Identification of Potential Bisphenol A (BPA) Exposure Biomarkers in Ovarian Cancer and to Predict the Consequences of SNPs on Biomarkers of Ovarian Cancer

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by

Aeman ZAHRA

College of Health and Life Sciences

BRUNEL UNIVERSITY LONDON

Abstract

There is a growing concern to public health posed by endocrine disrupting chemicals (EDCs). EDCs have been reported to exert a diverse range of health problems, as they mimic, interfere and subsequently alter endocrine signalling pathways. EDCs are linked with deleterious effects on both male and female reproductive systems e.g. infertility, PCOS, endometriosis, precocious puberty; and spermatogenesis. EDCs are commonly found in our food and consumer products, with bisphenol A (BPA) being a common culprit. Numerous studies have confirmed that BPA has xenoestrogenic activity and can exert adverse effects in female reproductive system. Currently, a significant knowledge gap remains regarding the role of BPA at ovarian level in health and disease.

Thus, a deeper understanding of the molecular and cellular mechanisms describing the effect of BPA in ovarian cancer is urgently needed. To tackle this challenge, we analysed public data from ovarian cancer patients and studied the changes in the transcriptional landscape for genes known to have differential expression pattern upon exposure to BPA. Our results point at a small group of genes (namely GBP5, IRS2, KRT4, LINCOO707, MRPL55, RRS1 and SLC4A11) with potential predictive power for overall survival based on their expression pattern. Then I embarked on analyses on the association of these biomarkers with any phenotypes and mutations indicative of involvement in female cancers and subsequently predicted the structural and functional consequences of those SNPs using in silico tools. In this study I have demonstrated that a R831C/R804C mutation in the SLC4A11 gene is deleterious with predicting $\Delta\Delta G$ values suggestive of reduction in protein stability due to this mutation.

I have then studied the impact of BPA in normal human ovaries using Epithelial Ovarian Cells (HOSEpiC) as an experimental in vitro model. HOSEpiC cells were treated with environmentally relevant concentrations of BPA (10nM and 100nM) and differentially expressed genes (DEGs) were identifid following RNAsequencing. Among the DEGs identifid in both groups, 76 genes were found to be commonly dysregulated irrespective of the level of BPA exposure. Biological pathways associated with the exposure of the different environmental doses of BPA included oocyte meiosis, cellular senescence and transcriptional dysregulation in cancer.

Finally, during the peak of COVID pandemic in 2020, I have also contributed in an

article arguing for a potential link between BPA and the severity of COVID-19. This is due to the fact that BPA is known to promote a wide spectrum of comorbidities that can be associated with severe COVID-19. In this study, I have provided evidence of co-expression of SARS-CoV-2 cell entry mediators (e.g. ACE2, TMPRSS2) with estrogen receptors that can be targeted by BPA. Collectively all these studies provide a better insight into the detrimental role of BPA in human reproduction and its involvement in the severity of other diseases (e.g., COVID-19). My data provides the basis for further research using more clinically-relevant models to study ovarian function and also lead to potentially new guidelines for reducing EDC exposure in high COVID-19 risk groups.

List of Abbreviations

CTC	**C**irculating **T**umor **C**ell
BPA	**B**is **P**henol-**A**
BPF	**B**is **P**henol-**F**
BPS	**B**is **P**henol-**S**
DDT	**D**ichloro**D**iphenyl**T**richloroethane
DES	**D**iethol**s**tilbestrol
E1	**E**strone
E2	**E**stradiol
E3	**E**striol
E4	**E**stretrol
ERs	**E**strogen **R**eceptors
EDC	**E**ndocrine **D**isrupting **C**hemicals
EGFR	**E**pidermal **G**rowth **F**actor **R**eceptor
FSH	**F**ollicle-**S**timulating **H**ormone
GnRH	**G**onadotropin **R**eleasing **H**ormone
GPR30	**G** **P**rotein-coupled **R**eceptors
HPOA	**H**ypothalamic-**P**ypothalamic-**O**varian-**A**xis
IHC	**I**mmuno**H**isto**C**hemistry
IVF	**I**n-**V**itro **F**ertilization
LH	**L**uteinizing **H**ormone
MAPK	**M**itogen-**A**ctivated **P**rotein **K**inase
MMP	**M**atrix **M**etallo**P**roteinases
OC	**O**varian **C**ancer
OS	**O**verall **S**urvival **R**ate
PCB	**P**oly**C**hlorinated**B**iphenyls
PCOS	**P**oly**c**sytic **O**varies **S**yndrome
PI3K	**P**hosphat**i**dylinositol **3**-**K**inase
ROVAR	**R**isk of **O**varian **C**ancer **R**elapse
TCDD	2,3,7,8-**t**etra**c**hloro**d**ibenzo-p-**d**ioxin
TCS	**T**riclosan
TF	**T**ranscription **F**actor
VEGF	**V**ascular **E**ndothelial **G**rowth **F**actor
WHO	**W**orld **H**ealth **O**rganization

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Chapter 1

Introduction

Endocrine disrupting chemicals (EDCs) are widespread in the environment e.g. in manufacturing and packaging materials, can accumulate all throughout the food chain, with the potential of disturbing the endocrine system of humans. They are lipophilic chemicals and has inability to be metabolized by the body [3]. EDCs are present in the form of compounds and may work additively or cumulatively, they barely found alone in nature, leading to an additive effect if they work at the same target [4]. According to the world health organization (WHO) nearly 800 chemicals are now known to have the probability of interfering with hormone receptors and causes disruption or conversion of hormones. Most of these chemicals have not been appropriately investigated [5]. In a recent report to the WHO it was emphasised that "there are many gaps in the available chemical test methods for screening chemicals for endocrine disrupting effects on female reproduction" (data sourced from [5]). Therefore, it is important to understand how EDCs exert their effects in reproductive organs both in diseased and healthy human body.

Ovarian cancer (OC) is the sixth most common cancer among females in the UK, accounting for 4% of all new cases of cancer. Every year over 7,500 women are diagnosed with ovarian cancer and It is projected that 10,501 new cases will be diagnosed in the UK in 2035 [6][7]. The total amount of OC costs in the NHS is £460 million per year, with the cost per patient being £65,740 [8]. Oestrogen plays the important role in growth, development, invasion and metastasis of OC [9], and is also responsible for the development and regulation of the female reproductive system.

Oestrogen exerts its effect by binding and activating the multiple oestrogen receptors (ERs). There have been many theories about the relative roles of ERα and ERβ in the development of ovarian cancer disease [9][10]. Recent study has shown that

numerous endocrine disrupting chemicals (EDCs) specifically Bisphenol-A (BPA) affects oestrogen signalling by interacting with two oestrogen receptors ERα and ERβ [11][12][13]. These findings show that there is a link between EDCs and ovarian cancer.

1.1 Physiological role and anatomy of the ovaries

The female reproductive system comprises of vagina, uterus, fallopian tube and two ovaries on each side of the uterus that are located in the area of the body called the pelvis (see Figure 1.1).

