science, College of Health Sciences, University of Kwa-Zulu Natal, under the supervision of Professor A.A. Chuturgoon, and Prof R. Naidoo.

4. Signed _____  Date 25th August 2021

PREFACE

The idea for this study came from my personal interest in toxicology and several reports in the lay media regarding the health effects of Bisphenol A (BPA). At the time I had a young child that was still bottle-feeding and when looking for plastic bottles I was advised to choose BPA free options. I realized that in South Africa we had almost no regulation regarding the presence of BPA in food and consumer products. Additionally, we had no idea of what BPA exposure was like in our population, especially, in the populations supposedly most vulnerable to the effects of BPA- pregnant women and neonates. Also, none of the major academic clinical laboratories including the national occupational health laboratories were measuring BPA in any type of human fluids at the time of the conception of this study. It represented to me an important knowledge gap in South Africa. Considering that NHANES reported that the presence of BPA in urine represented an increased overall risk in morbidity and mortality – this issue needed further scientific research.

.

This PhD thesis is presented as 7 chapters:

- Chapters 1 and 2 are the literature review and methods chapters, respectively.
- Chapters 3 to 6 contain the results of the studies undertaken for this PhD, with each chapter written in the format of a research publication.
- Chapters 3 and 4 has been published as papers, whilst the other 2 chapters have been submitted and are currently under review by the respective journals.
- Chapter 7 comprises of the conclusions and recommendations for further work.

DEDICATION

In memory of my late mum.

ACKNOWLEDGEMENTS

I would like to thank Drs Pragalathan Naidoo and Terisha Ghazi and Ms Kareshma Asharam for their assistance. I would also like to thank Prof Jaya George and Ms Tracy Snyman from the Department of Chemical Pathology at the University of the Witwatersrand who assisted with the 25 hydroxy Vitamin D analysis.

Thank you to my supervisors Professor A. Chuturgoon and Professor R. Naidoo for their guidance.

PUBLICATIONS ARISING FROM THIS THESIS

1. Gounden V, Zain Warasally M, Magwai T, Naidoo R, Chuturgoon A. A pilot study: Bisphenol-A and Bisphenol-A glucuronide levels in mother and child pairs in a South African population. Reproductive Toxicology. 2019; 89: 93-99. (see Appendix 2)

2. Gounden V, Warasally MZ, Magwai T, Naidoo R, Chuturgoon A. A pilot study: Relationship between Bisphenol A, Bisphenol A glucuronide and sex steroid hormone levels in cord blood in A South African population. Reproductive Toxicology. 2021; 100:83-89. (see Appendix 2)

Publications in review:

3. Gounden V, Warasally MZ, Magwai T, Naidoo R, Chuturgoon A, Relationship Between Bisphenol A, Bisphenol-Glucuronide and Total 25 Hydroxy Vitamin D in Maternal-Child Pairs in A South African Population. Submitted to: Toxicology Communications; Manuscript number 216036280

4. Gounden V, Ghazi T, Naidoo P, Naidoo R, Chuturgoon A. DNA methylation changes in the promoter regions of enzymes *CYP1B1* and *CYP3A4* and their relationship with steroid hormone, Bisphenol-A and Bisphenol-A glucuronide concentrations in maternal child pairs. In process of submission.

PRESENTATIONS ARISING FROM THIS THESIS

1. Gounden V, Warasally Z, Magwai T, Chuturgoon A. Development and validation of a LC-MS/MS method for the detection of serum Bisphenol A and Bisphenol glucuronide concentrations. Poster presentation Pathcape Congress, Cape Town, South Africa, August, 2018. Awarded best poster prize at congress.

2. Gounden V, Warasally Z, Magwai T, Naidoo R, Chuturgoon A. Determination of Bisphenol A and Bisphenol A glucuronide in the maternal and child pairs in a South African cohort. Poster presentation at the Euromed Lab Congress Barcelona, Spain, May 2019.

3. Gounden V, Warasally Z, Magwai T, Naidoo R, Chuturgoon A. A pilot study – the relationship between serum Bisphenol A and sex steroid hormone levels in maternal and child pairs in a South African population. Poster presentation at American Association of Clinical Chemistry (AACC) annual congress (virtual) December 2020.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

11DOC	11-deoxycorticosterone
17OHP	17-hydroxy progesterone
25OHD	25 hydroxy Vitamin
25OHD2	25 hydroxy Vitamin D2
Andro	Androstenedione
BMI	Body mass index
BPA	Bisphenol A
BPA-g	Bisphenol A glucuronide
Bw	body weight
CDK4	Cyclin-dependent kinase 4
C_q	Quantitation cycle
CpG	Cytosine guanine dinucleotide
CYP	cytochrome P450
DDT	dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DHT	Dihydrotestosterone
E2	Oestradiol
EDC	Endocrine disrupting chemicals
ED	Endocrine disruptor
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
ER	oestrogen receptors
ERK	extracellular regulated kinase
ERR	oestradiol related receptor
Etc	etcetera
FSH	Follicle Stimulating Hormone
GnRH	Gonadotrophin releasing hormone
GEPR	G-protein coupled oestrogen receptors (GEPR)
HPLC	High performance liquid chromatography
HSD3B1	hydroxy-delta-5-steroid dehydrogenase,3 beta-and steroid delta-isomerase 1
Kt	Kilotonnes
LC-MS/MS	Liquid chromatography tandem mass-spectrometry
LH	Luteinising hormone
LOD	Limit of detection
LOQ	Limit of quantitation
MAPK	mitogen-activated protein kinase
mER	membrane estradiol receptor
miRNA	micro RNA
mRNA	messenger RNA
ND	not detectable
NHANES	National Health and Nutrition Examination Survey
PCOS	Polycystic ovary syndrome
Prog	Progesterone
qPCR	Quantitative polymerase chain reaction
S/N	Signal to noise
StAR	Steroidogenic acute regulatory protein
STAT3	Signal transducer and activator of transcription 3
TDI	tolerable daily intake
TNF-α	Tumour necrosis factor alpha
TT	Total testosterone

UGT	Uridine 5'-diphospho-glucuronsyl transferase

ABSTRACT

Bisphenol A (BPA) is an endocrine disruptor that has become ubiquitous in our environment. It is utilised in numerous consumer products related to the manufacture of plastics. Exposure to BPA has been linked to a wide range of disease including disorders of immune, reproductive and neurological development as well as malignancy. The in-utero stage is particularly vulnerable to the effects of BPA exposure. Maternal exposure has been shown to be positively correlated to BPA levels in the foetus and in early infancy. There is a paucity of data on the extent of exposure to BPA in sub-Saharan populations. As an endocrine disruptor BPA has been shown to affect steroid hormone function and production. However, the mechanism of BPAs action on steroid hormones have not been fully elucidated. The objectives of this study were to describe the extent of BPA exposure in maternal-child pairs in a local cohort, to determine the effect BPA exposure on their steroid hormone concentrations and to elucidate further mechanisms of BPA action via methylation studies of promoter regions of enzymes involved in steroid metabolism.

Method: Matched maternal and cord blood samples collected as part of the Maternal and Child Environment birth cohort study were utilised for the purpose of the study. BPA and its metabolite BPA glucuronide (BPA-g) were analysed in the serum maternal and cord blood samples using an in-house developed liquid chromatography tandem mass spectrometry (LC-MS/MS) method. Samples were also analysed for nine sex steroid hormones namely-: oestradiol (E2), total testosterone (TT), 11-deoxycorticosterone (11DOC), Dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS) androstenedione (Andro), 17-OH progesterone (17OHP), dihydrosterone (DHT) and progesterone (Prog) using LC-MS/MS. 25 hydroxy-Vitamin D (D2 and D3) concentrations were determined in the study cohort using high performance liquid chromatography (HPLC). The degree of the methylation status of the promoter regions of the *CYP1B1* and *CYP3A4* was assessed using quantitative PCR. A p value of <0.05 was considered significant. Statistical analysis was performed on Medcalc statistical software program version 18.11 (Medcalc, Belgium).

Results: Significant exposure to BPA was described in this cohort with more than 75 percent of maternal and cord blood samples exhibiting detectable BPA and/or BPA-g levels. This study demonstrated a statistically significant positive correlation of maternal BPA and BPA-g concentrations with cord blood samples as well as a significant association with cord blood oestradiol and testosterone. A significant negative relationship with cord ($p=0.03$) and maternal BPA-g levels ($p=0.04$) and cord total 25OHD levels was noted. No significant association with *CYP1B1* and *CYP3A4* promoter methylation status and BPA concentrations was identified.

Conclusion. This study is the first in South Africa to describe the extent of BPA exposure in a human cohort and in maternal-child pairs. It is also the first in Africa and one of the few studies worldwide to describe the relationship between steroid hormones and BPA in maternal and cord blood samples. The significant BPA exposure noted in this study has important implications with regards to public health

strategies to limit BPA exposure as well as to prevent, identify and manage associated disease conditions.

CHAPTER 1
INTRODUCTION

1.1 Introduction, Aims and Objectives

Bisphenol A has been shown to have significant effects on human health ranging from reproductive, neurodevelopmental and metabolic pathologies as well as the promotion of tumorigenesis. Its presence in the environment has become ubiquitous (1,2,3). Steroid hormone function has been shown to be affected by BPA and the effect of BPA exposure is particularly deleterious in the developmental in-utero stage based on animal and in-vitro studies (4,5,6). Maternal exposure and the subsequent effects on progeny have been demonstrated to be not just through direct action of toxins on tissue but as a result of epigenetic changes (7,8).

Although there are several studies examining the levels of exposure to Bisphenol A in populations from industrialised or developed nations, there is a significant paucity of data with regards to the developing world and no data available with regards to maternal and child pair exposure for the continent of Africa and sub-Saharan Africa. There are no studies in Africa reviewing the association of steroid hormones including 25-hydroxy vitamin D with BPA exposure. Additionally, the mechanisms of BPA effect on health have still to be fully elucidated.

This study will attempt to address the three main knowledge gaps identified: 1) lack of data regarding BPA exposure in South Africa, 2) effect of BPA exposure on steroid hormones in vulnerable populations namely maternal child pairs, and 3) further elucidate mechanism of BPA health effects by exploring epigenetic effects on steroid metabolising enzymes.

Hypothesis: The hypothesis to be tested in this study is that there is significant BPA exposure in maternal/infant pairs and this contributes to alteration in steroid hormone concentrations.

Aims and objectives

Aim

The aim of this study was to describe the degree and effect of BPA exposure on serum steroid hormones in a maternal-child cohort.

Objectives

The objectives of this study were fourfold

1) Firstly, to develop an in-house method for the determination of serum BPA and BPA-g concentration.
2) Secondly to describe the extent of BPA exposure in maternal-child pairs in a local cohort.
3) Thirdly to determine the effect BPA exposure on their steroid hormone concentrations and,

4) Lastly to attempt to elucidate further mechanisms of BPA action via methylation studies of promoter regions of enzymes involved in steroid metabolism and possible mechanisms thereof.

1.2 Thesis Framework

The layout of the thesis is as follows with each chapter addressing specific objectives of the study.

- Chapter 1 This chapter gives a brief introduction and rationale of the study. Aims and objectives of the study are summarised in this chapter
- Chapter 2 – This chapter contains the literature review that informs the conduct of the study.
- Chapter 3 - provides a detailed description of the study population and methodologies utilised for all laboratory analysis performed during the course of this study
- Chapters 4-7 are the data chapters
- Chapter 4 – addresses the first two objectives of the study. This chapter describes the method developed and validated for the analysis of BPA and BPG levels in serum and the findings on analysis of the cohort of maternal-child pairs.
- Chapter 5 and 6 address the third objective of the study. These chapters detail the measurement of the serum concentrations of sex steroid hormones and 25-hydroxy-Vitamin D in the maternal-child pairs and describe any association with BPA and/or BPA-g levels
- Chapter 7 addresses the last objective to examine possible epigenetic changes (in this case DNA methylation) of the promoter regions of the steroid hormone metabolising enzymes that would further elucidate the mechanism of the action of BPA
- Chapter 8 Synthesis chapter. This is the concluding chapter, the important findings of this study are highlighted and limitations are further discussed. This chapter also serves to place the results of this study in the larger context of the health consequence of environmental exposures.

References

1. EPA report on endocrine disruptors https://www.epa.gov/endocrine-disruption. Accessed 17 September 2016
2. Kandarakis ED, Bourguignon JP, Giudice L et al, Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement Endocrine Reviews 2009; 30: 293–342.
3. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. Human exposure to bisphenol A (BPA). Reproductive Toxicology. 2007; 24: 139–77.
4. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins G, Soto AM, et al Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement. Endocrine Reviews 2009;30: 293-342.
5. Liu Q. Effects of Environmental Endocrine-Disrupting Chemicals on Female Reproductive Health. Advances in Experimental Medicine and Biology 2021; 1300: 205-229.
6. Barbagallo F, Condorelli RA, Mongioi LM, Cannarella R, Aversa A, Calogero AE. Effects of Bisphenols on Testicular Steroidogenesis. Frontiers in Endocrinology 2020; 11: 373.
7. Skinner MK. Endocrine Disruptors and Epigenetic Transgenerational Disease Etiology. Paediatric research 2007; 61: 48-50.
8. Lombó, M., Fernández-Díez, C., González-Rojo, S. et al. Genetic and epigenetic alterations induced by Bisphenol A exposure during different periods of spermatogenesis: from spermatozoa to the progeny. Scientific Reports 2019; 9: 18029.

CHAPTER 2
LITERATURE REVIEW

2.1 Endocrine disruptors

Environmental endocrine disruptors (EDCs) are environmental chemicals that can interfere with endocrine hormone signalling to alter cellular function. An endocrine-disrupting compound is defined by the United States (US) Environmental Protection Agency (EPA) as "an exogenous agent that interferes with the synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process (1). EDCs can enter the environment mainly through industrial and urban discharges, agricultural run-off and the burning and release of waste. Human exposure can occur via the ingestion of food, dust and water, inhalation of gases and particles in the air, and skin contact. In humans EDCs have been linked to adverse effects on reproductive systems including development of malignancy (for example breast and testicular cancers), abnormal neuronal development as well as intra-uterine growth restriction. Apart from the direct effect at the level of hormone signalling affecting the individual exposed to EDCs it has been suggested that exposure to EDCs can cause transgenerational epigenetic effects (1-5). Currently, a definitive list of endocrine disrupting chemicals does not exist (6). The reported endocrine disruptors do not appear to share any structural similarity except that most have a small molecular mass (< 1000 da) (7).

Substances found to have endocrine disrupting potential form a diverse group that may occur naturally in the environment and food or synthetically manufactured. Phyto-oestrogens such as genistein and coumesterol found commonly in food products containing soya are also considered to be potential endocrine disruptors. Synthetic chemicals include plastics such as Bisphenol A which is recognised as a synthetic xenoestrogen, plasticisers (phthalates), industrial solvents and their by-products polychlorinated biphenyls (PCB), pesticides [methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane (DDT)], fungicides for example vinclozolin which has antiandrogen properties, dioxins and pharmaceutical agents [diethylstilbestrol (DES)] (2,8). Table 2.1 below lists the common classes of endocrine disruptors and source of exposure.

Table 2.1 Summary of Common EDCs and Potential sources of exposure [Adapted from Michałowicz J] (8)

Chemical	Potential sources of exposure
Bisphenol A	Food, drinking water, toys, cosmetics • Used in the manufacture of epoxy resins used to coat food cans, bottle tops and water supply lines • Used in the manufacture of Polycarbonate plastics • Used in the manufacture of rigid plastics used for food storage and water containers, plastic tableware, coatings for wine storage vats • Toys made from rigid plastics especially in case of young children • Migration from the plastics used to package cosmetics • Medical devices made from plastics
Polychlorinated biphenyls (PCB) (persistent organic pollutant)	Food, drinking water, contaminated indoor air • Were widely used as coolant and lubricants in transformers, capacitors and other electrical equipment – released into the environment (soil, air and water) from poorly maintained hazardous waste sites that contain PCB, via illegal or improper dumping of PCB waste. • Environmental contamination taken up by plants, fish and other aquatic animals • Skin contact • Flame retardants
Polybrominated biphenyls	Use discontinued in the United States of America in 1970's. Contaminated food, air, water and soil • Used as flame retardant is a wide variety of consumer products including computer monitors, televisions, textiles
Phthalates	Food, air, dust, toys, medical devices • Eating and drinking foods that have been in contact with containers and products containing phthalates • Breathing in indoor air containing phthalate vapours or dust contaminated with phthalate particles originating from flooring, wall covering, lacquers, varnishing and coatings • Cosmetics, personal care products, plastic toys • Coatings on nutritional supplements
Pesticides	Contaminated food and water
Fungicides (for example vinclozolin)	Contaminated food and water
Polychlorinated dibenzodioxins (persistent organic pollutant)	Food (especially dairy products, meat and fish), soil Produced as a by-product of industrial processes but can also result from natural processes for example forest fires
Phytoestrogens	Plant derived found in a variety of foods most notably soy and soy related products including many processed foods such as hotdogs and other meat products, ice cream, infant formula, sports drinks, imitation dairy products

2.2 Bisphenol A

Bisphenol A (BPA) is a synthetic phenol that is utilised in the production of polycarbonate plastics and epoxy resins. It is found in a wide range of consumer products globally. These include toys, water pipes, drinking containers, eyeglass lenses, sport safety equipment, medical equipment and tubing, and consumer electronics (1,2,9). It is the most widely used synthetic compound in the world, with annual production estimated to reach over 7000 tons by the year 2023, owing to a significant growth in production and demand in Asia (10). Figure 2.1 below depicts the worldwide production of BPA over the period of 1993-2005 showing the highest producing regions. Total global production of BPA in 2015 was reported to be around 7.7 million metric tons (11).

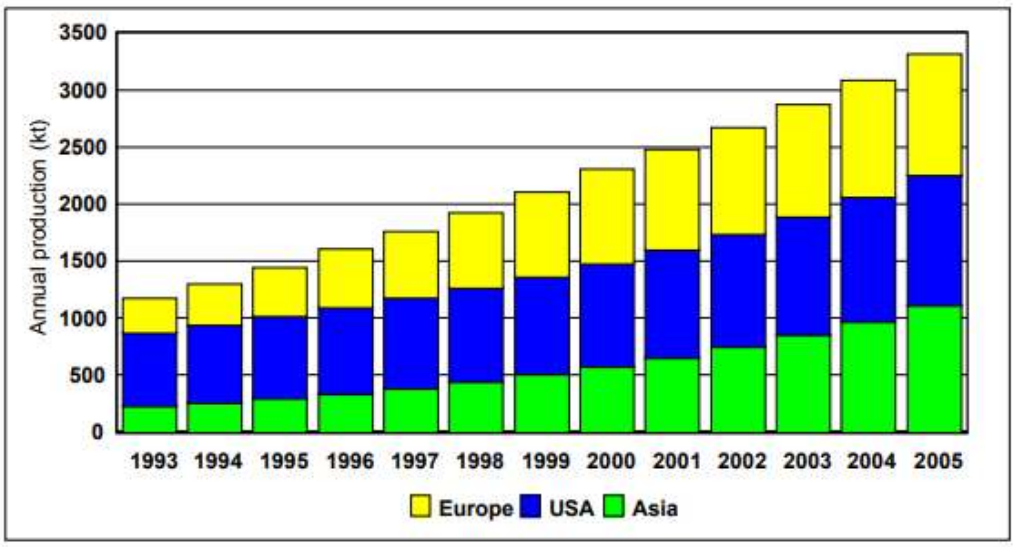

Figure 2.1 Worldwide production of BPA (1993-2005) in kilotonnes (kt) (12)

2.2.1 Physicochemical properties

BPA has a molecular weight of 228.29 g/mol and has a white crystalline solid structure. The mechanical properties of polymers made from BPA include thermal stability and low adsorption, making it a versatile choice to be used in the manufacture of a wide range of consumer products and devices (13).

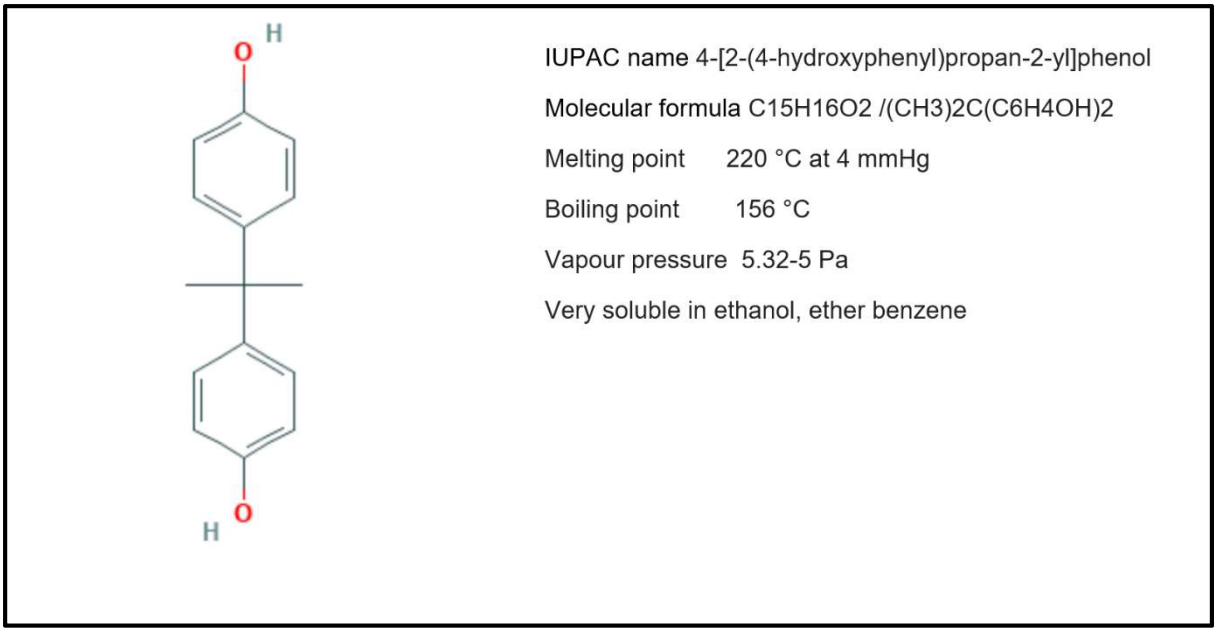

Figure 2.2 Structure and chemical properties of Bisphenol A (13) (Source: National Center for Biotechnology Information. PubChem Compound Summary for CID 6623, Bisphenol A. https://pubchem.ncbi.nlm.nih.gov/compound/Bisphenol-A)

2.2.2 Sources and routes of exposure

The release and migration of BPA monomers that are formed during the production and treatment of BPA material or plastic polymer degradation constitute the main origins of environmental BPA (14). BPA may enter an organism via various routes (15, 16):

- The digestive tract
- Absorption, by the skin
- The respiratory tract

Significant concentrations of BPA have been detected in amniotic fluid, cord blood and breast milk, as BPA is able to cross cell membranes due to its strong lipophilic properties. As such, growing foetuses and infants are continuously exposed to this synthetic phenol (17-20).

Sources

a) Food and water

Dietary intake constitutes the primary source of BPA intake amongst humans. This can occur via intake of animal and plant material exposed to BPA. Table 2.2 below indicates the BPA concentrations of various food items as reported by different regional studies.

Table 2.2 Concentrations of BPA reported worldwide in different foods and water

Fish	Meat/meat products	Fruits and vegetables	Grains	Water	Region	Reference
1.2 µg/kg	0.24 µg/kg	0.19 µg/kg	0.11 µg/kg		Norway	Sakhi et al, 2014 (21)
	419.26µg/kg	6.15 µg/kg			Taiwan	Chen et al, 2016 (22)
	36.9 µg/kg	9.92 µg/kg			Spain	Martinez et al, 2018 (23)
6.26 µg/kg	ND-12.7 µg/kg	ND-2.21 µg/kg	ND		Nigeria	Babalola, 2019 (24)
				3.3-8.8 ng/L (bottled water)	Saudi Arabia	Elobeid et al, 2012 (25)
				Asia 33.16 ng/L Europe 5.32 ng/L North America 0.65 ng/L (surface water)	mixed	Corrales et al, 2015 (26)
				0.0173 -1.468 µg/L (effluent water)	Eastern Cape , South Africa	Farounbi et al, 2020 (27)
				<0.0002 µg/L to < 0.002 ug/L (Europe) <0.002 µg/L to <1 µg/L (North America <0.014 µg/L- 0.026 µg/L (Asia)	Mixed	Arnold et al, 2012 (28)

ND-not detectable

BPA released from plastic food containers and linings of cans also contributes significantly to dietary ingestion. The amount of BPA released from the polymer lined food containers also depends on pH, usage time and temperature. In a study by Lorber et al (29), it was demonstrated that BPA from canned food accounts for a large proportion of the daily BPA intake. Elobeid et al (25), reported significantly increased levels of BPA in plastic waste bottles when bottles were exposed to outside temperatures versus room temperature.

Breast milk is also a source of BPA. This is due to its high lipophilic property and accumulation in mammary gland fat. The mean amount of BPA exposure has been reported to be around 0.3 µg/kg bw/day for exclusively breast-fed infants (15).

b) Air and dust

The respiratory route is estimated to account for more than 70% of non-dietary BPA exposure. BPA can be released into the air during the use of products containing BPA synthetic polymers. The degree of release is also directly related to the duration of usage of the product in question. Products that have been reported to release BPA into the air include commonly used household products, such as epoxy-based floorings, adhesives and electronic equipment (23). Liu et al (30) and Loganathan et al (31) reported the accumulation of BPA in indoor dust. This in turn can be inhaled, ingested or absorbed via the skin. Alkasir et al (32) reported BPA concentration of 0.05 to 3.87 µg/g in several different household dust samples. A further study in Belgium estimated mean BPA concentrations in dust of around 1.46 µg/g (33). BPA in dust and air may also be routes of occupational exposure (34).

c) Other sources

Thermal paper is used for cash receipts, boarding passes and various tickets. Exposure is higher in those groups who utilise thermal paper in their occupations, like cashiers for example (14). Bernier et al, estimated BPA exposure from handling thermal paper to be 0.0511µg/kg/bw/day (35). Dental materials have been shown to contribute minimally to total BPA exposure (15, 36).

2.2.3. Metabolism

Once BPA is ingested it is rapidly absorbed via the digestive tract and has a short half-life in humans of less than 6 hours. Following absorption, it is metabolised primarily by the liver and is conjugated via the action of the enzyme UDP glucuronyl-transferase (UGT) into BPA-glucuronide (BPA-g). BPA-g represents the major metabolite of BPA with other conjugates such as sulphates produced by the action of sulfotransferases, comprising the remainder. The water-soluble metabolites of BPA then enter the circulation and are excreted via urine (14). Most of the studies examining human exposure to BPA including the large epidemiological studies such as NHANES have measured urinary BPA concentrations (37). Due to the rapid rate of clearance via urine this may not represent an adequate picture of long term exposure. In humans, levels of the enzyme UGT have been shown to be lower in the foetal liver than the adult liver. The concentration of BPA in the placenta has been found to be much higher than that of maternal plasma (38). This indicates the likelihood of increased BPA exposure for the foetus even when maternal BPA concentrations are not significant.

2.2.4 Adverse Health Effects

Exposure to BPA is of the greatest health concern in early life where exposure in utero produces prenatal and postnatal adverse effects on multiple tissues, including the brain. Animal and human studies have demonstrated that BPA readily crosses the placenta and has been detected in amniotic fluid (39-42).

Whilst studies demonstrating causation in humans have been relatively limited, studies in animal models have associated BPA exposure to the development of breast and prostate cancers, polycystic ovarian syndrome, preterm delivery, intrauterine growth restriction and low birth weight (1,7,9,14). Recent studies have also associated elevated serum and urine BPA levels with cardiovascular disease, obesity and type 2 diabetes mellitus (43-45). Figure 3 below summarises the reported human pathologies associated with BPA exposure.

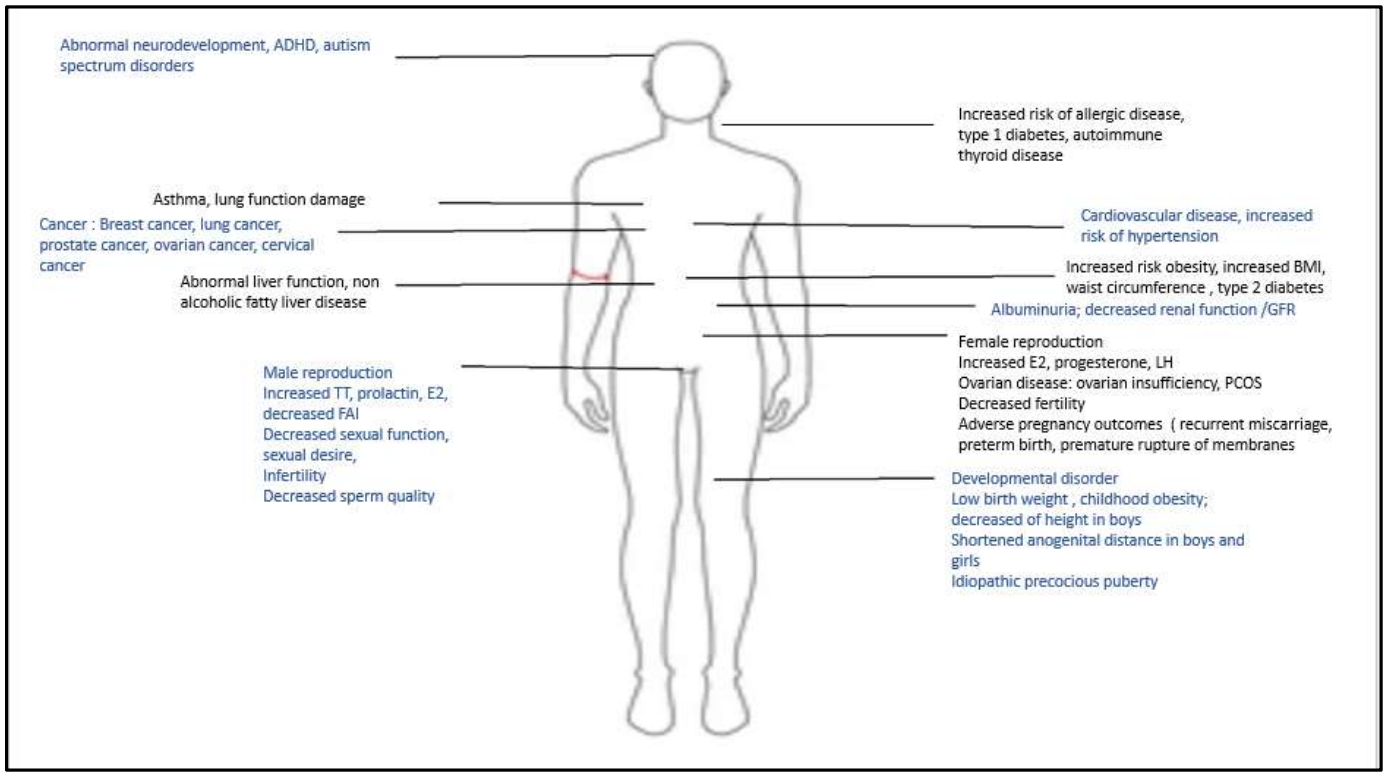

Figure 2.3: Summary of pathologies associated with BPA exposure in humans [adapted from Ma et al, 2019 (14)]

2.2.4.1 Effects on Reproduction

Disorders or disruption of the reproductive system have been reported to be associated with BPA exposure in several epidemiological studies. BPA is an endocrine disruptor and exerts its effects on the reproductive system by reducing or increasing the concerned regulatory hormones or interrupting the action of these hormones in some manner (14). BPA has been reported to increase serum oestradiol, progesterone, luteinising hormone (LH) and total testosterone levels (46). Other studies report a decrease in testosterone concentration with increased oestradiol and an altered serum oestradiol: testosterone ratio (47). Increased BPA concentrations have been reported in women diagnosed with polycystic ovarian disease (48,49). Various reproductive cancers such as endometrial, ovarian and prostate have been associated with BPA exposure (1,7,9,14). In males, infertility has been linked to BPA exposure, together with decreased sperm quality, sexual drive and erectile dysfunction (50, 51). Poor pregnancy outcomes such as miscarriage, premature rupture of membranes, preterm birth have been reported to be associated with BPA exposure (52, 53).

a) Precocious puberty

Precocious puberty is defined as the onset of features of puberty before the age of 8 years old in girls and before the age of 9 in boys (54). Many studies have reported an increasing trend in lowering of age of onset of puberty in girls in the last few decades. The effect of environmental exposure has been suggested as a possible causative factor (55-57). BPAs structural homology to 17□-oestradiol as well as its ability to have agonistic and antagonistic effects at the level of the ER alpha and ER beta receptors results in its potential to cause early puberty as well as infertility. The mechanism of precocious puberty caused by BPA is linked to its oestrogen like activity and ability to trigger positive feedback at the level of hypothalamus and pituitary eventually resulting in increased FSH and LH levels which induces puberty onset (58). Additionally, BPA is also associated with increased risk of obesity. Elevated body mass index (BMI) is a known trigger for early puberty particularly in girls. High levels of urinary BPA have been demonstrated in obese children and adolescents (59, 60).

b) Polycystic ovary syndrome (PCOS) and BPA

PCOS is characterised by reproductive and metabolic dysfunction in females of reproductive age. Infertility and hyperandrogenism are the most common clinical presentations. PCOS has been strongly associated with increased insulin resistance and obesity, similar to the metabolic syndrome. The pathogenesis of this PCOS remains to be fully elucidated but the growing incidence makes exposure to environmental toxins an important consideration (61). Several in vitro and animal studies have cited exposure to EDCs such as BPA as risk factors for PCOS development (62-64). However due to the mixed exposure in the environment, it is difficult to assess the individual effects of BPA. Thus epidemiological data from human studies remain limited. A study by Takeuchi et al (65) showed higher concentrations of BPA in biological fluids in women with PCOS and confirmed biochemical

hyperandrogenism. Higher levels of BPA have also been shown in women with PCOS versus healthy controls (62). A combination of genetic predisposition and exposure to BPA and other EDCs in critical development stages may induce a PCOS phenotype. Prenatal exposure to BPA may alter foetal programming and promote PCOS development later in life. High doses of BPA in the neonatal period in rat models resulted in the following changes in adulthood consistent with a PCOS-like picture: - increased serum testosterone and oestradiol levels, decreased progesterone and presence of ovarian cysts (64).

c) Malignancy

A significant proportion of the tumour inducing effects of BPA is as a result of its oestrogenic like properties. Hence many of the associations with malignancy are often with tumours predominantly found in females (66,67).

Ovarian cancer: Elevated levels of oestrogen receptors expression have been noted in approximately half of ovarian epithelial cancer cells when compared to normal or benign tumour cells (68). Some reports have shown an association of oestrogen replacement therapy and ovarian cancer in post-menopausal women (69,70). Exposure to higher or inappropriate levels of oestrogen are thought to stimulate tumour progression and proliferation. Since BPA mimics oestrogen it may have the same effects. In animal models, neonatal BPA exposure has been associated with abnormal ovarian morphology, hyperplasia cystic lesions and endometriosis (71,72). BPA has been shown to further promote ovarian tumorigenesis by altering the expression of several genes in ovarian tissues including altering mRNA expression of mRNAs such as CDK4, cyclin D1 and A (73-76).

Breast Cancer: The development of mammary glands and breast tumorigenesis is strongly influenced by oestrogen exposure and oestrogen signalling pathways. Several reports have demonstrated that foetal exposure to low doses of BPA alters cell proliferation, apoptosis, and development of mammary glands as well as increasing ductal density and sensitivity to oestrogens (77,78,79). All of these changes can be associated with breast cancer development. The exact mechanism of these changes have not been fully elucidated. Studies in rats exposed to BPA during intrauterine development reported an increase in hyperplastic ducts and incidence of mammary tumours (80,81). The increasing incidence of breast cancer over the last 40 years have resulted in strong case for the role of environmental factors in tumorigenesis. Human studies on exposure and cancer development are very limited. A study from South Korea collected blood samples between 1994 and 1997 from 167 adult females. These were then followed up after 10 years for development of breast cancer and the stored serum samples were analysed for BPA. They reported some association with BPA levels and risks of breast cancer in age matched subjects however overall there was no significant difference ($p=0.42$) in BPA levels between cases and controls (82).

Prostate Cancer: The role of steroid hormones in the development of these tumours has been well described, with both androgens and oestrogens being of significance. Rodent studies have demonstrated that BPA exposure affects prostate cells and cancer development, regulating proliferation and migration of prostate cancer cells (83-85).

2.2.4.2 Cardiac and metabolic effects

Metabolic syndrome, increased insulin resistance and overt type 2 diabetes mellitus have been associated with BPA exposure (43-45). Teppala et al (86) reported a positive association with elevated urinary BPA levels and the presence of metabolic syndrome. Their group also showed a positive correlation with urine BPA levels and the presence of hypertension and cardiovascular disease (87,88). Obesity and increased abdominal fat deposition have also been associated with higher BPA concentrations. BPA and its metabolites have been described as obesogens with evidence demonstrating the direct action of BPA on adipocyte differentiation and the expression of genes involved in adipogenesis (89). Mackay et al reported that female mice fed a high fat diet and exposed to BPA had higher feed intake and gained more weight than control mice fed the same diet. This supports evidence of neurological effects affecting weight and satiety with BPA exposure (90). Increased blood pressure in pregnancy and preeclampsia has been directly correlated with higher concentrations of urinary BPA (91,92).