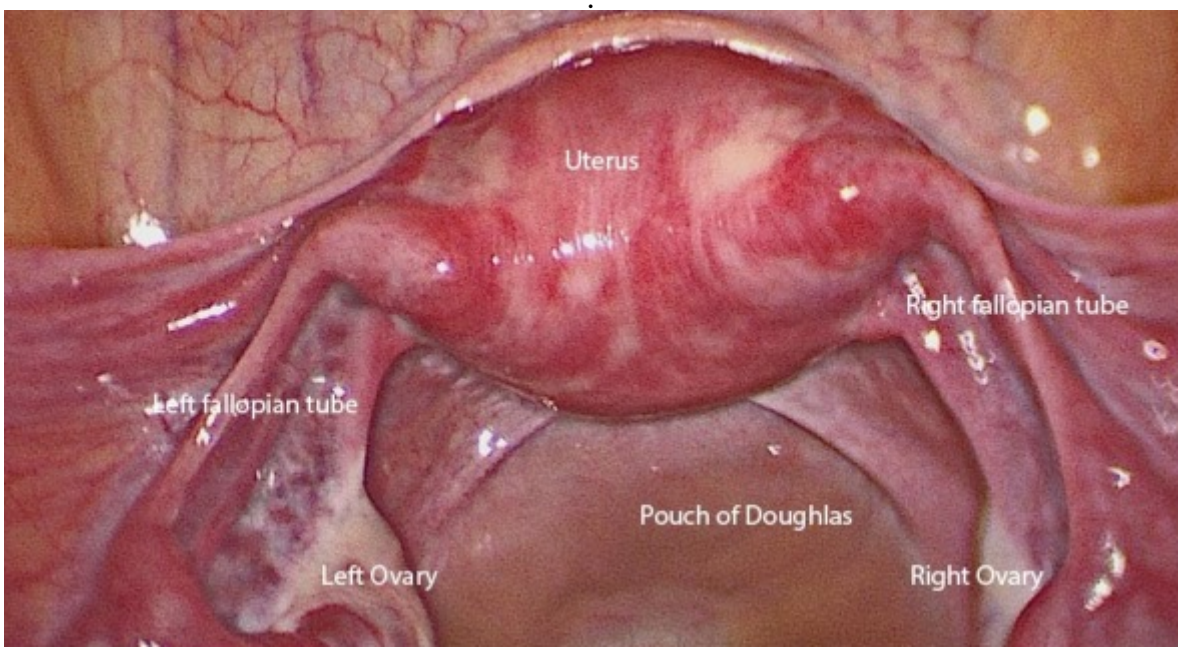

FIGURE 1.1: Laparoscopic view of normal pelvis [14].

Each ovary is attached to the uterus by its utero ovarian ligament, usually the ovaries are found lateral from the uterus, sometimes the ovaries are asymmetrical and mobile and change their position as the uterus changes its own position with the degree of urinary bladder repletion [15][16]. The ovary has an oval shape. The mean measurements of the ovary are 30/15/15 mm, and the volume is 1.8-5.7 cm [16]. The ovaries produce female sex hormones and these are oestrogen and progesterone.

These hormones help in controlling the menstrual cycle [17]. The ovarian function is regulated by a complex control system comprises of hypothalamus, pituitary and ovaries itself, and its main functions are follicular maturation, ovulation and corpus luteum formation. These organs communicate via positive and negative feedback signals, hypothalamus produces gonadotropin releasing hormone (GnRH) hormone- this induces synthesis and release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (see Figure 1.2). After binding to their specific receptors at the ovary FSH and LH helps in follicular maturation, ovulation and corpus luteum formation [18][19].

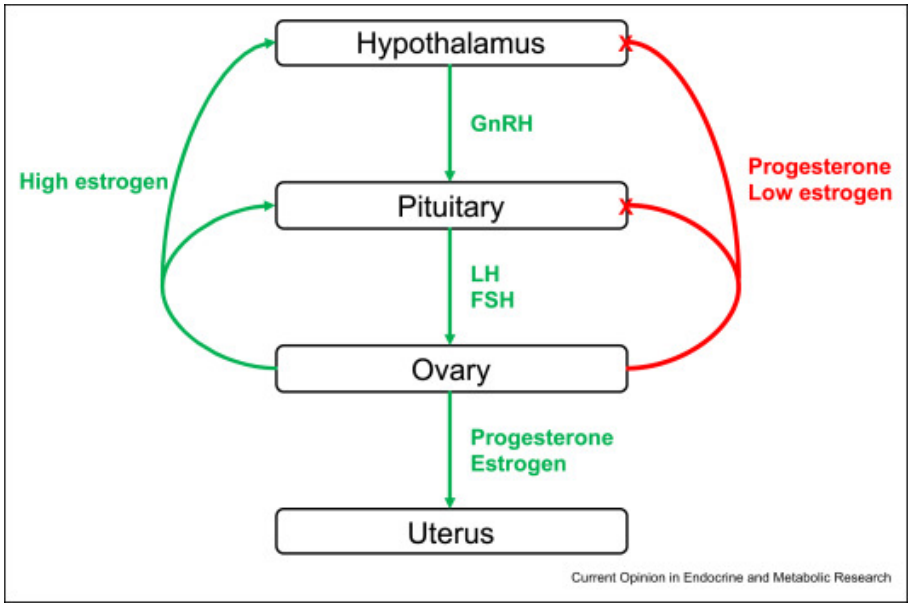

FIGURE 1.2: In females, the feedback effects will depend on the phase of the menstrual cycle. At the beginning of the cycle, increased FSH will stimulate growth and differentiation of the follicles, which are at different stages of development. As a consequence, ovarian steroid production increases; this requires both LH and FSH. At the late follicular phase, when circulating oestradiol has reached a critical concentration, the negative feedback is switched to a positive one. These effects lead to the pre-ovulatory LH surge and a smaller FSH rise. Ovulation occurs 9-12 hr after the LH surge. In the absence of fertilisation, progesterone and oestradiol levels drop. The loss of the negative feedback induces a selective rise in FSH, more follicles are recruited and a new cycle begins [20].

1.2 Ovarian Cancer

1.2.1 Incidence

Ovarian cancer is the sixth most common female cancer and its diagnostic rate is increasing day by day of about 7,495 people every year in the UK [21]. Despite a slight decrease in the number of new cases per year in the UK over the past 20 years, the gap between incidence and deaths remains unchanged, suggesting little improvement in overall survival rates [22]. Poor survival is mainly because of the late diagnosis at stages III and IV in 70% cases [23][24], where metastatic spread makes treatment options limited. The most common type of ovarian cancer is epithelial ovarian cancer as compare to the other types of ovarian cancers (e.g. germ cell ovarian cancer, stromal cell ovarian cancer and Small cell ovarian carcinoma), accounting for only 10% of cases [25] (see Figure 1.3).

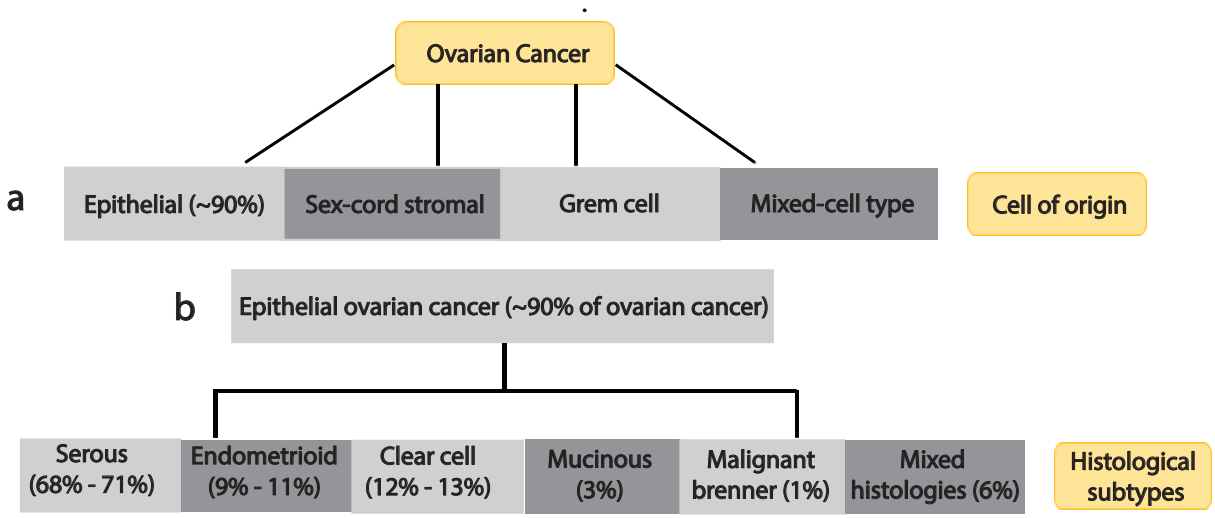

FIGURE 1.3: Histological subtypes of OC and widely accepted epithelial OC classification paradigm based on clinicopathologic and molecular evidence. Adapted from [26][27][28].

Ovarian cancer incidence increases with age as it is primarily a post-menopausal disease (see Figure 1.4).

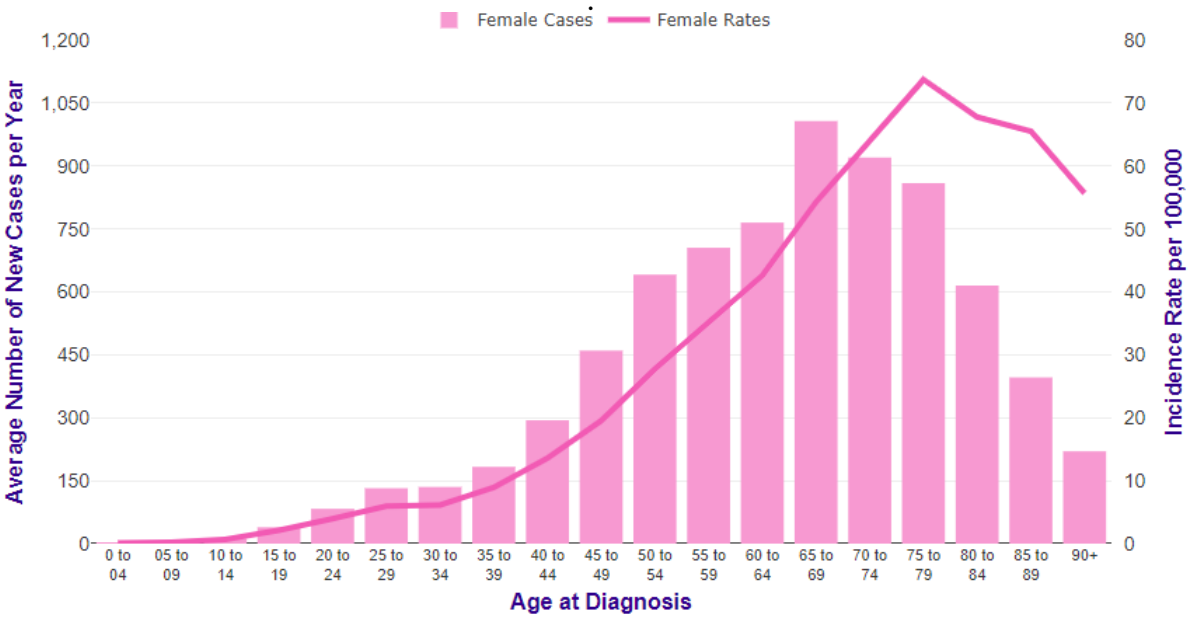

FIGURE 1.4: The average number of new ovarian cancer cases and incidence rate by age group. This also shows that ovarian cancer is on peak in post-menopausal group of women between 70 to 74 years of age (Ovarian Cancer Incidence Statistics., 2016-2018. Data extracted from CRUK [6]).

1.2.2 Classification

There are four different stages of OC (see Figures **??**, **??**, **??** and **??**) [23]. Stage I is described by the presence of the cancer inside only one or both ovaries. Stage II is characterised by the growth of cancer outside the ovary/ovaries. Stage III sees the cancer tumour grown up to the lymphatic system, and stage 4 is defined by the spread of cancer to other organs of the body. Stage IV it is a last stage of cancer [23].

Stages

The stage of a cancer is about, how far it has grown and if it has spread in the vicinity or distant organs. Clinicians use a simple 1 to 4 staging system which is called the FIGO (Federation International of Gynaecological Oncologists) system for ovarian cancer [29] (see Table 1.1).

TABLE 1.1: Ovarian cancer stages.

Stages	**Description**
Stage I	Tumour is only in the ovaries [28].
Stage IA	The cancer is completely inside one ovary.
Stage IB	The cancer is completely inside both ovaries.
Stage IC	Some tumour cells are on the surface of an ovary/ fluid taken from inside abdomen.
Stage II	Tumour cells have grown outside the ovaries [29].
Stage IIA	The tumour has grown into the fallopian tubes/womb.
Stage IIB	The tumour has grown into other tissues in the pelvis, for instance the bladder or rectum.
Stage IIC	The tumour has grown into other tissues in the pelvis and there are cancer cells in fluid taken from inside the abdomen.
Stage III	The tumour cells has spread outside the pelvis into the abdominal cavity, found in the lymph nodes in your upper abdomen, groin or behind the womb [30].
Stage IIIA	Tumour growths are found in tissue samples taken from the lining of the abdomen.
Stage IIIB	Tumour growths are found on the lining of the abdomen and their size is about 2cm or smaller.
Stage IIIC	Tumour growths bigger than 2cm and are found on the lining of the abdomen, and also it can be found in lymph nodes in the upper abdomen, groin and/or behind the womb.
Stage IV	The tumour has spread to other body organs some distance away from the ovaries such as liver or lungs [31].

In addition to the stages, patients will be given a grade that defines the level of differentiation of the tumour cells. Grades are defined as shown in Table 1.2.

TABLE 1.2: Ovarian cancer grading (CRUK [34]).