2.2.4.3 Neurodevelopmental disorders

Steroid hormones play a critical role in the central nervous system development, brain organization of the neuroendocrine circuitry and behaviour. The developing brain contains receptors for steroid hormones and exposure to different levels of hormones in-utero co-ordinate sex specific physiology and behaviour. EDs may alter any of these developmental processes as well as the alteration of activity of appetite and satiety neurons. In addition to BPAs interaction with steroid hormone receptors in the central nervous system, it has also been demonstrated to effect thyroid hormone signalling. Several studies in both animals and humans have demonstrated associations between BPA exposure and neurobehavioral sequelae.

Animal studies have shown changes in the following systems due to BPA exposure

-dopaminergic system in the forebrain

-sex specific anxiety, depression and hyperactivity in rodent models

-memory impairment

-effect on gene expression involved in socio-sexual behaviour (93)

Braun et al, showed a positive association between gestational BPA levels and aggression and hyperactivity in children, with this association being stronger in female children (94). Another study in

a cohort of Korean children also demonstrated a positive relationship between child urinary BPA concentrations and attention problems (95).

2.2.4.5 BPA and bone

Oestrogens play a pivotal role in bone formation and resorption. The cellular components important for bone metabolism, osteoblasts and osteoclasts have shown to be affected by BPA exposure. Studies have reported the inhibition of differentiation and apoptosis of both cells when exposed to BPA in vitro (96, 97). NHANES studies have reported an association with higher urinary BPA levels and lower serum 25 hydroxy-Vitamin D concentrations (98). Additionally, as a steroid hormone vitamin D receptor and binding proteins may be affected by the binding of BPA. Studies reviewing bone mineral density in pre and post-menopausal did not show any association with BPA levels (99, 100).

2.2.5 Worldwide extent of BPA exposure

Population based studies have been performed in countries across the globe with notably fewer from Africa. Reported measured levels of BPA appear to vary, however the extent of exposure appears to be ubiquitous across age groups, geographical location, gender and race.

Estimated daily intake of BPA: In 2018, Huang et al reviewed urinary BPA concentration data from over 140 peer reviewed published studies originating from 30 different countries (101). Utilising the provided urine concentration data which was input into a previously published estimation equation they were able to calculate the daily BPA intake in ng/kg body weight (bw)/ day (102). Predictably, estimated values were overall higher in Europe and North America, with significant exposure in Asian nations as well. The highest estimation of BPA intake in adults was reported to be from Italy with an estimated 64.75 ng/kg bw/day. However, Ghana was ranked 8th in terms of adult estimated intake at around 40 ng/kg bw/day. It should be noted that the data from Ghana was based on a small sample number of 35. The estimated average global BPA intake was reported to be 30.76 mg/kg bw/day. Reports of human BPA intake via inhalation ranged from 0.008 to 9.9 ng/kg bw/ day whilst those in tap water ranged from 0.03- 15 ng/kg bw/day.

Findings in terms of estimated exposure in children and infants: Only 18 of the 30 countries had published data pertaining to childhood urinary BPA levels. Taiwan had the highest estimated child BPA daily intake with 201 ng/kg bw/day with Italy conversely having the lowest. Huang et al (101) did not show a linear relationship between adult and child BPA exposure in the nations that reported for both groups. When only reports with sample number (n) ≥1000 was utilised a linear relationship was observed ($R=0.956$ $p=<0.05$). Exposure in pregnant women and infants was also estimated. Of note the global estimated BPA daily intake in pregnant women was 42.03 ng/ kg bw/day which was higher than

that for the adult group. Limited data was available for estimation of infant BP exposure. This ranged from 7.93 ng/kg bw/day to 475.80 ng/kg bw/day. Notably, they reported the global estimate of BPA intake by children and pregnant women to be 1.4-2 times the general adult population (101).

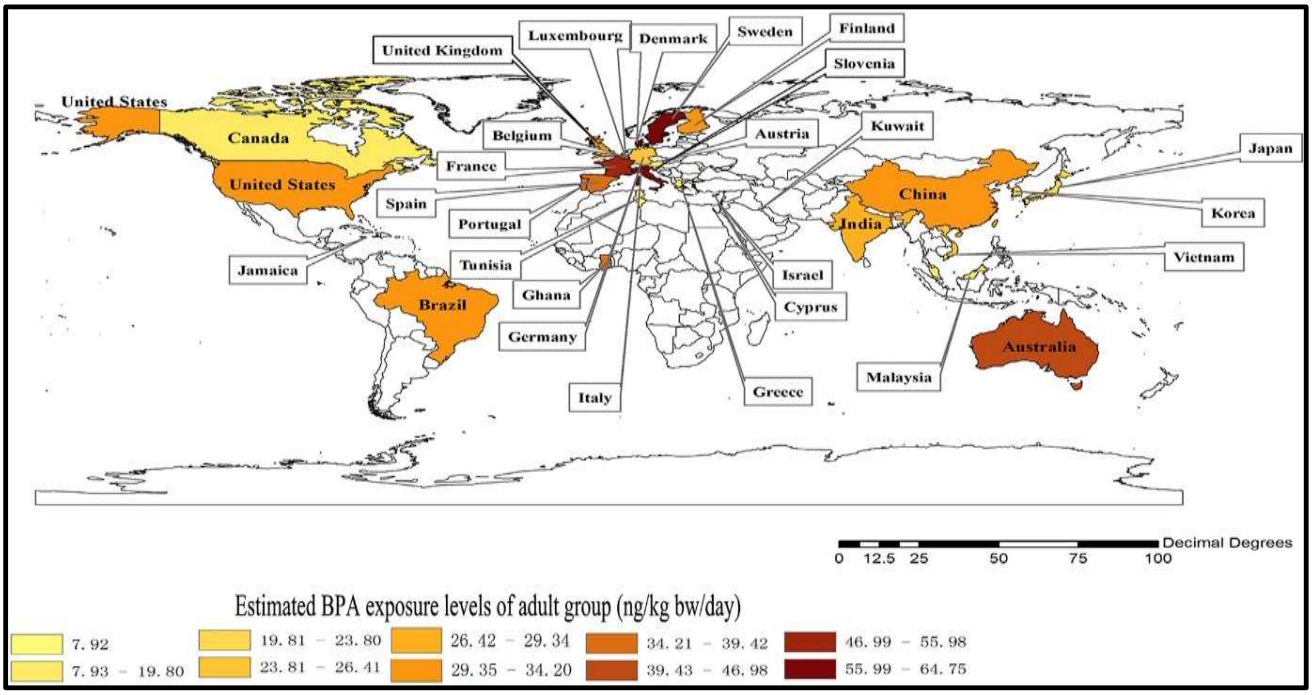

Figure 2.4 Estimated global BPA exposure amongst adults (101)

In a recent systematic review Rotimi et al (103) retrieved 42 publications arising from African countries describing measured BPA estimates in the environment, food sources and human biological fluids. Only 10 studies were retrieved that looked at human BPA levels either in urine, serum or other fluids. The range of BPA levels observed in the African studies were lower than that reported by Huang et al (101). Of the 10 studies five originated from Egypt. The highest values were seen in a study performed in obese children in Egypt with urine BPA concentrations ranging from 23.2 to 208.55 ng/mL (104).

2.2.6 Regulatory control of BPA exposure

Regulations banning or limiting the use of BPA in consumer products, in particular those used for infants, have been introduced in the United States, European Union and Canada, however use of BPA products remains largely unregulated across Africa. The recommended tolerable daily intake (TDI) of BPA varies depending on regulatory authority. In 2015 the European Food Safety Authority reduced their TDI from 50 to 4 μg/kg bw/day. In contrast South Korea and Japan still recommend a TDI of 50

μg/kg bw/day (105-107). As of 2012 the American Food and Drugs administration (FDA) banned the use of BPA in infant products such as baby bottles and formula packaging (108).

2.2.7 Mechanisms of BPA action

Molecular mechanisms that underlie the neurodevelopmental toxicity and sex-specific effects of BPA are not well understood (4,5). Most of the work examining the mechanisms of action have been performed in animal or in vitro models and this may not be directly translatable to humans.

2.2.7.1 Hormonal disruption

a) Receptor binding

BPA shares structural homology to endogenous steroid hormones in particular oestradiol.

BPA is a selective oestrogen receptor (ER) modulator that binds both, ERα and ERβ. BPA effects are typically attributed to its estrogenic or anti-oestrogenic action, however this action cannot completely account for the adverse effects of low potency BPA at the low-dose exposures that are commonly seen. BPA binds to these receptors with significantly lower affinity than oestradiol. Thus it is thought that BPA binding to receptors may actually induce alternative pathways that facilitate its actions at low concentrations (109). BPA has also been demonstrated to bind to the non-classical ER receptors, G-protein coupled oestrogen receptors (GEPR) and oestradiol related receptor (ERR) -□ (110,111). Takayanagi et al (112) reported that binding to the ERR- □ receptor is 80 times more potent that the ERα receptor. Of note it has also been reported that the concentration of ERR -□ in the placenta is higher than other oestrogen receptors and this may provide the explanation for the high BPA accumulation and its in-utero effects (112). BPAs action via the ER has been shown to induce cellular proliferation and thus promote tumorigenesis (113,114). Activation of calcium signalling pathways following binding to the membrane ER (mER) has been demonstrated to be an action of BPA as well as the promotion of cellular proliferation or apoptosis by binding to mER (115,116). Binding of BPA to GPER activates various secondary pathways including MAPK, STAT3 and ERK2 (117,118). BPA has been shown to act as an antagonist at the level of the androgen receptor. This has been shown to affect the functioning of endogenous androgens (119).

b) Steroidogenesis

BPA has been reported to interfere with ovarian steroidogenesis. The ovary is the key source of oestrogens. Cholesterol is the precursor of all steroids, and its transport from intracellular sources into the mitochondria is facilitated by the steroidogenic acute regulatory protein (STaR). This is the rate-limiting step in steroid biosynthesis. Cholesterol is acted upon by CYP11A1 and converted to pregnenolone and then acted upon by various other enzymes to form the various hormones produced by the ovary (120). Figure 2.5 below summarises the production of hormones within the ovary and steps in the pathway where BPA may interfere. Within the theca cells of the ovarian follicles, androgens are

produced and transported into the developing antral follicle where it is acted upon by CYP19A1 and converted to oestrogens (121). This pathway may be affected by BPA action (121).

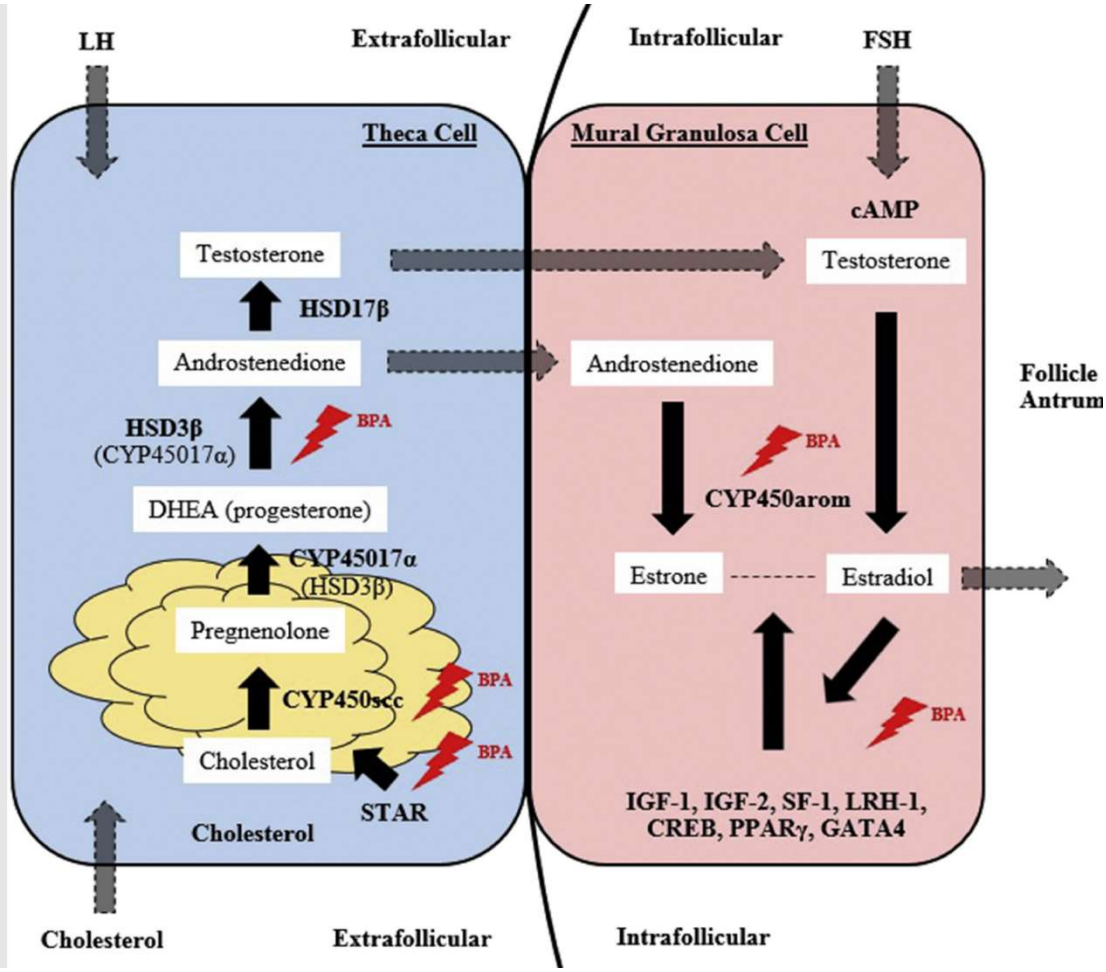

Figure 2.5: Production of hormones within the ovarian follicle and different enzymes and receptors that may be affected by BPA exposure, Bloom et al (122)

Key cAMP =cyclic adenosine monophosphate; CREB = cAMP-dependent response element binding protein; CYP450scc =P450 cholesterol side chain cleavage enzyme (CYP11A1); CYP45017a= 17a-hydroxylase-17,20-desmolase (CYP17A1); CYP450arom= cytochrome P450 aromatase (CYP19A1); HSD3b = 3b-hydroxysteroid dehydrogenase; HSD17b = 17b-hydroxysteroid dehydrogenase; IGF = insulin-like growth factor; LRH-1, liver receptor homologue 1; PPAR-g, peroxisome proliferator–activated receptor g; SF-1, steroidogenic factor 1; STAR, steroid acute regulatory protein

The effects of BPA on steroidogenesis are largely dependent on the dose and duration of exposure and also the developmental period at which exposure occurred. Most of the knowledge regarding BPAs effect on steroidogenesis comes from animal studies. Reports vary depending on several factors- animals used, dose and developmental stage. Fernandez et al (64), reported an increase in oestradiol and decrease in progesterone levels in rats exposed to BPA in the postnatal period. Other studies where

exposure occurred in utero did not show similar results (122). Low dose BPA exposure in adult rats reduced testosterone and oestradiol concentrations and decreased steroidogenic acute regulatory protein (StAR) and CYP19a1 expression (123). In vitro studies where mouse follicles were exposed to BPA resulted in a reduction of several sex steroid hormones being produced including progesterone, oestradiol and testosterone as well as decreasing StAR, CYP11a1, hydroxy-delta-5-steroid dehydrogenase and 3 beta-and steroid delta-isomerase 1 (HSD3B1) transcript expression (124). In humans, samples of oocytes obtained from women undergoing IVF showed that the extent of BPA exposure was negatively associated with oestradiol serum concentrations.

A positive correlation with elevated androgen levels and BPA has been demonstrated in several human studies. It has been proposed that ovarian theca-interstitial cells are stimulated to secrete androgens by the action of BPA (61,64). BPA has also been demonstrated to affect binding of sex hormone binding globulin to testosterone. This in turn results in higher levels of free testosterone/androgens in circulation and disrupts the circulatory oestradiol: testosterone ratios (125). The BPA metabolising hormone UDP-GT found in the liver is down-regulated by the action of androgens. Thus higher androgen levels may lead to decreased metabolism of BPA and further increase in androgens synthesis (126).

c) Hypothalamic -pituitary gonadal axis

A study examining BPA exposed and unexposed female factory workers in China revealed a significant positive correlation with urinary BPA levels and elevated serum prolactin and progesterone (127). Gonadotrophin releasing hormone (GnRH) is produced by the hypothalamus and acts on the anterior pituitary to secrete FSH and LH which are important with regards to onset of puberty as well as regulating normal ovulation, fertility, steroid hormone and sperm production. Various studies have demonstrated an effect of BPA exposure on gonadotrophin releasing hormone as well as FSH and LH in both genders (128,129). One study demonstrated an increase in GnRH following BPA exposure leading to inhibition of LH release (130,131). The mechanism for this effect on GnRH is thought to be related to the effect of BPA in regulating kisspeptin expression (14). Kisspeptin is a peptide produced by the hypothalamus that acts upstream of GnRH and plays a regulatory role in onset of puberty, sex hormone dependent secretion of gonadotrophins and fertility (132). BPA has also been shown to disrupt the hypothalamic pituitary adrenal and thyroid axes. BPA binds to corticoid receptors competitively and can regulate the expression of these receptors. BPA can also disrupt the release of TSH via its action on the pituitary (14).

d) Obesogen

BPA has been described as an obesogen with various actions of altering lipid and fat storage, disruption of energy balance and promotion of fat accumulation. BPA has shown to decrease adiponectin secretion (133). In animal studies, pregnant mice subjected to BPA had significantly higher body weights for

four months after delivery that their matched controls. Furthermore, exposure during pregnancy led to the development of insulin resistance and weight increase in these mice (134,135).

BPA has been reported to influence the expression of 11 □hydroxy-steroid dehydrogenase type 1 (11□HSD1) (which promotes adipogenesis) and to promote lipid accumulation in adipocytes. In a study examining omental fat from children that required abdominal surgery it was shown the BPA concentrations was associated with higher 11□HSD1 mRNA expression (136). BPA may also have a direct impact on pancreatic cell function in turn affecting glucose homeostasis. Pancreatic beta cells also express endocrine receptors (ERs) (137). Binding of BPA or oestradiol to the pancreatic ERs results in activation of ERK 1/2 signalling and regulates insulin content (138,139). Additionally, BPA affects the activity of K-ATP channels which are integral for the secretion of insulin from the beta cells. These effects in turn have been shown to cause a 2-fold increase in release of insulin following glucose stimulation (140).

2.2.7.2 Inflammation and oxidative stress

Murine studies have demonstrated an upregulation of pro-inflammatory cytokines TNF-α and interleukin 6 when microglial cells were exposed to BPA. The same study also demonstrated that BPA activates the pro-inflammatory transcription factor NF-kB (141). Cellular components of both the innate and adaptive immune system have been shown to be affected by BPA exposure during key developmental periods. This may accelerate the development of autoimmune disease in particular type 1 diabetes in females (142).

2.2.7.3 DNA adduct formation

A study performed in rats reported the conversion of BPA to Bisphenol-o-quinone by the action of microsomal p450 enzymes in the rat liver. The bisphenol-o-quninone acts as a DNA adduct, forming in the rat liver and mammary gland (14). The formation of DNA adducts can interfere with DNA transcription and replication resulting in mutation induction in affected tissues and the formation of tumours. DNA adducts may also induce germ cell mutations that have be associated with developmental defects (143).

2.2.7.4 BPA induced DNA damage

In vitro studies in animal and human cells have shown evidence of the direct DNA damaging effects of BPA. When normal breast cells were exposed to BPA an upregulation of DNA repair genes and downregulation of apoptotic genes was reported (14).

2.2.7.5 Epigenetic modifications

Recent research suggests that long-lasting effects of prenatal BPA exposure likely involve the disruption of epigenetic programming during development (144,145). Epigenetics refers to a change of gene expression that is independent of the DNA sequence. It is essentially a change in phenotype expressed without a change in genotype. The mechanism by which this occurs may be as a result of chemical modifications of the DNA (for example, methylation of CpG dinucleotides within gene promoters) or the physical accessibility of the DNA by virtue of its association with histones (for example histone acetylation), non-histone proteins, or noncoding RNA. Epigenetic changes are capable of being passed on to somatic daughter cells and in some cases to offspring via the germline. This is known as translational genetic inheritance. Changes in the epigenome occurring during embryonic development will have a much greater impact on the overall epigenetic status of the organism since these changes can be transmitted over consecutive mitotic divisions (146,147). Exposure to BPA has been shown to induce several epigenetic modifications in both animal and human cells. BPA may not only affect the methylation patterns for several genes but could also directly influence the genes responsible for methylation (148).

Table 2.3 Summary of evidence for BPA role in epigenetic changes in animal and human studies

Finding	Reference
In pregnant mice BPA exposure has been demonstrated to induce upregulation of DNA methyl transferase expression	Ye et al, 2019 (149)
In animal studies, BPA has been shown to affect DNA methylation of genes involved in brain development and function, such as Bdnf, Fkbp5, and Grin2b	Xu et al, 2010 (150) Kitraki et al, 2015 (151)
Histone acetylation (H3K9Ac and H3K27Ac) enhanced in spermatozoa and embryos from male zebrafish exposed BPA	Lombo et al, 2019 (152)
Hypermethylation of Erα and Erβ in testes in rat studies	Doshi et al, 2011 (153)
Murine studies hypomethylation of maternally imprinted genes Igf2r, in BPA exposed group	Trapphoff et al, 2013 (154)
Porcine studies upregulation and downregulation of miRNAS in females affecting genes involved in insulin signalling	Savabieasfahani et al, 2006 (155)
Murine: reduced DNA methylation of genes involved in fatty acid and cholesterol metabolism in liver	Ke et al, 2016 (156)
Human breast epithelial cell line genome wide changes in DNA methylation and global gene expression changes, hypermethylation of cancer related genes BRCA1, CCNA, CDKN2A	Fernandez et al, 2012 (157); Qin et al, 2012 (158)

2.2.8 Challenges with measurement of BPA

There are several challenges with the measurement of BPA in humans.

- Rapid inactivation of ingested BPA and clearance via urine.
  This has possibly led to discrepancies between pharmacokinetic models and the results of epidemiologic studies.

- Different bodily fluids used for BPA analysis.
  Urine is easily available but does not indicate long term exposure and would need to be repeated over a period of time to establish ongoing levels of BPA exposure. The same applies to serum specimens. However, serum is able to show the presence of active BPA in circulation. Other matrices such as amniotic fluid may require more technically demanding and time-consuming sample preparation in order for measurements to be made.

- Analytical methodologies

Earlier studies utilised the less specific and less sensitive immunoassay (ELISA) based techniques. These methods are subject to greater interferences than HPLC and mass spectrometry methods. There is also currently a lack of standardisation of methods for the measurement of BPA and this results in non-transferability of results across different units.

- Exogenous contamination of BPA in biological specimens
  Due to the ubiquitous presence of BPA in the environment, contamination of specimens during routine collection and analysis is a concern. The use of plastic tubes, lab containers and other plastic containing equipment used in the laboratory and specimen processing may contribute to contamination (159,160).

Hence, careful laboratory procedures and analysis with sensitive and specific methodologies are required to obtain valid data regarding the extent of BPA exposure.

.

2.3 References

1. EPA report on endocrine disruptors https://www.epa.gov/endocrine-disruption. (Accessed 17 September 2016)
2. Kandarakis ED, Bourguignon JP, Giudice L et al, Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement Endocrine Reviews 2009; 30: 293–342,
3. Skinner MK. Endocrine Disruptors and Epigenetic Transgenerational Disease Etiology. Paediatric research 2007;61: 48-50.
4. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. Human exposure to bisphenol A (BPA). Reproductive Toxicology 2007; 24:139–77.
5. National Toxicology Program. NTP-CERHR Expert Panel Report on the Reproductive and Developmental Toxicity of Bisphenol A. Durham:Research Triangle Park; 2007
6. Snyder SA,Westerhoff P, Yoon Y and Sedlak DL. Pharmaceuticals, personal care products and endocrine disruptors in water. Implications for the water industry. Environmental Engineering Science 20; 2003: 449-469.
7. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins G, Soto AM, et al. Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement, Endocrine Reviews 2009; 30:293–342.
8. Michałowicz J. Bisphenol A--sources, toxicity and biotransformation. Environmental Toxicology and Pharmacology 2014; 37: 738-58.
9. Jalal N, Surendranath AR, Pathak JL, Yu S, Chung CY. Bisphenol A (BPA) the mighty and the mutagenic. Toxicology Reports 2018; 5:76-84.
10. A, Bisphenol (BPA) Chemical Profile Asia Phenol; 2016 http://www/icis.com/resources/news/2016 (last accessed 14 Jan 2018)
11. Industry experts (2016). Bisphenol A- A global market overview. Available: https://industry-experts.com/verticals/chemicals-and-materials/bisphenola-a-global-market-overview. Accessed 12 October 2021
12. Groshart CP, Okkeman PC, Pijnenburg AM. Chemical Study on Bisphenol A. Rapprtnr; 2001: 027.2001
13. National Center for Biotechnology Information. PubChem Compound Summary for CID 6623, Bisphenol A. https://pubchem.ncbi.nlm.nih.gov/compound/Bisphenol-A. Accessed June. 21, 2021.
14. Ma Y, Liu H, Wu J, Yuan L, Wang Y, Du X et al. The adverse health effects of Bisphenol A and related toxicity mechanisms. Environmental research 2019; 176:108575.
15. EFSA Panel on Food contact materials and Aids 2015. Scientific opinion on the risks to public health related to the presence of Bisphenol A (BPA) in foodstuffs 2015;13: 3978.

16. Geens T, Aerts D, Berthot C, Bourguignon JP, Goeyens L, Lecomte P et al. A review of dietary and non-dietary exposure to Bisphenol A. Food Chem Toxicol. 2012; 50: 3725-3740.
17. Mendonca K, Hauser R, Calafat AM, Arbuckle TE, Duty SM. Bisphenol A concentrations in maternal breast milk and infant urine. International Archives of Occupational and Environmental Health 2014; 87:13-20.
18. Ashley-Martin J, Dodds L, Levy AR, Platt RW, Marshall JS, Arbuckle TE. Prenatal exposure to phthalates, bisphenol A and perfluoroalkyl substances and cord blood levels of IgE, TSLP and IL-33. Environmental Research 2015; 140: 360-8.
19. Teeguarden JG, Waechter JM, Clewell HJ, Covington TR, Barton HA, Evaluation of Oral and Intravenous Route Pharmacokinetics, Plasma Protein Binding, and Uterine Tissue Dose Metrics of Bisphenol A: A Physiologically Based Pharmacokinetic Approach, Toxicological Sciences 2005;85: 823–838.
20. Pinney SE, Mesaros CA, Snyder NW, Busch CM, Xiao R, Aijaz S, Ijaz N, Blair IA, Manson JM. Second trimester amniotic fluid Bisphenol A concentration is associated with decreased birth weight in term infants. Reproductive Toxicology 2017; 67:1-9.
21. Sakhi AK, Lillegaard IT, Voorspoels S, Carlsen MH, Løken EB, Brantsæter AL, et al.Concentrations of phthalates and Bisphenol A in Norwegian foods and beverages and estimated dietary exposure in adults. Environment International 2014; 73:259-69.
22. Chen W, Shen Y and Chen S, Assessing Bisphenol A (BPA) exposure risk from long-term dietary intakes in Taiwan. Science of the Total Environment 2016; 543 :140-146.
23. Martinez MA, Rovira J, Sharma RP, Nadal M, Schumacher M, Kumar V. Comparing dietary and non-dietary source contribution of BPA and DEHP to prenatal exposure. A Catalonia (Spain) case study. Environmental Research 2018; 166:25-34.
24. Babalola, B.A. Bisphenol-A (BPA) in Foods commonly consumed in Southwest Nigeria and its Human Health Risk. Scientific Reports 2019;9, 17458.
25. Elobeid MA, Almarhoon ZM, Virk P, Hassan ZK, Omer SA, ElAmin M et al. Bisphenol A Detection in Various Brands of Drinking Bottled Water in Riyadh, Saudi Arabia using Gas chromatography/mass spectrometer. Tropical Journal of Pharmaceutical Research 2012; 11: 455-459.
26. Corrales J, Kristofco LA, Steele WB, et al. Global Assessment of Bisphenol A in the Environment: Review and Analysis of Its Occurrence and Bioaccumulation. Dose Response. 2015; 13:1559325815598308.
27. Farounbi, A.I., Ngqwala, N.P. Occurrence of selected endocrine disrupting compounds in the eastern cape province of South Africa. Environmental Science and Pollution Research 2020;27**:** 17268–17279.

28. Arnold SM, Clark KE, Staples CA, et al. Relevance of drinking water as a source of human exposure to Bisphenol A. Journal of Exposure Science and Environmental Epidemiology 2013; 23:137-144.
29. Lorber M, Schecter A, Paepke O, Shropshire W, Chirstensen K, Birnbaum L. Exposure assessment of adult intake of bisphenol A (BPA) with emphasis on canned food dietary exposures. Environment International 2015; 77:55-62.
30. Liu M, Jia S, Dong T, Han Y, Xue J, Wanjaya ER et al. The occurrence of bisphenol plasticizers in paired dust and urine samples and its association with oxidative stress. Chemosphere 2019; 216:472-478.
31. Loganathan SN, Kannan K. Occurrence of bisphenol A in indoor dust from two locations in the eastern United States and implications for human exposures. Archives of Environmental Contamination and Toxicology 2011; 61:68-73.
32. Alkasir RS, Rossner A, Andreescu S. Portable colorimetric paper based biosensing device for the assessment of bisphenol A indoor dust. Environmental Science and Technology. 2015;49: 9889-9897.
33. Geens T, Roosens L, Neels H, Covaci A. Assessment of human exposure to Bisphenol A, Triclosan, and Tetrabromobisphenol A through indoor dust intake in Belgium. Chemosphere 2009; 76:755-760.
34. Hines CJ, Jackson MV, Deddens JA, Clark JC, Ye X, Chistianson AL et al. Urinary bisphenol A (BPA) concentrations amongst workers in industries that manufacture and use BPA in the USA. Annals of Work Exposure and Health 2017; 61: 164-182.
35. Bernier MR, Van den Berg LN. Handling of thermal paper. Implications for dermal exposure to Bisphenol A and its alternatives, PLoS One 2017;6: e0178449.
36. Becher R, Wellendorf H, Sakhi AK, Samuelson JT, Thomsen C, Bolling AK et al. Presence and leaching of Bisphenol A (BPA) from dental materials. Acta Biomaterialia Odontologica Scandinavica 2018; 4: 56-62.
37. Bao W, Liu B, Rong S, Dai SY, Trasande L, Lehmler H. Association Between Bisphenol A Exposure and Risk of All-Cause and Cause-Specific Mortality in US Adults. Journal of American Medical Association Network Open 2020; e2011620.
38. Ikezuki, Y., Tsutsumi, O., Takai, Y., Kamei, Y. & Taketani, Y. Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. Human reproduction *2002;* 17: 2839–2841.
39. Leclerc F, Dubois MF, Aris A. Maternal, placental and fetal exposure to bisphenol A in women with and without preeclampsia. Hypertension and Pregnancy 2014; 33: 341–348
40. Cao XL, Zhang J, Goodyer CG, et al. Bisphenol A in human placental and fetal liver tissues collected from Greater Montreal area (Quebec) during 1998-2008. Chemosphere. 2012; 89: 505–511.

41. Edlow AG, Chen M, Smith NA, et al. Fetal Bisphenol A exposure: concentration of conjugated and unconjugated Bisphenol A in amniotic fluid in the second and third trimesters. Reproductive Toxicology 2012; 34: 1–7.
42. Philippat C, Wolff MS, Calafat AM, et al. Prenatal exposure to environmental phenols: concentrations in amniotic fluid and variability in urinary concentrations during pregnancy. Environmental Health Perspectives 2013; 121: 1225–1231.
43. Hwang, S., Lim, Je., Choi, Y. et al. Bisphenol A exposure and type 2 diabetes mellitus risk: a meta-analysis. BMC Endocrine Disorders 2018; 18**:** 81
44. Shankar A, Teppala S. Relationship between urinary bisphenol A levels and diabetes mellitus. Journal of Clinical Endocrinology and Metabolism 2011; 96: 3822-6.
45. LaKind JS, Goodman M and Mattison DR. Bisphenol A and indicators of obesity, glucose metabolism/type 2 diabetes and cardiovascular disease: A systematic review of epidemiologic research, Critical Reviews in Toxicology 2014; 44: 121-150
46. Wisniewski, P., Romano, R.M., Kizys, M.M., Oliveira, K.C., Kasamatsu, T., Giannocco, G, et al. Adult exposure to bisphenol A (BPA) in Wistar rats reduces sperm quality with disruption of the hypothalamic-pituitary-testicular axis. Toxicology 2015; 329:1–9.
47. Ferguson, K.K., Peterson, K.E., Lee, J.M., Mercado-Garcia, A., Blank-Goldenberg, C Tellez-Rojo, M.M., et al. Prenatal and peripubertal phthalates and bisphenol A in relation to sex hormones and puberty in boys. Reproductive Toxicology 2014; 47: 70–76.
48. Akin, L., Kendirci, M., Narin, F., Kurtoglu, S., Saraymen, R., Kondolot, M., et al. The endocrine disruptor Bisphenol A may play a role in the aetiopathogenesis of polycystic ovary syndrome in adolescent girls. Acta Paediatrica 2015; 104: e171–177.
49. Vahedi, M., Saeedi, A., Poorbaghi, S.L., Sepehrimanesh, M., Fattahi, M. Metabolic and endocrine effects of Bisphenol A exposure in market seller women with polycystic ovary syndrome. Environmental Science and Pollution Research International 2016;23: 23546–23550.
50. Ji, H., Miao, M., Liang, H., Shi, H., Ruan, D., Li, Y., et al. Exposure of environmental Bisphenol A in relation to routine sperm parameters and sperm movement characteristics among fertile men. Scientific Reports 2018; 8: 17548.
51. Li, D., Zhou, Z., Qing, D., He, Y., Wu, T., Miao, M., et al. Occupational exposure to Bisphenol-A (BPA) and the risk of self-reported male sexual dysfunction. Human Reproduction 2010; 25: 519–527.
52. Ferguson KK, Chin HB. Environmental chemicals and preterm birth: Biological mechanisms and the state of the science. Current Epidemiology Reports 2017;4: 56-71.
53. Zhang Y, Mustieles V, Williams PL, Yland J, Souter I, Braun MJ et al. Prenatal urinary concentrations of phenols and risk of preterm birth: exploring windows of vulnerability, Fertility and Sterility 2021; 3:53.

54. Available on Rare Diseases website: https://rarediseases.org/rare-diseases/precocious-puberty/ Accessed on 6th July 2020

55. Sørensen K, Mouritsen A, Aksglaede L, Hagen C, P, Mogensen S, S, Juul A: Recent Secular Trends in Pubertal Timing: Implications for Evaluation and Diagnosis of Precocious Puberty. Hormone Research in Paediatrics 2012; 77:137-145.

56. Parent AS, Teilmann G, Juul A, Skakkebaek NE, Toppari J, Bourguignon JP. The timing of normal puberty and the age limits of sexual precocity: variations around the world, secular trends, and changes after migration. Endocrine Reviews 2003; 24: 668–93.

57. Gluckman PD, Hanson MA. Changing times: the evolution of puberty. Molecular and Cellular Endocrinology 2006;254–255:26–31.

58. Leonardi A, Cofini M, Rigante D, et al. The Effect of Bisphenol A on Puberty: A Critical Review of the Medical Literature. International Journal of Environmental Research and Public Health 2017; 14:1044.

59. Trasande L., Attina T.M., Blustein J. Association Between Urinary Bisphenol A Concentration and Obesity Prevalence in Children and Adolescents. Journal of American Medical Association 2012; 308:1113.

60. Li D.-K., Miao M., Zhou Z., Wu C., Shi H., Liu X., Yuan W. Urine bisphenol-A level in relation to obesity and overweight in school-age children. *PLoS ONE.* 2013; 8: e65399. doi: 10.1371/journal.pone.0065399.

61. Norman RJ, Dewailly D, Legro RS, Hickey TE, Polycystic ovary syndrome, The Lancet, 2007;370: 685-697.

62. Kandaraki E, Chatzigeorgiou A, Livadas S, Palioura E, Economou F, Koutsilieris M, et al. Endocrine disruptors and polycystic ovary syndrome (PCOS): elevated serum levels of Bisphenol A in women with PCOS. Journal of Clinical Endocrinology and Metabolism 2011;96: E480-4.

63. Iram AK, Akbar M, Mohd AG, Qudsia F, Humira J, Saika M, et al Bisphenol A (BPA) acts as an endocrine disruptor in women with Polycystic Ovary Syndrome: Hormonal and metabolic evaluation, Obesity Medicine 2019; 14: 100090

64. Fernandez, M., Bourguignon, N., Lux-Lantos, V., and Libertun, C. Neonatal exposure to bisphenol A and reproductive and endocrine alterations resembling the polycystic ovarian syndrome in adult rats. Environmental Health Perspectives 2010; 118: 1217–1222.