Grade	**Description**
I	Differentiated
II	Moderately Differentiated
III	Poorly Differentiated

1.2.3 Mortality Rate

Around 7,500 women are diagnosed with ovarian cancer in the UK each year [21], this makes ovarian cancer the sixth most common cause of cancer death among females in the UK, thus has high mortality rate [21]. Only one third of patients diagnosed with an ovarian cancer survive after 5 years in UK [35]. Moreover, women who are diagnosed with advanced ovarian cancer are less likely to survive in the UK than in the developing countries around the world [35]. Survival rate of the patients depends upon the different factors specially the stage at which cancer is reached after diagnosis [35].

1.2.4 Diagnosis

To increase the survival rate, early diagnosis or screening of ovarian cancer is crucial. Unfortunately, to date there is no clear and specific screening test for the most aggressive ovarian cancer. All available screening tests take a lot of time and are still ineffective. The most commonly used screening tests are trans-vaginal ultrasound and serum cancer antigen 125 (CA-125). CA-125 is a protein that is not only produced by cancer cells (not just OC) but also when patients have other non-cancer related irritants (e.g. infection, fluid, post abdominal surgery) in their abdomen making its levels a non-reliable diagnostic marker [36]. Recent study has shown that CA125 is not very specific for routine screening because even a benign condition can elevate CA125 [37]. Additionally, in early OC, 50% of the patients will have normal CA-125 levels. In fact CA125 was initially developed to monitor people who previously diagnosed with ovarian cancer; it's not a perfect method for early diagnosis of ovarian cancer [38]. Even in recurrent OC it may take 4-6 months until CA-125 levels may rise again to indicate that the cancer has come back [39]. Moreover, recent study has shown that the level of serum human epididymis protein 4 (HE4) can be a useful preoperative test for predicting the benign or malignant nature of pelvic masses, it seems to have a promising role in the prediction of clinical and surgical outcomes [40]. Therefore, HE4 seems to better predict recurrence in comparison to CA-125, but as very often happens with new biomarkers, the audit of clinical outcomes (e.g. improved survival and cost-benefit ratio) represents the major challenge.

1.2.5 Treatment

The treatment for ovarian cancer will totally depend on few factors e.g. what is the size and type of ovarian cancer, where exactly it is located, if it has spread and also depends on the general health of the patient [41]. Most common treatments for the ovarian cancer patients are chemotherapy and a surgery to remove ovaries along with lymph node if it is at stage 3 or advanced [42]. Other treatments are targeted and hormone therapy [41].

In ovarian cancer, angiogenesis has been shown to have a central role in both disease progression and prognosis. A direct relationship has been demonstrated between the expression of biomarkers for angiogenesis such as VEGF, the degree of neovascularization and the behaviour of epithelial ovarian cancers [43][44]. These data suggest that pharmacological inhibitors of angiogenesis may have the capacity to arrest tumour progression. Several phase II trials of different antiangiogenic drugs and therapies have been reported to demonstrate activity against relapsed ovarian cancer [45][46].

1.2.6 Relapse

Recurrent ovarian cancer is lethal, the status of recurrent ovarian cancer is heterogeneous but limited patients can be cured depending upon the site of recurrence [47]. Period up to first relapse differs extensively from few months to 5 years, many of these patients will receive three or more lines of chemotherapy but will ultimately become resistant to standard therapies [47].

In recent study [48], the risk of ovarian cancer relapse (ROVAR) algorithm has been designed to predict risk of relapse after first-line treatment for ovarian cancer patient by using 4 variables: stage, tumour grade, CA-125 level and posttreatment computerized tomography (CT) scan. The ROVAR score is a useful tool for follow-up support for ovarian cancer patients. However, the major limitation of ROVAR algorithm is that it has 10% chance of inaccurate prediction for the patient's risk of relapse and also it requires careful prospective validation in a large sample of ovarian cancer patients before it is fully implemented. Table 1.3 represents the percentage of primary site of recurrence in the ovarian cancer patients.

TABLE 1.3: Site distribution at first relapse of ovarian cancer [47].

Primary site recurrence	**Recurrence rate**
Abdominal cavity	33 (29.4%)
Pelvic cavity	29 (25.9%)
Vaginal stump	17 (15.2%)
Retroperitoneal lymph node	8 (7.1%)
Superficial lymph node	7 (6.3%)
Liver, spleen	7 (6.3%)
Bladder	3 (2.7%)
Bone	3 (2.7%)
Brain	2 (1.8%)
Lung	2 (1.8%)
Adrena	l 1 (0.9%)

1.2.7 Genetic and Epigenetic Events

Recurrence of OC with acquired chemoresistance is the eventual cause of mortality in the majority of patients. Therefore, the urgent investigation of the molecular events that drives the resistance to the certain therapies is required. High grade serous carcinomas is the most common type of OC and is blamed for treatment failure [47], therefore, gene expression studies have mostly focussed on this subgroup. A recent study has shown the Promoter hypermethylation and associated gene silencing of BRCA1 is the most common canonical epigenetic defect in High grade serous carcinomas [48]. At the genomic level, the most common molecular defect is the TP53 mutation in High grade serous carcinomas, the majority of these mutations are missense, frameshift, nonsense or splice junction variants [48]. High grade serous carcinomas may be sub-classified into three main groups: BRCA1 loss (genetic), BRCA1 loss (epigenetic), and no BRCA1 loss. Tumours in these groups show different molecular abnormalities/alterations involving the PI3K/AKT and p53 pathways [49]. Interruption of epigenetic regulators frequently leads to loss of transcriptional control and disruption of apoptotic and proliferation pathways and these epigenetic alterations are particularly promising targets for therapy as they are largely reversible [50]. Another study has shown that mutations in genes encoding epigenetic regulators are mutated in ovarian cancer, driving tumourigenesis and resistance to treatment. Several epigenetic modifiers have arose as promising drug targets for ovarian cancer therapy and most of them are in clinical trial phases [51].

1.3 Endocrine System

Endocrine system is composed of tissues and glands that secretes hormones for managing and regulating vital biological processes in the body e.g. function of the reproductive system, development of the brain and nervous system, balancing blood sugar level, growth and metabolism [49][50]. Major glands of the endocrine system are the ovaries, testes, pituitary, thyroid, and adrenal that produce tissue-specific hormones [50]. The word "hormone" is derived from the Greek *hormone* - meaning set in motion [51]. Hormones are the chemical messengers that travels from one to another organ via bloodstream to regulate and control the physiological activities including growth, development, metabolism, appetite, puberty, mood and fertility [52]. A schematic representation of the mechanisms of hormonal action is shown in Figure 1.5.

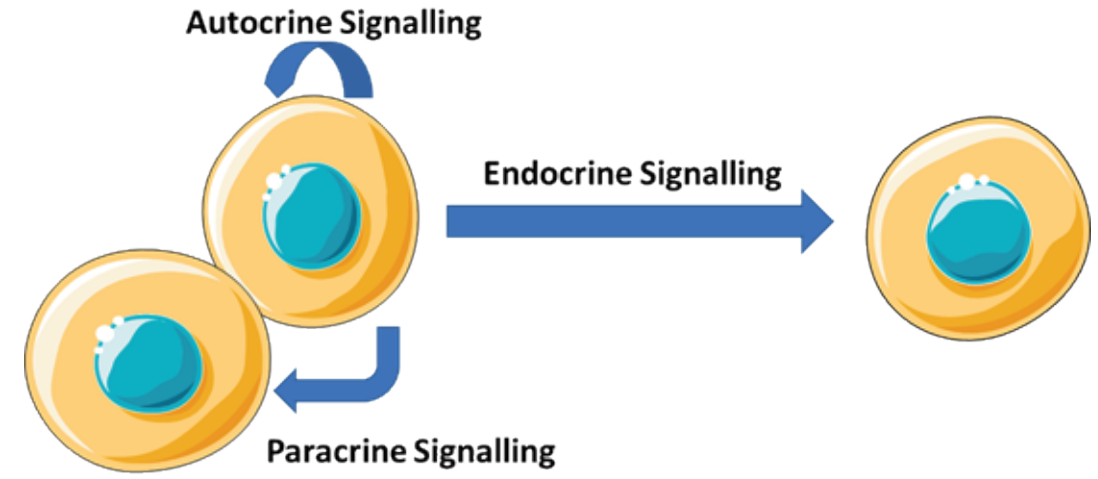

FIGURE 1.5: Mechanisms of hormonal action [52]: **Autocrine**: chemical acts on same cell. **Paracrine**: chemical released by one cell acts on neighbouring cells within a tissue. **Endocrine**: the chemical released by specialised group of cells into the circulation and acting on a distant target.

1.3.1 Endocrine Disrupting Chemicals

Endocrine disrupting chemical (EDC) is "an exogenous substance or mixture that alters function of the endocrine system and consequently causes adverse health effects

in an intact organism, or its progeny, or sub-populations" [53]. EDCs are environmental chemicals found in the manufacturing, packaging materials and can accumulate throughout the food chain, with the potential of disrupting the endocrine system of living organisms (specifically humans). EDCs are widespread in the environment, and due to long halflives that are commonly found in these lipophilic chemicals and an inability of these compounds to be metabolized by the body [54]. However, metabolised EDCs are even more toxic than the original chemical itself [55] for endocrine target organs..

1.4 Windows of Exposure to EDCs

In the late 1980s and throughout the 1990s, the idea was developed also known as "Barker Hypothesis" that adult diseases can be caused by the impairments in development happening in utero [56][57]. Early pregnancy exposure to EDCs may impact the maternal immune system, which may lead to poor infant birth weight and gestational age [58][59] as shown in the Figure 1.6. EDCs exposure in developmental windows are particularly harmful because of organogenesis and the development of tissues occur during that time, and these events are controlled by finely regulated molecular and biochemical processes [60]. Prescription of DES to pregnant women led to reproductive cancers development in daughters e.g. breast cancer, as well as problems during pregnancy or even stillbirth [61]. Finally, phthalate exposure in rodents was associated with a hormonal profile similar to PCOS later in life [62].

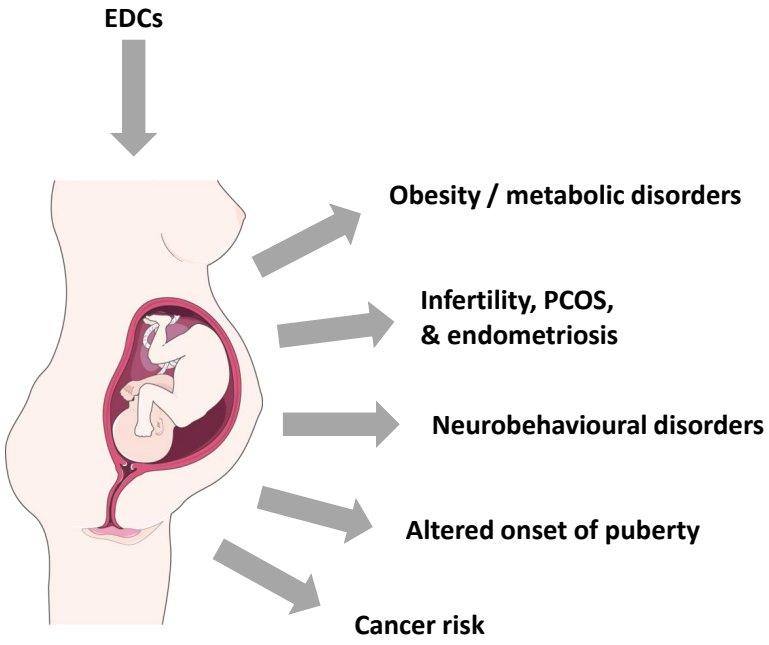

FIGURE 1.6: EDC exposure in utero can lead to diseases and developmental problems later in life.

1.4.1 Different Types of EDCs

Depending upon the structure, function and role of different EDCs they can be organized into different groups. There are thousands of different EDCs but most common are bisphenol-A (BPA), polychlorinated biphenyls (PCBs), phthalates, triclosan (TCS), dichloro-diphenyltrichloroethane (DDT), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and dietholstil-bestrol (DES) [55], shown in (Table 1.4). Research has shown that the EDCs can have toxic effects that impact human health (see Figure 1.7).

TABLE 1.4: Different types of EDCs with their sources and effects. Adapted from: [63][64][65][66][67].

EDCs	Source	Effect
BPA	Mostly polycarbonate plastics and metal cans	Memory problems, learning difficulty, anxiety, endometriosis.

DES	Effective pharmaceutical artificial oestrogen widely prescribed previously from 1938-1971 for anti-abortive agent [71][66].	Reproductive cancers, vaginal clear-cell adenocarcinoma in female offspring, genital malformations, infertility. DES exposure in early gestation is associated with an increased risk of depression in women [72].
DDT	Organochlorine insecticide [73]. It has been banned because of environmental issues, but it is still in use in some countries for malaria, head lice and as pesticide [74][75].	Unprompted movements, increased vulnerability to external stimuli. The current findings suggest that the increased serum levels of DDT is associated with the risk of breast cancer in South-eastern women of Iran [76].
PCB	Plasticizers, transformer oil/ fluids, lubricants, industrial solvents, dyes, rubbers and pesticides [68].	Disrupted hypothalamic ER distribution, memory issues & learning problems, neurological and immunological systems [67]. PCBs may increase the risk of initiating endometriosis [70].

		PCBs have been associated to a mass of pathologies in humans, ranging from developmental pathologies, endocrine system pathologies, cardiovascular diseases and disorders, immunological, neurological and reproductive effects, as well as being linked to some cancers [77].
Phthalates	Cosmetic, pesticides and frequently used as plasticizers in the manufacturing of polyvinyl chloride products [78].	Hyperactivity, low IQ and poor communication skills, altered pubertal timing in girls. A study has shown that phthalates exert an ovarian toxicity, with a focus on the effects on folliculogenesis and steroidogenesis [78].
TCDD	Highest in food contaminant, byproduct of burning fossil fuels, bleaching during paper production, preservative for wood, textiles, paint, glue, plastic production etc. [66].	Disturbed thyroid hormone action, reduced male sex behaviour. Chemical plant explosion in 1976 near Italy causes the greatest identified exposure to TCDD, women living near the site have been carefully observed and shown a modest increased risk of endometriosis and infertility [79].