65. Takeuchi T, Tsutsumi O, Ikezuki Y, Takai Y, Taketani Y.Positive relationship between androgen and the endocrine disruptor, bisphenol A in normal women and women in ovarian dysfunction. Endocrine Journal 2004;51; 165-9

66. Bai W, Oliveros-Saunders B, Wang Q, Acevedo-Duncan ME, Nicosia SV. Estrogen stimulation of ovarian surface epithelial cell proliferation. In Vitro Cellular and Developmental Biology -Animal. 2000; 36: 657–666

67. O'Donnell AJ, Macleod KG, Burns DJ, Smyth JF, Langdon SP. Estrogen receptor-alpha mediates gene expression changes and growth response in ovarian cancer cells exposed to estrogen. Endocrine Related Cancer. 2005; 12: 851–866.
68. Soto AM and Sonnenschein C. Environmental causes of cancer: endocrine disruptors as carcinogens. Nature Reviews Endocrinology 2010; 6: 363–370.
69. Lacey JV, Jr, Mink PJ, Lubin JH, Sherman ME, Troisi R, Hartge P, Schatzkin A, Schairer C. Menopausal hormone replacement therapy and risk of ovarian cancer. Journal of American Medical Association 2002; 288: 334–341.
70. Suzuki A, Sugihara A, Uchida K, Sato T, Ohta Y, Katsu Y, Watanabe H, Iguchi T. Developmental effects of perinatal exposure to Bisphenol-A and diethylstilbestrol on reproductive organs in female mice. Reproductive Toxicology 2002; 16: 107–116.
71. Rodríguez HA, Santambrosio N, Santamaría CG, Muñoz-de-Toro M, Luque EH. Neonatal exposure to bisphenol A reduces the pool of primordial follicles in the rat ovary. Reproductive Toxicology 2010; 30: 550–557.
72. Markey CM, Coombs MA, Sonnenschein C, Soto AM. Mammalian development in a changing environment: Exposure to endocrine disruptors reveals the developmental plasticity of steroid-hormone target organs. Evolution and Development 2003; 5: 67–75.
73. Ptak A, Gregoraszczuk EL. Bisphenol A induces leptin receptor expression, creating more binding sites for leptin, and activates the JAK/Stat, MAPK/ERK and PI3K/Akt signalling pathways in human ovarian cancer cell. Toxicology Letters 2012; 210: 332–337.
74. Bredhult C, Bäcklin BM, Olovsson M. Effects of some endocrine disruptors on the proliferation and viability of human endometrial endothelial cells in vitro. Reproductive Toxicology. 2007;23: 550–559
75. Hall JM, Korach KS. Endocrine disrupting chemicals promote the growth of ovarian cancer cells via the ER-CXCL12-CXCR4 signaling axis. Molecular Carcinogenesis 2013;52: 715–725.
76. Cavallaro U, Christofori G. Multitasking in tumor progression: Signaling functions of cell adhesion molecules. Annals of the New York Academy of Science 2004;1014: 58–66.
77. Gao H, Yang BJ, Li N, et al. Bisphenol A and hormone-associated cancers: current progress and perspectives. Medicine (Baltimore*)* 2015;94: e211.
78. Fenton SE. Endocrine-disrupting compounds and mammary gland development: Early exposure and later life consequences. Endocrinology. 2006;147(Suppl):S18–S24.
79. Fernandez SV, Russo J. Estrogen and xenoestrogens in breast cancer. Toxicologic Pathology. 2010; 38: 110–122.
80. Acevedo N, Davis B, Schaeberle CM, Sonnenschein C, Soto AM. Perinatally administered bisphenol a as a potential mammary gland carcinogen in rats. Environmental Health Perspectives 2013; 121: 1040–1046.

81. Hofseth LJ, Raafat AM, Osuch JR, Pathak DR, Slomski CA, Haslam SZ. Hormone replacement therapy with estrogen or estrogen plus medroxyprogesterone acetate is associated with increased epithelial proliferation in the normal postmenopausal breast. Journal of Clinical Endocrinology and Metabolism 1999; 84: 4559–4565
82. Yang M, Ryu JH, Jeon R, Kang D, Yoo KY. Effects of bisphenol A on breast cancer and its risk factors. Archives of Toxicology 2009; 83:281-5.
83. Keri RA, Ho SM, Hunt PA, Knudsen KE, Soto AM, et al. An evaluation of evidence for the carcinogenic activity of bisphenol A. Reproductive Toxicology 2017; 24: 240–252
84. Tse LA, Lee PMY, Ho WM, Lam AT, Lee MK, Ng SSM, et al. Bisphenol A and other environmental risk factors for prostate cancer in Hong Kong. Environment International. 2017; 107:1-7.
85. Huang, DY., Zheng, CC., Pan, Q. *et al.* Oral exposure of low-dose bisphenol A promotes proliferation of dorsolateral prostate and induces epithelial–mesenchymal transition in aged rats. Scientific Reports 2018; 8**:** 490.
86. Teppala, S., Madhavan, S., Shankar, A. Bisphenol A and metabolic syndrome: Results from NHANES. International Journal of Endocrinology 2012; 10: 598180.
87. Shankar A and Teppala S. Urinary bisphenol A and hypertension in a multi-ethnic sample of US adults. Journal of Environment and Public Health. 2012; 10: 481641
88. Shankar A, Teppala S and Sabanayagam C. Bisphenol A and peripheral arterial disease: Results from the NHANES. Environmental Health Perspectives 2012 ;120: 1297–1300.
89. Rubin BS, Schaeberle CM, Soto AM. The Case for BPA as an Obesogen: Contributors to the Controversy Frontiers in Endocrinology 2019; 10: 30.
90. Mackay H, Patterson ZR, Khazall R, Patel S, Tsirlin D, Abizaid A. Organizational effects of perinatal exposure to Bisphenol-A and diethylstilbestrol on arcuate nucleus circuitry controlling food intake and energy expenditure in male and female CD-1 mice. Endocrinology 2013;154 :1465-75.
91. Ye Y, Zhou Q, Feng L, Wu J, Xiong Y, Li X. Maternal serum Bisphenol A levels and risk of pre-eclampsia: A nested case-control study. European Journal of Public Health 2017; 27:1102–1107.
92. Ye Y, Tang Y, Xiong Y, Feng L, Li X. Bisphenol A exposure alters placentation and causes preeclampsia-like features in pregnant mice involved in reprogramming of DNA methylation of 72. Federation of American Societies for Experimental Biology Journal 2019; 33: 2732–2742.
93. Schug TT, Blawas AM, Gray K, Heindel J, and Lawler CP. Elucidating the Links Between Endocrine Disruptors and Neurodevelopment Endocrinology 2015; 156: 1941–1951.
94. Braun JM, Kalkbrenner AE, Calafat AM, et al. Impact of early-life Bisphenol A exposure on behaviour and executive function in children. Pediatrics 2011; 128: 873-882.

95. Yoo SJ, Joo H, Kim D, Lim MH, Kim E, Ha M, et al. Associations between Exposure to Bisphenol A and Behavioral and Cognitive Function in Children with Attention-deficit/Hyperactivity Disorder: A Case-control Study. Clinical Psychopharmacology and Neuroscience 2020; 18: 261-269.

96. Suzuki N, Hattori A, Bisphenol A suppresses osteoclastic and osteoblastic activitiesin the cultured scales of goldfish. Life Sciences 2003;73: 2237–2247.

97. Chamorro-García R, Kirchner S, Li X, Janesick A, Casey SC, Chow C,et al, Bisphenol A diglycidyl ether induces adipogenic differentiation of multipotent stromal stem cells through a peroxisome proliferator-activated receptor gamma-independent mechanism, Environmental Health Perspectives 2012;120:984–989.

98. Johns LE, Ferguson KK, Meeker JD. Relationships Between Urinary Phthalate Metabolite and Bisphenol A Concentrations and Vitamin D Levels in U.S. Adults: National Health and Nutrition Examination Survey (NHANES), 2005-2010. Journal of Clinical Endocrinology and Metabolism 2016; 101: 4062-4069.

99. Zhao HY, Bi YF, Ma LY, Zhao L, Wang TG, Zhang LZ. The effects of Bisphenol A (BPA) exposure on fat mass and serum leptin concentrations have no impact onbone mineral densities in non-obese premenopausal women. Clinical Biochemistry 2012:45; 1602–1606

100. Kim DH, Oh CH, Hwang YC, Jeong IK, Ahn KJ, Chung HY, Chang JS, Serum Bisphenol a concentration in postmenopausal women with osteoporosis. Journal of Bone Metabolism 2012; 19: 87–93.

101. Huang R, Liu Z, Yuan S, Yin H, Dang Z, Wu P, Worldwide human daily intakes of bisphenol A (BPA) estimated from global urinary concentration data (2000–2016) and its risk analysis. Environmental Pollution 2017; 230: 143-152.

102. LaKind J, Levesque J, Dumas P, et al. Comparing United States and Canadian population exposures from National Biomonitoring Surveys: Bisphenol A intake as a case study. Journal of Exposure Science and Environmental Epidemiology 2012; 22**:** 219–226.

103. Rotimi OA, Olawole TD, De Campos OC, Adelani IB, Rotimi SO. Bisphenol A in Africa: A review of environmental and biological levels. Science of the Total Environment 2021; 764: 142854.

104. Abo El-Atta HM, El-Mansoury AM, El-Hawary AK, Adbel-Naby ME, Helmy MA. Bisphenol Aand risk of obesity among a sampley of Egyptian children: role of adiponectin as a biomarker of exposure. Mansoura Journal of Forensic Medicine and Clinical Toxicology 2018; 26: 39-52.

105. https://www.efsa.europa.eu/en/topics/topic/bisphenol#:~:text=Exposure%20from%20the%20diet%20or,kg%20of%20bw%2Fday). Accessed 30 June 2021

106. KFDA (Korea Food and Drug Administration). (2008). Standard limits and regulations for utensils, containers, and packing materials. KFDA No. 2008–111. (Korean) Retrieved from https://fse.foodnara.go.kr Accessed on 3 July 2021

107. Masuyama, K. The amendment of the specification on plastic packages. Japanese Food Sanitation Research,1994; 44: 9– 27. (in Japanese)

108. Federal Drug Administration(FDA) website: https://www.fda.gov/food/food-additives-petitions/bisphenol-bpa. Accessed on 4 January 2021

109. Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, Sonnenschein C, et al. In vitro molecular mechanisms of Bisphenol A action. Reproductive Toxicology, 2007; 24: 178– 198.

110. Watson CS, Bulayeva NN, Wozniak AL and Alyea RA. Xenoestrogens are potent activators of nongenomic estrogenic responses. Steroids, 2007;72: 124– 134.

111. Takayanagi S, Tokunaga T, Liu X, Okada H, Matsushima A, Shimohigashi Y. Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor γ (ERRγ) with high constitutive activity. Toxicology Letters 2006; 167: 95– 105.

112. Takeda Y, Liu X, Sumiyoshi M, Matsushima A, Shimohigashi M and Shimohigashi Y. Placenta expressing the greatest quantity of bisphenol A receptor ERRγ among the human reproductive tissues: Predominant expression of type-1 ERRγ isoform. Journal of Biochemistry 2009; 146:113– 122.

113. Hall JM, Korach KS. Endocrine disrupting chemicals promote the growth of ovarian cancer cells via the ER-CXCL12-CXCR4 signaling Axis. Molecular Carcinogenesis 2013;52 :715–725.

114. Lee GA, Hwang KA, Choi KC. Inhibitory effects of 3,3'-diindolylmethane on epithelial-mesenchymal transition induced by endocrine disrupting chemicals in cellular and xenograft mouse models of breast cancer. Food and Chemical Toxicology 2017; 109:284–295.

115. Chevalier N, Vega A, Bouskine A, Siddeek B, Michiels JF, Chevallier D et al. GPR30, the non-classical membrane G protein related estrogen receptor, is overexpressed in human seminoma and promotes seminoma cell proliferation. PLoS One 2012; 7: e34672.

116. Gonzalez-Rojo S, Lombo M, Fernandez-Diez C, Herraez MP. Male exposure to bisphenol a impairs spermatogenesis and triggers histone hyperacetylation in zebrafish testes. Environmental Pollution 2019; 248:368–379.

117. Castillo Sanchez, R., Gomez, R., Perez Salazar, E. Bisphenol A induces migration through a GPER-, FAK-, Src-, and ERK2-dependent pathway in MDA-MB-231 breast cancer cells. Chemical Research and Toxicology 2016; 29: 285–295.

118. Thomas P and Dong J. Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: A potential novel mechanism of endocrine disruption. Journal of Steroid Biochemistry and Molecular Biology 2006;102: 175–179.
119. Wang H, Ding Z, Shi QM, Ge X, Wang HX, Li MX, et al. Anti-androgenic mechanisms of Bisphenol A involve androgen receptor signaling pathway. Toxicology 2017;387: 10–16.
120. Bose HS, Lingappa VR, Miller WL. Rapid regulation of steroidogenesis by mitochondrial protein import. Nature 2002; 417:87-91
121. Hillier SG, Whitelaw PF, Smyth CD. Follicular oestrogen synthesis the two-cell two gondotrophin model revisited. Molecular and Cellular Endocrinology 1994; 100:51-4
122. Bloom MS, Mok-Lin E and Fujimoto VY. Bisphenol A and ovarian steroidogenesis. Fertility and Sterility 2016:106:857-863
123. Zhou W, Liu J, Liao L, Han S, Liu J. Effect of bisphenol A on steroid hormone production in rat ovarian theca-interstitial and granulosa cells. Molecular and Cellular Endocrinology 2008; 283:12–18
124. Peretz J, Flaws JA. Bisphenol A down-regulates rate-limiting Cyp11a1 to acutely inhibit steroidogenesis in cultured mouse antral follicles. Toxicology and Applied Pharmacology 2013; 271:249–256
125. Dechaud H, Ravard C, Claustrat F, de la Perriere AB. Pugeat M. Xenoestrogen interaction with human sex hormone-binding globulin. Steroids 1999; 64:328-34
126. Takeuchi T, Tsutsumi O, Ikezuki Y, Kamei Y, Osuga Y, Fujiwara T et al. Elevated serum bisphenol A levels under hyperandrogenic conditions may be caused by decreased UDP-glucuronosyl-transferase activity. Endocrine Journal 2006; 53:485-91.
127. Miao M, Yuan W, Yang F, Liang H, Zhou Z, Li R, et al. Associations between bisphenol A exposure and reproductive hormones among female workers. International Journal of Environmental Research and Public Health 2015; 12:13240–50.
128. Xu G, Han A, Xu N, Su P. Effects of maternal exposure to bisphenol A during pregnancy on puberty in advance and hypothalamo-pituitary gonadal axis hormones levels in female offspring. Wei Sheng Yan Jiu 2018; 47:425-431.
129. Kurian JR, Keen KL, Kenealy BP, Garcia JP, Hedman CJ, Terasawa E. Acute influences of bisphenol A exposure in hypothalamic release of gonadotropin-releasing- hormone and kisspeptin in female rhesus monkeys. Endocrinology 2015;1572563-2570
130. Giesbrecht GF, Fjaredar M, Liu J, Thomas J, Letourneau N, Campbell T. Prenatal Bisphenol A exposure and dysregulation of infant hypothalamic pituirary-adrenal axis:Findings from the APrON cohort study. Environmental Health 2017; 16: 47

131. Naule L, Picot M, Martini M, Parmentier C, Hardin-Pouzet H, Keller M et al Neuroendocrine and behavioural effects of maternal exposure to oral bisphenol A in female mice. Journal of Endocrinology 2014; 220:375-388

132. Skorupskaite K, George JT, Anderson RA. The kisspeptin-GnRH pathway in human reproductive health and disease. Human Reproduction Update 2014; 20:485-500.

133. Hugo ER, Brandebourg TD, Woo JG, Loftus J, Alexander JW, Ben-Jonathan N. Bisphenol A at environmentally relevant doses inhibits adiponectin release from human adipose tissue explants and adipocytes. Environmental Health Perspectives 2008; 116:1642-1647.

134. Alonso-Magdalena P, Vieira E, Soriano S, Menes L, Burks D, Quesada I,et al, Bisphenol A exposure during pregnancy disrupts glucose homeostasisin mothers and adult male offspring, Environmental Health Perspective 2010;118:1243e1250

135. Alonso-Magdalena P, Garcia-Arevalo M, Quesada I, Nadal A, Bisphenol-A treatment during pregnancy in mice: a new window of susceptibility for the development of diabetes in mothers later in life. Endocrinology 2015; 156:1659e1670.

136. Wang B, Sun M, Hou X, Pan X Li. The environmental obesogen bisphenol A promotes adipogenesis by increasing the amount of 11β-hydroxysteroid dehydrogenase type 1 in the adipose tissue of children. International Journal of Obesity 2013; 37:999-1005

137. Nadal A, Alonso-Magdalena P, Soriano S, Ripoli C, Fuentes E, Quesada I. Role of estrogen receptors alpha, beta and GPER1/GPR30 in pancreatic beta-cells. Frontiers in Bioscience 2011; 16:251-260

138. Alonso-Magdalena P, Morimoto S, Ripoll C, Fuentes E, Nadal A. The estrogenic effect of bisphenol A disrupts pancreatic beta-cell function in vivo and induces insulin resistance, Environmental Health Perspectives 2006; 114:106e112

139. Wong WP, Tiano JP, Liu S, Hewitt SC, Le May C, Dalle S et al Extranuclear estrogen receptor-alpha stimulates NeuroD1 binding to the insulin promoter and favors insulin synthesis, Proceedings of National Academy of Science U.S.A 2010; 107:13057e13062

140. Soriano S, Alonso-Magdalena P, Garcia-Arevalo M, Novials A, Muhammed SJ, Salehi A, et al, Rapid insulinotropic action of low doses of bisphenol-A on mouse and human islets of Langerhans: role of estrogen receptor beta, PloS One 2012; 7: e31109.

141. Zhu J, Jiang L, Liu Y, Qian W, Liu J, Zhou J, et al. MAPK and NF-κB pathways are involved in bisphenol A-induced TNF-α and IL-6 production in BV2 microglial cells. Inflammation. 2015; 38: 637-48.

142. Xu J, Huang G, Nagy T, Teng Q, Guo TL. Sex-dependent effects of bisphenol A on type 1 diabetes development in non-obese diabetic (NOD) mice. Archives in Toxicology 2019; 93**:** 997–1008

143. Hwa Yun B, Guo J, Bellamri M, Turesky RJ. DNA adducts: Formation, biological effects and new biospecimens for mass spectrometric measurements in humans. Mass Spectrometry Reviews 2020; 39: 55-82.

144. Kundakovica M, Gudsnuka K, Franksa B, Madrida J, et al. Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure. Proceedings of National Academy of Science 2013; 110:9956–9961

145. Zhang X and Ho S. Epigenetics meets endocrinology Journal of Molecular Endocrinology 2011; 46: R11–R32.

146. Weinhold B. Epigenetics: The Science of Change. Environmental Health Perspectives 2006: 114: A160–A167.

147. Kaelin WG and McKnight SL Influence of Metabolism on Epigenetics and Disease Cell 2013; 153:53-69.

148. Cariati F, Carbone L, Conforti A, Bagnulo F, Peluso SR, Carotenuto C, et al Bisphenol A-Induced Epigenetic Changes and Its Effects on the Male Reproductive System. Frontiers in Endocrinology 2020; 11: 453.

149. Ye Y, Tang Y, Xiong Y, Feng L, Li X. Bisphenol A exposure alters placentation and causes preeclampsia-like features in pregnant mice involved in reprogramming of DNA methylation of 72. Federation of American Societies for Experimental Biology Journal 2019; 33: 2732–2742.

150. Xu XH, Zhang J, Wang YM0, Ye YP and Luo QQ. Perinatal exposure to bisphenol-A impairs learning-memory by concomitant down-regulation of N-methyl-D-aspartate receptors of hippocampus in male offspring mice. Hormones Behaviour 2010; 58, 326–33.

151. Kitraki E, Nalvarte I, Alavian-Ghavanini A and Ruegg, J. Developmental exposure to bisphenol A alters expression and DNA methylation of Fkbp5, an important regulator of the stress response. Molecular and Cellular Endocrinology 2015; 417, 191- 199.

152. Lombó, M., Fernández-Díez, C., González-Rojo, S. *et al.* Genetic and epigenetic alterations induced by bisphenol A exposure during different periods of spermatogenesis: from spermatozoa to the progeny. Scientific Reports 2019; 9**,** 18029.

153. Doshi T, Mehta SS, Dighe V, Balasinor N, Vanage G.Hypermethylation of estrogen receptor promoter region in adult testis of rats exposed neonatally to bisphenol A. Toxicology 2011; 289:74–82.

154. Trapphoff T, Heiligentag M, El Hajj N, Haaf T, Eichenlaub-Ritter U. Chronic exposure to a low concentration of bisphenol A during follicle culture affects the epigenetic status of germinal vesicles and metaphase II oocytes. Fertility and Sterility 2013; 100:1758–67.

155. Savabieasfahani M, Kannan K, Astapova O, Evans NP, Padmanabhan V. Developmental programming: differential effects of prenatal exposure to bisphenol-A or methoxychlor on reproductive function. Endocrinology 2006; 147:5956–66.

156. Ke ZH, Pan JX, Jin LY, Xu HY, Yu TT, et al. Bisphenol A exposure may induce hepatic lipid accumulation via reprogramming the DNA methylation patterns of genes involved in lipid metabolism. Scientific Reports 2016; 6: 31331.

157. Fernandez SV, Huang Y, Snider KE, Zhou Y, Pogash TJ, Russo J. 2012. Expression and DNA methylation changes in human breast epithelial cells after bisphenol A exposure. International Journal of Oncology 2012; 41: 369–77.

158. Qin XY, Fukuda T, Yang L, Zaha H, Akanuma H, et al. Effects of bisphenol A exposure on the proliferation and senescence of normal human mammary epithelial cells. Cancer Biology and Therapy 2012; 13:296–306.

159. Vandenberg LN, Gerona RR, Kannan K, et al. A round robin approach to the analysis of Bisphenol a (BPA) in human blood samples. Environmental Health 2014;13**,** 25.

160. vom Saal FS, Welshons WV. Evidence that bisphenol A (BPA) can be accurately measured without contamination in human serum and urine, and that BPA causes numerous hazards from multiple routes of exposure. Molecular and Cellular Endocrinology 2014; 398:101-113.

CHAPTER 3
RESEARCH DESIGN AND METHODS

This was a cross-sectional birth cohort study.

3.1 Population and study sample

A subset of samples and data collected as part of the Mother and Child in the Environment (MACE) birth cohort study were utilized for this study. The MACE study population consisted of pregnant females recruited from antenatal clinics in the Durban South Basin area (Merebank, Bluff, Austerville and Wentworth) and other clinics in the North Durban area (KwaMashu and Newlands East and West), Durban, KwaZulu-Natal, South Africa. The Durban south basin is an area where large communities are located within heavily polluted large scale industrial enterprises. The overall objective of the MACE study was to describe birth outcomes among pregnant mothers in communities exposed to industrial pollution compared to communities without such exposure. Detailed information regarding occupational and environmental exposure as well as dietary history was collected by trainer interviewers via use of standardized interviews. Details regarding pregnancy outcome for participants had also been collected during course of the MACE study. Enrolment for the MACE study took place from April 2013 through to March 2018.

Written informed consent was obtained from the pregnant females in the MACE study cohort. The Biomedical Research Ethics Committee (BREC) of the University of KwaZulu Natal approved the MACE study protocol (ethics clearance number BF263/12) and approved this current study (ethics approval number BE597/16) (See appendix 1 and 2).

3.1.1 *Sample size and selection of sample*

For the current study subset of matched maternal and cord serum and extracted DNA samples collected as part of the MACE study were used. This study would be regarded as a pilot study and the sample number is 90 maternal cord pairs (180 samples). For sample size calculation the equation described by Viechtbauer et al for sample size determination in pilot studies, was utilised (1). A confidence level of 95% and probability estimate of 0.05 was input into the equation below:

$$n = \frac{\ln(1-\gamma)}{\ln(1-\pi)}.$$

(where γ refers to the confidence internval ; π to probability and n sample size)

To determine levels of probability, reference was made to previous studies that described the prevalence of detectable serum BPA levels. These ranged from 17%-84%, thus a conservative estimate of 5% was utilised (2,3). A minimum sample size of 59 samples was estimated for the purpose of this pilot study. A statistician was further consulted to verify the sample size determination.

3.1.2 Inclusion and exclusion criteria

The inclusion criteria were as per the MACE study and are listed below

Inclusion criteria

(a) The recruited participants had to be residing in the geographical area within which the clinic is located, and had to live in this area for the full duration of the pregnancy and follow-up period.

(b) Pregnant females had to preferably be less than 20 weeks of gestational age on entry although those presenting before the onset of the 3rd trimester were not excluded.

Exclusion criteria

Participants with the following conditions were excluded from the samples utilised for this current study: Pre-eclampsia, Hypertension, Placenta Previa, Diabetes mellitus, genital tract infection and multiple pregnancies.

3.2 Sample collection

Venous blood samples were taken during the third trimester (completed 27 weeks to term) from the pregnant individuals. Cord bloods were taken at delivery. Maternal and cord bloods were collected in 5 ml ethylenediaminetetraacetic acid (EDTA), sodium citrate and plain serum tubes.

For serum: samples were centrifuged at 1000 x *g* for 10 minutes, separated and serum stored in cryo-vials at -80 degrees Celsius until analysis. DNA was extracted from whole blood and stored at -80 degrees Celsius until analysis.

3.3 Biochemical Methods

3.3.1 Determination of serum Bisphenol A and Bisphenol glucuronide

Determination of serum levels of BPA and its conjugate BPA-g were performed using a liquid chromatography tandem mass spectrometry (LC-MS/MS) methodology.

Chromatographic separations were carried out using the AB Sciex 4500 triple quadrupole mass spectrometer (AB Sciex LLC, Framingham, USA) equipped with an Agilent 1260 Ultra high-performance liquid chromatography (uHPLC) system (Agilent Technologies,Santa Clara, USA). Analytes of interest were separated on a Phenomenex (Phenomenex,Torrance, USA) C18 column (2.1 x 50 mm, 1.6 µm). A 3-minute linear gradient elution was used from 10-100% of acetonitrile in water followed by a hold for 1 minute at a flow rate of 0.4 ml/min. Total run time was 4 minutes. Injection volume utilised was 20µl. Serum sample was prepared using 50 µl of serum mixed with 100 µl acetonitrile containing the internal standards deuterated 5 ng/ml BPA (d6BPA, Cambridge Isotope Laboratories, Andover, MA) and 5 ng/ml $^{13}C_{12}$ BPA-g (Sigma-Aldrich Gmbh, Munich, Germany). Electrospray ionisation in negative modes was used for the measurement of each analyte. The following

single reaction monitoring (SRM) transitions were used; for BPA mass to charge ratio (m/z) 227/212 (quantifier) and m/z 227 to 133 (qualifier); for d6BPA m/z 233 to115 (quantifier) and m/z 233 to 113 (qualifier); for BPA-g m/z 403 to 227 (quantifier) and m/z 403 to 113 (qualifier); for $^{13}C_{12}$ BPA-g m/z 415 to 239 (quantifier) and m/z 415 to 113 (qualifier). Five levels of standards (including blank) (ranging from 0 ng/ml to 25 ng/mL) and 2 levels of serum based internal quality control materials (level 1 BPA and BPA-g- 2 ng/mL; level 2 BPA and BPA-g -10 ng/mL) were analysed at the beginning and end of each run. A procedural blank was analysed with each run. Samples were only processed when blank showed no detectable BPA levels. Recovery, linearity, imprecision, carry over, assessment of system contamination for BPA and sample stability studies were performed. The limit of detection (LOD) and limit of quantification (LOQ) for BPA and BPA-g were calculated based on signal-to-noise (S/N) ratios of 3:1 and 10:1, respectively, were obtained by injecting standard dilutions with the corresponding S/N ratio. In order to assess system contamination: pools of charcoal stripped serum (Sigma Aldrich Gmbh, Munich Germany) neat and spiked with known concentrations of BPA were passed through the entire analytical procedure to calculate recoveries of BPA through the analytical method. BPA ≥ 99% purity (Sigma Aldrich Gmbh, Munich, Germany) was utilised for recovery studies and preparation of internal quality control material. HPLC grade water was also analysed through the system to assess level of BPA contamination of water utilised for sample processing. Precision studies were performed following the Clinical and Laboratory Standards Institute (CLSI) EP15-A3 protocol (4).

3.3.2 Determination of serum steroid hormone levels

The following nine steroid hormones were analysed in maternal serum and cord blood using LC-MS/MS methodology: oestradiol (E2), total testosterone (TT), 11-deoxycorticosterone (11DOC), Dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS) androstenedione (Andro), 17-OH progesterone (17OHP), dihydrosterone (DHT) and progesterone (Prog). There hormones were selected as they cover the major sex steroid hormones in humans.

A commercially available kit the MassChrom Steroid Panel 2 kit (Chromosystems Instruments and Chemicals GmbH, Germany) was utilised for analysis of steroid hormones. Sample/calibrator/control preparation was as per manufacturer's instructions. Briefly, samples and reconstituted controls/calibrators are added to the provided kit extraction buffer. This initiates the protein interactions be removed to enable the bound and free steroids to be measured as total steroid. A solid phase extraction (SPE) was performed using individual SPE columns. The prepared sample/control/calibrant is then eluted using the provide buffer solution. Samples are evaporated to dryness using Nitrogen gas and reconstituted with the reconstitution buffer. An injection volume of 40µl was utilised.

Chromatographic separations were carried out using the AB Sciex 4500 triple quadrupole mass spectrometer equipped with an Agilent 1260 Ultra high-performance liquid chromatography (uHPLC) system. Analytes of interest were separated on the provided kit column- C18 column (2.1 x 50 mm, 1.6 um). A linear gradient was used from 10-100% of acetonitrile in water followed by a hold for 1 minute at a flow rate of 0.4 ml/min. Total run time was 12.5 minutes. The following multiple reaction monitoring (MRM) transitions were used: Androstenedione 287>109,287>97; DHEA 289>213, 271>213; DHEAS 271>213, 271>197; DHT 291>255; 291>159; E2 255>159, 255>133; 17 OHP 331>109, 331>97; Progesterone 315>97, 315>109; Testosterone 289>97, 289>109; 11DOC. Deuterated internal standards were utilised for each analyte of interest and were added to samples prior to extraction. Six levels of commercially available standards (including blank) (values as per table 1 below) and 2 levels of serum based internal quality control materials (ranges as per table 1 below) were analysed at the beginning, middle and end of each run. A procedural blank was analysed with each run. Within run coefficient of variation (CV) for the steroid hormones ranged from 0.48% to 12.73%, and for the between-run CVs ranged from 1.6-10.2 percent (%) across the three internal quality control (IQC) levels.

3.3.3 Determination of serum 25-hydroxy Vitamin D levels

Determination was performed as per previously described by George et al (5). Serum maternal and cord blood 25OH Vitamin D was measured using the Clin Rep high performance liquid chromatography (HPLC) kit (Recipe, Munchen, Germany). In this analytical method, 25(OH)D was determined from plasma using HPLC with a photodiode array (PDA) detector. Prior to HPLC analysis a short liquid-liquid extraction was performed. The liquid-liquid extraction includes a precipitation step that denatured the protein bond and extracted the 25(OH)D out of the lipid layer into a clear upper layer. Fifty microliters of the eluent were injected onto the HPLC system where the analytes were separated on the appropriate C18 analytical column (Recipe, Munchen, Germany) using an isocratic mobile phase elution. The separated 25(OH)D2 and 25(OH)D3 were detected at a wavelength of 264nm. The concentration was calculated using a standard curve with two sets of controls run every 20 samples. The standards and controls were human based lyophilized samples that are traceable to the National Institute of Standards and Technology (NIST) 25(OH)D standard. The chromatograms were integrated using peak height. Total vitamin D (25(OH)D) was taken as the sum of 25(OH)D2 and 25(OH)D3. The intra-assay and inter–assay coefficient of variation (CV) for 25(OH)D3 for controls at a mean of 61.7 nmol/L ranged from 0.36-9.4%, and for controls at a mean of 222 nmol/L ranged from 2.1-5.5%. For 25(OH)D2 the CV for the low control (mean 49.2 57 nmol/L) was 6.8-9.7% and for the high control (mean 199 nmol/L) ranged from 1.1-5.7%. The limit of quantification was 6.5 nmol/L for 25(OH)D3 and 11.0 nmol/L for 25(OH)D2. Any samples with 25(OH)D2 below the limit of quantification was assigned a nominal value of zero.

3.4 Molecular methods

3.4.1 Isolation of Genomic DNA

Genomic DNA was extracted from the blood samples using the FlexiGene® DNA isolation kit (Qiagen, Germantown, USA). DNA samples were banked and stored at -80ºC for DNA methylation studies.

3.4.2 DNA Methylation studies:

Following extraction of genomic DNA from whole blood, the isolated DNA was then eluted in nuclease-free water and purified using the Zymo Research DNA Clean and Concentrator™-5 Kit (Zymo Research, Irvine, USA), as per manufacturer's instructions. DNA concentration was determined using the Nanodrop 2000 spectrophotometer (Thermo-Fischer Scientific, Waltham, USA) and standardized to 4 ng/µl. DNA purity was assessed using the A260/A280 absorbance ratios. A value of between 1.8-2.0 was considered to be an acceptable A260/A280 ratio (6).

The promoter methylation of the candidate genes *CYP1B1* and *CYP3A4* were assessed using the OneStep qMethyl Kit (Zymo Research, Irvine, USA) as per manufacturer's instructions. In brief, 20 ng of DNA was subjected to a test and reference reaction containing specific primers (Table 1). Cycling conditions were as follows: digestion by methyl sensitive restriction enzymes (37°C, 2 h), initial denaturation (95°C, 10 min), followed by 45 cycles of denaturation (95°C, 30 s), annealing (58 °C 60 s), extension (72°C, 60 s), final extension (72°C, 60 s), and a hold at 4°C. Quantitative PCR (qPCR) experiments were conducted using the CFX96 Real Time PCR System (Bio-Rad, Bio-Rad Life Sciences, Hercules, USA) and analyzed using the Bio-Rad CFX Manager™ Software version 3.1 (Bio-Rad, Bio-Rad Life Sciences, Hercules, USA).

The percentage methylation was calculated using the formula (stated below) as supplied in the OneStep qMethyl kit:

Quantification of percentage promoter methylation formula (7)**:**

Methylation (%) = 100 X $2^{-\Delta Ct}$, where ΔCt = Ct (test reaction) – Ct (reference reaction)

The reference reaction utilises a human non-methylated DNA standard as provided in the kit.

Table 3.1: qPCR primer sequences for promotor methylation and annealing temperatures

Gene	Sense Primer (5'→3')	Anti-Sense Primer (5'→3')	Annealing Temperature (°C)
CYP1B1	TTTGTGTGCCCAAGCACTGTC	CACAACTGGAGTCGCAGAA	58
CYP3A4	CATGCCCTGTCTCTCCTTTAGC	CCTTTCAGCTCTGTGTTGCTC	58

Note samples for which the DNA concentration were < 4 ng/µl following DNA clean up were not utilised for qPCR analysis. Sample runs with quantification cycle (C_q) values >40 were not included in analysis of results because of the implied low efficiency (8).

3.5 Statistical analysis

Data was assessed for normality using the Shapiro- Wilk test. Non-parametric tests Kruskal Wallis test, Spearman's correlation or Wilcoxon signed rank test for univariate analysis. Passing Bablock regression analysis was performed for non-parametric data. Analysis of covariance (ANCOVA) was used to assess for confounding variables and for multiple regression analysis A p value of <0.05 was considered significant. Statistical analysis was performed on Medcalc statistical software program version 18.11 (Medcalc, Belgium). A detailed description of statistical analyses performed per study component has been included in the method section of each data chapter.

References

1. Viechtbauer W, Smits L, Kotz D, Bude L, Spigt M, Serroyen J et al. A simple formula for the calculation of sample size in pilot studies. Journal of Clinical Epidemiology 2015; 68: 1375-1379
2. He Y, Miao M, Herrinton LJ, Wu C, Yuan W, Li DK. Bisphenol A levels in blood and urine in a Chinese population and the personal factors affecting the levels. Environmental Research 2009; 109: 629-633
3. Lee HJ, Ryu HY, Kim HK, Min CS, Lee JH, Kim E, et al. Maternal and fetal exposure to Bisphenol A in Korea. Reproductive Toxicology 2008; 25: 413-419
4. CLSI. User Verification of Precision and Estimation of Bias; Approved Guideline-Third Edition CLSI document EP15-A3. Wayne, PA: Clinical and Laboratory Standards Institute; 2014
5. George JA, Norris SA, van Deventer HE, Crowther NJ. The association of 25 hydroxyvitamin D and parathyroid hormone with metabolic syndrome in two ethnic groups in South Africa. *PLoS One*. 2013;8: e61282.
6. Glasel JA. Validity of nucleic acid purities monitored by A260/A280 Absorbance ratios. Biotechniques 1995; 18: 62-63
7. OneStep qMethyl kit instruction manual. https://files.zymoresearch.com/protocols/_d5310_onestep_qmethy_kit.pdf. Accessed 5 May 2021
8. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE Guidelines: *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments, Clinical Chemistry 2009;55: 611–622.

CHAPTER 4

A PILOT STUDY: BISPHENOL–A AND BISPHENOL-A GLUCURONIDE LEVELS IN MOTHER AND CHILD PAIRS IN A SOUTH AFRICAN POPULATION

Gounden V[1,2], Warasally MZ[2], Magwai T[2], Naidoo R[3], Chuturgoon A[4]
[1]Department of Chemical Pathology, University of KwaZulu-Natal and [2]National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital, Durban, South Africa
[3]Department of Occupational Health, University of KwaZulu-Natal, Durban, South Africa
[4]Department of Medical Biochemistry, University of KwaZulu-Natal, Durban, South Africa

Corresponding author: Verena Gounden
Department of Chemical Pathology, University of KwaZulu-Natal and National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital
2nd Floor Pathology Building Inkosi Albert Luthuli Central Hospital
800 Vusi Mzimela Road, Cato Manor, Durban, 4058
Email: verenagounden@yahoo.com

Keywords: Bisphenol A; liquid chromatography tandem mass spectrometry; mother-child pairs; cord blood

Reproductive Toxicology. 2019 ;89:93-99.
DOI:10.1016/j.reprotox.2019.07.008

Abstract

Exposure to Bisphenol A (BPA) during early development particularly in-utero has been linked to a wide range of pathology. The aim of this study was to determine serum levels of BPA and its naturally occurring metabolite BPA-glucuronide (BPA-g) in South African mother-child pairs.

Method: Third-trimester serum maternal samples and matching cord blood samples were analysed for BPA and BPA-g using LC-MS/MS.

Results: Ninety maternal and child pairs were analysed. BPA was detectable in more than 25% of maternal and cord blood samples. Spearman correlation demonstrated significant positive correlation between maternal and child BPA and BPA-g levels with correlation coefficients of 0.892 and 0.744, respectively. A significant positive association between cord BPA levels and child birth-weight ($p=0.02$) as well as with maternal BMI ($p=0.04$) was noted.

Conclusion: This is the first study to describe the presence of detectable BPA levels using LC-MS/MS methodology in a South African population.

Introduction

Environmental pollutants have been shown to have significant effects on human health. Steroid hormone function has been shown to be affected by some of these pollutants in particular a class known as, endocrine disruptors (EDs) (1). The United States environmental protection agency has defined endocrine disrupting chemicals or EDs as "exogenous agents that interfere with the normal function of endogenous hormones responsible for the maintenance of homeostasis and the regulation of developmental processes" (2). The possible deleterious effects on human health associated with EDs have ranged from effects on reproductive health in particular sperm health, carcinogenesis, and increasingly effects on other endocrine and metabolic functions such as thyroid disease and obesity (1,3,4). Deleterious neurodevelopmental effects have also been described in animal and human studies (5,6,7). The effect of EDs are most significant when exposure occurs during developmental stages as such the foetus is particularly vulnerable to these effects (8). Thus, exposure of the pregnant mother to EDs may have adverse effects on the developing foetus. Examples of environmental EDs that have been targeted for adverse effects on reproductive systems in humans and other animals are pesticides [e.g. dichlorodiphenyltrichloroethane (DDT)], fungicides (e.g. vinclozolin), insecticides (e.g. trichlorfon), herbicides (e.g. atrazine) and plastics (e.g. phthalates and Bisphenol A) (1, 9). Bisphenol A (BPA) is found in a vast array of plastic consumer products including lining of tin cans, food and water containers, medical devices and toys (1, 10). Apart from exposure due to use of these products, environmental exposure may occur by other means. BPA has been measured in soil, river water, indoor air and dust as well as landfill leachates (11,12). Animal and human studies have demonstrated that BPA readily crosses the placenta and has been detected in amniotic fluid (13, 14, 15). Exposure to BPA has been linked to prenatal and postnatal adverse effects on multiple tissues, including the reproductive system and neurodevelopment. Whilst studies in humans have been limited, studies in animal models have associated BPA exposure to development of breast and prostate cancers, polycystic ovarian syndrome, preterm delivery, intrauterine growth restriction and low birth weight (1). Recent studies have also associated elevated serum and urine BPA levels with cardiovascular disease, obesity and type 2 diabetes mellitus (16,17,18).

BPA exerts it varied effects by several different mechanisms. It has been described to have oestrogenic, anti-oestrogenic, androgenic and anti-androgenic properties (1,19). With regards to the interaction of BPA with oestrogen receptors it has been defined as a selective oestrogen receptor modulator which binds to oestrogen receptors (ERs) to act as an agonist or antagonist in a tissue specific manner. The effect of interaction depends on BPA binding to either ER alpha or ER beta and its relative concentration (19,20). Apart from interaction with the classical ERs, BPA also been described to bind to non-traditional ERs associated with the cell membrane. This activates intracellular pathways that can change

cell function via a more rapid and greater extent that binding to the traditional nuclear oestrogen receptor (21).

BPA has also been shown to interact with thyroid receptors. Interaction with membrane associated G protein-coupled oestrogen receptor located in the hypothalamus and pituitary has been described (20,21). Recently Kojima *et al* demonstrated effects of BPA and its related analogues against the following human nuclear receptors: oestrogen, androgen, glucocorticoid, pregnane-X and constitutive androstane receptors (22). BPA and most of its other related analogues showed agonistic and antagonistic effects on these afore-mentioned receptors (22). Another mechanism of action of BPA has been reported via in-vitro studies which involves epigenetic alterations. Atkinson et al showed a metabolite of BPA (BPA-o-quinone) being able to covalently bind to DNA and in the presence of peroxidase produce toxic adducts. The resultant toxic adduct affects reproductive tissue organogenesis and can result in gene modifications and chromosomal mutations (23).

Epidemiological surveys performed in the United States reported that over 90% of the Americans that were sampled had detectable BPA levels in their urine (24). Due to growing health concerns regarding BPA exposure there has been increased use of BPA substitutes such as bisphenol F and S (25). A follow up to the initial NHANES data for the period 2013-2014 showed that exposure to these substitutes have become ubiquitous, however the reported median urine BPA levels in the population tested were still found to be higher than BPF and BPS levels (26). In the developing world we have little information or research regarding the extent of this issue. Previous studies have reported measurable levels of BPA in pregnant populations both in serum and urine (27-30). Urine measures which usually consist of BPA metabolites provide an index of exposure to BPA but do not necessarily indicate circulating levels. The presence of unconjugated BPA in circulation indicates internal exposure to the parent compound (31) In pregnant women it may provide a more direct indication of likelihood of foetal exposure than maternal urine.

Naturally occurring BPA metabolites, BPA glucuronide (BPA-g) and sulfate conjugates are the major phase II metabolites produced by the liver following dietary exposure to BPA (32). BPA-g, which is the major metabolite of BPA was widely thought of inert as it is unable to bind to oestrogen receptors however recent evidence in animal studies suggest that it may de-conjugate to expose the foetus to BPA even though adequate conjugation of BPA has occurred after maternal intake (33). Animal studies have shown that whilst BPA-g levels in maternal serum may be highly variable, levels in cord serum remain steady and are thought to reflect the cumulative dose of BPA received by the foetus during late pregnancy (34). Acute single- human experiments have provided evidence for the recognised short half -life of BPA, which is cleared within 24 hours of exposure/consumption (35). However, some authors have reported that frequent exposure together with accumulation in certain tissues such as adipose

results in ongoing release into the circulation (36). This is key to consider when examining likely exposure of foetus to BPA via maternal routes.

To the authors knowledge no previous studies have been published that have examined maternal and foetal exposure to BPA in an African population. The aims of the study were two-fold a) to determine the serum levels of common environmental endocrine disruptor Bisphenol A and its glucuronide metabolite (BPA-g) in South African mother child pairs and b) to develop and validate an in-house method of the determination of BPA and BPA-glucuronide levels in serum utilising liquid chromatography tandem mass spectrometry (LC-MS/MS).

Methods

Population and study samples

Blood samples and data collected as part of the Mother and Child in the Environment (MACE) birth cohort study were utilized for this study. The MACE study population consists of pregnant females recruited from antenatal clinics in Durban, South Africa from the "South Basin" area and other clinics in the North Durban area. The Durban South basin is an area where large communities are located within heavily polluted large-scale industrial enterprises. Mothers were recruited during the third trimester of pregnancy (between 27 completed to 40 weeks of pregnancy). Overall objective of the mace study was to describe birth outcomes among pregnant mothers in communities exposed to industrial pollution compared to communities without such exposure. Detailed information regarding occupational and environmental exposure as well as dietary history was collected by trainer interviewers via use of standardized interviews. Details regarding pregnancy outcome for participants were also collected during the course of the MACE study. Venous blood samples were taken during the third trimester from the pregnant individuals. Cord bloods were taken at delivery. Blood were collected from maternal participants at one of their regularly scheduled ante-natal appointments. Maternal and cord bloods collected in serum polypropylene vacutainer tubes were later analysed for BPA and BPA-g. These samples were centrifuged, separated and serum stored at -80 degrees Celsius until analysis. Maternal /cord paired samples with sufficient serum volumes (n=90) were utilized for the current study.

Inclusion and exclusion criteria were as per MACE study

Inclusion criteria: pregnant females >18 years in third trimester of pregnancy attending one of the antenatal clinics as described earlier. Exclusion criteria: presence of any of the following clinical conditions pre-eclampsia, hypertension, placenta praevia, diabetes, genital tract infection and multiple pregnancies.

Bisphenol A and Bisphenol glucuronide analysis

Determination of serum levels of BPA and its conjugate BPA-g were performed. Chromatographic separations were carried out using the AB Sciex 4500 triple quadrupole mass spectrometer equipped with an Agilent 1260 Ultra high-performance liquid chromatography (uHPLC) system. Analytes of interest were separated on a Phenomenex C18 column (2.1 x 50 mm, 1.6 um). A 3-minute linear gradient was used from 10-100% of acetonitrile in water followed by a hold for 1 minute at a flow rate of 0.4 ml/min. Total run time was 4 minutes. Injection volume utilised was 20µl. Serum sample was prepared using 50 µl of serum mixed with 100 µl acetonitrile containing the internal standards deuterated 5 ng/ml BPA (d6BPA, Cambridge Isotope Laboratories, Andover, MA) and 5 ng/ml $^{13}C_{12}$ BPA-g (Sigma-Aldrich Gmbh, Munich, Germany. Electrospray ionisation in negative modes was used for the measurement of each analyte. The following single reaction monitoring (SRM) transitions were used; for BPA mass to charge ratio (m/z) 227/212 (quantifier) and m/z 227 to 133 (qualifier); for d6BPA m/z 233 to115 (quantifier) and m/z 233 to 113 (qualifier); for BPA-g m/z 403 to 227 (quantifier) and m/z 403 to 113 (qualifier); for $^{13}C_{12}$ BPA-g m/z 415 to 239 (quantifier) and m/z 415 to 113 (qualifier). Five levels of standards (including blank) (ranging from 0 ng/ml to 25 ng/mL) and 2 levels of serum based internal quality control materials (level 1 BPA and BPA-g- 2 ng/mL; level 2 BPA and BPA-g -10 ng/mL) were analysed at the beginning and end of each run. A procedural blank was analysed with each run. Samples were only processed when blank showed no detectable BPA levels. Recovery, linearity, imprecision, carry over, assessment of system contamination for BPA and sample stability studies were performed. The limit of detection (LOD) and limit of quantification (LOQ) for BPA and BPA-g were calculated based on signal-to-noise (S/N) ratios of 3:1 and 10:1, respectively, were obtained by injecting standard dilutions with the corresponding S/N ratio. In order to assess system contamination: pools of charcoal stripped serum (Sigma Aldrich Gmbh, Munich Germany) neat and spiked with known concentrations of BPA were passed through the entire analytical procedure to calculate recoveries of BPA through the analytical method. BPA ≥ 99% purity (Sigma Aldrich Gmbh, Munich, Germany) was utilised for recovery studies and preparation of internal quality control material.

HPLC grade Milli-Q water was also analysed through the system to assess level of BPA contamination of water utilised for sample processing. Precision studies were performed following the Clinical and laboratory standards institute (CLSI) EP15-A3 protocol (37).

Statistical analysis

Data was assessed for normality using the Shapiro- Wilk test. Non-parametric tests Kruskal Wallis test, Spearman's correlation or Wilcoxon signed rank test for univariate analysis. Passing Bablock regression analysis was performed for non-parametric data. A p value of <0.05 was considered significant. Statistical analysis was performed on Medcalc statistical software program version 18.11 (Medcalc, Belgium). For further analysis of data, maternal participants were categorised into different groups

based on gestational age at the time of blood collection namely, category A=27-29 weeks' gestation; category B=30-32 weeks; category C=33-35 weeks; category D=36-38 weeks; category E= >38 weeks.

Ethical approval

The research has complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee. Ethical clearance for this study was obtained from the Biomedical Research and Ethics Committee (BREC) of the University of KwaZulu-Natal (Ethics Clearance Certificate BE 597/16).

Results

BPA and BPA-g assay

The performance characteristics of the LC-MS/MS developed assay for BPA and BPA-g measurement are summarised in Table 4.1 below.

Table 4.1 Performance characteristics of BPA and BPA-g LC-MS/MS assay

	BPA		BPA-g	
	Level 1 IQC	Level 2 IQC	Level 1	Level 2
Within run CV (n=5)	6.4%	5.4%	4.3%	3.8%
Between Day CV (n=15)	9.4%	6.2%	4.3%	4.2%
Recoveries	91-118%			
Limit of Quantitation (LOQ)	0.4 ng/mL		0.4 ng/mL	
Linearity	LOQ – 25 ng/mL		LOQ -25 ng/mL	

Note: IQC: internal quality control; LOQ: limit of quantitation; CV: coefficient of variation

The limit of detection (LOD) for both BPA and BPA-g LOD was 0.12 ng/mL

Results for sample stability studies used spiked serum: There was a <5% difference in results and <2% difference in results from Day 0 and Day 30 aliquots for samples stored at -20 and -70 °C respectively. All spiked samples analysed for contamination showed recoveries between 91-118%. Analysis of water showed undetectable BPA levels. Figure 4.1 below demonstrate selective reaction monitoring (SRM) chromatogram for a maternal serum sample analysed for BPA.

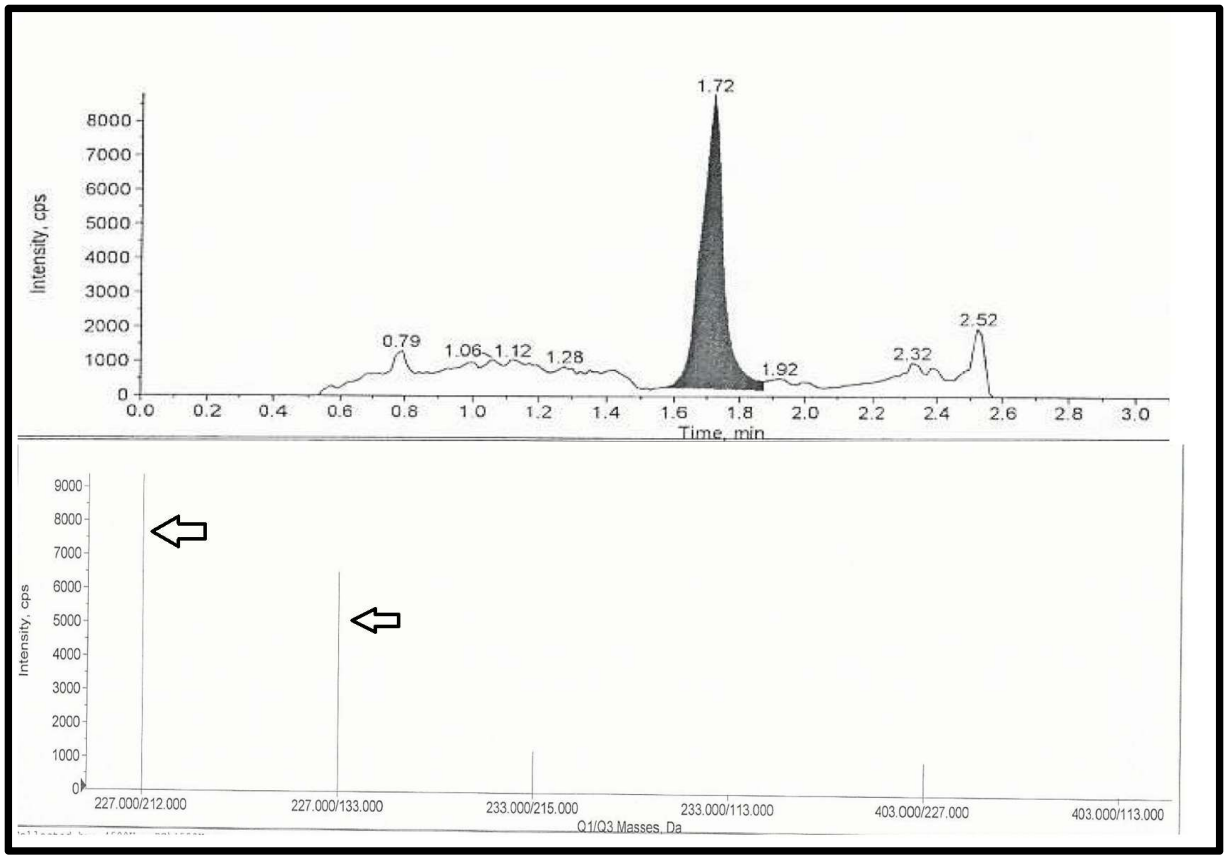

Figure 4.1 LC-MS/MS chromatogram obtained in negative ion mode showing the SRM transition of maternal serum sample analysed for BPA. Mass spectrum shown above with qualifier and quantifier transitions for BPA indicated by arrows.

3.2 BPA and BPA-g levels

Samples from a total of 90 maternal and child pairs were analysed. There was a mean time interval of 8.1 weeks (± 3 weeks) between maternal sample collection and cord blood collection.

Participant characteristics are summarised in Table 4.2 below.

Table 4.2 Summary of Participant characteristics

	Median (range) / Mean (± SD)
Maternal Age (years)	25.5 years (16-41) (n=90)
Maternal BMI (kg/m^2) per gestation at sample collection	
Total	30.0 kg/m^2 (18.6-61.1) (n=90)
Category A (27-29 weeks gestation)	30.0 kg/m^2 (18.6-61.0) (n=43)
Category B (30-32 weeks gestation)	30.5 kg/m^2 (21.5-61.1) (n=28)
Category C (33-35 weeks gestation)	29.0 kg/m^2 (22.2-39.6) (n=9)
Category D (36-38 weeks gestation)	31.8 kg/m^2 (26.3-58.0) (n=9)
Category E (>38 weeks gestation)	n=1 BMI 38.1 kg/m^2
Gestational length (weeks)	38 weeks (32-42) (n=90)
Birthweight(grams) per gestational length and child sex	
Total	2768 g (± 529) (n=90)
Male	2768 g (± 538) (n=53)
Female	2769 g (±523) (n=37)
32-35 weeks gestation	
All	2011 g (±303) (n=7)
Male	1998 g (n=4)
Female	2030 g (n=3)
36-40 weeks gestation	
All	2822 g (±±487) (n=78)
Male	2802 g (n=46)
Female	2852 g (n=32)
>40 weeks gestation	
All	2981g (±632) (n=5)

Male	3272g (n=3)
Female	2545 g (n=2)
Maternal BPA levels per gestation at sample collection	
Total	0.95 ng/mL (0.4- 15.3) (n=26)
Category A (27-29 weeks gestation)	1.11 ng/mL (0.4-15.3) (n=14)
Category B (30-32 weeks gestation)	0.99 ng/mL (0.4-6.4) (n=10)
Category C (33-35 weeks gestation)	1.08 ng/mL (n=1)
Category D (36-38 weeks gestation)	0.59 ng/mL (n=1)
Category E (>38 weeks gestation)	N=0
Maternal BPA-g levels per gestation & sample collection	
Total	4.71 ng/mL (0.48-21.8) (n=75)
Category A (27-29 weeks gestation)	3.5 ng/mL (0.4-14.8) (n=36)
Category B (30-32 weeks gestation)	4.34 ng/mL (0.4-21.8) (n=26)
Category C (33-35 weeks gestation)	3.90 ng/mL (2.77) (n=2)
Category D (36-38 weeks gestation)	4.95 ng/mL (3.13) (n=8)
Category E (>38 weeks gestation)	5.02 ng/mL (n=1)
Cord blood (child) BPA levels (ng/mL)	
Total	0.92 ng/mL (0.4-13.2) (n=24)
Male	0.54 ng/mL (0.4-13.2) (n=13)
Female	1.25 ng/mL (0.4-8.3) (n=11)
Cord blood (child) BPA-g levels (ng/mL)	
Total	4.21 ng/mL (0.4-26) (n=78)
Male	4.2 ng/mL (0.4-26.0) (n=44)
Female	4.1 ng/mL (0.65-21.3) (n=34)

#Non-parametric data presented as median (range); normally distributed data presented as mean (±SD).

Kruskal Wallis analysis showed no statistically significant difference of maternal BMI across the different gestation categories (p=0.79). Most maternal samples with detectable BPA levels were collected in the early third trimester (<33 weeks; n=24 of 26). Third trimester maternal BPA concentrations ranged from the limit of detection 0.4 ng/mL to 15.3 ng/mL, median 0.95 ng/mL; the majority of mothers n=64 (71%) did not have detectable BPA levels. However, most mothers n=75 (83%) had detectable BPA-g levels, range 0.48 ng/mL -21.8 ng/mL with a median of 4.71 ng/mL. Both maternal BPA (p=0.88) and BPA-g (p=0.85) were not significantly different across different the gestation categories for time of maternal sample collection. Analysis via ANCOVA when corrected for, maternal age, parity and gestation at time of blood collection showed no significant relationship between maternal BMI and maternal BPA and BPA-g levels with p values of 0.06 and 0.11 respectively. Child (cord blood) levels correlated with maternal findings with BPA levels ranging from 0.4-13.2 ng/mL, median 0.92 ng/mL; with majority of samples (n=66; 73%) being undetectable. Child BPA-g levels ranged from 0.4-26 ng/mL, median 4.21 ng/mL; with 13% of samples having undetectable levels. Kruskal Wallis analysis shows no significant difference in cord BPA (p=0.25) and BPA-g levels (p=0.25) between male and female infants on analysis. When taking into consideration gestational length and sex of infant there was not significant association of these with BPA levels. When taking into consideration gestational length there was no significant association with child gender and birth weight (p=0.38).

A significant positive correlation between maternal and child BPA and BPA-g levels were observed with Spearman rank correlation coefficients of 0.892 (95% CI 0.736 -0.958) and 0.744 (95%CI 0.623-0.830) respectively. Passing-Bablock regression analysis data for relationship between maternal and cord BPA and BPA-g levels respectively is shown in Figures 4.2 and 4.3.

On ANCOVA analysis maternal BPA and (p<0.001) and maternal BPA-g levels (p<0.001) had the most significant associations with cord BPA and BPA-g levels respectively. In addition, child-birth-weight (p=0.02) and maternal BMI (p=0.04) also had significant positive associations with cord BPA levels but not cord BPA-g levels. Other factors included in the ANCOVA analysis: child gender, infant gestational length and maternal gestation at collection of samples for analysis, weeks interval between collection of maternal and cord sample as well maternal parity did not have a significant association with cord BPA and BPA-g levels.

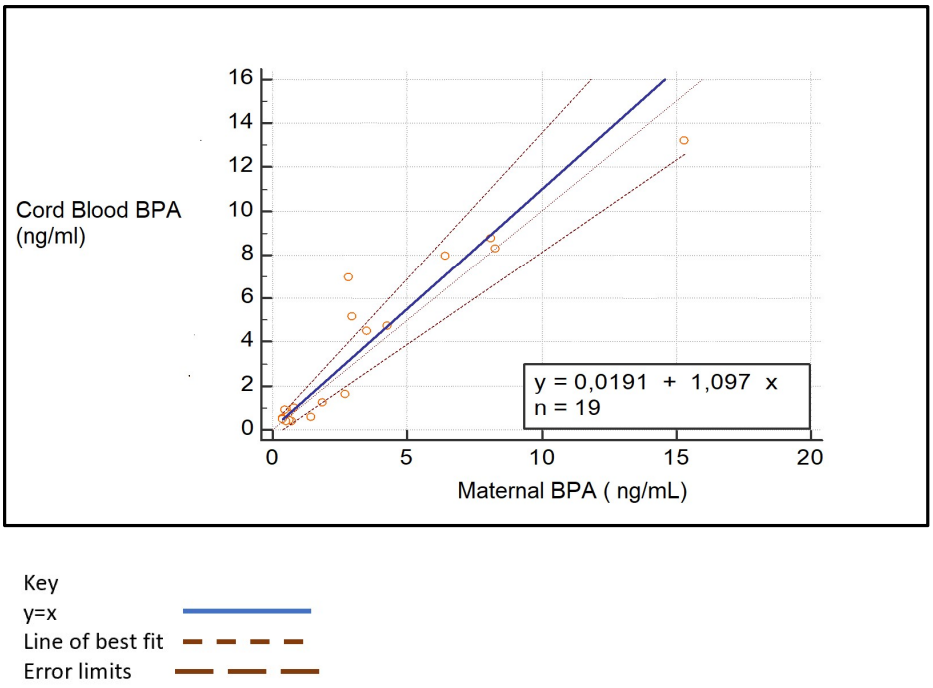

Figure 4.2: Passing-Bablock regression analysis between maternal and child (cord) BPA levels

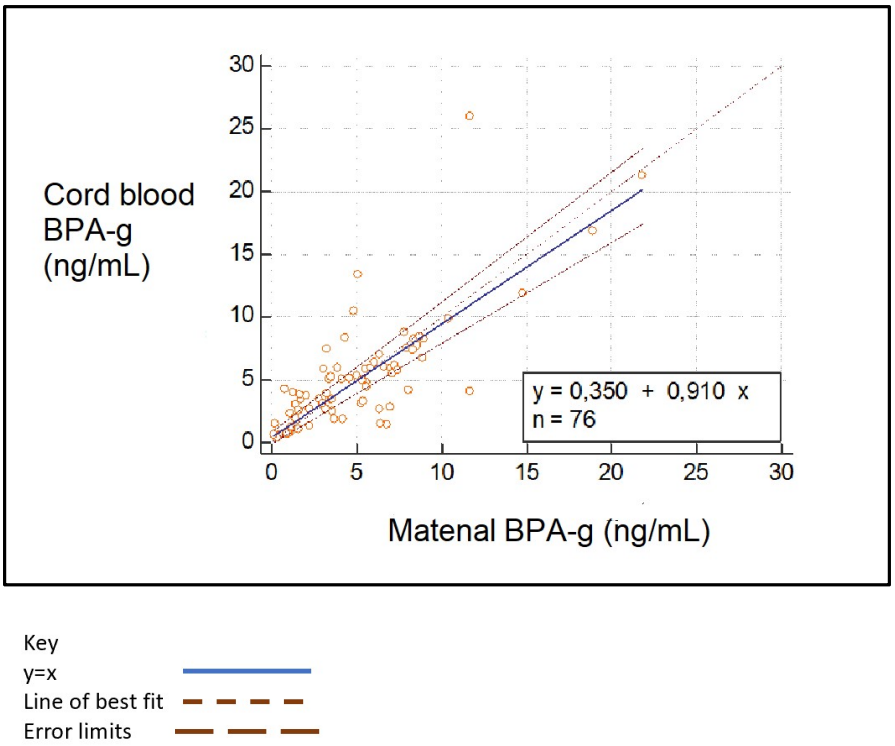

Figure 4.3: Passing-Bablock regression analysis between maternal and child (cord) BPA-g levels

Wilcoxon paired sample analysis (pairs n=19) showed no significant differences between maternal and cord blood pairs median BPA values (p=0.4). (Refer to Figure 4). However, whilst there was correlation

between maternal BPA and cord BPA-g levels, paired sample analysis showed significant difference between medians (p=0.007) with a higher median seen in cord blood. (Refer Figure 4.5).

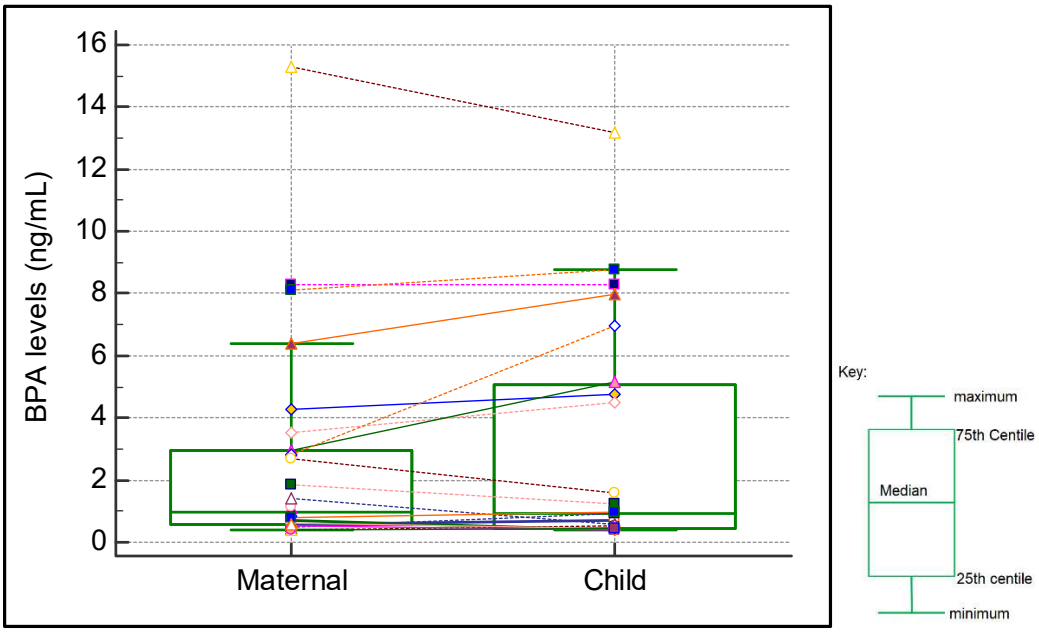

Figure 4.4 Box and whiskers plot- showing paired maternal and child (cord) BPA levels

Note each line represents a maternal-child pair.

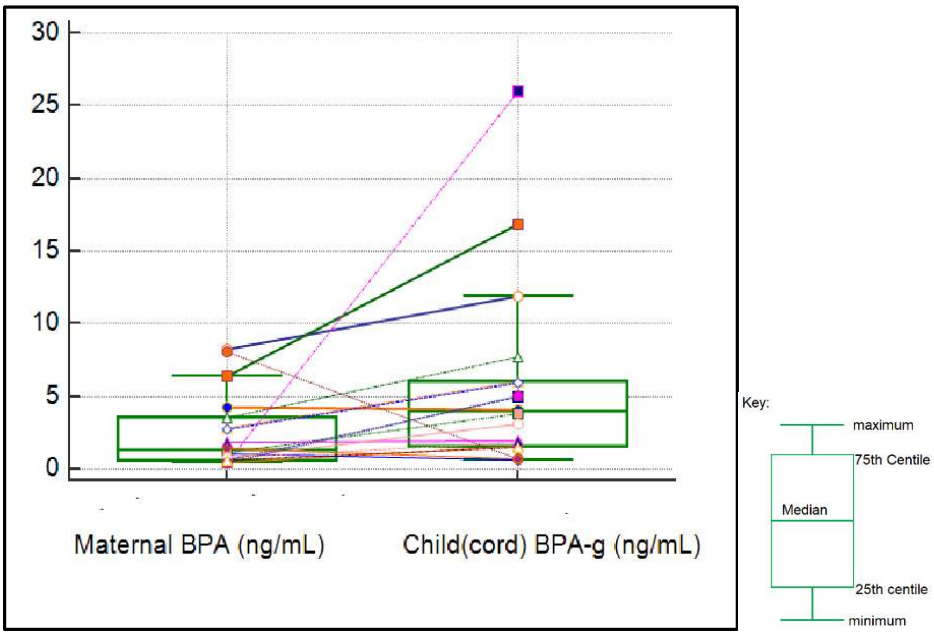

Figure 4.5 Box and whiskers plot- showing paired maternal BPA and child(cord) BPA-g levels (Note each shape represents a maternal-child pair)

Effect of BPA and BPA-g levels on child birth weight:

On ANCOVA analysis following correction for gestational length there was no significant association between child birth weight and the following: maternal serum BPA levels (p=0.09); maternal BPA-g levels (p=0.83); cord BPA levels (p=0.06) and cord BPA-g levels (p=0.65). However, it should be noted that both maternal and cord BPA levels were close to significant p value of 0.05. When further corrected for sex of child levels of significance did not change.

Discussion

Research examining levels of BPA exposure in developing countries has been identified as an important research need (35). Research over several years on animal models has shown that BPA exposure has significant adverse effects on the health of the subject and progeny exposed in-utero. The crux of the current public health issue around BPA is centred around determining what levels of exposure and for what time period would affect human health. Animal studies and the smaller number of human studies have indicated that exposure in the perinatal period is of particular significance (1).

The findings of this study have demonstrated lower median levels of maternal BPA (0.95 ng/mL) however a similar range of values (0.4-15.3 ng/mL) were seen as previous studies performed in industrialised nations. Padmanabhan *et al* reported values of serum maternal BPA ranging from undetectable (<0.5 ng/mL) to 22.3 ng/mL with a mean value of 5.9 ng/mL in a US study cohort (27). Another study in German women reported serum maternal BPA levels between 0.3 to 18.9 ng/mL with median values of 3.1 ng/mL (38). A larger study of 300 participants in South Korea investigating serum BPA levels in maternal and cord blood reported BPA concentrations from non-detectable to 66.48 ng/mL in pregnant women and from non-detectable to 8.86 ng/mL in umbilical cord blood (39). Previous studies using chromatographic methodologies to measure BPA in term cord blood have reported similar findings to the current study with mean levels ranging from 0.13 ng/mL to 1.13 ng/mL (current study median cord serum BPA 0.92 ng/mL) (39,40). We reported the majority of both maternal serum (71%) and cord blood samples (73%) had BPA levels lower than the detectable limit of the assay. Detection rates in other studies ranged from 14 to 17% above lower limit of detection for serum maternal BPA (41,42). In the current study where serum BPA was detectable in maternal/child pairs, median maternal levels were not statistically significant from the median cord bloods when analysed using Wilcoxon paired analysis (p=0.4). A positive correlation was found between maternal and foetal BPA concentrations, correlation coefficient 0.892. The child birth weight and maternal BMI also showed statistically significant association with cord BPA levels but to a lesser degree than maternal BPA levels. However maternal BMI had no significant association with maternal serum BPA or BPA-g levels. This may be due to the relatively homogenous distribution of BMI with very few maternal

participants having normal or low BMIs. The current study also displayed highly variable BPA levels in maternal and cord blood as has been previously reported (27-31).

Serum BPA levels of greater than 0.23 ng/mL has been reported as the lowest observed adverse effect concentration (LOEC) where in vitro effects on human tissues are observed (11,43). In this study all the detectable cord blood BPA levels were 0.4 ng/mL or greater indicating that in those infants where BPA was present it was present in significant levels to possibly impact on health. In the current study we did not demonstrate any significant association of either BPA and BPA-g levels in maternal serum or cord blood on child birth weight, when birth weight was analysed as the dependent variable. However, effects of BPA exposure such as the endocrine, metabolic and neurodevelopmental sequelae will only present later in childhood, puberty or even adulthood. Molecular mechanisms that underlie the neurodevelopmental toxicity and sex-specific effects of BPA are still not completely elucidated (35, 44). Recent research suggests that long-lasting effects of prenatal BPA exposure likely involve disruption of epigenetic programming during development (44). In particular, DNA methylation and histone modifications are the epigenetic processes that are sensitive to environmental disruptors (46). This further underlines the importance of exposure of progeny in-utero due to possibility of generational effects. Unfortunately, at this stage we are unable to assess these outcomes as part of the study.

Controversy exists with regards to contamination of samples with BPA during sample collection, preparation and measurement processes. The finding that the majority of our samples (maternal serum and cord blood) had BPA values that were below the detection level of our assay is highly suggestive that BPA contamination if indeed present was minimal/non-detectable. Wherever possible glass equipment including flasks, vials, pipettes were used during sample preparation and analysis. Polypropylene tubes not containing BPA were used for sample collection. Water used for sample preparation was also run and found not to have detectable BPA levels. Additionally, blanks were run with each batch to further ensure contamination was not present from the sample preparation and handling phases. We also demonstrated that storage of blank serum samples and BPA spiked samples for several months in polyethylene tubes did not show any significant increase in BPA concentrations indicating leaching was not appreciable. Recovery studies with charcoal stripped serum also indicate that leaching from any plastic equipment used was minimal (not detectable from instrument noise) and the soft ionisation extraction techniques utilised for sample analysis and preparation did not result in deconjugation of BPA-g to BPA in the analysis process. It is conceivable that small amounts of BPA may have contaminated samples such that samples that would have otherwise been lower than the detectable limit are now just above the limit. In the case of this study only 3 samples for the maternal samples were close to BPA LOQ or less than 10% above it and for cord blood samples 3 were at LOQ. The rest of both maternal serum and cord blood samples were 20% or more higher than the LOQ. In a review of recent studies involving BPA measurement vom Saal *et al* indicated that there appears to be

no, or little significant contamination noted with current procedures of analysis and sample handling (47). Demonstration of detectable BPA-g levels in the vast majority of maternal child pairs with detectable BPA levels is also an indicator that BPA contamination of samples in this study was not to a significant degree. For maternal serum there was only 1 participant were BPA was detectable and BPA-g was undetectable in serum. For cord blood there was only 2 participants where BPA was detectable, and BPA-g was undetectable in serum. BPA levels in both patients was close to limit of quantitation and BPA-g just below.

Another limitation of this study that could be cited is that maternal BPA measurements from earlier in pregnancy were not performed as part of the study. The early intra-uterine period, specifically the 8-14 weeks when organogenesis occurs, is generally considered the most sensitive period for any interference from toxin exposure. However, exposure in late pregnancy has also been shown to be of clinical significance by animal studies (48). Since repeating sampling was not performed for each maternal /child pair the findings of this study represent only a snapshot of possible exposure. Unfortunately, data for exact time of day of maternal collection was not available and further analysis could not be performed to determine the effect of time of day on maternal BPA serum levels. However, the clinics at which collection of samples occurred routinely see individuals from 8 am to around 12 pm., thus samples were taken during mid-morning for the majority of volunteers.