TCS	TCS is an antimicrobial chemical present in toothpaste, mouthwash, hand sanitizer, and surgical soaps [69]. Antimicrobial nature of Triclosan (TCS) causes over 75% of the US population to be exposed to this chemical via consumer and personal care products [69].	Causes reproductive problems. Studies observed an association between an increase in TCS exposure and birth defects [80][69].

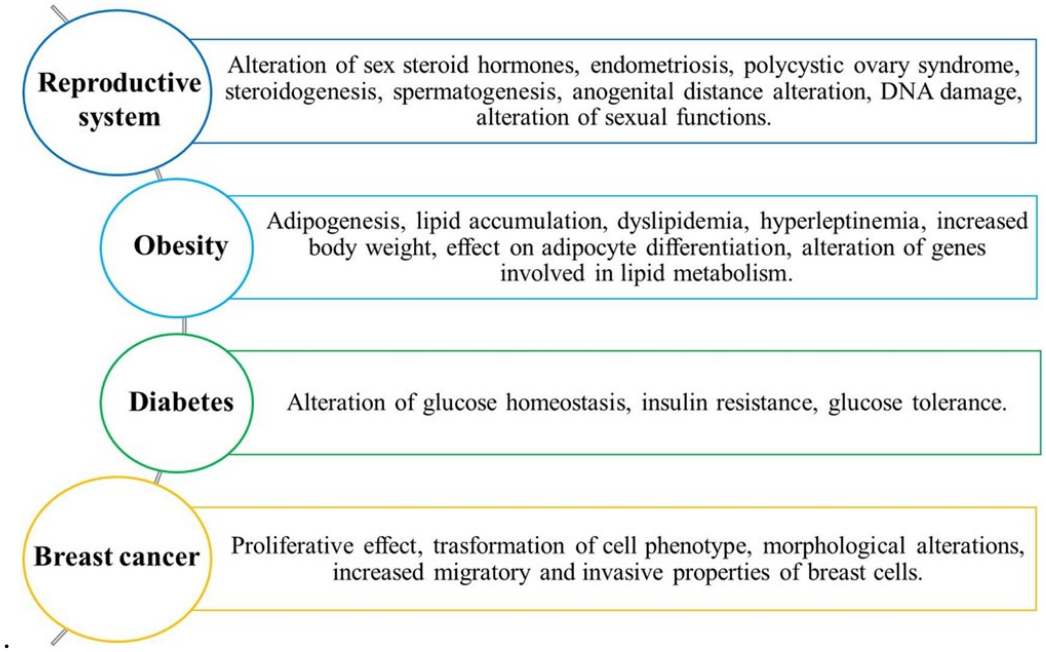

FIGURE 1.7: The effects of toxicity of EDCs in relation to human general health problems [69].

Bisphenol-A (BPA)

BPA is a compound first produced in 1891 and is one of the most developing pollutants which is commonly detected in the environment [85][86][87]. It is widely used

in a variety of products e.g. the lining of aluminium cans, plastics, and thermal receipts [88]. BPA is an EDC, it effect the oestrogen signalling by interacting with two oestrogen receptors ERα and ERβ [11].

Exposure of Bisphenol A (BPA) has been linked with severe endocrine disrupting effects in humans and wildlife. Toxicological studies suggested that BPA increases the body mass and disrupts normal cardiovascular physiology by interacting with endogenous hormones in rodents [89][53]. Previous research has shown that the BPA has significant proliferative effect on epithelial ovarian cancer cells (EOC) in-vitro [90]. Numerous experimental studies have shown the potentially detrimental effect of BPA on reproduction [91][92]. For example, BPA causes alterations in the ovary, uterus, and mammary glands, and also affects hypothalamus which controls the estrous cyclicity [93][94]. Following are few examples of BPA effects on reproductive system.

Implantation Failure, Infertility and Dysregulation of the Hypothalamic-Pituitary-Ovarian Axis (HPOA)

Polycystic Ovaries Syndrome (PCOS)

A role of BPA as an endocrine disruptor in the pathogenesis of PCOS has been recently proposed, this study has reported the high level of BPA in women suffering from PCOS as compared to the normal ovulating women [98][99]. Women with the suffering from PCOS may be more vulnerable to exposure to the BPA [100].

Uterine changes and Endometriosis

Endometriosis is the gynaecological disorder that occurs when the lining of the uterus called the endometrium, grows outside the uterine cavity, such as the fallopian tubes, ovaries or along the pelvis. It has the ability to interact with hormonal signalling specially oestrogen ER, due to this BPA may be involved in the oestrogen dependent pathologies [101][102].

When BPA was administered in a rodent model, it resulted in increasing the thickness of uterine epithelia, reduced epithelial apoptosis and downregulation of ERα in epithelial cells in adult female offspring [103].

Placentation

The placenta plays a vital role during pregnancy, it is the interface between mother and fetus, and this organ is liable for nutrient and waste exchange [104]. Continuous low doses of BPA has potential to changes the physiology of the human placenta by upregulating oestrogen receptor α, initiating an increase of cell proliferation which may result in the development of metabolic diseases [104]. BPA exposure has been associated with certain placenta-related complications e.g. preeclampsia, fetal growth restriction, miscarriage, and preterm birth [105].

1.5 Oestrogen: An Introduction

Oestrogen was first discovered in 1900, and are mainly synthesised by the ovaries, as well as by the fatty tissues and adrenal glands [106]. Mainly there are four types of oestrogens called estrone (E1), oestradiol (E2), estriol (E3) and estretrol (E4) [107]. Chemical composition of each oestrogen is similar e.g. one benzene ring, a phenolic hydroxyl group, and a ketone group in E1 or hydroxyl group in the rest as shown in the Figure 1.8 [106]. However, the term oestrogen is commonly used for oestradiol (E2), due to its physiological significance and prevalence during reproductive years and menopause [106]. All four oestrogens have different affinity but can bind to both membrane and nuclear oestrogen receptors [106]. In humans oestradiol (E2) is mainly produced by the granulosa cells of the ovarian follicles [106].

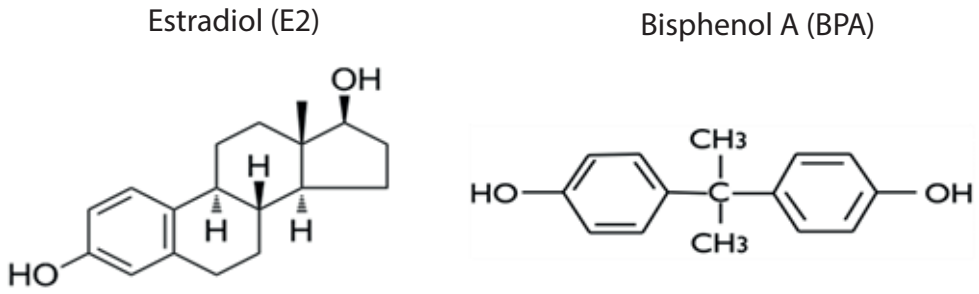

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FIGURE 1.8: Similarity between BPA and E2. Oestradiol is a type of oestrogen and is an estrane steroid with 2 hydroxyl groups. BPA is a diphenylmethane derivative with two hydroxyphenyl groups [106].