The current study is the first to the authors' knowledge to measure and demonstrate significant BPA and BPA-g levels amongst a maternal/child cohort in the African continent. This illustrates that the issue of BPA exposure is not reserved to western industrialised nations. Additionally, analyses of BPA and BPA-g were performed utilising the specific and sensitive liquid chromatography tandem mass spectrometry methodology with deuterated internal standards as opposed to less specific methods such as immunoassay and spectrophotometry. The method utilises small serum volumes of 50 µl with only 20 µl injection volume required for analysis making it a relatively sensitive method given minimal sample volumes used. This together with the short run time and simple sample preparation would allow for easier implementation in other centres.

Competing interests: The authors have no competing interests to declare

Funding: This research was funded by the University of KwaZulu Natal, National Research Foundation (NRF) Thuthuka grant, National Health Laboratories Service Research Trust Grant and Loreal Unesco Women in Science PhD fellowship award.

Author Contributorship: VG researched literature and conceived the study with AC and RN. VG, AC, RN was involved in protocol development, gaining ethical approval. VG, MW and TM development of BPA assay and data analysis. VG wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript

Acknowledgements: We would like to thank Dr Pragalathan Naidoo, Dr Terisha Ghazi and Ms Kareshma Asaram for their assistance.

Honorarium: none to declare

References

1. Diamanti- Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM et al. Endocrine -Disrupting Chemicals: An Endocrine Society Scientific Statement. Endocrine Reviews 2009; 30: 293-342.
2. EPA report on endocrine disruptors https://www.epa.gov/endocrine-disruption. Accessed 17 September 2016
3. Moriyama K, Tagami T, Akamizu T, Usui T, Saijo, Kanamoto N, et al. Thyroid Hormone action is disrupted by Bisphenol A as an antagonist. Journal of Clinical Endocrinology and Metabolism 2002;87;5185-5190.
4. Gore AC, Chappell VA, Fenton SE, et al. EDC-2: The Endocrine Society's Second Scientific Statement on endocrine-disrupting chemicals. Endocrine Reviews 2015; 36: E1–150.
5. Inadera H. Neurological Effects of Bisphenol A and its analogues. International Journal of Medical Sciences 2015;12: 926-936.
6. Lim YH, Bae S, Kim BN, Shin CH, Lee YA, Kim JI. Prenatal and postnatal Bisphenol A exposure and social impairment in 4-year old children. Environmental Health 2017; 16: 79.
7. Braun JM, Kalkbrenner AE, Calafat AM, Yolton K, Ye X, Dietrich KN, Lanphear BP. Impact of early-life bisphenol A exposure on behavior and executive function in children. Pediatrics. 2011; 128: 873–82.
8. Sheehan DM, Willingham EJ, Bergeron JM, Osborn CT,Crews D. No threshold dose for estradiol-induced sex reversal of turtle embryos: how little is too much? Environmental Health Perspectives 1999;1 07:155–159.
9. Skinner MK. Endocrine Disruptors and Epigenetic Transgenerational Disease Etiology. Paediatric research 2007; 61: 48-50.
10. National Institute of Environmental Health Sciences. Accessed 18 December 2018 https://www.niehs.nih.gov/health/topics/agents/sya-bpa/index.cfm
11. vom Saal FS, Huges C. An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. Environmental Health Perspectives 2005;113: 926–933.
12. Khim JS, Lee KT, Villeneuve DL, Kannan K, Giesy JP, Koh CH. In vitro bioassay determination of dioxin-like and estrogenic activity in sediment and water from Ulsan Bay and its vicinity, Korea. Archives of Environmental Contamination and Toxicology 2001; 40: 151–160.
13. Domoradzki JY, Pottenger LH, Thornton CM, Hansen SC, Card TL, Markham DA, et al. Metabolism and pharmacokinetics of bisphenol A (BPA) and the embryo-fetal distribution of BPA and BPA-monoglucuronide in CD Sprague-Dawley rats at three gestational stages. Toxicological Sciences 2003; 76: 21–34.

14. Takahashi O and Oishi S. Disposition of orally administered 2,2-Bis (4-hydroxyphenyl) propane (Bisphenol A) in pregnant rats and the placental transfer to foetuses. Environ Health Perspect. 2000; 108:931-935
15. Balakrishnan B, Henare K, Thorstensen EB, Ponnampalam AP, Mitchell MD. Transfer of bisphenol A across the human placenta. American Journal of Obstetrics and Gynecology. 2010 ;202:393.e1-7.
16. Gao X, Wang HS. Impact of Bisphenol A on the cardiovascular system- epidemiological and experimental evidence and molecular mechanisms. International Journal of Environmental Research and Public Health 2014; 11:8399-841.
17. Bertoli S, Leone A and Battezzati A. Human bisphenol A exposure and the Diabesity phenotype. Dose Response 2015; 13:1-12
18. Hwang S, Lim J, Choi Y and Jee SH. Bisphenol A exposure and type 2 diabetes mellitus risk: a meta-analysis. BMC Endocrine Disorders 2018; 18: 81.
19. Jalal N, Surendranath AR, Pathak JL, Yu S, and Chung CY. Bisphenol A the mighty and the mutagenic. Toxicology Reports 2018;5: 76-84.
20. L Yin, Burns KA and Korach KS. Estrogen receptor (ER)-mediated activation by endocrine disrupting chemicals (EDCs): A comparison between synthetic and natural compounds. Diabetes Metabolism Journal 2019; 43:46-48
21. Watson CS, Jeng YJ and Kochukov MY. Nongenomic signaling pathways of estrogen toxicity. Toxicological Sciences 2010; 115: 1–11.
22. Kojima H, Takeuchi S, Sanoh S, Okuda K, Kitamura S, Uramaru N et al. Profiling bisphenol A its eight analogues on transcriptional activity via human nuclear receptors. Toxicology 2019; 413:48-55.
23. Atkinson A and Roy D. In vivo DNA adduct formation by bisphenol A. Environmental and Molecular Mutagen 1995; 26: 60-6.
24. Calafat AM, Kuklenyik Z, Reidy JA, Caudill SP, Ekong J, Needham LL. Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population. Environmental Health Perspectives 2005; 113: 391–95.
25. Liu B, Lehmler HJ, Sun Y, Xu G, Liu Y, Zong G et al. Bisphenol A substitutes and obesity in US adults:analysis of a population based, cross sectional study. Lancet Planet Health 2017; 1: e114-e122.
26. Lehmler HJ, Liu B, Gadogbe M and BaoW. Exposure to Bisphenol A, Bisphenol F and Bisphenol S in US Adults and Children: The National Health and Nutrition Examination Survery 2013-2014. ACS Omega 2018; 30:6523-6532.
27. Padmanabhan V, Siefert K, Ransom S, Johnson T, Pinkerton J, Anderson L, et al. Maternal bisphenol-A levels at delivery: A looming problem. Journal of Perinatology 2008; 28: 258-263

28. Cantonwine DE, Ferguson KK, Mukherjee B, McElrath TF and Meeker JD. Urinary Bisphenol A levels during pregnancy and risk of preterm birth. Environmental Health Perspectives 2015; 123: 895-901.
29. Wan Y, Choi K, Kim S, Ji K, Chang H, Wiseman S et al. Hydroxylated Polybrominated Diphenyl Ethers and Bisphenol A in pregnant women and their matching foetuses: Placenta transfer and Potential Risks. Environmental Science and Technology 2010; 44: 5233-5239.
30. Gerona RR, Woodruff TJ, Dickenson CA, Pan J, Schwartz JM, Sen S. Bisphenol-A (BPA), BPA glucuronide and BPA sulfate in mid-gestation umbilical cord serum in a Northern and Central California population. Environmental Science and Technology 2013; 47: 40276.
31. Van den Berg L, Chahoud I, Heindel JJ, PadmanabhanV, Paumgartten FJR and Schoenfelder G. Review:Urinary, circulating and tissue biomonitoring studies indicate a widespread exposure to Bisphenol A. Cienc, saude Coletiva 2012; 17; 407-434.
32. Ho KL, Yuen KK, Yau MS, Murphy MB, Wan Y, Fong BMW. Glucuronide and Sulfate Conjugates of Bisphenol A: Chemical Synthesis and Correlation Between Their Urinary Levels and Plasma Bisphenol A Content in Voluntary Human. Archives of Environmental Contamination and Toxicology 2017; 73: 410-420.
33. Nishikawa M, Yanagisawa R, Koike N, Inoue H, Yokota H. Placental transfer of conjugated bisphenol A and subsequent reactivation in the rat fetus. Environmental Health Perspectives 2010; 118:1196–203.
34. Gauderat G, Picard-Hagen N, Toutain PL, Servien R, Viguie C, Puel S et al. Prediction of human prenatal exposure to bisphenol A and bisphenol A glucuronide from an ovine semi-physiological toxicokinetic model. Nature Scientific reports 2017; 7: 15330.
35. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. Human exposure to bisphenol A (BPA). Reproductive Toxicology 2007; 24: 139–77.
36. Stahlhut RW, Welshons WV and Swan SH. Bisphenol A Data in NHANES suggest longer than expected half-life. Substantial non-food exposure or both. Environmental Health Perspectives2009;117: 784-789
37. CLSI EP15-A3 protocol volume 34, number 12. User verification of precision and estimation of bias: approved guideline 3rd edition Sept 2014
38. Schonfelder G, Wittfoht W, Hopp H, Talsness CE, Paul M, Chahoud I. Parent bisphenol A accumulation in the human maternal-fetal-placental unit. Environmental Health Perspectives 2002; 110: A703–A707.
39. Lee YJ, et al. Maternal and fetal exposure to bisphenol A in Korea. Reproductive Toxicology. 2008; 25:413–9.
40. Zhang T, Sun H, Kannan K. Blood and Urinary Bisphenol A Concentrations in Children, Adults, and Pregnant Women from China: Partitioning between Blood and Urine and Maternal and Fetal Cord Blood. Environmental Science and Technology 2013; 47:4686–94.

41. Teeguarden JG, Calafat AM, Ye X, Doerge DR, Churchwell MI, Gunawan R. Twenty-four hour human urine and serum profiles of Bisphenol A during High-Dietary Exposure. Toxicological Sciences 2011; 123:48-57.
42. Y He, M Miao, LJ Herrinton, C Wu, W Yuan, Z Zhou, et al. Bisphenol A levels in blood and urine in a Chinese population and the personal factors affecting the levels. Environmental Research 2009; 109: 629-633.
43. Welshons WV, Nagel SC, vom Saal FS. Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. Endocrinology. 2006;147: S56–69.
44. National Toxicology Program. NTP-CERHR Expert Panel Report on the Reproductive and Developmental Toxicity of Bisphenol A. Durham:Research Triangle Park; 2007.
45. Kundakovica M, Gudsnuka K, Franksa B, Madrida J, et al. Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure. Proceedings of the National Academy of Science 2013;110: 9956–9961.
46. Reik W. Stability and flexibility of epigenetic gene regulation in mammalian development. Nature. 2007; 447: 425–432
47. Vom Saal FS and Welshons WV. Evidence that Bisphenol A can be accurately measured without contamination in human serum and urine and that BPA causes numerous hazards from multiple routes of exposure. Molecular and Cellular Endocrinology 2014; 398:101-113.
48. Ohtani N, Suda K, Tsuji E, Tanemura K, Yokota H, Inoue H, et al. Late pregnancy is vulnerable for exposure to BPA. Journal of Veterinary Medical Science 2018; 80:536-543

CHAPTER 5

A PILOT STUDY: RELATIONSHIP BETWEEN BISPHENOL A, BISPHENOL A GLUCURONIDE AND SEX STEROID HORMONE LEVELS IN CORD BLOOD IN A SOUTH AFRICAN POPULATION

Gounden V[1,2], Warasally MZ[2], Magwai T[2], Naidoo R[3], Chuturgoon A[4]
[1]Department of Chemical Pathology, University of KwaZulu-Natal and [2]National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital, Durban, South Africa
[3]Department of Occupational Health, University of KwaZulu-Natal, Durban, South Africa
[4]Department of Medical Biochemistry, University of KwaZulu-Natal, Durban, South Africa

Corresponding author: Verena Gounden
Department of Chemical Pathology, University of KwaZulu-Natal and National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital
2nd Floor Pathology Building Inkosi Albert Luthuli Central Hospital
800 Vusi Mzimela Road, Cato Manor, Durban, 4058
Email: verenagounden@yahoo.com

Keywords:
Bisphenol-A; liquid chromatography tandem mass spectrometry; mother-child pairs; cord blood; estradiol; testosterone

Reproductive Toxicology. 2021;100:83-89.

DOI: 10.1016/j.reprotox.2021.01.004.

Abstract

Exposure to Bisphenol A (BPA) during early development particularly in- utero has been linked to a wide range of pathology. The aim of this study was to examine the relationship of BPA and its naturally occurring metabolite BPA-glucuronide (BPA-g) with sex steroid hormone levels in South African mother-child pairs.

Third-trimester serum maternal samples and matching cord blood samples were analyzed for BPA, BPA-g and nine sex steroid hormones using liquid chromatography tandem mass spectrometry (LC-MS/MS).

Sixty maternal and child pairs were analyzed. Rank correlation demonstrated a significant positive relationship between cord blood estradiol and cord blood BPA ($p=0.002$) and maternal BPA levels ($p=0.02$) respectively. Cord blood testosterone from male infants showed a negative Spearman's correlation ($r=-0.5$, $p=0.02$) with maternal BPA-g. There was no statistical difference in total testosterone levels in cord blood from male and female infants.

The findings of the current study indicate a significant relationship between some key sex steroid hormones namely testosterone, dihydro-testosterone and estradiol. and fetal exposure BPA.

Introduction

The United States environmental protection agency has defined endocrine disrupting chemicals or endocrine disruptors (EDs) as "exogenous agents that interfere with the normal function of endogenous hormones responsible for the maintenance of homeostasis and the regulation of developmental processes. These agents act by disrupting the synthesis release, transport, metabolism binding action or elimination of natural hormones in the body"(1). Over the past 15–20 years both the scientific literature and then in turn the lay media have reported on the possible deleterious effects of these exogenous agents on fish, wildlife, the environment and human health. The possible deleterious effects on human health associated with endocrine disruptors has ranged from effect on reproductive health in particular sperm health, their role as carcinogens, and increasingly reports on effects on other endocrine and metabolic functions, such as, thyroid disease and obesity.

Bisphenol A (BPA), an endocrine disruptor, is found in a vast array of plastic consumer products including lining of tin cans, food and water containers, medical devices and toys (1,2). Exposure to BPA has been linked to prenatal and postnatal adverse effects on multiple tissues, including the reproductive system and neurodevelopment. Animal studies and the smaller number of human studies have indicated that exposure in the perinatal period is of particular significance (1). BPA effects are typically attributed to its estrogenic or anti-estrogenic action however this action can not completely account for the adverse effects of low potency BPA at the low-dose exposures that are commonly seen (1,3). BPA has also been reported interact with androgen, glucocorticoid and thyroid receptors (4). Previous reports have described effects of BPA on steroid hormone levels and metabolism. In vitro studies of exposure of BPA to human granulosa cells have shown changes in progesterone and estradiol synthesis with effect on enzyme levels (5). Animal studies have also demonstrated that BPA exposure at some doses affects ovarian follicle numbers as well and progesterone and estradiol levels (6). Takeuchi et al in an earlier study described significant correlation between BPA levels and total and free testosterone levels in humans (7).

BPA-glucuronide (BPA-g) is a major metabolite of BPA metabolism. Levels in cord blood remain steady and are thought to reflect the cumulative dose of BPA received during late pregnancy (8). BPA-g was widely thought of inert as it is unable to bind to oestrogen receptors however recent evidence in animal studies suggest that it may deconjugate to expose the foetus to BPA even though adequate conjugation of BPA has occurred after maternal intake (33/9). In context of the short half-life of BPA, BPA-g acts as a surrogate marker of BPA exposure (9).

In a previously published study, we reported BPA was detectable in more than 25% of maternal and cord blood samples. We demonstrated significant positive correlation between maternal and child BPA and BPA-g levels with correlation coefficients of 0.892 and 0.744, respectively (10).

As part of a larger study examining the effect of BPA on maternal and child-pairs we examined the relationship between BPA and BPA-g levels on sex steroid and related hormone levels in maternal and child pairs.

Methods

Population and study samples

Blood samples and data collected as part of the mother and child in the environment (MACE) birth cohort study were utilized for this study. The MACE study population consists of pregnant females recruited from antenatal clinics in Durban, South Africa from the "South basin" area and other clinics in the North Durban area. The Durban South basin is an area where large communities are located within heavily polluted large-scale industrial enterprises. Mothers were recruited during the third trimester of pregnancy (between 27 completed to 40 weeks of pregnancy). Details regarding pregnancy outcome for participants were also collected during the course of the MACE study. Venous blood samples were taken during the third trimester from the pregnant individuals. Cord bloods were taken at delivery. Blood were collected from maternal participants at one of their regularly scheduled ante-natal appointments. Maternal and cord bloods collected in serum polypropylene vacutainer tubes were later analyzed for BPA and BPA-g. These samples were centrifuged, separated and serum stored at -80 degrees Celsius until analysis. Maternal /cord paired samples with sufficient serum volumes were utilized for the current study. Inclusion and exclusion criteria were as per MACE study which has been described previously (10).

Bisphenol A and Bisphenol glucuronide analysis

The methods used for determination of BPA and BPA-g levels has been previously described in detail (10).

Determination of steroid hormone levels

The following nine steroid hormones were analyzed in maternal serum and cord blood using LC-MS/MS methodology: estradiol (E2), total testosterone (TT), 11-deoxycorticosterone (11DOC), Dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS) androstenedione (Andro), 17-OH progesterone (17OHP), dihydrotestosterone (DHT) and progesterone (Prog). There hormones were selected as they cover the major sex steroid hormones in humans.

A commercially available kit the MassChrom Steroid Panel 2 kit (Chromosystems Instruments and Chemicals GmbH, Germany) was utilized for analysis of steroid hormones

Sample/calibrator/control preparation was as per manufacturer's instructions. Chromatographic separations were carried out using the AB-Sciex 4500 triple quadrupole mass spectrometer equipped

with an Agilent 1260 Ultra high-performance liquid chromatography (uHPLC) system. Analytes of interest were separated on the analytical column provided in the commercially available kit. A linear gradient was used from 0-70% of Mobile phase B in Mobile phase A followed by a hold for 1 minute at a flow rate of 0.8 ml/min. Total run time was 12.5 minutes. Injection volume utilized was 30µl.
The following single reaction monitoring (SRM) quantifier and qualifier transitions were used: Androstenedione 287>109,287>97; DHEA 289>213, 271>213; DHEAS 271>213, 271>197; DHT 291>255; 291>159; E2 255>159, 255>133; 17 OHP 331>109, 331>97; Progesterone 315>97, 315>109; Testosterone 289>97, 289>109; 11DOC 331>109, 331>91.

De-deuterated internal standards were utilized for each analyte of interest and were added to samples prior to extraction. Six levels of commercially available standards (including blank) and three levels of serum based internal quality control materials were analyzed at the beginning, middle and end of each run. MassChrom® steroid serum calibrators and controls were purchased from Chromsystems Instruments & Chemicals GmbH (Munich, Germany). A procedural blank was analyzed with each run. Within run coefficient of variation (CV) for the steroid hormones ranged from 0.48% to 12.73%, and for the between-run CVs ranged from 1.6-10.2 percent (%) across the three internal quality control (IQC) levels.

Statistical analysis

Data was assessed for normality using the Shapiro-Wilk test. Non-parametric tests Kruskal Wallis test, Spearman's correlation or Wilcoxon signed rank test for univariate analysis. A p value of <0.05 was considered significant. Analysis of covariance (ANCOVA) was used to assess for confounding variables and for multiple regression analysis. Statistical analysis was performed on Medcalc statistical software program version 18.11 (Medcalc, Belgium).

Ethical approval

The research has complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee. Ethical clearance for this study was obtained from the Biomedical Research and Ethics Committee (BREC) of the University of KwaZulu-Natal (Ethics Clearance Certificate BE 597/16).

Results

Following exclusion of maternal-child pairs where all analytes could not be measured due to insufficient sample volume data analysis of sixty pairs of maternal and cord blood samples were performed and are described here.

Table 5.1 summarizes information regarding hormone levels found in maternal samples, and cord blood samples for male and female newborns. Kruskal-Wallis analysis was performed to determine if statistically significant difference could be identified between the two genders for cord blood hormone levels. There was no noted statistically significant difference between the two genders for the medians of cord blood steroid hormones measured. E2 missed statistical significance with a p value of 0.08. It was noted that the upper range of androgen levels (TT; DHT, Andro, DHEA; DHEAS) were higher for male infant cord blood than for females. As most parameters had a non-gaussian distribution, non-parametric statistical methods were used to analyze data. Spearman's rank correlation showed no statistically significant correlation between maternal estradiol (p=0.55) and testosterone levels (p=0.56).

Relationship maternal, cord blood BPA and steroid hormone levels

Estradiol

Expectedly a strong positive correlation between maternal and cord blood estradiol levels was observed with p <0. 0001. Following ANCOVA analysis with inclusion of other confounding variables such as infant gender, birth weight, gestational age and maternal BMI a statistical significant correlation between maternal and cord estradiol was still present. None of the other variables assessed displayed a significant correlation on ANCOVA with cord blood estradiol this included maternal and cord blood BPA-G level. Cord estradiol levels showed a significant relationship with maternal BPA-g values (p=0.002) when maternal estradiol was excluded from the ANCOVA analysis. Spearman's rank correlation showed a positive correlation of cord blood estradiol with both maternal (p =0.001) and cord blood (p=0.01) BPA- g levels. ANCOVA analysis showed significant correlation (p<0.0001) between maternal estradiol and maternal BPA-g levels independent of maternal body mass index (BMI), age, gestation of pregnancy and fetal gender. Spearman's rank correlation between cord blood estradiol with cord blood BPA and maternal BPA levels were 0.7 (p=0.002) and 0.6 (p=0.02) respectively. ANCOVA analysis of the relationship of the steroid hormones with maternal and cord BPA levels to determine the effect of confounding variables could not be performed due to the limited number of samples that had detectable BPA levels (n=15).

Total Testosterone

There was no statistically significant correlation between maternal and cord blood testosterone levels (p=0.09). Cord blood testosterone levels showed statistically significant negative correlation (p=0.009) with both maternal BPA-g and cord BPA-g levels. Cord blood testosterone from male infants showed a negative Spearman's correlation (r=-0.5, p=0.02) with maternal BPA-g levels. Following ANCOVA analysis variables such as birth weight, infant gender, gestational age had no significant relationship with cord testosterone levels. Figure 5.1 illustrates the lack of statistically significant difference of cord

blood testosterone concentrations between both sexes. Overall cord blood testosterone levels correlated negatively with cord (r=-0.60; p=0.02) and maternal BPA levels (r=-0.52; p=0.05).

Androstenedione

Cord blood androstenedione levels showed statistically significant negative correlation with both maternal BPA-g (p=0.01) and cord BPA-g levels (p=0.01). Following ANCOVA analysis both maternal BPG (p=0.01) and maternal androstenedione (p=0.001) had a statistically significant relationship with cord blood androstenedione levels. None of the confounding variables (gestational age, infant birth weight and gender) had a significant influence on the relationship between cord androstenedione and BPA-g levels. Maternal androstenedione levels had a significant correlation (p=0.02) with maternal BPA-g levels. Cord blood androstenedione levels were positively correlated with maternal androstenedione levels (r=0.62; p<0.001).

11DOC

Cord blood 11–DOC levels showed a trend for negative correlation with maternal BPA-G levels but this missed statistical significance (p=0.05), however, the negative correlation with cord BPA- levels was statistically significant on Spearman's correlation analysis (p=0.01).

On ANCOVA analysis gestational age, infant birth weight and gender did not show an association with cord 11 DOC levels.

DHT

Cord blood DHT levels showed a negative statistically significant correlation with cord blood BPA-g (p=0.01) but just missed statistical significance for maternal BPA-g levels (p=0.05). There was no statistically significant difference between DHT cord blood levels in male versus female infants (p=0.98).

DHEA

Cord blood DHEA levels showed no statistically significant correlation with maternal BPA-g (p=0.11) and cord blood BPA-g (p=0.72).

DHEAS

Cord blood DHEA levels showed no statistically significant correlation with maternal BPA-g (p=0.65) and cord blood BPA-g (p=0.3).

17 -hydroxyprogesterone (17OHP)

Cord blood 17 hydroxyprogesterone levels did not show a significant relationship with maternal BPA-g (p=0.09), however cord blood 17OHP levels did display a statistically significant negative relationship

with maternal BPA-g (p=0.002). Analysis examining relationship with cord blood BPA-g levels showed no statistically significant findings.

Progesterone

Cord blood progesterone levels showed statistically significant negative correlation with both maternal BPA-g (p=0.03) and cord BPA-g levels (p=0.0027). However, when corrected for other confounder variables -birth weight and gestational age the correlation was not statistically significant.

Testosterone and estradiol ratio

Testosterone/estradiol (T/E) ratios, expressed in nmol/nmol ranged from 0.0008 to 202 (median 4.80) in male cord bloods. In female cord blood samples, the T/E ratios ranged from 0.001 to 25.10 nmol/nmol with a median of 2.15 nmol/nmol. (refer Figure 2)

There was a negative correlation between male cord T/E ratios and cord blood BPA-g levels (r=-0.617; p=0.0003) and maternal BPA-levels r=-0.63; p=0.0001). Kruskal-Wallis analysis between T/E ratios in male and female cord blood samples did not reach statistical difference (p=0.17).

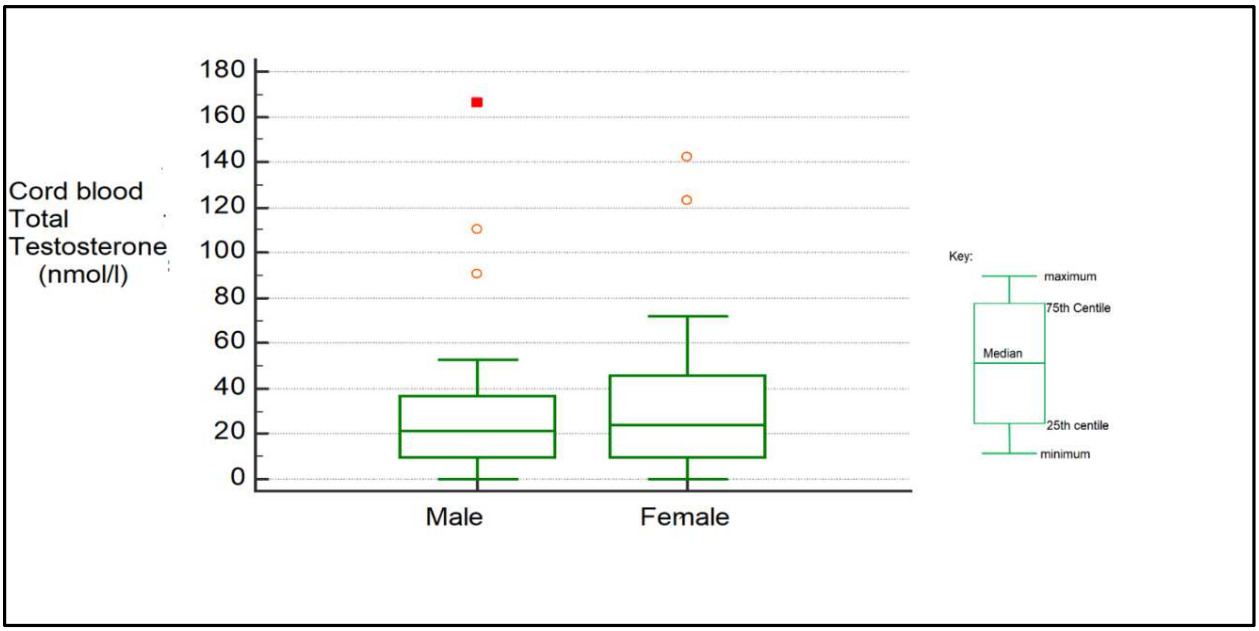

Figure 5.1 Box and whiskers diagram depicting the range of cord blood testosterone levels in male and female infants

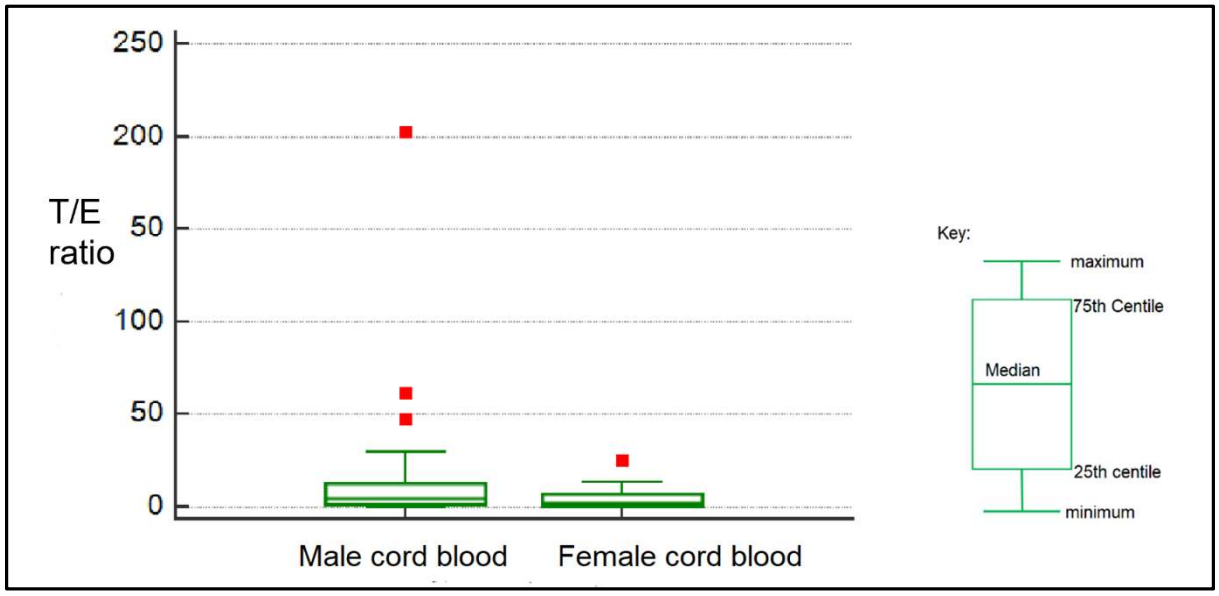

Figure 5.2 Box and whiskers diagram depicting the range of cord blood testosterone/estradiol ratios in male and female infants

Table 5.1 Summary data of maternal and cord blood pairs

Maternal	Age	BMI kg/m^2 Mean (SD)	BPA(ng/mL) Median (range)	BPA-g (ng/mL) Median (range)	E2 (pmol/L) Median (range)	TT (nmol/L) Median (range)	11-DOC (nmol/L) Median (range)	DHEA (nmol/L) Median (range)	DHEAS (nmol/L) Median (range)	Andro (nmol/L) Median (range)	17-OHP (nmol/L) Median (range)	DHT (nmol/L) Median (range)	Prog (nmol/L) Median (range)
N=60	25 (16- 40)	32.2 (7.7)	1.16 (0.4-15.3) N=15	4.64 (0.15-21.8) N=54	7985 (219-468000)	12.7 (0.34-85.2)	1.44(0.22-28)	133 (6.6-764)	1390 (170-9250)	72.6 (1.71-1570)	27.6 (2.61-465)	2.3(0.4-37.8)	299.5 (45.6-3810)
Cord blood	Gestational Age (weeks) Median (range)	Birth weight (grams) Mean (SD)	BPA (ng/mL) Median (range)	BPA-g (ng/mL) Median (range)	E2 (pmol/L) Median (range)	TT (nmol/L) Median (range)	11-DOC (nmol/L) Median (range)	DHEA (nmol/L) Median (range)	DHEAS (nmol/L) Median (range)	Andro (nmol/L) Median (range)	17-OHP (nmol/L) Median (range)	DHT (nmol/L) Median (range)	Prog (nmol/L) Median (range)
Male (N=36)	38 (35-41)	2910 (475)	0.53 (0.4-8.3) N=9	4.4 (0.4-16.9) N=30	5950 (33-268000)	21.5 (0.16-166)	4.7 (1.0-128)	121 (6.29-741)	1840 (194-8080)	146 (0.29-965)	49.3 (12-315)	2.58 (0.32-7.72)	869 (228-2950)
Female (N=24)	38 (36-41)	2936 (452)	1.09 (0.5-13.2) N=6	4.64 (0.65-21.3) N=22	14700 (1160-121000)	24.1 (0,17-142)	4.7 (1.98-55.8)	91.8 (14.6-450)	1795 (5.1-8230)	126 (49-814)	54.1 (16.6-302)	2.4 (0.51-7.54)	1385 (63.7-3490)
					P=0.085	P=0.52	P=0.73	P=0.71	P=0.99	P=0.4	P=0.12	P=0.98	P=0.22

Non parametric data presented as median (range); normally distributed data presented as mean (±SD)

Discussion

The presence of BPA in the environment is ubiquitous. There has been much research on how the intrauterine environment and exposure of an individual preconception and pregnant females during the gestational period affect eventual infant outcomes and disease occurrence throughout life (11). The distribution of disease that can result from the interaction of the fetus with its environment range from poor birth outcome, disorders of anatomical development, reproductive health, malignancy, neuro-behavioral, metabolic and endocrine related disorders. Several studies in animal models and humans have described the association of BPA exposure with all of the afore-mentioned pathologies (12,13,14).

The inter-play of steroid hormones is a vital element to the normal functioning of the feto-maternal-placental unit. Abnormalities of these hormones may lead to disorders of conception, abnormal fetal development, poor birth outcomes and pathology later in life. Aberrant progesterone levels have been associated with poor placental implantation, pregnancy loss and pre-eclampsia (15,16,17). Elevated testosterone and elevated estradiol levels in the fetus have been linked to autism (18,19). Abnormal levels of testosterone have been associated with abnormal reproductive organ development in the male and female fetus (20). Low levels of testosterone in -utero have also been linked to increased incidence of obesity, diabetes and hypertension in adulthood (21). High levels of androgen exposure in-utero have been associated with pre-eclampsia as well as polycystic ovarian disease later in life (16,22,23). The number of studies reviewing the relationship between BPA and steroid hormones in maternal-child pairs is limited. A study by Kolatorova *et al*, which evaluated BPA and steroid hormones in 27 maternal-child pairs reported no association with maternal BPA and cord blood steroids (24). A further larger study by Minatoya *et al*, reported findings that suggest that fetal BPA levels may be associated with changes in certain reproductive hormones (25). To the authors knowledge this is the first study to examine the relationship of BPA as well as one of its major metabolites (BPA-g) with steroid reproductive hormones in maternal-child pairs.

It should be highlighted that for all steroid hormones analysed no statistically significant differences were noted between the cord blood levels between female and male infants. For hormones like estradiol, which is high in both genders in cord blood and then declines soon after birth there is conflicting data with regards to whether female or male infants have the higher cord blood levels. In a review by Kuijper *et al*, none of the studies analyzed showed a statistical significance between estradiol cord blood levels between both sexes (26). This is in keeping with the current study findings which shows a trend for higher estradiol values in the cord blood of female infants but is not statistically significant. We demonstrated a significant positive correlation between BPA-g levels in maternal and

cord blood with cord blood estradiol levels. The study by Minatoya *et al*, reviewing cord blood BPA levels and reproductive hormones in neonates demonstrated that cord BPA level showed weak positive association with testosterone, estradiol, and progesterone levels in boys (25). Their findings suggested that foetal BPA levels might be associated with changes in certain reproductive hormone levels of neonates in a sex-specific manner. Whilst the present study showed trends for increased levels of certain sex steroid hormones, for example estradiol in female infants these did not approach statistical significance.

Studies by Barry *et al* and Robinson *et al*, have reported significantly lower total or bioavailable testosterone in cord blood of healthy female infants versus male infants (27, 28). Of particular note, is that the current study did not show a significant difference between cord blood total testosterone levels and T/E ratios in female and male infants. In a metanalysis of 18 studies reviewing sex differences in testosterone in cord blood revealed significantly higher cord TT in boys than girls at a moderate effect size. However, the authors also reported significant heterogeneity between the 18 studies (27).
In the current study a significant negative relationship between testosterone levels in cord blood and maternal serum BPA-g levels was described. Liu *et al* in their study examining urine maternal bisphenol levels also reported lower cord testosterone levels and lower T/E ratios in male newborns with high urine BPA levels. They reported total testosterone levels in male cord blood ranging from 0.8 to 65 nmol/L (median 10.5 nmol/l) (29). We report male cord blood testosterone values ranged from 0.16-166 nmol/L (median 21.5 nmol/L).

The current study does not demonstrate significant correlation between maternal and child testosterone levels. The data with regards to normal testosterone ranges in infant cord blood is varied, conflicting and depends on the methodology used (immunoassay based or chromatography/mass spectrometry) to assess testosterone and the different forms of testosterone (total, bioavailable or free) measured.
High androstenedione levels were observed in both maternal and cord blood with maternal blood median of 72.6 nmol/L and male and female cord blood medians of 146 nmol/L and 126 nmol/l, respectively. In this study we have reported higher androstenedione levels in maternal and cord blood than have been previously reported in healthy newborns and mothers at full term (26). Following removal of possible outliers, median androstenedione values remained significantly elevated with values of 68 nmol/L and 128 nmol/l for maternal and cord blood respectively. The presence of an interference/s causing falsely elevated androstenedione values in the cord blood samples is a possible consideration. This has been described in another study measuring steroid hormones in newborns utilizing an LC-MS/MS method with a similar extraction technique (protein precipitation and solid phase extraction) as the current study. Whilst the interferent was not identified in the study by Lima-Valassi *et al*, a change in sample extraction technique was able to reduce the androstenedione values observed (30).

DHT like TT displayed a negative correlation with maternal and cord blood BPA-g levels. Additionally, both hormones displayed no statistical difference between levels of these hormones in male and female cord blood. A previous study using gas chromatography tandem mass spectrometry (GC-MS/MS) reported DHT levels of 2.4 fold higher in male cord blood. The same study reported a smaller difference in male and female cord blood for total testosterone with values in males being 1.3-fold higher (31). The lack of a significant difference between male and female cord blood levels for DHT and TT in the current study is thus a significant finding, particularly in view of the fact that androgen exposure plays a vital role in development of the fetus in the intra-uterine period as well as later in life. Exposure of male fetuses to anti-androgen EDs results in feminization and exposure of female fetuses to androgens leads to their masculinization (32). Both insufficient and excessive androgen levels have sequelae on development of both sexes affecting reproductive, metabolic and neurological development.
BPA has been reported to affect the action of the 5 alpha reductase enzymes responsible for synthesis of DHT from circulating testosterone. Animal studies have shown decreased expression of some 5-alpha reductase isoenzymes in certain tissues following exposure to BPA. DHT may also be decreased due to possible downregulation of TT synthesis mediated by BPA exposure (33, 34).

The mechanisms of BPAs actions as an endocrine disruptor has been linked to its agonistic and/or agonistic actions on hormone receptors including estradiol, androgen and thyroid hormone receptors (35). Exposure to BPA has also been shown to significantly decrease expression CYP2A1 and steroid acute regulatory protein (StAR). Both of which are responsible for rate-limiting steps in the synthesis of steroid hormones (36). Additionally, BPA has been demonstrated to affect the catabolism of steroid hormones (37). BPA exposure may cause epigenetic dysregulation of specific genes involved in steroid hormone production or metabolism which may also affect steroid hormone levels and balance between the estrogens and androgens (38, 39).

The clinical effects of ED exposure are difficult to fully quantify in humans. Exposure occurs in -utero and via the environment and there is exposure to multiple possible EDs with additive effects and variable and ill-defined cut-offs for pathology inducing doses. Numerous studies have shown fetuses, neonates, infants and children to be greater than 10-fold more sensitive than adults to the effect of EDs (40,41). This is further made more complex by reported exposure/s in-utero only developing pathology much later in life for example infertility in adult males. Additionally, the sequalae of ED exposure during fetal development is different from exposure as an adult. One of the more obvious clinical consequences of anti-androgenic exposure with BPA in-utero-is interference with masculinization and neurodevelopment. However, several other clinical effects on for example immune maturation, or metabolic function may not be so obvious a result of ED exposure. Clinical evaluation is further made difficult by the lack of sensitive markers of these ED exposure events (42).

There are limitations to the current study. This includes the cross-sectional nature of the study and the small sample size. Additionally, as BPA and BPA-g levels were not followed over time during the course of the pregnancy we cannot be certain to what degree the measured maternal serum and cord blood BPA/BPA-g levels reflect the actual exposure throughout pregnancy and to what extent cord sex steroid hormone reflects the actual hormonal levels throughout the entire intra-uterine period. The advantages of this study is the use of LC-MS/MS methodology to measure both BPA and steroid hormones in maternal and cord blood. BPA-g, a metabolite of BPA was also measured as a surrogate for exposure.

Cord blood is a particularly challenging medium to analyse. Previous methodologies for measurement of steroid hormones that were primarily immunoassay based suffered from significant cross-interference between steroids and their metabolites. The use of liquid chromatography coupled with tandem mass spectrometry reduces the likelihood of interference. Another limitation of the current study was that biologically active fractions of testosterone such as bioavailable testosterone or free testosterone were not estimated or measured directly.

This is the first study to the authors knowledge to examine the relationship between BPA as well as BPA-g with steroid hormones in maternal-child pairs. Our findings suggest a possible relationship between fetal/cord blood steroid hormone levels in particular estradiol (positive) and testosterone (negative) with maternal and cord BPA-g levels a surrogate marker for BPA exposure). Due to the cross-sectional nature of the current study causality or associations with development of disease cannot be shown in the BPA exposed infants. Future studies monitoring the presence of disease in a similar cohort from birth into later adulthood examining the impact of fetal and environmental exposure are required. Also, studies defining the mechanisms for BPA disturbance of steroid hormones levels in humans are needed.

Funding: This research was funded by the University of KwaZulu-Natal, National Research Foundation (NRF) Thuthuka grant, National Health Laboratories Service Research Trust Grant and Loreal Unesco Women in Science PhD fellowship award.

Acknowledgements: We would like to thank Dr Pragalathan Naidoo, Dr Terisha Ghazi and Ms Kareshma Asaram for their assistance.

Author Contributorship: VG researched literature and conceived the study with AC and RN. VG, AC, RN was involved in protocol development, gaining ethical approval. VG, MW and TM development of BPA and steroid assay and data analysis. VG wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

References

1. Diamanti- Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, et al. Endocrine -Disrupting Chemicals: An Endocrine Society Scientific Statement. Endocrine Reviews 2009; 30: 293-342
2. National Institute of Environmental Health Sciences. Accessed 18 December 2018 https://www.niehs.nih.gov/health/topics/agents/sya-bpa/index.cfm
3. Jalal N, Surendranath AR, Pathak JL, Yu S and Chung CY. Bisphenol A the might and the mutagenic. Toxicology reports 2018:5;76-84
4. Kojima H, Takeuchi S, Sanoh S, Okuda K, Kitamura S, Uramaru N et al. Profiling bisphenol A its eight analogues on transcriptional activity via human nuclear receptors. Toxicology 2019; 413:48-55
5. Abdallah M, Michal A, Gil Y, Ariel H, et al Does BPA alter steroid hormone synthesis in human granulosa cell in vitro? Human Reproduction, 2016;31: 1562–1569.
6. Patel S, Brehm E, Gao L, Rattan S et al. Bisphenol A Exposure, Ovarian Follicle Numbers, and Female Sex Steroid Hormone Levels: Results From a CLARITY-BPA Study *Endocrinology* 2017;158: 1727–1738.
7. Takeuchi T, Tsutsumi O. Serum bisphenol A concentrations showed gender differences, possibly linked to androgen levels. Biochemical and Biophysical Research communications 2002; 291: 76-78.
8. Gauderat G, Picard-Hagen N, Toutain PL, Servien R, Viguie C, Puel S, et al. Prediction of human prenatal exposure to bisphenol A and bisphenol A glucuronide from an ovine semi-physiological toxicokinetic model. Nature Scientific report 2017; 7: 155330.
9. Nishikawa M, Yanagisawa R, Koike N, Inoue H, Yokota H. Placental transfer of conjugated bisphenol A and subsequent reactivation in the rat fetus. Environmental Health Perspectives 2010; 118:1196–1203.
10. Gounden V, Zain Warasally M, Magwai T, Naidoo R, Chuturgoon A. A pilot study: Bisphenol-A and Bisphenol-A glucuronide levels in mother and child pairs in a South African population. Reproductive Toxicology 2019;89:93-99.
11. Barker DJ. The origins of the development origins theory. Journal of Internal Medicine 2007; 261: 412-417.
12. Giulivo, M., Lopez de Alda, M., Capri, E., Barcelo, D.et al. Human exposure to endocrine disrupting compounds: their role in reproductive systems, metabolic syndrome and breast cancer. A review. Environmental Research 2016: 151, 251–264.
13. Pinney SE, Mesaros CA, Snyder NW, Busch CM, Xiao R, Aijaz S, et al. Second trimester amniotic fluid bisphenol A concentration is associated with decreased birth weight in term infants. Reproductive Toxicology 2017; 67: 1–9.

14. Snijder CA, Heederik D, Pierik FH, Hofman A, Jaddoe VW, Koch HM, et al. Fetal growth and prenatal exposure to bisphenol A: the generation R study. Environmental Health Perspectives 2013: 121: 393–398.
15. Kiprono LV, Wallace K, Moseley J, Martin J, Lamarca B. Progesterone blunts vascular endothelial cell secretion of endothelin-1 in response to placental ischemia. American Journal of Obstetrics and Gynecology 2013; 209: 1-6.
16. Lissauer, D. *et al*. Progesterone promotes maternal-fetal tolerance by reducing human maternal T-cell polyfunctionality and inducing a specific cytokine profile. European Journal of Immunology 2015;45: 2858-2872.
17. Tamimi R, Lagion P, Vatten LJ. Pregnancy hormones, preeclampsia and implication for breast cancer risk in the offspring: Cancer epidemiology, biomarker and prevention. New England Journal of Medicine 2003; 12: 647-50.
18. Auyeung B, Lombardo MV, Baron-Cohen S. Prenatal and postnatal hormone effects on the human brain and cognition. *Pflugers Archives* 2013; 465: 557–571.
19. Simon Baron-Cohen, Alexandros Tsompanidis, Bonnie Auyeung, Bent Nørgaard-Pedersen, et al. Foetal oestrogens and autism. Molecular Psychiatry 2019; 10: s41380.
20. Juul, A., Almstrup, K., Andersson, A. *et al.* Possible fetal determinants of male infertility. Nature Reviews Endocrinology 2014; 10: 553–562.
21. K. R. Kilcoyne, L. B. Smith, N. Atanassova, S. Macpherson,et al . Fetal programming of adult Leydig cell function by androgenic effects on stem/progenitor cells. Proceedings of the National Academy of Sciences, 2014; 10: 132753511.
22. Abbott DH, Kraynak M, Dumesic DA, Levine JE. In utero Androgen Excess: A Developmental Commonality Preceding Polycystic Ovary Syndrome. Frontiers in Hormone Research 2019; 53: 1-17.
23. Filippou P, Homburg R. Is foetal hyperexposure to androgens a cause of PCOS? Human Reproduction Update 2017; 23; 421.
24. Kolatorova L, Vitku J, Hampl R, Adamcova K, Skodova T, Simkova M . Exposure to bisphenols and parabens during pregnancy and relations to steroid changes. Environmental Research 2018; 163: 115-122.
25. Minatoya A, Sasaki S, Araki A, Miyashita C, Itoh S, Yamamoto J, et al. Cord Bisphenol A levels and reproductive and thyroid hormone levels in neonates. The Hokkaido Study on Environment and Childrens Health. Epidemiology 2017; 28: S3-S9.
26. Kuijper EAM, Ket JCF, Caanen MR and Lambalk CB. Reproductive hormone concentrations in pregnancy and neonates: a systemic review. Reproductive medicine 2013;27: 33-63.

27. Barry JA, Hardiman PJ, Siddiqui MR, Thomas M. Meta-analysis of sex difference in testosterone levels in umbilical cord blood. Journal of Obstetrics and Gynaecology 2011; 8: 697-702.

28. Robinson M, Whitehouse A, Jacoby P, Mattes E, Sawyer M, Keelan J. Umbilical Cord Blood Testosterone and Childhood Internalizing and Externalizing Behavior: A Prospective Study. Plos One 2013;8: e59991.

29. Liu C, Xu X, Zhang Y, Li W , Huo X. Associations between maternal phenolic exposure and cord sex hormones in male newborns. Human Reproduction 2016; 31: 648-656.
30. Lima-Valassi HP, Takitaine J, Silva MR, Alves ANL, Hayashi GY, Hadachi SM. Interference in serum Androstenedione measured by LC-MS/MS in newborn samples. Journal of Endocrine Society 2020;4
31. Lundell AC, Ryberg H, Vandenput L, Rudin A, Ohlsson C, Tivesten Å. Umbilical cord blood androgen levels in girls and boys assessed by gas chromatography-tandem mass spectrometry. Journal of Steroid Biochemistry and Molecular Biology 2017; 171: 195-200.
32. Welsh M. Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. Journal of Clinical Investigation 2008; 118: 1479-1490.
33. Sánchez P, Castro B, Torres JM, Olmo A, del Moral RG, Ortega E. Bisphenol A modifies the regulation exerted by testosterone on 5 α -reductase isozymes in ventral prostate of adult rats. Biomedical Research International 2013; 2013: 629235.
34. Castro B, Sánchez P, Torres JM, et al. Bisphenol A exposure during adulthood alters expression of aromatase and 5*α*-reductase isozymes in rat prostate. *PLoS ONE*. 2013;8: e55905.
35. Acconcia F, Pallottini V and Marino M. Molecular mechanisms of Action of BPA. Dose Response 2015;13.
36. Peretz J, Flaws JA. Bisphenol A down-regulates rate-limiting Cyp11a1 to acutely inhibit steroidogenesis in cultured mouse antral follicles. Toxicology and Applied Pharmacology 2013; 271: 249–256.
37. Tabb MM, Blumberg B. New modes of action for endocrine-disrupting chemicals. Molecular Endocrinology 2006; 20: 475–482.
38. Ueda K. Effect of environmental chemicals on the genes and the gene expression. Yakugaku Zasshi 2009; 129: 1501–1506.
39. Mileva G, Baker SL, Konkle AT, Bielajew C. Bisphenol-A: epigenetic reprogramming and effects on reproduction and behavior. International Journal of Environmental Research and Public Health 2014; 11: 7537–7561.
40. Renwick AG, Lazarus NR. Human variability and noncancer risk assessment–an analysis of the default uncertainty factor. Regulatory Toxicology and Pharmacology 1998; 27: 3-20.

41. Hattis D, Baird S, Goble R. A straw man proposal for a quantitative definition of the RfD. Drug Chemical Toxicology 2002; 25: 403-436.
42. Barbara Demeneix, Laura N Vandenberg, Richard Ivell, R Thomas Zoeller, Thresholds and Endocrine Disruptors: An Endocrine Society Policy Perspective. Journal of the Endocrine Society 2020; 4*:* bvaa085.

CHAPTER 6

RELATIONSHIP BETWEEN BISPHENOL A, BISPHENOL-GLUCURONIDE AND TOTAL 25 HYDROXY VITAMIN D IN MATERNAL-CHILD PAIRS IN A SOUTH AFRICAN POPULATION.

Gounden V[1,2], Naidoo R[3], Chuturgoon A[4]
[1]Department of Chemical Pathology, University of KwaZulu-Natal and [2]National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital, Durban, South Africa
[3]Department of Occupational Health, University of KwaZulu-Natal, Durban, South Africa
[4]Department of Medical Biochemistry, University of KwaZulu-Natal, Durban, South Africa

Corresponding author: Verena Gounden
Department of Chemical Pathology, University of KwaZulu-Natal and National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital
2nd Floor Pathology Building Inkosi Albert Luthuli Central Hospital
800 Vusi Mzimela Road, Cato Manor, Durban, 4058
Email: verenagounden@yahoo.com

Submitted to Journal: Toxicology Communications; Manuscript number 216036280

Keywords:
Bisphenol A; Vitamin D; high performance liquid chromatography; mother-child pairs; cord blood;

Abbreviations:

Bisphenol-A: BPA
Bisphenol glucuronide: BPA-g
25OHD:25 hydroxy vitamin D

Funding: This research was funded by the University of KwaZulu Natal, National Research Foundation (NRF) Thuthuka grant, National Health Laboratories Service Research Trust Grant and Loreal Unesco Women in Science PhD fellowship award.

The Mother and Child in the Environment (MACE) cohort study is funded by the following research funding agencies in South Africa: National Research Foundation (NRF) (grant number: 90550), Medical Research Council (MRC), the University of KwaZulu-Natal's Flagship Funding and the AstraZeneca Research Trust.

Acknowledgements: We would like to thank Drs Pragalathan Naidoo and Terisha Ghazi and Ms Kareshma Asharam for their assistance. We would also like to thank Professor Jaya George and Ms Tracy Snyman for assistance with 25 hydroxy Vitamin D analysis.

Author Contributorship: VG researched literature and conceived the study with AC and RN. VG, AC, RN was involved in protocol development, gaining ethical approval. TS 25 OHD method optimization, analysis and data analysis. VG wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

Abstract

Exposure to Bisphenol A (BPA) during early development particularly in- utero has been linked to a wide range of pathology. Over the last two decades the importance of vitamin D in maternal and child health has been highlighted. The aim of this study was to examine the relationship of BPA and its naturally occurring metabolite BPA-glucuronide (BPA-g) with 25 hydoxy-vitamin D (25OHD) levels in South African mother-child pairs.

Third-trimester serum maternal samples and matching cord blood samples were analyzed for BPA and BPA-g using liquid chromatography tandem mass spectrometry (LC-MS/MS) and 25OHD3 and 25OHD2 using high performance liquid chromatography

A total of 58 maternal and child pairs were analyzed. More than fifty percent of maternal-child pairs were noted to be vitamin D deficient or insufficient using the Endocrine Society Practice guidelines cut-off of 50 nmol/L. Analysis of covariance showed a significant relationship between cord BPA-g levels and cord 25OHD levels ($p=0.03$) as well as between maternal BPA-g levels ($p=0.04$) and cord total 25OHD levels ($p=0.04$).

The findings of the current study confirm the significant direct relationship between foetal and maternal vitamin D levels and indicate a possible relationship with BPA/BPA-g and foetal/early infant Vitamin D levels that needs to be further investigated in this population.

Background

Vitamin D is a cholesterol derived prohormone that is available in two common storage forms, the plant derived, ergo-cholecalciferol (25-hydroxy vitamin D2) and the animal derived cholecalciferol (25 hydroxy vitamin D3) (1,2). In humans, vitamin D3 is converted to the active form 1,25 hydroxy vitamin D which acts via vitamin D binding receptors in various tissues (2, 3,4,5). Classically vitamin D has long been associated with bone and skeletal health, including calcium and phosphate metabolism (2). There are numerous studies that support the importance of vitamin D in other aspects of health including the development and maintenance of the immune system, neurodevelopment and development of reproductive organs. Inadequate levels of Vitamin D have been associated with tumorigenesis, abnormalities in glucose metabolism, cardiovascular disease, obesity and development of autoimmune disease (6,7).

Vitamin D levels during the intrauterine and neonatal period are largely dependent on maternal vitamin D levels (8-10). Maternal vitamin D deficiency (VDD) can result in neonatal hypocalcaemia. In addition, maternal VDD has been associated with other gestational pathologies including poor placentation and maintenance of the pregnancy, maternal obstetric complications such as preeclampsia, preterm birth as well as deleterious effect on foetal immune, neural and metabolic development. Maternal VDD may also negatively affect the anthropometric parameters in the neonate and increase the risk for asthma and type 1 diabetes in later life (11-14). Recent literature from different regions have reported significant proportions of neonates with insufficient 25 hydroxy-vitamin D (25OHD) levels and a similar prevalence in the mothers (15-22). However, the issue of VDD is clouded by lack of consensus for optimal cut offs to denote deficiency in young infancy and the possibility of different cut offs based on racial groups. Limited data is available on maternal and neonatal vitamin D status in the African continent and associated deleterious effects. A study performed in a Kenyan pregnant cohort reported a 51% Vitamin D insufficiency and a 21 % deficiency (using Endocrine Society Guidelines) (23). However, a study in a Zimbabwean cohort did not show similarly high levels of deficiency/insufficiency (24).

The intra-uterine period is highly susceptible to the effect of endocrine disruptors on foetal health. Bisphenol A (BPA), an environmental chemical and endocrine disruptor, is found in a vast array of plastic consumer products including lining of tin cans, food and water containers, medical devices and toys (25,26). Exposure to BPA has been linked to prenatal and postnatal adverse effects on multiple tissues, including the reproductive system and neurodevelopment. BPA effects are typically attributed to its estrogenic or anti-estrogenic action however this action can not completely account for the adverse effects of low potency BPA at the low-dose exposures that are commonly seen (25,27). BPA has also been reported to interact with other steroid hormone receptors including androgens and glucocorticoids

(28). The structural homology of vitamin D with sex steroid hormones like estradiol and testosterone, make it possible that BPA may also disrupt the actions of Vitamin D as well. A 2016 report from the US National Health and Nutrition Examination Survey (NHANES) examined the relationship between urinary BPA (uBPA) and 25OHD levels in a large cohort of pregnant women. Their findings showed an inverse association with uBPA and total 25OHD levels. BPA was significantly associated with a 20% increase in the odds of VDD at 26 weeks' gestation in the women (29).

BPA-glucuronide (BPA-g) is a major metabolite of BPA metabolism. Levels in cord blood remain steady and are thought to reflect the cumulative does of BPA received during late pregnancy (30). BPA-g, was widely thought of inert however recent evidence from animal studies suggest that it may deconjugate to expose the foetus to BPA (31). In context of the short half-life of BPA, BPA-g acts as a surrogate marker of BPA exposure (31).

In a previously published study, we reported BPA was detectable in more than 25% of maternal and cord blood samples in a South African cohort. We demonstrated significant positive correlation between maternal and child BPA and BPA-g levels with correlation coefficients of 0.892 and 0.744, respectively (32). As part of a larger study examining the effect of BPA on maternal and child-pairs we examined the relationship between BPA and BPA-g levels on 25-hydroxy Vitamin D levels in maternal and child pairs. As a secondary objective in this study we also examined the relationship between 25 OH Vitamin D levels and birth parameters.

Methods

Population and study samples

Blood samples and data collected as part of the mother and child in the environment (MACE) birth cohort study were utilized for this study. This is described in detail in another publication (33). The MACE study population consists of "healthy" pregnant females recruited from antenatal clinics in industrially dense south Durban, South Africa and other clinics in the north Durban area. The south Durban is an area where large communities are located within heavily polluted large-scale industrial enterprises. The north communities, although of similar socio-economic profile, is less industrially active. Mothers were recruited during the first trimester of pregnancy. Details regarding pregnancy outcome for participants were also collected during the course of the MACE study (33). Venous blood samples were taken during the third trimester (between 27 completed to 40 weeks of gestation) from the pregnant individuals. Cord bloods were taken at delivery. Bloods were collected from maternal participants at one of their regularly scheduled ante-natal appointments. Maternal and cord bloods collected in serum polypropylene vacutainer tubes were later analyzed for BPA and BPA-g. These samples were centrifuged, separated and serum stored at -80 degrees Celsius until analysis. Maternal

/cord paired samples with sufficient serum volumes were utilized for the current study. Vitamin D measurement and supplementation in pregnancy is not part of routine practice guidelines in the South African public health care system. Inclusion and exclusion criteria were as per MACE study which has been described previously (32).

Bisphenol A and Bisphenol glucuronide analysis

The methods used for determination of BPA and BPA-g levels has been previously described in detail (32). Briefly BPA and BPA-g levels were carried out using the AB Sciex 4500 triple quadrupole mass spectrometer equipped with an Agilent 1260 Ultra high-performance liquid chromatography (uHPLC) system. Analytes of interest were separated on a Phenomenex C18 column (2.1 x 50 mm, 1.6 um). A 3-minute linear gradient was used from 10-100% of acetonitrile in water followed by a hold for 1 minute at a flow rate of 0.4 ml/min. Serum samples were prepared using 50 µl of serum mixed with 100 µl acetonitrile containing the internal standards deuterated 5 ng/ml BPA (d6BPA, Cambridge Isotope Laboratories, Andover, MA) and 5 ng/ml $^{13}C_{12}$ BPA-g (Sigma-Aldrich Gmbh, Munich, Germany. Electrospray ionisation in negative modes was used for the measurement of each analyte. Qualifier and quantifier single reaction monitoring (SRM) transitions were used for both BPA and BPA-g.

Determination of 25 hydroxy Vitamin D levels

Maternal serum and cord blood that were analysed for BPA and BPA-G were then analysed for vitamin D. Fifty-eight pairs of the initial 90 pairs for which BPA was measured were sufficient for Vitamin D analysis. 25 OHD3 and 25 OHD2 were measured in maternal and cord serum by high performance liquid chromatography using a commercial kit, ClinRep (Recipe, Munich, Germany). Total25 OHD is the sum of the measured D3 and D2. The intra assay CV for 25(OH)D ranged from 0.9–4.9% and the inter-assay CV ranged from 3.0– 4.9%. The limit of detection was 2.5 nmol/l for 25(OH)D_3 and 7.5 nmol/L for 25(OH)D_2. Further details on this method have been previously published (34). Total Vitamin D levels of < 50 nmol/L were considered to be deficient and values between 50 and 75 nmol/l were classified as insufficient as defined by Endocrine Society practice guidelines (35). (Refer to Table 6.1).

Table 6.1: Endocrine Society Practice guidelines classification of vitamin D status in relation to 25(OH) D concentrations

25(OH) Vitamin D Concentration (nmol/L)	**Classification**
< 50 nmol/L	Deficient
>50 - <75 nmol/L	Insufficient
≥ 75 nmol/L	Sufficient

Statistical analysis

Univariate analyses were performed for maternal and newborn characteristics, including means and standard deviations or median and range for continuous variables. Data was assessed for normality using the Shapiro-Wilk test. Non-parametric tests Kruskal Wallis test, Spearman's correlation or Wilcoxon signed rank test for performed for the univariate analysis. Kruskal-Wallis analysis (or one-way ANOVA for parametric data) was performed to determine if any statistically significant difference could be identified between the two genders for cord blood BPA, BPA-g and total 25OHD levels. A p value of <0.05 was considered significant. Analysis of covariance (ANCOVA) was used to assess for confounding variables, with the dependent continuous variable being total 25OHD levels and independent variable either maternal or cord BPA or BPA-g levels. Statistical analysis was performed on Medcalc statistical software program version 18.11 (Medcalc, Belgium).

Ethical approval

The research has complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration and has been approved by the authors' institutional review board or equivalent committee. Ethical clearance for this study was obtained from the Biomedical Research and Ethics Committee (BREC) of the University of KwaZulu-Natal (Ethics Clearance Certificate BE 597/16).

Results

A total of 58 maternal-cord pairs were analysed for 25OHD. Table 6.2 summarises the demographic data, associated 25OHD levels and other baseline characteristics of the cohort. There was no statistically significant sex difference for any of the parameters presented.

Table 6.2 Baseline characteristics of cohort

	Maternal (n=58)		Child (n=58; male n=36; female=22)	Male	Female	*p value*
Age (years) (range)	25.0 (17-40)	Gestation (weeks)	38 (33-41)	Male 38 (32-41)	Female 38 (33-41)	p=0.87
BMI (kg/m^2) (SD)	32.8 (9.4)	Birth weight (grams)	2695 (541)	Male 2588 (502)	Female 287 (569)	p=0.06
Gestation when samples taken (weeks) (SD)	30 (3)	Length (cm)	49 (33-56)	Male 49 (40-53)	Female 49 (33-56)	p=0.78
		Head circumference (cm)	33.5 (27-47)	Male 34 (27-44)	Female 33 (30-47)	p=0.95
Total 25OHD levels (nmol/L) (SD)	55.7 (12.9)	Cord blood Total 25 OHD levels (nmol/L)	54.7 (19.1)	Male 56.6 (19.8)	Female 51.5 (18.0)	p=0.34
BPA (ng/mL) (n=17)	0.8 (0.4-6.4)	Cord blood BPA (ng/mL) (n=15)	0.91 (0.4-8.0)	Male(n=9) 0.7 (0.4-7.9)	Female(n=6) 1.3 (0.4-6.9)	p=0.60
BPA-g(ng/mL)	3.9 (0.15-21.8)	Cord blood BPA-g (ng/mL)	4.1 (0.34-26)	Male 3.7 (0.34-26.0)	Female 4.3 (0.65-21.3)	p=0.74

Data shown with mean (SD) for normally distributed parameters and with median (range) values for non- normally distributed parameters

Maternal and cord total 25OHD levels

Only four (2 pairs) of the 116 serum samples had detectable 25 OHD 2 levels. Thus analysis performed examined total 25OHD levels. Maternal total 25OHD levels ranged from 29.5 to 94.4 nmol/L and cord levels from < than detectable to 100.6 nmol/L. Thirty-four percent (n=20) of maternal samples and 50% (n=29) of cord blood samples had deficient (i.e. < 50 nmol/L) 25OHD levels. Twenty-one percent (n=12) of maternal-child pairs were 25OHD deficient and 33% of the pairs were considered insufficient (>50-75 nmol/L). Table 6.3 below provides further information regarding the categorisation of 25OHD levels across the maternal-child pairs.

Table 6.3 Categorisation of 25OHD levels (based on Endocrine Society Practice Guidelines) across the maternal-child pairs (n=58 pairs)

	Both maternal and cord blood samples deficient (<50 nmol/L)	**Both maternal and cord blood samples insufficient (<75 nmol/L)**	**Only maternal sample deficient (<50 nmol/L)**	**Only cord sample deficient (< 50 nmol/L)**	**Both maternal and cord blood sufficient ≥ 75 nmol/L**
N (%)	12 (21%)	19 (33%)	8(14%)	17 (29%)	2 (3%)
Serum BPA-g levels per 25OHD categorisation					
	Deficient	Insufficient		Sufficient	p value
Maternal BPA-g (ng/mL)	4.58 (1.03-11.7)	3.29 (0.15-21.8)		3.5 (3.23-6.35)	P=0.75
Cord BPA-g (ng/mL)	4.14 (0.357-26)	3.91 (0.34-9.85)		2.62 (2.15-3.4)	P=0.36

Relationship between maternal, cord blood BPA/BPA-g and Vitamin D levels

Spearman's rank correlation showed a positive correlation of cord blood and maternal BPA-g levels (r=0.74 p <0.001) as well as with cord blood and maternal BPA levels (p=0.008). BPA levels were detected in a smaller percentage of the cohort as compared to BPA-g (maternal n=17; cord n=15). Cord blood 25OHD directly correlated with maternal 25OHD levels (Spearman's correlation coefficient (r=0.5 p=0.002) (see figure 6.1).

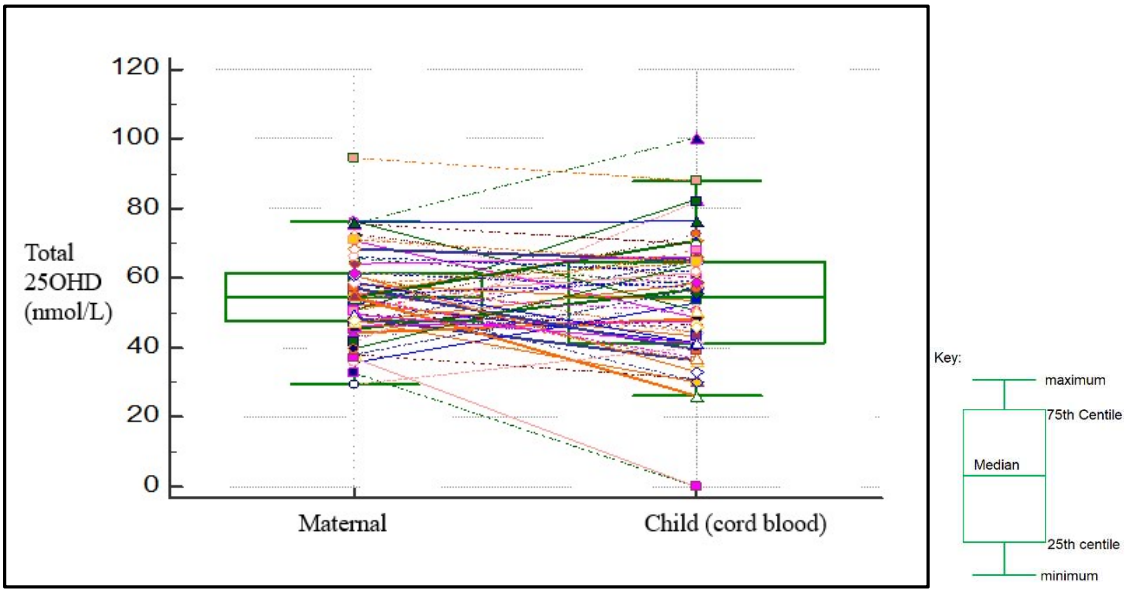

Figure 6.1: Box and whiskers plot- showing paired maternal and child (cord blood) total 25OHD levels. Note each shape represents a maternal-child pair

On rank correlation maternal BPA (slope=-1; p=0.18) and cord BPA (slope =-1,2 p=0.4) showed a negative but not statistically significant relationship with cord total 25OHD levels. Due to the small number of samples with detectable BPA levels further analysis was not performed. Spearman rank correlation also showed a slight negative relationship between cord total 25OHD levels and maternal (slope -0.2 p=0.6) and cord BPA- g (slope -0.3 p=0.6) levels but these were not statistically significant. Kruskal Wallis analysis of maternal and cord BPA-g levels across total 25OHD categories was performed (refer Figure 6.2). This showed no statistical difference of BPA-g median levels (for maternal BPA-g p=0.75 and cord levels p=0.36) between study participants classed as having deficient, insufficient or sufficient vitamin D.

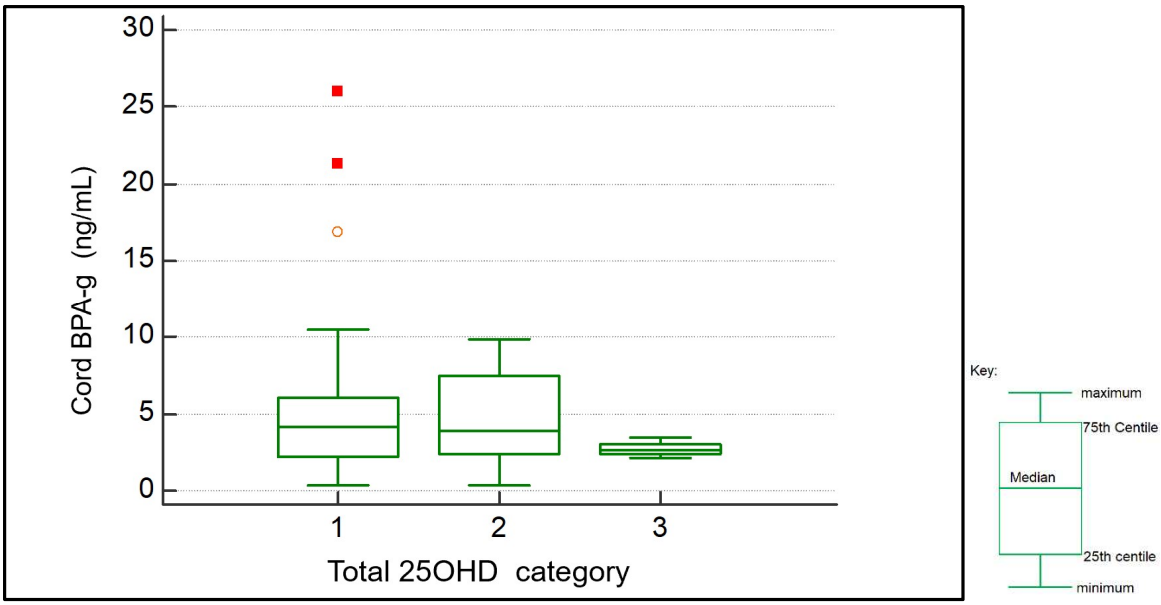

Figure 6.2: Box and whiskers plot- showing BPA-g levels per total 25OHD category as found in cord blood samples (1=deficient; 2=insufficient; 3= sufficient)

Notably a trend for higher BPA-g levels was observed in both maternal and cord bloods for those with deficient and insufficient 25OHD (Table 3). Analysis of covariance was performed to determine the effect of other confounders on this relationship. We wished to examine for any significant association with the presence of BPA or its metabolite on cord or maternal total 25OHD levels (continuous variable) when controlling for these confounders. Of note cord (p=0.033) and maternal BPA-g (p=0.04) levels showed statistically significant associations with cord total 25OHD levels. Maternal BMI, gestational age of infant and infant sex did not show any statistically significant relationship with cord total 25OHD levels on ANCOVA analysis.

On ANCOVA analysis maternal total 25OHD levels showed no significant relationship with BMI (p=0.9), age (p=0.7), weeks of gestation (p=0.9) or maternal BPA-g levels (p=0.8).

Infant anthropometric parameters and Vitamin D levels

On Spearman's rank correlation neither head circumference nor length were significantly correlated with either maternal (p=0.24 and p=0.40 respectively) or cord blood (p=0.7 and p=0.2 respectively) total 25OHD levels. Whilst on ANCOVA analysis birth weight showed a statistically significant association with cord total 25OHD levels (p=0.05), Spearman's rank correlation was not significant (p=0.15).

Influence of seasonal variation

Based on the month samples were taken, data was classified with regards to season [winter (June-August), summer (December-February), spring (September- November) and autumn(March-May)].

The mean values for samples (n=5 pairs) taken in autumn were higher for both cord and maternal total 25OHD versus the other seasons, with levels ranging from 15-30% higher. Seasonal variation lacked statistical significance on one-way ANOVA analysis with regards to association with cord blood vitamin D levels (p=0.07) and maternal vitamin D levels (p=0.06).