1.5.1 Oestrogen Receptor Structure

Oestrogenes exert their functions by activating their cogrnate hormone receptors. Oestrogen receptors (ERs) are comprised of distinct domains that are structurally and functionally conserved like many other nuclear receptors (NRs) [106]. These include, the DNA binding domain (DBD) which is the most conserved domain among the others, the C - terminal ligand-binding domain (LBD) and the NH2-terminal domain which is the most variable domain in sequence and length [108]. Activation function (AF) regions are located within the DBD and LBD and are responsible for regulating and recruiting the coregulatory proteins to the receptor when bound to DNA, as well as regulating the transcriptional activity of ERs [109][110]. Though each of the two ERs are coded by distinct genes which are located on different chromosomes, they have a similar affinity to E2 and binds to the same DNA elements [110][111]. ERα is composed of 595 amino acids, whereas ERβ is 530 amino acids long [112]. The structurally distinct amino terminal A/B domains share a 17% amino-acid identity between the ERs. The DNA-binding domain C region shows 97% homology. The flexible hinge D domain (36%) contains a nuclear localization signal and links the C domain to the ligand-binding domain (E) domain, which shows 56% amino-acid homology between the ERs. The carboxyl-terminal F domain shares an 18% amino-acid identity [112][109], as shown in the Figure 1.9.

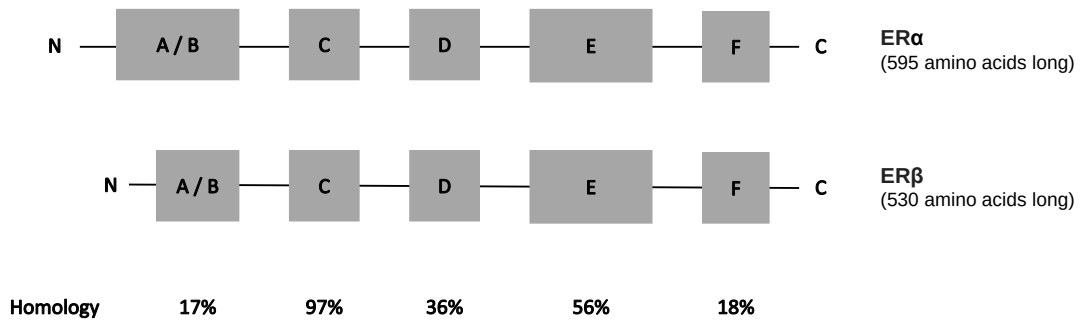

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FIGURE 1.9: Schematics of the oestrogen receptor ERα and ERβ structural regions. Domain A/B: transactivation mediation in the absence of ligand. Domain C: Binding sites of EREs. Domain D: hinge region. Domains E and F: oestrogen and oestrogenic compound binding sites [113].

1.5.2 BPA and Oestrogen Signalling

Oestrogen receptors (ERα and ERβ), can act as transcription factors upon activation with oestrogen. There is a high order of complexity of ER signalling. Upon ligand binding, ERs dimerise, translocate to the nucleus and bind to specific oestrogen response elements (ERE) on DNA promoter regions, where they can also interact with other transcription complexes, thereby influencing the transcription of genes unspecific for the binding of ligand bound ERs [110] as shown in the Figure 1.10.

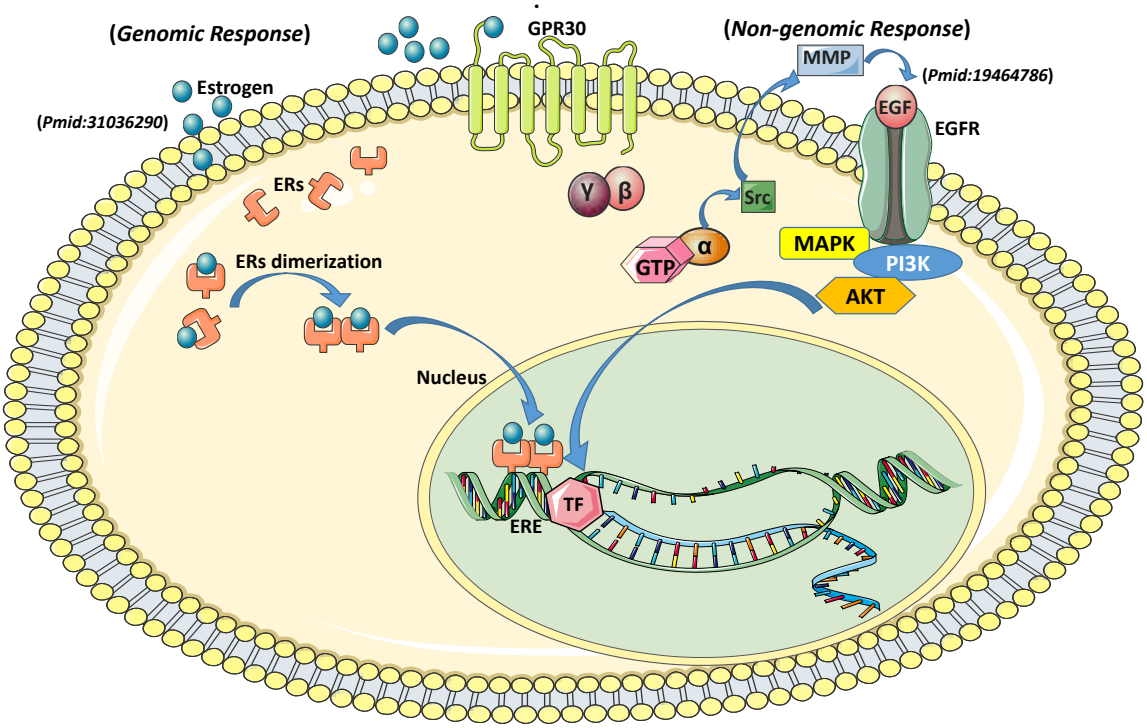

FIGURE 1.10: Schematic diagram summarising the genomic (ERs) and non-genomic (GPR30 and EGFR) oestrogen signalling. **Genomic signalling** involve migration of the dimerized ligand bound ERs to the cell nucleus, and direct interaction with chromatin at specific DNA sequences known as oestrogen response elements (EREs) [106]. **Non-genomic signalling** of oestrogen involve stimulation and activation of signal-transduction mechanisms with the consequent production of intracellular second messengers [106]. ERs represents oestrogen receptors, MAPK represents Mitogen-activated protein kinase, GPR30 represents G protein-coupled oestrogen receptor, AkT represents Protein kinase B, PI3K represents phosphatidylinositol 3-kinase, MMP represents Matrix metalloproteinases, EGFR represents epidermal growth factor receptor and TF represents transcription factor.