Discussion

The most striking finding in our study was the association (after correction for confounders) between a known endocrine disruptor (BPA metabolite BPA-g) and cord total 25OHD levels. Two previous studies have described a significant negative relationship with urine BPA metabolites and 25OHD in women, pregnant women and cord blood (29,36). These studies did not examine serum BPA or BPA-g levels in their cohorts. BPA-g reflects the cumulative dose of exposure of both the foetus to maternal BPA in late pregnancy, as well as environmental BPA exposure in the mothers (30). As such is more likely a better surrogate of in-utero exposure than maternal urinary BPA or isolated serum BPA levels. However, no studies to date have reported on the relationship of serum BPA metabolites including BPA-g on total 25OHD levels. Furthermore, the relationship between maternal and cord levels support the evidence for antenatal BPA exposure being transferred to the new-born (32), while maternal 25OHD levels influence that of the growing infant (11-14).

The significant role 25OHD plays in maternal, foetal and childhood health and beyond, has been previously described (2-10). An inverse relationship between vitamin D and serum BPA has been reported, across both adult males and females, in a single study (37). However, BPA-g levels were also not measured in this instance. BPA-g, which is the major metabolite of BPA was widely thought of inert as it is unable to bind to steroid receptors however recent evidence in animal studies suggest that it may deconjugate to expose the foetus to BPA even though adequate conjugation of BPA has occurred after maternal intake (31).

The presence of BPA in the environment is ubiquitous. There has been much research on how the intrauterine environment and exposure of individuals' preconception and pregnant females during the gestational period affect eventual infant outcomes and disease occurrence throughout life (25). There is sparse data arising from the limited mechanistic studies examining the effect of BPA on the vitamin D endocrine system. Animal studies have demonstrated that BPA can disturb calcium metabolism by influencing the expression of vitamin D–dependent calcium binding protein (38,39,41). The study by Otsuka et also showed an inverse relationship with serum calcium levels and BPA in pregnant mice (39). Another possible mechanism of BPA on Vitamin D metabolism is via an effect on metabolising enzymes; either by changing the expression of cytochrome P450 enzymes responsible for steroid metabolism or affecting messenger RNA (mRNA) expression (40,41). A recent study examining the effect of BPA exposure on male and female rats beginning from post-natal day 9, for 91 days

demonstrated that BPA increased urinary excretion of 25OHD3 thus decreasing vitamin D levels in serum (42). This is further suggestive of mechanistic effect of BPA on vitamin D metabolism.
The findings of this study demonstrated that a significant proportion of pregnant women and neonates have suboptimal 25OHD levels. More than 50% of the maternal and cord blood samples were either deficient or had insufficient levels of total 25OHD. Whilst there was a direct and significant correlation between maternal and cord blood total 25 OHD levels, the correlation was moderate (R=0.5 p=0.002) in this study as compared to some previous studies. Jacquemyn *et al* reported a correlation of R=0.91 in a multi-ethnic cohort from Belgium (43). One possibility to be considered to explain the moderate correlation in this study is the effect of BPA exposure on the cohort.

The current study utilised the Endocrine Society practice guidelines for determination of cut-offs for interpretation of Vitamin D levels in the maternal cord pairs (35). There is currently no consensus with regards optimal cut-offs in pregnant women or infants and various cut -offs recommended by the various authorities and societies. The Institute of Medicine (IOM) guidelines stipulate values ≥ 50 nmol/L as being sufficient (2). Using this cut off: 31% (n=18/58) of mothers and 41% of new-borns (n=24/58) would be vitamin D insufficient. However, these cut points are based solely on sufficiency for adequate bone health and not for the other aspects of health and physiological functions that have been linked to vitamin D status (44). These functions include neurodevelopment, immune, cardio-metabolic, reproductive function and protection against cancer (44-47). BPA exposure has itself been linked to negative sequelae affecting the same physiological systems as well as in the development of malignancy (25,48). These cut-off guidelines have also been largely developed in European/North American populations and have not been verified in other populations with randomised controlled trials (44).
Some seasonal variation was also noted with higher total 25OHD values noted in autumn. This is in keeping with previous reports in southern hemisphere cohorts (34, 49).

One of the strengths of this study is the use of the specific and sensitive HPLC methodology to measure the 25 hydroxy vitamin D2 and D3 levels. Many of the previous studies examining the relationship between maternal and cord Vitamin D utilised immunoassay based techniques. Immunoassay is more susceptible to interferences from compounds of similar structure and from heterophile antibodies (50). Race has been reported as a significant confounder on Vitamin D levels - the current study cohort was homogenous with regards to race as all participants were Black African.

There are limitations to the current study. This includes the cross-sectional nature of the study and the small sample size. As BPA and BPA-g levels were not followed over time during the course of the pregnancy we cannot be certain to what degree the measured maternal serum and cord blood BPA/BPA-g levels reflect the actual exposure throughout pregnancy. The current study did not take into account the possible confounding effect of dietary food intake and use of sunscreen on the maternal vitamin D

levels. However, in this population cohort due to low socio-economic status use of both sunscreen and vitamin D supplementation is unlikely. Additionally, the almost absence of detectable 25OHD2 levels amongst this study population is an indication that vitamin D supplementation did not occur during pregnancy.

This is the first study to the authors' knowledge to examine the relationship between serum BPA as well as BPA-g with 25-hydroxy vitamin D levels in maternal-child pairs. Our findings suggest a relationship between BPA exposure and Vitamin D levels in the intra-uterine period. Follow up is required to understand causality or associations with development of disease in the BPA exposed infants. Further studies are required to examine the mechanistic relationship of exposure to endocrine disruptors like BPA and its effect on Vitamin D, in order to better evaluate and understand the health consequences in humans.

References

1. Moon RJ, Davies JH, Cooper C, Harvey NC. Vitamin D and maternal and child health.Calicified Tissue International 2020;106-30-46
2. Institute of Medicine (US) Committee to Review Dietary Reference Intakes for Vitamin D and Calcium; Ross AC, Taylor CL, Yaktine AL, et al., editors. Dietary Reference Intakes for Calcium and Vitamin D. Washington (DC): National Academies Press (US); 2011. 3, Overview of Vitamin D. Available from: https://www.ncbi.nlm.nih.gov/books/NBK56061
3. Haussler MR, Jurutka PW, Mizwicki M, Norman AW. Vitamin D receptor (VDR)-mediated actions of1alpha,25(OH)(2)vitamin D genomic and non-genomic mechanisms. Best Practice and Research Clinical Endocrinology and Metabolism 2011; 25: 543–559.
4. Ding C, Gao D, Wilding J, Trayhurn P, Bing C. Vitamin D signalling in adipose tissue. British Journal of Nutrition 2012; 108: 1915–1923.
5. Bischoff HA, Borchers M, Gudat F, Duermueller U, Theiler R,Stahelin HB et al In situ detection of 1,25-dihydroxyvitaminD3 receptor in human skeletal muscle tissue. Histochemistry Journal 2001; 33: 19–24.
6. Christakos S, Hewison M, Gardner DG, et al. Vitamin D: beyond bone. Annals of New York Academy of Science 2013; 1287: 45-58.
7. Lopez AG, KerlanV, Desailloud R.Non-classical effects of vitamin D: Non-bone effects of vitamin D. Annales d'Endocrinologie 2021;82:43-51.
8. Grant CC, Stewart AW, Scragg R, Milne T, Rowden J, Ekeroma A, et al. Vitamin D during pregnancy and infancy andinfant serum 25-hydroxyvitamin D concentration. Pediatrics 2013; 133: e143–e153.
9. Kiely M, O'Donovan SM, Kenny LC, Hourihane JO, Irvine AD, Murray DM. Vitamin D metabolite concentrations in umbilical cord blood serum and associations with clinical characteristics in large prospective mother-infant cohort in Ireland. Journal of Steroid Biochemistry and Molecular Biology 2017; 167:162–8.
10. Hollis BW, Wagner CL. New insights into the vitamin D requirements during pregnancy. Bone Research 2017; 5: 17030.
11. Roth DE, Leung M, Mesfin E, Qamar H, Watterworth J, Papp E. Vitamin D supplementation during pregnancy: state ofthe evidence from a systematic review of randomised trials. British Medical Journal 2017; 359: j5237.
12. Marshall I, Mehta R, Petrova A. Vitamin D in the maternal-fetal-neonatal interface: clinical implications and requirements for supplementation. Journal of Maternal Fetal and Neonatal Medicine 2013; 26: 633–8.
13. Kaushal M, Magon N. Vitamin D in pregnancy: a metabolic outlook. Indian Journal of Endocrinology and Metabolism 2013; 17: 76–82.

14. Thorne-Lyman A, Fawzi W. Vitamin D during pregnancy and maternal, neona-tal and infant health outcomes: a systematic review and meta-analysis. Pediatric Perinatal Epidemiology 2012; 26: 75–90.
15. Halicioglu O, Aksit S, Koc F, Sezin A, Akman SA, Albudak E, et al. Vitamin D deficiency in pregnant woman and their neonates in spring time in western Turkey. Paediatric Perinatal Epidemiology 2012; 26: 53–60.
16. Kılıcaslan AÖ, Kutlu R, Kilinc I, Ozberk DI. The effects of vitamin D sup-plementation during pregnancy and maternal vitamin D levels on neonatal vitamin D levels and birth parameters. Journal of Maternal Fetal and Neonatal Medicine 2017; 25: 1–8.
17. Sathish P, Raveendran S, Padma R, Balakrishnan D, Muthusami M. Correlation between maternal and neonatal blood vitamin D levels and its effect on the newborn anthropometry. International Journal of Reproduction Contraception Obstetrics and Gynecology 2016; 5: 2983–8.
18. Gellert S, Ströhle A, Bitterlich N, Hahn A. Higher prevalence of vitamin D deficiency in German pregnant women compared to non-pregnant women. Archives of Gynecology and Obstetetrics 2017; 296: 43–51.
19. Skowrońska-Jóźwiak E, Lebiedzińska K, Smyczyńska J, Lewandowski KC, Głowacka E, Lewiński A. Effects of maternal vitamin D status on pregnancy outcomes, health of pregnant women and their offspring. Neuroendocrinology Letters 2014; 35:367–72.
20. Domaracki P, Sadlecki P, Odrowąż-Sypniewska G, Dzikowska E, Walentowicz P, Siodmiak J, et al. Serum 25(OH) vitamin D levels in Polish women during pregnancies complicated by hypertensive disorders and gestational diabetes. International Journal of Molecular Science 2016; 17:1574.
21. Rodriguez A, García-Esteban R, Basterretxea M, Lertxundi A, Rodríguez- Bernal C, Iñiguez C, et al. Associations of maternal circulating 25- hydroxyvitamin D3 concentration with pregnancy and birth outcomes. British Journal of Obstetrics and Gynaecology 2015; 122: 1695–704.
22. Karras SN, Shah I, Petroczi A, Goulis DG, Bili H, Papadopoulou F, et al. An observational study reveals that neonatal vitamin D is primarily determined by maternal contributions: implications of a new assay on the roles of vitamin D forms. Nutrition Journal 2013; 12: 77.
23. Toko EN, Sumba OP, Daud II, et al. Maternal Vitamin D Status and Adverse Birth Outcomes in Children from Rural Western Kenya. Nutrients 2016; 8: 794.
24. Chikwati RP, Musarurwa C, Duri K, Mhandire K, Snyman T, George JA. Maternal plasma vitamin D levels and associated determinants in late pregnancy in Harare, Zimbabwe: a cross-sectional study. BMC Pregnancy Childbirth 2019; 19: 218.

25. Diamanti- Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM et al. Endocrine -Disrupting Chemicals: An Endocrine Society Scientific Statement. Endocrine Reviews 2009; 30: 293-342.
26. National Institute of Environmental Health Sciences. Accessed 18 December 2018 https://www.niehs.nih.gov/health/topics/agents/sya-bpa/index.cfm
27. Jalal N, Surendranath AR, Pathak JL, Yu S and Chung CY. Bisphenol A the might and the mutagenic. Toxicology reports 2018:5;76-84
28. Kojima H, Takeuchi S, Sanoh S, Okuda K, Kitamura S, Uramaru N, et al. Profiling bisphenol A its eight analogues on transcriptional activity via human nuclear receptors. Toxicology 2019; 413: 48-55.
29. Johns LE, Ferguson KK, Cantonwine DE, McElrath TF, Mukherjee B, Meeker JD. Urinary BPA and Phthalate Metabolite Concentrations and Plasma Vitamin D Levels in Pregnant Women: A Repeated Measures Analysis. Environmental Health Perspectives 2017; 125:087026.
30. Gauderat G, Picard-Hagen N, Toutain PL, Servien R, Viguie C, Puel S, et al. Prediction of human prenatal exposure to bisphenol A and bisphenol A glucuronide from an ovine semi-physiological toxicokinetic model. Nature Scientific report 2017; 7: 155330
31. Nishikawa M, Yanagisawa R, Koike N, Inoue H, Yokota H. Placental transfer of conjugated bisphenol A and subsequent reactivation in the rat fetus. Environmental Health Perspectives 2010; 118:1196–203.
32. Gounden V, Zain Warasally M, Magwai T, Naidoo R, Chuturgoon A. A pilot study: Bisphenol-A and Bisphenol-A glucuronide levels in mother and child pairs in a South African population. Reproductive Toxicology. 2019; 89: 93-99.
33. Jeena, P.M., Asharam, K., Mitku, A.A. *et al.* Maternal demographic and antenatal factors, low birth weight and preterm birth: findings from the mother and child in the environment (MACE) birth cohort, Durban, South Africa. *BMC* Pregnancy Childbirth 2020; 20: 628.
34. George JA, Norris SA, van Deventer HE, Crowther NJ. The association of 25 hydroxyvitamin D and parathyroid hormone with metabolic syndrome in two ethnic groups in South Africa. *PLoS One*. 2013; 8: e61282.
35. Holick M.F., Binkley N.C., Bischoff-Ferrari H.A., Gordon C.M., Hanley D.A., Heaney R.P., Murad M.H., Weaver C.M. Evaluation, Treatment, and Prevention of Vitamin D Deficiency: An Endocrine Society Clinical Practice Guideline. Journal of Clinical Endocrinology and Metabolism 2011; 96: 1911–1930.
36. Lauren E. Johns, Kelly K. Ferguson, John D. Meeker. Relationships Between Urinary Phthalate Metabolite and Bisphenol A Concentrations and Vitamin D Levels in U.S. Adults: National Health and Nutrition Examination Survey (NHANES), 2005–2010. The Journal of Clinical Endocrinology & Metabolism 2016; jc.2016-2134.

37. Erden ES, Genc S, Motor S, Ustun I, Ulutas KT, Bilgic HK, et al. Investigation of serum bisphenol A, vitamin D, and parathyroid hormone levels in patients with obstructive sleep apnea syndrome. Endocrine 2014; 45: 311-8.
38. Kim S, An BS, Yang H, Jeung EB. Effects of octylphenol and bisphenol A on the expression of calcium transport genes in the mouse duodenum and kidney during pregnancy. Toxicology 2013; 303: 99–106.
39. Otsuka H, Sugimoto M, Ikeda S,Kume S. Effects of bisphenol A administration to pregnant mice on serum Ca and intestinal Ca absorption. Animal Science 2012; J83: 232–237.
40. Liu C, Zhao L, Weil L. DEHP reduces thyroid hormones via interacting with hormone synthesis-related proteins, deiodinases, transthyretin, receptors, and hepatic enzymes in rats. Environmental Science and Pollution Research International 2015; 22: 12711–12719.
41. Quesnot N,Bucher S,Fromenty B,Robin MA.Modulation of metabolizing enzymes by bisphenolA in human and animal models. Chemical Research in Toxicology 2014; 27:1463–1473.
42. Kim JK, Khan A, Cho S, Na J, et al. Effect of developmental exposure to bisphenol A on steroid hormone and vitamin D3 metabolism, Chemosphere 2019: 237: 124469.
43. Jacquemyn Y, Ajaji M, Karepouan N. Vitamin D levels in maternal serum and umbilical cord blood in a multi-ethnic population in Antwerp, Belgium. Facts Views Vis Obgyn 2013; 5 :3-5.
44. Vieth R, Holick MF.Chapter 57B - The IOM—Endocrine Society Controversy on Recommended Vitamin D Targets: In Support of the Endocrine Society Position, Editor(s): David Feldman, Vitamin D (Fourth Edition) Academic Press, 2018:1091-1107.ISBN 9780128099650,
45. Khazai N, Judd SE, Tangpricha V. Calcium and vitamin D: skeletal and extraskeletal health. Current Rheumatology Reports 2008; 10: 110-117.
46. Jia Guo and Zhangsuo Liu. The Multiple Roles of Vitamin D Besides Calcium-Phosphorus Metabolism, A Critical Evaluation of Vitamin D - Basic Overview, Sivakumar Gowder, IntechOpen, Available from: https://www.intechopen.com/books/a-critical-evaluation-of-vitamin-d-basic-overview/the-multiple-roles-of-vitamin-d-besides-calcium-phosphorus-metabolism . Accessed 4 April 2021
47. Khammissa RAG, Fourie J, Motswaledi MH, Ballyram R, Lemmer J, Feller L. The Biological Activities of Vitamin D and Its Receptor in Relation to Calcium and Bone Homeostasis, Cancer, Immune and Cardiovascular Systems, Skin Biology, and Oral Health", BioMedical Research International 2018: 9276380.
48. Mathieu-Denoncourt J,Wallace SJ, deSolla SR, Langlois VS. Plasticizer endocrine disruption:Highlighting developmental and reproductive effects in mammals and non-mammalian aquatic species. General and Comparative Endocrinology 2015; 219: 74– 88.

49. Norval M, Coussens AK, Wilkinson RJ, Bornman L, Lucas RM, Wright CY. Vitamin D Status and Its Consequences for Health in South Africa. International Journal of Environmental Research and Public Health 2016; 13: 1019.
50. Tate J, Ward G. Interferences in immunoassay. Clinical Biochemist Reviews 2004; 25: 105-120.

CHAPTER 7

DNA METHYLATION CHANGES IN THE PROMOTER REGIONS OF ENZYMES *CYP1B1* AND *CYP3A4* AND THEIR RELATIONSHIP WITH STEROID HORMONE, BISPHENOL-A AND BISPHENOL-A GLUCURONIDE CONCENTRATIONS IN MATERNAL CHILD PAIRS

Gounden V[1,2], Ghazi T[3], Naidoo P[3], Naidoo R[4], Chuturgoon A[3]
[1]Department of Chemical Pathology, University of KwaZulu-Natal and [2]National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital, Durban, South Africa
[3]Department of Medical Biochemistry, University of KwaZulu-Natal, Durban, South Africa
[4]Department of Occupational Health, University of KwaZulu-Natal, Durban, South Africa

Corresponding author: Verena Gounden
Department of Chemical Pathology, University of KwaZulu-Natal and National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital
2nd Floor Pathology Building Inkosi Albert Luthuli Central Hospital
800 Vusi Mzimela Road, Cato Manor, Durban, 4058
Email: verenagounden@yahoo.com

Manuscript in the process of submission

Abstract

Background: The wide spectrum of health effects resulting from exposure to Bisphenol A (BPA) has been well described. This includes the effect on steroid hormone concentrations and steroidogenesis. Animal studies have demonstrated the susceptibility of progeny to adverse effects of BPA when exposed in the prenatal period and early infancy. However, the precise mechanism of BPAs action and effect on different human tissues has not been fully elucidated. We postulated that a possible additional mechanism of the interaction of BPA with steroid hormones is via its interaction with steroid hormone metabolising enzymes. We further postulated that this may occur via an epigenetic mechanism namely changes in DNA methylation of the promoter region of these enzymes. The aim of this study was to assess presence of methylation changes in the promoter region of two key cytochrome 450 metabolising enzymes *CYP1B1* and *CYP3A4* and determine if there was an association with BPA exposure.

Method: Serum and stored genomic DNA samples from the maternal and matched cord blood pairs collected as part of the Mother and Child in the environment (MACE) birth cohort study were utilized for this study. The promoter methylation of the candidate genes *CYP1B1* and *CYP3A4* were assessed using the OneStep qMethyl Kit (Zymo Research, Irvine, USA). Serum concentrations of BPA and BPA-glucuronide (BPA-g, a metabolite of BPA), as well as 9 sex steroid hormones were measured using liquid chromatography tandem mass spectrometry.

Results: A total of forty-four samples which included sixteen matched mother-child pairs were analysed. There were no statistically significant associations identified for BPA/BPA-g serum concentrations and percentage of methylation for both promoter regions for maternal and cord samples. Only DHEAS showed a significant ($p=0.01$) negative association with *CYP1B1* promoter methylation status.

Conclusion: Further studies are required to determine the effect of BPA exposure on steroid metabolising hormones.

Background

Bisphenol-A (BPA) is a synthetic polymer and endocrine disruptor that is a ubiquitous pollutant in our environment (1,2). The relationship between BPA and its negative impact on health have been well described in animal studies. BPA acts on classical and non-classical oestrogen receptors and may have agonist and antagonistic actions on different tissues depending on duration and levels of exposure. In humans, exposure to BPA particularly in-utero or early childhood has been associated with a host of pathologies from neurodevelopmental anomalies, cardiometabolic disease and malignancy. In addition, BPA has been demonstrated to have deleterious effects on normal reproductive development and fertility (3,4). BPA concentrations have been demonstrated to be 3-4 times higher in amniotic fluid as compared to maternal serum (5). This together with the process of organogenesis and cell differentiation occurring within the foetus, makes in-utero exposure to BPA even more of a concern. Animal studies have demonstrated that exposure to environmentally relevant doses of BPA can result in advanced puberty, increased prostatic growth and altered mammary gland development (6,7,8). Further in rodent studies exposure to BPA in-utero has been demonstrated to permanently alter the morphology and the functionality of female reproductive organs (9).

The mechanisms that underlie the toxicity and sex-specific effects of BPA are not well understood (10, 11). BPA is a selective oestrogen receptor (ER) modulator that binds both, ERα and ERβ. BPA effects are typically attributed to its estrogenic or anti-oestrogenic action, however this action can not completely account for the adverse effects of low potency BPA at the low-dose exposures that are commonly seen (12). Recent research suggests that long-lasting effects of prenatal BPA exposure likely involve disruption of epigenetic programming during development (13, 14). Epigenetics refers to a change of gene expression that is independent of the DNA sequence. It is essentially a change in phenotype expressed without a change in genotype. The mechanism by which this occurs may be as a result of changes in chemical modifications of the DNA for example, methylation of cytosine-guanine dinucleotides (CpG) within gene promoters or the physical accessibility of the DNA by virtue of its association with histones (for example histone acetylation), nonhistone proteins, or noncoding RNA. Epigenetic changes are capable of being passed on to somatic daughter cells and in some cases to offspring via the germline this is known as translational genetic inheritance. Changes in the epigenome occurring during embryonic development will have a much greater impact on the overall epigenetic status of the organism since these changes can be transmitted over consecutive mitotic divisions (15,16). Changes in methylation of these aggregates of CpGs or CpG islands in the promoter region of genes can regulate gene transcription and thus gene expression. Hormonal exposures in early life have been demonstrated to affect DNA methylation in reproductive tract tissues and thus impact on normal development. Alterations in DNA methylation have been linked to cancer initiation and promotion (17,18). Additionally, abnormal hormone exposure in early life can alter DNA methylation patterns and

thus protein expression (19,20). Various epigenetic changes have been proposed to be result of BPA exposure (21-23).

Normal reproductive development is dependent on the specific interaction of steroid hormones with their receptors at the specific tissues. Normal development and growth of other organs such as the brain and the central nervous system are also dependent on the appropriate hormonal milieu particular at the periods of growth such as in-utero, infancy and puberty. Concentrations and effect of these steroid hormones are dependent on their synthesis, availability of receptor sites and degradation. The cytochrome P450 group of enzymes play a key role in the metabolism of steroid hormones and are responsible for hydroxylation as well as conjugation with glucuronide and sulfate leading to steroid hormone inactivation. The concentrations of hormones both in circulation and at the receptor level are determined by the regulation of steroid synthesis and degradation. In addition, binding to hormone binding proteins and metabolic conversion to inactive forms of the hormone all effect the concentration of bioactive hormone. These processes in turn are affected by various enzymes involved in the synthesis of hormones and proteins and their metabolism (24,25,26). The expression of these metabolising enzymes may be increased or suppressed by exposure to various xenobiotics. Several studies have examined the effect of DNA methylation on this group of metabolising enzymes. In rodent studies, Peretz et al and Ziv-Gal et al reported that the exposure of ovarian follicles to BPA inhibited growth and decreased production of steroid hormones due to the effect on transcription expression of the relevant metabolising enzymes including *StAR* and *CYP11a1* (27,28). However, reports by Zhou et al, where differing BPA concentrations were used in vitro resulted in increased testosterone synthesis by theca interstitial cells (29). In animal studies it appears that both the dose of BPA and the cell lines utilised influence steroidogenesis and metabolising enzymes.

In a study examining colon cancer cells, *CYP1B1* and *CYP3A4* genes were upregulated by treatment with a demethylating agent caused by hypermethylation of CpG sites in the 5 promoter regions (30). The *CYP1B1* enzyme is involved in particular in oestrogen metabolism and may play an important role in susceptibility to oestrogen dependent cancers such as breast and ovarian cancer. Beedanagari et al showed the effect of epigenetic related methylation changes in the promoter region of CYP1B1 affected enzyme inducibility (31). CYP3A enzymes metabolise almost 50% of currently used drugs as well as endogenous and exogenous corticosteroids. Inter-individual variability in expression of *CYP3A* enzymes are high due to epigenetic regulatory mechanisms. Different DNA methylation patterns have been found between primary hepatocytes and hepatocyte cell lines (32,33).

A study by Nahar et al examine the effect of BPA exposure on human tissues (placenta, foetal liver and kidney). BPA concentrations, BPA-specific metabolizing enzyme gene expression, and global DNA methylation were characterized across three matched tissues from elective second trimester pregnancy

terminations. The findings of this study demonstrated that BPA-specific metabolism gene expression of the *GUSB*, *UGT2B15*, *STS*, and *SULT1A1* differed across each tissue type. Additionally, total BPA concentrations were positively associated with global methylation for the placenta which suggested organ-specific biological effects after foetal exposure (35).

We postulated that a possible additional mechanism of the interaction of BPA with steroid hormones apart from the described interaction with hormone receptors, is via BPAs interaction with steroid hormone metabolising enzymes. We further postulated that this may occur via an epigenetic mechanism namely changes in DNA methylation of the promoter region of these enzymes.

Methods

Sample population

Blood samples and data collected as part of the Mother and Child in the environment (MACE) birth cohort study were utilized for this study. The MACE study population consists of pregnant females recruited from antenatal clinics in Durban, South Africa from the "South Basin" area and other clinics in the North Durban area. The Durban south basin is an area where large communities are located within heavily polluted large-scale industrial enterprises. Mothers were recruited during the third trimester of pregnancy (between 27 completed to 40 weeks of pregnancy). Details regarding pregnancy outcome for participants were also collected during the course of the MACE study. Inclusion and exclusion criteria were as per MACE study which has been described previously (36).

Sample collection

Venous blood samples were taken during the third trimester from the pregnant individuals. Cord bloods were taken at delivery. Bloods were collected from maternal participants at one of their regularly scheduled ante-natal appointments. Whole blood samples were collected in potassium EDTA tubes. Genomic DNA was extracted from the whole blood samples collected using the FlexiGene® DNA isolation kit (Qiagen, Germantown, USA). DNA samples were stored at -70ºC until analysis performed.

Maternal and cord bloods collected in serum polypropylene vacutainer tubes were later analyzed for BPA and BPA-g. These samples were centrifuged, separated and serum stored at -80 degrees Celsius until analysis. Maternal /cord paired samples with sufficient serum volumes were utilized for the current study.

DNA Methylation studies:

Following extraction of genomic DNA from whole blood the isolated DNA was then eluted in nuclease-free water and purified using the DNA Clean and Concentrator™-5 Kit (Zymo Research, Irvine, USA),

as per manufacturer's instructions. DNA concentration was determined using the Nanodrop 2000 spectrophotometer (Thermo-Fischer Scientific, Walthahm, USA) and standardized to 4 ng/µl. DNA purity was assessed using the A260/A280 absorbance ratios (a ratio of 1.8-2.0 was considered acceptable). The promoter methylation of the candidate genes *CYP1B1* and *CYP3A4* were assessed using the OneStep qMethyl Kit (Zymo Research, Irvine, USA), as per manufacturer's instructions. In brief, 20 ng DNA was subject to a test and reference reaction containing specific primers (see Table 1). Cycling conditions were as follows: digestion by methyl sensitive restriction enzymes (37°C, 2 h), initial denaturation (95°C, 10 min), followed by 45 cycles of denaturation (95°C, 30 s), annealing (58 °C 60 s), extension (72°C, 60 s), final extension (72°C, 60 s), and a hold at 4°C. Quantitative PCR (qPCR) experiments were conducted using the CFX96 Real Time PCR System (Bio-Rad, Bio-Rad Life Sciences, Hercules, USA) and analyzed using the Bio-Rad CFX Manager™ Software version 3.1 (Bio-Rad, Bio-Rad Life Sciences, Hercules, USA).

The percentage methylation was calculated using the formula below as supplied in the OneStep qMethyl kit:

Quantification of promoter methylation formula:

Methylation (%) = 100 X $2^{-\Delta Ct}$, where ΔCt = Ct (test reaction) – Ct (reference reaction)

Table 7.1: qPCR primer sequences for promotor methylation and annealing temperatures

Gene	Sense Primer (5'→3')	Anti-Sense Primer (5'→3')	Annealing Temperature (°C)
CYP1B1	TTTGTGTGCCCAAGCACTGTC	CACAACTGGAGTCGCAGAA	58
CYP3A4	CATGCCCTGTCTCTCCTTTAGC	CCTTTCAGCTCTGTGTTGCTC	58

Note samples for which the DNA concentration were < 4 ng/ul following DNA clean up were not utilised for qPCR analysis. Sample runs with quantitation cycle (C_q) values >40 were not included in analysis of results because of the implied low efficiency (37).

Steroid hormone analysis

Steroid hormone analysis has been described in detail in a previous publication (38). Briefly the following nine steroid hormones were analyzed in maternal serum and cord blood using LC-MS/MS methodology: estradiol (E2), total testosterone (TT), 11-deoxycorticosterone (11DOC), Dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS) androstenedione (Andro), 17-OH progesterone (17OHP), dihydrotestosterone (DHT) and progesterone (Prog). There hormones were selected as they cover the major sex steroid hormones in humans. A commercially available kit the MassChrom Steroid Panel 2 kit (Chromosystems Instruments and Chemicals GmbH,

Germany) was utilized for analysis of steroid hormones. Chromatographic separations were carried out using the AB-Sciex 4500 triple quadrupole mass spectrometer equipped with an Agilent 1260 Ultra high-performance liquid chromatography (uHPLC) system. A linear gradient was used from 0-70% of Mobile phase B in Mobile phase A followed by a hold for 1 minute at a flow rate of 0.8 ml/min. Total run time was 12.5 minutes. Injection volume utilized was 30µl.De-deuterated internal standards were utilized for each analyte of interest and were added to samples prior to extraction. Six levels of commercially available standards (including blank) and three levels of serum based internal quality control materials were analyzed at the beginning, middle and end of each run. MassChrom® steroid serum calibrators and controls were purchased from Chromsystems Instruments & Chemicals GmbH (Munich, Germany). Within run coefficient of variation (CV) for the steroid hormones ranged from 0.48% to 12.73%, and for the between-run CVs ranged from 1.6-10.2 percent (%) across the three internal quality control (IQC) levels.

Ethical approval

The research has complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration and has been approved by the authors' institutional review board or equivalent committee. Ethical clearance for this study was obtained from the Biomedical Research and Ethics Committee (BREC) of the University of KwaZulu-Natal (Ethics Clearance Certificate BE 597/16).

Statistical analysis

Statistical analysis was performed on Medcalc statistical software program version 18.11 (Medcalc, Belgium). The Shapiro-Wilk test was used to determine normality. Kruskal Wallis analysis across groups and Spearman's rank correlation was utilised for non-parametric data. Statistical significance was considered at $p < 0.05$.

Results

Baseline characteristics for CYP1B1 promoter methylation studies

A total of forty-four samples were analysed which included sixteen maternal-child pairs. The baseline characteristics of the cohort for which *CYP1B1* promoter methylation studies were performed are detailed in Table 7.2. There was not statistically significant difference in methylation status between maternal and cord blood samples. Cord blood BPA and BPA-g concentrations correlated with levels found in maternal blood.

Table 7.2 Baseline characteristics of cohort for *CYP1B1* promoter methylation studies

	BPA (ng/L)	BPA-g (ng/L)	DOC (nmol/L)	DHEAS (nmol/L)	Prog (nmol/L)	17OHP (nmol/L)	DHT (nmol/L)	DHEA (nmol/L)	Andro (nmol/L)	Oestradiol (pmol/L)	TT (nmol/L)	25OH Vitamin D (nmol/L)	*CYP1B1* methylation
Maternal (n=21)	3.1 (3.1)	4.8 (0.2-21.8)	2.5 (0.28-28.0)	978 (300-7260)	303 (38.5-3810)	16 (2.7-110)	2.5 (0.5-9.2)	53.5 (3.8-234)	82.3 (0.77-1570)	9720 (412-468000)	10.4 (0.7-45.5)	59.5 (16.8)	2.4 (0.14-71.3)
Cord blood (n=23)	3.19 (3.5)	4.11 (0.7-13.4)	4.2 (0.8-55.8)	1850 (194-8230)	883 (270-3440)	60.5 (12-315)	2.3 (1.5)	68.3 (1.5-569)	143 (0.3-905.0)	17300 (1160-241000)	16.1 (0.18-90.4)	58.1 (13.4)	0.5 (0.1-56.1)
													P=0.21

Normal data presented as mean (SD); non-parametric data presented as median (range)

CYP1B1 analyses

Kruskal Wallis analysis showed no significant difference (p=0.21) in percentage methylation of the *CYP1B1* promoter between maternal and cord samples. (refer Figure 7.1)

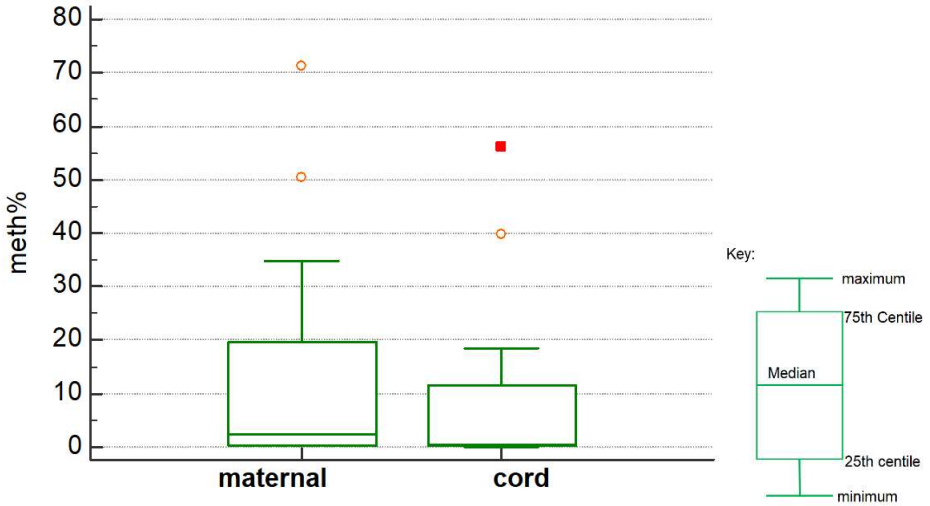

Figure 7.1: Box and whiskers plot of percentage methylation (meth%) for *CYP1B1* promoter region for maternal and cord samples

BPA: Samples were further divided into two categories -:1) those with detectable serum BPA concentrations and 2) those with below detectable BPA concentrations. Kruskal Wallis analysis to determine difference in methylation status between the two categories of BPA levels showed no statistically significant difference (p= 0.38).

BPA-g: Spearman's correlation showed no significant correlation (p=0.86) between methylation percentage and BPA-g concentrations across maternal and cord blood samples.

Steroid hormones: Spearman's rank correlation showed no significant relationship between any of the steroid hormones concentrations and *CYP1B1* promoter methylation status with the exception of DHEA (r=-0.42; p=0.01).

Baseline characteristics for CYP3A4 promoter methylation studies

A total of thirty-five samples were analysed, of which included sixteen maternal-child pairs.

Figure 7.2 below shows RT PCR melting curve analyses.

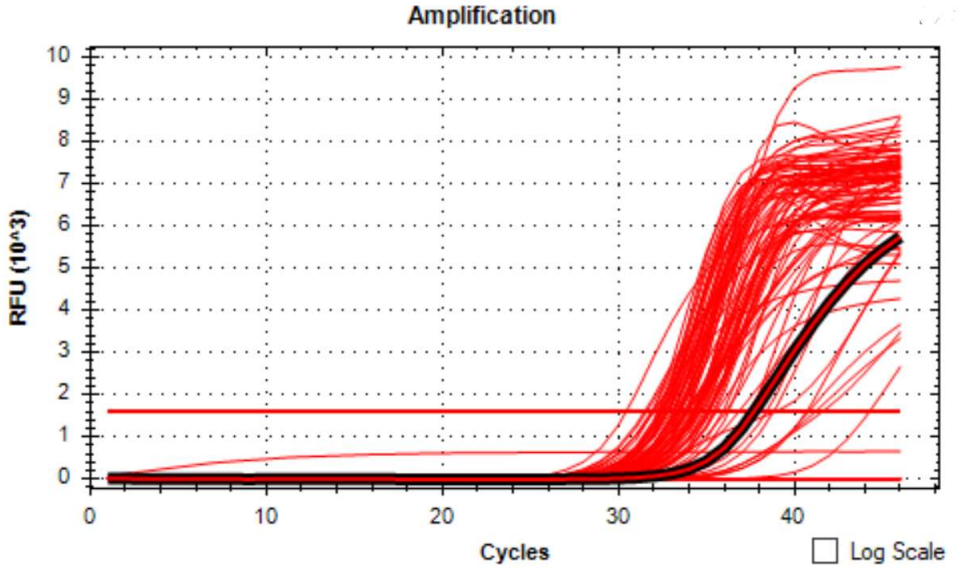

Figure 7.2. *CYP3A4* promoter region methylation studies melting curve analysis

Table 7.3 Baseline characteristics of cohort for *CYP3A4* promoter methylation studies

	BPA (ng/L)	BPA-g (ng/L)	DOC (nmol/L)	DHEAS (nmol/L)	Prog (nmol/L)	17OHP (nmol/L)	DHT (nmol/L)	DHEA (nmol/L)	Andro (nmol/L)	Oestradiol (pmol/L)	TT (nmol/L)	25OH Vitamin D (nmol/L)	*CYP3A4* methylation
Maternal (n=18)	3.0 (3.1)	4.4 (0.18-21.8)	2.34 (0.28-6.27)	1109 (302-7260)	283 (38-2490)	17.8 (2.7-110)	2.54 (0.49-9.2)	62.7 (6.4-234)	92.7 (0.8-1570)	9415(412-468000)	10.3(0.7-38.2)	59.4 (17.4)	38.1 (11.4-94.7)
Cord blood (n=17)	3.1 (3.2)	5.0 (0.7-13.4)	4.22 (0.81-55.8)	1510 (194-8080)	767 (351-2950)	53.8 (12-315)	2.03 (0.11-5,8)	94.4 (1.5-569)	133 (0.3-905)	17300(1160-241000)	12.3 (0.2-90.4)	61.1 (40.5-87.8)	49.3 (2.8-95.1)
													P=0.28

Normal data presented as mean (SD); non-parametric data presented as median (range)

BPA: Kruskal Wallis analysis to determine difference in methylation status between the two categories of BPA levels showed no statistically significant difference (p= 0.62).
BPA-g: Spearman's correlation showed no significant correlation (p=0.43) between BPA-g concentrations across maternal and cord blood samples and *CYP3A4* promoter methylation status.
Steroid hormones: Spearman's rank correlation showed no significant relationship between any of the sex steroid hormones or 25 OH vitamin D and *CYP3A4* promoter methylation status.

Discussion

The current study did not demonstrate any significant correlation with BPA, BPA-g concentrations and the degree of methylation of the promoter regions for either *CYP1B1* or *CYP3A4* enzymes. We reported the significant finding of a negative association with methylation status of the promoter region of *CYP1B1* and the concentrations of DHEA levels in maternal and cord blood. No significant association was found between the methylation status of the promoter region of *CYP1B1* and maternal or cord BPA/BPA-g concentrations. Additionally, there was no statistically significant correlation between DHEA concentrations and either maternal or cord BPA/ BPA-g concentrations. In humans DHEA is produced by the adrenal gland and is taken up by several tissues. Apart from its role as a precursor to other sex hormones including oestradiol and testosterone and development of secondary sexual characteristics DHEA has also been shown to play a role in the development and maintenance of immune-competence (39, 40). In animal studies DHEA has been reported to regulate the expression of various metabolising enzymes including CYP1A1, CYP1A2 and CYP1B1 (41, 42). The significance of the finding of an association with DHEA concentrations and *CYP1B1* promoter methylation status is uncertain.

There are limitations to the current study. The small sample size being the first. Unfortunately analyses of more samples and further relevant metabolising enzymes promoter regions could not be undertaken due to limitations with number of samples with sufficient concentrations of genomic DNA. Additionally, this study did not review tissue specific DNA following exposure, as evidenced by previous studies the effect of BPA exposure on DNA methylation is often tissue specific and may not be generally identified.

The findings of this study are inconclusive with regards to the effect of BPA on methylation status of steroid metabolising enzymes. Further studies examining a larger cohort, a wider array of possibly significant enzymes and other mechanisms of epigenetic changes are required.

References

1.Dodds EC, Lawson W. Synthetic oestrogenic agents without phenanthrene nucleus. Nature 1936; 137: 996–7.

2. EPA report on endocrine disruptors https://www.epa.gov/endocrine-disruption. Accessed 17 September 2016

3. Kandarakis ED, Bourguignon JP, Giudice L et al, Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement Endocrine Reviews 2009; 30: 293–342.

4. Ma Y, Liu H, Wu J, Yuan L, Wang Y, Du X et al. The adverse health effects of Bisphenol A and related toxicity mechanisms. Environmental research 2019; 176:108575.

5. Yamada H, Furuta I, Kato EH, et al. Maternal serum and amniotic fluid Bisphenol A concentrations in the early second trimester. Reproductive Toxicology 2002; 16: 735–9.

6. Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenbergh JG. vom Saal FS. Environmental toxins: exposure to bisphenol A advances puberty. Nature 1999; 401: 763–4.

7. Timms BG, Howdeshell KL, Barton L, Bradley S, Richter CA, vom Saal FS. Estrogenic chemicals in plastic and oral contraceptives disrupt development of the fetal mouse prostate and urethra. Procedures of the National Academy of Science USA 2005; 102: 7014–9.

8. Munoz-de-Toro M, Markey CM, Wadia PR, et al. Perinatal exposure to bisphenol-A alters peripubertal mammary gland development in mice. Endocrinology 2005; 146: 4138–47.

9. Markey CM, Wadia PR, Rubin BS, Sonnenschein C, Soto AM. Long-term effects of fetal exposure to low doses of the xenoestrogen Bisphenol-A in the female mouse genital tract. Biology of Reproduction 2005; 72: 1344–51.

10. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. Human exposure to Bisphenol A (BPA). Reproductive Toxicology 2007; 24: 139–77.

11. National Toxicology Program. NTP-CERHR Expert Panel Report on the Reproductive and Developmental Toxicity of Bisphenol A. Durham: Research Triangle Park; 2007.

12. Jalal N, Surendranath AR, Pathak JL, Yu S, Chung CY. Bisphenol A (BPA) the mighty and the mutagenic. Toxicology Reports 2018; 5:76-84.

13. Kundakovica M, Gudsnuka K, Franksa B, Madrida J et al, Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure. Procedures of the National Academy of Science USA 2013; 110: 9956–996.

14. Zhang X and Ho S. Epigenetics meets endocrinology. Journal of Molecular Endocrinology 2011; 46: R11–R32.

15.Weinhold B. Epigenetics: The Science of Change. Environmental Health Perspectives 2006; 114: A160–A167.

16. Kaelin WG. and McKnight SL Influence of Metabolism on Epigenetics and Disease. Cell 2013; 153: 53-69.

17. Esteller M. Aberrant DNA methylation as a cancer-inducing mechanism. Annual Review of Pharmacology and Toxicology 2005; 45: 629–56.
18.Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. Nature Reviews 2006; 7: 21–33.
19. Yegnasubramanian S, Kowalski J, Gonzalgo ML, et al. Hypermethylation of CpG islands in primary and metastatic human prostate cancer. Cancer Research 2004; 64: 1975–86.
20. Enokida H, Shiina H, Urakami S, et al. Multigene methylation analysis for detection and staging of prostate cancer. Clinical Cancer Research 2005; 11: 6582–8.
21.Onuzulu CD, Rotimi OA, Rotimi SO. Epigenetic modifications associated with in utero exposure to endocrine disrupting chemicals BPA, DDT and Pb. Reviews in Environmental Health 2019, 34, 309–325.
22. Alavian-Ghavanini, A and Ruegg, J. Understanding epigenetic effects of endocrine disrupting chemicals: From mechanisms to novel test methods. Basic Clinical Pharmacology and Toxicology 2018; 122: 38–45.
23.Chianese R., Troisi J, Richards S, Scafuro M., Fasano S, Guida, M, et al. Bisphenol A in reproduction: Epigenetic effects. Current Medicinal Chemistry 2018; *25*: 748–770.
24. Simpson ER. Aromatization of androgens in women: current concepts and findings. Fertility and Sterility 2002; 77: 6 –10.
25. Simpson ER. Sources of estrogen and their importance. Journal of Steroid Biochemistry and Molecular Biology 2003; 86: 225–230.
26. Conley A, Hinshelwood M. Mammalian aromatases. Reproduction 2001; 121: 685– 695.
27. Peretz J, Gupta RK, Singh J, Hernandez-Ochoa I, Flaws JA: Bisphenol A impairs follicle growth, inhibits steroidogenesis, and downregulates rate-limiting enzymes in the estradiol biosynthesis pathway. Toxicological Sciences 2011; 119: 209-217.
28. Ziv-Gal A, Craig ZR, Wang W, Flaws JA: Bisphenol A inhibits cultured mouse ovarian follicle growth partially via the aryl hydrocarbon receptor signaling pathway. Reproductive Toxicology 42, 58-67, 2013.
29. Zhou W, Liu J, Liao L, Han S, Liu J: Effect of bisphenol A on steroid hormone production in rat ovarian theca-interstitial and granulosa cells. Molecular and Cellular Endocrinology 2008; 283: 12-18.
30. Habano W, Gamo T, Sugai T, Otsuka K, Wakabayashi G, Ozawa S. CYP1B1, but not CYP1A1, is downregulated by promoter methylation in colorectal cancers. International Journal of Oncology 2009; 34: 1085–91.
31. Beedanagari SR, Taylor RT, Bui P, Wang F, et al. Role of epigenetic mechanisms in differential regulation of the dioxin-inducible human CYP1A1 and CYP1B1 genes. Molecular Pharmacology.2010;78:608-16.

32. Bodin, K, Bretillon L, Aden Y, Bertilsson L, Broome U, Einarsson C, et al. Antiepileptic drugs increase plasma levels of 4beta-hydroxycholesterol in humans: evidence for involvement of cytochrome p450 3A4. Journal of Biological Chemistry 2001; 276: 38685–38689.
33. Patki KC, Von Moltk, LL and Greenblatt DJ. In vitro metabolism of midazolam, triazolam, nifedipine, and testosterone by human liver microsomes and recombinant cytochromes p450: role of cyp3a4 and cyp3a5. Drug Metabolism and Disposal 2003;31: 938–944.
34. Matthaei J, Bonat WH, Kerb R, Tzvetkov MV, Strube J, Brunke S, et al. Inherited and Acquired Determinants of Hepatic CYP3A Activity in Humans. Frontiers in Genetics 2020; 11: 944.
35. Nahar MS, Liao C, Kannan K, Harris C, Dolinoy DC. In utero Bisphenol A concentration, metabolism, and global DNA methylation across matched placenta, kidney, and liver in the human fetus, Chemosphere 2015;124: 54-60.
36. Jeena PM, Asharam K, Mitku AA et al. Maternal demographic and antenatal factors, low birth weight and preterm birth: findings from the mother and child in the environment (MACE) birth cohort, Durban, South Africa. BMC Pregnancy Childbirth 2020; 20; 628.
37. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al, The MIQE Guidelines: *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments. Clinical Chemistry 2009;55: 611–622,
38. Gounden V, Warasally MZ, Magwai T, Naidoo R, Chuturgoon A. A pilot study: Relationship between Bisphenol A, Bisphenol A glucuronide and sex steroid hormone levels in cord blood in A South African population. Reproductive Toxicology 2021; 100: 83-89.
39. Ebeling P, Koivisto VA. Physiological importance of dehydroepiandrosterone. Lancet. 1994; 343:1479-81.
40. Prall SP, Muehlenbein MP. DHEA Modulates Immune Function: A Review of Evidence. Vitamains and Hormones 2018;108:125-144.
41. Ciolino H, MacDonald C, Memon O, Dankwah M, Yeh GC. Dehydroepiandrosterone inhibits the expression of carcinogen-activating enzymes in vivo. International Journal of Cancer 2003;105:321-5.
42. Belic A, Tóth K, Vrzal R, Temesvári M, Porrogi P, Orbán E, et al. Dehydroepiandrosterone post-transcriptionally modifies CYP1A2 induction involving androgen receptor. Chemico Biological Interactions 2013;203:597-603.

CHAPTER 8
SYNTHESIS CHAPTER

This chapter summarises the results of the study, highlighting key points in Table 8.1 below, as well as discussing broader relevance of the study and suggestions for related future research.

8.1 Summary of findings

This study attempted to address the two main knowledge gaps identified 1) lack of data regarding BPA exposure in vulnerable populations maternal child pairs and its effect on steroid hormones and 2) further elucidate the mechanism of BPA health effects by exploring epigenetic effects on steroid metabolising enzymes.

Table 8.1 Consolidated Findings of study

Objectives	Chapter	Evidence
Develop and validate an in-house method using liquid chromatography tandem mass spectrometry for the measurement of serum Bisphenol A	3	An in house liquid mass tandem spectrometry method was developed for the determination of BPA and BPA-g in serum and cord blood. This was validated with CVs of 3.8-9.4%. The sample preparation method developed was simple dilution and protein precipitation and a final run time of less than 5 minutes was achieved.
Describe the extent of exposure to BPA by determining the serum levels of Bisphenol A and its commonly occurring metabolite Bisphenol glucuronide in a South African cohort of maternal- child pairs	3	Ninety maternal and child pairs were analysed. BPA was detectable in more than 25% of maternal and cord blood samples. Spearman's correlation demonstrated significant positive correlation between maternal and child BPA and BPA-g levels with correlation coefficients of 0.892 and 0.744, respectively. This is the first study to describe the presence of detectable BPA levels using LC-MS/MS methodology in a South African population.
Determine the serum levels of steroid hormones and describe any association with BPA/BPA-g levels	4 and 5	Third-trimester serum maternal samples and matching cord blood samples were analyzed for BPA, BPA-g and nine sex steroid hormones using liquid chromatography tandem mass spectrometry (LC-MS/MS). This study demonstrated a significant positive relationship between cord blood estradiol and cord blood

		BPA (p=0.002) and maternal BPA levels (p=0.02) respectively. Cord blood testosterone from male infants showed a negative Spearman's correlation (r=-0.5, p=0.02) with maternal BPA-g. There was no statistical difference in total testosterone levels in cord blood from male and female infants. The findings indicate a significant relationship between some key sex steroid hormones namely testosterone, dihydrotestosterone and estradiol and BPA exposure in the foetus.
Characterize the presence of epigenetic changes: DNA Methylation affecting genes coding for key enzymes (CYP3A44 and CYP1B1, CYP1A1, EPHX1, CYP2D6) responsible for steroid metabolism	6	Promoter methylation status of the genes for metabolising enzymes *CYP1B1 and CYP3A4* were analysed. No significant correlation was noted between BPA/BPA-g concentrations and promoter methylation status for either maternal or infant specimens.

In summary the study has highlighted the following key points:

1. Development of a local in-house validated method for the simultaneous analysis of BPA and BPA-g. This optimised methodology used minimal consumables and small sample volumes, involved simple sample preparation and had short run times. The method is highly amenable for use in larger epidemiological studies to determine extent of BPA exposure in the South African) and other low and middle income countries) context at large.
2. A high proportion of elevated BPA concentrations and the presence of BPA-g in maternal and matched cord blood pairs.
3. Positive correlation with maternal BPA /BPA-g concentrations and that found in cord bloods.
4. Demonstrated significant associations of infant sex steroid hormones in particular testosterone and oestradiol as well as 25 OH vitamin D to BPA exposure in-utero.
5. This study reported no association between the degree of promoter methylation for CYP1B1 and CYP3A4 enzymes and testosterone and oestradiol concentrations.

8.1.1 BPA: Extent of exposure

This is the first study in South Africa to determine and report on human exposure to Bisphenol A. Additionally it is the first study originating from Africa that examined the serum and cord blood concentrations of BPA and its common metabolite BPA-g in matched maternal-child pairs. It is also the first published study in Africa to develop and validate a liquid-chromatography tandem mass spectrometry method for measurement of BPA and BPA-g in serum and cord blood.

The extent of BPA exposure in this South African cohort is in keeping with previously reported studies from industrialised nations. The findings of this study have demonstrated median levels of maternal BPA (0.95 ng/mL) in keeping with values seen in previous studies performed in industrialised nations. A study in German women reported serum maternal BPA levels between 0.3 to 18.9 ng/mL with median values of 3.1 ng/mL (1). Many of the studies performed before the last 3-5 years utilised immunoassay based assays for determination of BPA levels and this showed higher values than the more specific chromatographic/mass spectrometry based methods. Previous studies using chromatographic methodologies to measure BPA in term cord blood have reported similar findings to the current study with mean levels ranging from 0.13 ng/mL to 1.13 ng/mL (current study median cord serum BPA 0.92 ng/mL) (2,3). In this study all the detectable cord blood BPA levels were 0.4 ng/mL or greater indicating that in those infants where BPA was present it was present in significant levels to possibly impact on health. This study reiterated the significant positive correlation between maternal and foetal BPA concentrations previously reported in other studies (2,4,5). Of significant concern from the findings of this study is that more than eighty percent of cord blood samples had detectable BPA-g levels. This is an indicator of significant levels of exposure occurring in-utero.

8.1.2 BPA exposure and birthweight

The child birthweight showed statistically significant negative association with cord BPA levels but to a lesser degree than maternal BPA levels. However maternal BMI had no significant association with maternal serum BPA or BPA-g levels. This may be due to the relatively homogenous distribution of BMI with very few maternal participants having normal or low BMIs.

8.1.3 BPA exposure and steroid hormones

This is one of the few studies worldwide that have examined the relationship between BPA concentrations and several sex steroid hormones in both maternal and cord blood samples.

The use of a reference methodology (LC-MS/MS) to measure the hormones, BPA and its metabolite BPA-g also allowed for specific and sensitive analyses of these substances. BPAs action as an endocrine disruptor has been attributed to its agonistic and antagonistic actions at the levels of hormone receptors including those of oestrogens and androgens. This action is also dependent on the dose of BPA exposure. This study demonstrated a significant positive relationship between cord blood estradiol and

cord blood BPA (p=0.002) and maternal BPA levels (p=0.02) respectively. Cord blood testosterone levels showed statistically significant negative correlation (p=0.009) with both maternal BPA-g and cord BPA-g levels. Cord blood testosterone from male infants showed a negative Spearman's correlation (r=-0.5, p=0.02) with maternal BPA-g levels even when corrected for confounders. The lack of statistically significant difference of cord blood testosterone concentrations between both sexes is clinically relevant as the normal expectation would be higher testosterone concentrations in the male cord blood specimens. Overall cord blood testosterone levels correlated negatively with cord (r=-0.6; p=0.02) and maternal BPA levels (r=-0.52; p=0.05). DHT like TT displayed a negative correlation with maternal and cord blood BPA-g levels. Additionally, DHT like testosterone displayed no statistical difference between levels of these hormones in male and female cord blood. A previous study in normal infants using gas chromatography tandem mass spectrometry (GC-MS/MS) reported DHT levels of 2.4 fold higher in male cord blood. The same study reported a smaller difference in male and female cord blood for total testosterone with values in males being 1.3-fold higher (6). The lack of a significant difference between male and female cord blood levels for DHT and TT in the current study is thus a significant finding, particularly in view of the fact that androgen exposure plays a vital role in development of the foetus in the intra-uterine period as well as later in life. Exposure of male fetuses to anti-androgen EDs results in feminization and exposure of female fetuses to androgens leads to their masculinization (7). Both insufficient and excessive androgen levels have sequelae on development of both sexes affecting reproductive, metabolic and neurological development.

8.2 Theoretical, local and national relevance

The burden of disease related to environmental exposure is a growing health concern. Research examining levels of BPA exposure in developing countries has been identified as an important research need (8). The ubiquitous presence of BPA in our environments and the growing body of literature describing the impact of exposure on physical and mental health in humans, highlights the need for further research in this topic. In particular, there is a need to define physiological relevant BPA levels and fully describe the pathological consequences of exposure in humans across different age groups as well as to determine the mechanisms of pathology caused by BPA in order to prevent or manage related disease. The findings of this study indicate a significant degree of BPA exposure in (late) pregnancy and in-utero. Given the severity and wide array of pathologies that have been linked to BPA exposure this is a significant public health concern. Our findings together with the current global literature should assist in guiding regulatory decisions regarding the use of BPA in consumer products in South Africa.

In this study we demonstrated the presence of elevated and likely physiologically relevant median BPA and BPA-g concentrations in the mother-child pairs. Recent literature has described the phenomenon of low dose health effects with BPA which refers to the development of pathology following exposure

to BPA doses below the Food and Drug Administration (FDA) determined lowest observed adverse effect level (LOAEL) (9). The implications of this is significant with regards to what may be considered safe exposure. Additionally, some human modelling studies have described significant long term foetal exposure to BPA and BPA-g even with short-lived increases in maternal concentrations (10). Furthermore, we have shown a significant relationship with BPA concentrations and key sex steroid hormones testosterone and oestradiol, which has implications for reproductive and neurodevelopment in the foetus and early childhood. The findings of the study highlight the vulnerability of the in-utero development period and the importance of ensuring adequate health measures preconception and during pregnancy to optimise maternal child health. The current study findings may be used to provide preliminary data to advocate for stronger government regulations limiting the use of BPA containing consumer products and implementation of health advisories at clinical contact level particularly for pregnant and breastfeeding women. Information derived from the current study may be used to further promote research in Sub-Saharan Africa regarding BPA exposure in this region.

8.3 Areas of future research

This was a cross sectional study the results presented cannot prove causality and larger longitudinal studies would be required to further investigate the clinical impact of a) BPA exposure in utero and b) the significant associations with certain steroid hormones as reported in this study. Further epidemiological studies are required to determine extent of exposure nationally and in different community setting namely rural, urban, occupational, low/high socio-economic.

8.3.1 Assessment of environmental BPA and other EDC exposure

Estimation of intake of BPA via different food sources, drinking water, dust and other environmental exposure is required in the South African context. Whilst there have been a few studies reviewing levels of BPA in potable and waste water, there have been no reports estimating total exposure for a particular community/geographical location. Future studies examining this will also need to carefully identify any other EDC exposure in the environment being evaluated. The presence of other EDCS may have synergistic, antagonistic or no effect on the health effects of BPA exposure.

8.3.2 Relationship between BPA exposure and long term health sequalae

Further longitudinal studies following up infants with identified in utero exposure (detectable BPA and BPA-g cord blood levels) are required to determine long term health sequalae. One of the major difficulties with establishing long term health sequalae following BPA exposure in early life is excluding the influence of other environmental exposures. Dosage relationships with regards to exposure and pathology development also need to be studied further.

8.3.3 Effect of BPA exposure and altered hormone levels on pregnancy outcomes

Another area for future research is the effect of BPA exposure and described associated changes in steroid hormones including 25 OH vitamin D on pregnancy, neonatal and infant outcomes.

8.3.4 Epigenetic and other molecular mechanisms of BPA action

An area of research pertaining to BPA that remains unexplored is the effect of BPA on post translational modification of the various proteins that may be impacted by BPA action. This includes the recently described effect of BPA acting as a potent calcium channel inhibitor of the Golgi and sarcoplasmic reticulum (11).

8.4 Limitations

As a pilot study one of the major limitations of this study is the small sample size. Due to the limitations in the access to the sample type used for this study namely cord blood, numbers in these types of studies are generally not as high as when carried out in a general adult population. The study was cross-sectional as such does not prove causality for the associations reported in this study. As BPA and BPA-g levels were not followed over time during the course of the pregnancy it would be difficult to ascertain to what degree the measured maternal serum and cord blood BPA/BPA-g levels reflect the actual exposure throughout pregnancy and to what extent cord sex steroid hormone reflects the actual hormonal levels throughout the entire intra-uterine period. Another limitation of this study that could be cited is that maternal BPA measurements from earlier in pregnancy were not performed as part of the study. The early intra-uterine period, specifically the 8-14 weeks when organogenesis occurs, is generally considered the most sensitive period for any interference from toxin exposure. However, exposure in late pregnancy has also been shown to be of clinical significance by animal and human studies (12,13). Since repeating sampling was not performed for each maternal /child pair the findings of this study represent only a snapshot of possible exposure. As stored genomic DNA was utilised for the epigenetic studies there were samples with insufficient DNA concentrations that could not be used for the RT- PCR analysis resulting in a smaller number of samples and promoter regions that could be assessed.

References

1. Schonfelder G, Wittfoht W, Hopp H, Talsness CE, Paul M, Chahoud I. Parent bisphenol A accumulation in the human maternal-fetal-placental unit. Environmental Health Perspectives 2002; 110: A703–A707
2. Lee YJ, et al. Maternal and fetal exposure to Bisphenol A in Korea. Reproductive Toxicology 2008; 25:413–9.
3. Zhang T, Sun H, Kannan K. Blood and Urinary Bisphenol A Concentrations in Children, Adults, and Pregnant Women from China: Partitioning between Blood and Urine and Maternal and Fetal Cord Blood. Environmental Science and Technology. 2013; 47:4686–94.
4. Aziz A. Estimation of Bisphenol A (BPA) concentrations in pregnant women, fetuses and nonpregnant women in Eastern Townships of Canada. Reproductive Toxicology 2014; 45:8-13
5. Zhang T, Sun H and Kannan K. Blood and Urinary Bisphenol A concentrations in children, adults and pregnant women from China: Partitioning between Blood and urine and Maternal fetal cord blood. Environmental Science and Technology 2013; 47: 4686–4694
6. Lundell AC, Ryberg H, Vandenput L, Rudin A, Ohlsson C, Tivesten Å. Umbilical cord blood androgen levels in girls and boys assessed by gas chromatography-tandem mass spectrometry. Journal of Steroid Biochemistry and Molecular Biology. 2017; 171:195-200.
7. Welsh M. Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. Journal of Clinical Investigation 2008; 118:1479-1490.
8. Thoene M, Rytel L, Nowicka N, Wojtkiewicz J. The state of bisphenol research in the lesser developed countries of the EU: a mini-review. Toxicology Research (Camb). 2018; 7: 371-380.
9. Vom Saal FS and Vandenberg LN. Update on the health effects of Bisphenol A: overwhelming evidence of harm. Endocrinology 2021; 163: bqaa171.
10. Gauderat G, Picard-Hagen N, Toutain PL, Servien R, Viguie C, Puel S, et al. Prediction of human prenatal exposure to Bisphenol A and bisphenol A glucuronide from an ovine semi-physiological toxicokinetic model. Scientific Reports 2017; 7: 15330
11. Pan MH, Wu YK, Liao BY, Zhang H, Li C, Wang JL et al. Bisphenol A exposure disrupts organelle distribution and functions during mouse oocyte maturation. Frontiers of Cellular and Developmental Biology 2021; 9: 661155.
12. Ohtani N, Suda K, Tsuji E, et al. Late pregnancy is vulnerable period for exposure to BPA. Journal of Veterinary Medical Science 2018; 80: 536-543.
13. Namat A, Xia W, Xiong C, Xu S, Wu C, Wang A, et al. Association of BPA exposure during pregnancy with risk of preterm birth and changes in gestational age: A meta-analysis and systematic review. Ecotoxicology and Environmental Safety 2021; 220:112400.

APPENDIX 1
Ethical clearances

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

01 August 2017

Dr V Gounden (983170122)
Discipline of Chemical Pathology
School of Laboratory Medicine and Medical Sciences
Verena.gounden@nhls.ac.za

Dear Dr Gounden

Protocol: Effect of environmental endocrine disruptor BPA on epigenetic regulation of steroid hormone metabolism and influence on associated levels of hormones among mother and child pairs.
Degree: PhD **BREC reference number: BE597/16**

EXPEDITED APPROVAL

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 03 November 2016.

The study was provisionally approved pending appropriate responses to queries raised. Your response received on 18 July 2017 to BREC letter dated 14 December 2016 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given **full ethics approval** and may begin as from 01 August 2017.

This approval is valid for one year from **01 August 2017**. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its next meeting taking place on **12 September 2017.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

[redacted]

Professor V Rambiritch
Deputy Chair: Biomedical Research Ethics Committee

cc supervisor: chutur@ukzn.ac.za cc postgraduate administrator: dudhrajhp@ukzn.ac.za

Biomedical Research Ethics Committee
Professor J Tsoka-Gwegweni (Chair)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 2488 **Facsimile:** +27 (0) 31 260 4609 **Email:** brec@ukzn.ac.za

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

02 July 2021

Dr V Gounden (983170122)
Discipline of Chemical Pathology
School of Laboratory Medicine and Medical Sciences
Verena.gounden@nhls.ac.za

Dear Dr Gounden

Protocol: Effect of environmental endocrine disruptor BPA on epigenetic regulation of steroid hormone metabolism and influence on associated levels of hormones among mother and child pairs.
Degree: PhD
BREC reference number: BE597/16

New Title: Effect of environmental endocrine disruptor BPA on epigenetic regulation of steroid hormone metabolism and influence on associated levels of hormones among mother and child pairs

We wish to advise you that your application for amendments received on 24 June 2021 to change the title to the above new title for the above study has been noted and approved by a subcommittee of the Biomedical Research Ethics Committee.

The committee will be notified of the above at its next meeting to be held on 10 August 2021.

Yours sincerely

[redacted]

Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

APPENDIX 2
Published papers
Paper 1

Reproductive Toxicology 89 (2019) 93–99

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

A pilot study: Bisphenol–A and Bisphenol-A glucuronide levels in mother and child pairs in a South African population

Verena Gounden[a,*], Mohamed Zain Warasally[b], Thabo Magwai[b], Rajen Naidoo[c], Anil Chuturgoon[d]

[a] Department of Chemical Pathology, University of KwaZulu-Natal and National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital, Durban, South Africa
[b] Department of Chemical Pathology, National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital, Durban, South Africa
[c] Department of Occupational Health, University of KwaZulu-Natal, Durban, South Africa
[d] Department of Medical Biochemistry, University of KwaZulu-Natal, Durban, South Africa

ARTICLE INFO

Keywords:
Bisphenol A
Liquid chromatography tandem mass spectrometry
Mother-child pairs
Cord blood

ABSTRACT

Exposure to Bisphenol A (BPA) during early development particularly in- utero has been linked to a wide range of pathology. The aim of this study was to determine serum levels of BPA and its naturally occurring metabolite BPA-glucuronide (BPA-g) in South African mother-child pairs.

Method: Third-trimester serum maternal samples and matching cord blood samples were analysed for BPA and BPA-g using LC–MS/MS.

Results: Ninety maternal and child pairs were analysed. BPA was detectable in more than 25% of maternal and cord blood samples. Spearman correlation demonstrated significant positive correlation between maternal and child BPA and BPA-g levels with correlation coefficient of 0.892 and 0.744, respectively. A significant positive association between cord BPA levels and child birth-weight ($p = 0.02$) as well as with maternal BMI ($p = 0.04$) was noted.

Conclusion: This is the first study to describe the presence of detectable BPA levels using LC-MS/MS methodology in a South African population.

1. Introduction

Environmental pollutants have been shown to have significant effects on human health. Steroid hormone function has been shown to be affected by some of these pollutants in particular a class known as, endocrine disruptors (EDs) [1]. The United States environmental protection agency has defined endocrine disrupting chemicals or EDs as "exogenous agents that interfere with the normal function of endogenous hormones responsible for the maintenance of homeostasis and the regulation of developmental processes" [2]. The possible deleterious effects on human health associated with EDs have ranged from effects on reproductive health in particular sperm health, carcinogenesis, and increasingly effects on other endocrine and metabolic functions such as thyroid disease and obesity [1,3,4]. Deleterious neurodevelopmental effects have also been described in animal and human studies [5–7]. The effect of EDs are most significant when exposure occurs during developmental stages as such the foetus is particularly vulnerable to these effects [8]. Thus, exposure of the pregnant mother to EDs may have adverse effects on the developing foetus. Examples of environmental EDs that have been targeted for adverse effects on reproductive systems in humans and other animals are pesticides [e.g. dichlorodiphenyltrichloroethane (DDT)], fungicides (e.g. vinclozolin), insecticides (e.g. trichlorfon), herbicides (e.g. atrazine), plastics (e.g. phthalates and bisphenol A) [1,9].

Bisphenol A (BPA) is found in a vast array of plastic consumer products including lining of tin cans, food and water containers, medical devices and toys [1,10]. Apart from exposure due to use of these products, environmental exposure may occur by other means. BPA has been measured in soil, river water, indoor air and dust as well as landfill leachates [11,12]. Animal and human studies have demonstrated that BPA readily crosses the placenta and has been detected in amniotic fluid [13–15].

Exposure to BPA has been linked to prenatal and postnatal adverse effects on multiple tissues, including the reproductive system and neurodevelopment. Whilst studies in humans have been limited, studies in animal models have associated BPA exposure to development of

* Corresponding author at: Department of Chemical Pathology, University of KwaZulu-Natal and National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital, 2nd Floor Pathology Building Inkosi Albert Luthuli Central Hospital, 800 Vusi Mkhwanazi Road, Cato Manor, Durban, 4058, South Africa.
E-mail address: verenagounden@yahoo.com (V. Gounden).

https://doi.org/10.1016/j.reprotox.2019.07.008
Received 5 February 2019; Received in revised form 3 July 2019; Accepted 10 July 2019
Available online 11 July 2019
0890-6238/

Paper 2

Reproductive Toxicology 100 (2021) 83–89

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

A pilot study: Relationship between Bisphenol A, Bisphenol A glucuronide and sex steroid hormone levels in cord blood in A South African population

Verena Gounden[a,*], Mohamed Zain Waraally[b], Thabo Magwai[b], Rajen Naidoo[c], Anil Chuturgoon[d]

[a] Department of Chemical Pathology, University of KwaZulu-Natal and National Health Laboratory Service, Inkosi Albert Luthuli Central Hospital, Durban, South Africa
[b] Department of Chemical Pathology, National Health Laboratory Service, Inkosi Albert Luthuli Central Hospital, Durban, South Africa
[c] Department of Occupational Health, University of KwaZulu-Natal, Durban, South Africa
[d] Department of Medical Biochemistry, University of KwaZulu-Natal, Durban, South Africa

ARTICLE INFO

Keywords:
Bisphenol-A
Liquid chromatography tandem mass spectrometry
Mother-child pairs
Cord blood
Estradiol
Testosterone

ABSTRACT

Exposure to Bisphenol A (BPA) during early development particularly in-utero has been linked to a wide range of pathology. The aim of this study was to examine the relationship of BPA and its naturally occurring metabolite BPA-glucuronide (BPA-g) with sex steroid hormone levels in South African mother-child pairs.

Third-trimester serum maternal samples and matching cord blood samples were analyzed for BPA, BPA-g and nine sex steroid hormones using liquid chromatography tandem mass spectrometry (LC-MS/MS).

Sixty maternal and child pairs were analyzed. Rank correlation demonstrated a significant positive relationship between cord blood estradiol and cord blood BPA ($p = 0.002$) and maternal BPA levels ($p = 0.02$) respectively. Cord blood testosterone from male infants showed a negative Spearman's correlation ($r = -0.5$, $p = 0.02$) with maternal BPA-g. There was no statistical difference in total testosterone levels in cord blood from male and female infants.

The findings of the current study indicate a significant relationship between some key sex steroid hormones namely testosterone, dihydrotestosterone and estradiol and fetal exposure BPA.

1. Introduction

The United States environmental protection agency has defined endocrine disrupting chemicals or endocrine disruptors (EDs) as "exogenous agents that interfere with the normal function of endogenous hormones responsible for the maintenance of homeostasis and the regulation of developmental processes. These agents act by disrupting the synthesis release, transport, metabolism binding action or elimination of natural hormones in the body" [1].

Over the past 15-20 years both the scientific literature and then in turn the lay media have reported on the possible deleterious effects of these exogenous agents on fish, wildlife, the environment and human health. The possible deleterious effects on human health associated with endocrine disruptors has ranged from effect on reproductive health in particular sperm health, their role as carcinogens, and increasingly reports on effects on other endocrine and metabolic functions, such as, thyroid disease and obesity.

Bisphenol A (BPA), an endocrine disruptor, is found in a vast array of plastic consumer products including lining of tin cans, food and water containers, medical devices and toys [1,2]. Exposure to BPA has been linked to perinatal and postnatal adverse effects on multiple tissues, including the reproductive system and neurodevelopment. Animal studies and the smaller number of human studies have indicated that exposure in the perinatal period is of particular significance [1]. BPA effects are typically attributed to its estrogenic or anti-estrogenic action, however this action can not completely account for the adverse effects of low potency BPA at the low-dose exposures that are commonly seen [1, 3]. BPA has also been reported interact with androgen, glucocorticoid and thyroid receptors [4]. Previous reports have described effects of BPA on steroid hormone levels and metabolism. In vitro studies of exposure

Abbreviations: BPA, Bisphenol-A; BPA-g, bisphenol glucuronide; E2, estradiol; TT, total testosterone; 11DOC, 11-deoxycorticosterone; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; Andro, androstenedione; 17OHP, 17-OH progesterone; DHT, dihydrotestosterone; Prog, progesterone.

* Corresponding author at: Department of Chemical Pathology, University of KwaZulu-Natal and National Health Laboratory Service, Inkosi Albert Luthuli Central Hospital, 2nd Floor Pathology Building Inkosi Albert Luthuli Central Hospital, 800 Vusi Mkhwebu Road, Cato Manor, Durban, 4091, South Africa.
E-mail address: verena.gounden@nhls.ac.za (V. Gounden).

https://doi.org/10.1016/j.reprotox.2021.01.004
Received 8 November 2020; Received in revised form 3 January 2021; Accepted 9 January 2021
Available online 13 January 2021