Furthermore, non-genomic pathways activated by oestrogens can also be mediated via the membrane-bound g-protein coupled receptor (GPR30) and epidermal growth factor receptor (EGFR) as shown in the Figure 1.10. GPR30 is a GPCR discovered in 1996 [114][115][116] and binds oestrogen with high affinity [117]. GPR30 plays a role in the physiology of the reproductive system, as well as being involved in reproductive cancers, osteoporosis, obesity, hypertension, autoimmune diseases ageing, and changes in metabolism [118].

1.6 Involvement of BPA in Hormone-Sensitive Cancers

Breast cancer is the most common cancer type among females, and the main risk factors are environmental exposures, inheritance and lifestyle [119]. *In vitro* data has suggested an association between increased incidence rate of breast cancer and BPA exposure at environmental doses of this EDC [120]. As mentioned, BPA mimics oestrogen, thus it can drive cell proliferation, migration and thereby, contributing to the hormone-sensitive cancers e.g. breast, ovary, and prostate [121]. BPA may also interact with other steroid receptors (such as androgen receptor) or the disruption of the centrosome amplification, and play a role in prostate cancer initiation [121][122].

1.6.1 BPA and Ovarian Cancer

Emerging data provides a strong link between ovarian cancer and BPA at transcriptional level, using the ER positive BG-1 ovarian adenocarcinoma cancer cell line as an experimental model [123]. Study has shown the effect of BPA on the transcriptional levels of altered genes in this study, treatment with BPA has increased the mRNA levels of responsive genes related to apoptosis, cell cycle, and signal transduction [123].

Furthermore, BPA induced cell migration by up-regulating the migration related factors metalloproteinases (MMPS) and cadherin *in vitro* [124]. Interestingly, the stimulatory effects of BPA on cell migration was eliminated by pre-treatment of the cells with inhibitors of the mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase pathways (PI3K) [124]. These result demonstrated that BPA can induce OVCAR-3 cells migration by activating MAPK and PI3K/Akt signalling pathways [124]. These data corroborated by another study showing that that BPA increased OVCAR-3 cell proliferation, by altering expression of certain genes

(e.g. CDK4, cyclin A, PCNA, E2F1, and E2F3) that were involved in apoptosis and cell cycle [125].

It is well documented that exposure to BPA in the prenatal period is associated with cystic endometrial hyperplasia, ovarian cysts, aneuploidy in oocytes and a reduction in the primordial pool of follicles in mouse ovaries, indicating an association between BPA and increased proliferation of ovarian cells mediated by the oestrogenic pathway [119][126][127].

BPA Actions on Normal Ovary

BPA has different effects on ovaries depending on the time of its exposure on this organ. Susiarjo et.al have shown [128], that pregnant mice exposed to BPA developed synaptic abnormalities e.g. partial or complete synaptic failure of a single chromosome pair, end to end associations between non-homologous chromosomes and an increased risk of aneuploidy. Similar studies suggested that the exposure to BPA causes increase in meiotic disturbances in mice, such as aneuploidy in oocytes [129][119].

Finally, BPA can exert harmful effects on ovarian function with an increased follicular depletion leading to an earlier age of menopause onset [130].

1.7 BPA and Severe COVID-19

Growing COVID-19 cases in 2020 affected mortality worldwide [131]. Data indicated that the risk of severe COVID-19 is increased by certain underlying comorbidities [132], including asthma, cancer, cardiovascular disease (CVD), hypertension, diabetes, immunosuppression and obesity [132] as shown in the Figure 1.11.

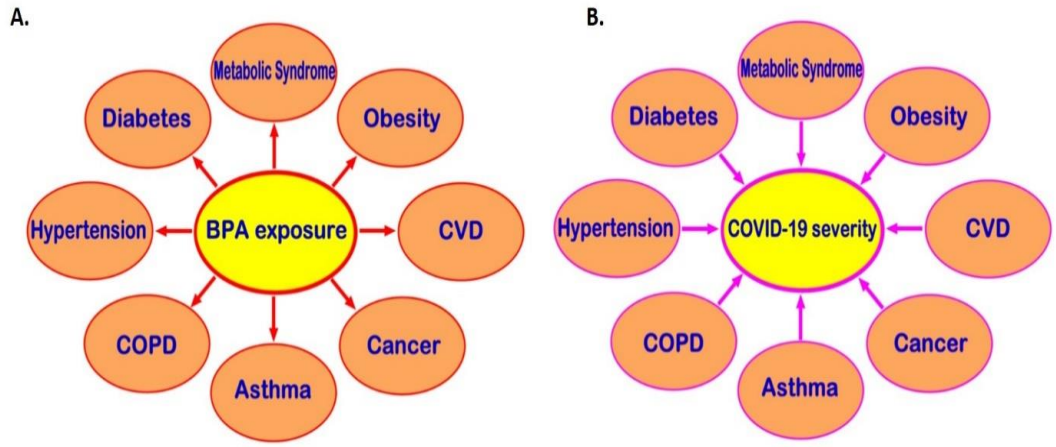

FIGURE 1.11: Exposure to BPA can promote the development of multiple diseases (A). These comorbidities incline to worse COVID-19 clinical outcomes (B). Potential links via which bisphenol A (BPA) could be indirectly increasing the risk for severe COVID-19 [135].

Particularly, exposure to hormonally active chemicals, so called, EDCs / BPA can promote such cardiovascular diseases [133] [134][135], endocrine-related [136], and cancers [137][138] etc, as shown in the Figure 1.11 and, therefore, may have association with risk of severe COVID-19 [139].

As more as COVID-19 data is becoming available and getting investigated, the number of risk factors are increasing, with a recent review demonstrating a strong link between EDCs and obesity [140], with immune function impairment [141] and have link to incline the complications observed in patients with severe COVID-19 [142].

Eventually, this can lead to new context and strategies for urgently reducing the exposure to EDCs/BPA, particularly in high risk COVID-19 groups (e.g. elderly people, as well as patients with comorbidities such as autoimmune, diabetes, hypertension, obesity and cancer).

1.8 Aims and Objectives

1.8.1 General Hypothesis

There is growing evidence that BPA, can affect male and female reproductive systems in humans and studies implicate this compound in many malignancies including cancer [143]. Although the connection between BPA exposure and some gynecological disorders is still under investigation, there is currently satisfactory evidence to prompt precautionary actions against excess exposure to BPA [144]. We hypothesise, therefore, that BPA might exert adverse effects at the ovarian level and be implicated in events leading to ovarian cancer.

In this study, we have used extensive bioinformatics/in-silico tools and databases for the transcriptional analysis, functional analysis and also to predict the proteins structural and functional analysis e.g. TCGA, GTEx, UK Biobank (Phenoscanner) and cBioPortal were used for data availability. Structural and functional consequences of alteration/SNPs on the proteins were predicted by PDB, UniProt, Phyre2, AlphaFold, Missense3D, Yasara and Pymol. RNA-seq processing pipeline was designed by using TopHat2, Bowtie2, Samtools, Cufflinks and Cuffdiff tools. Functional annotation was performed by using KEGG, CTD, Reactome, FunRich and GO consortium.

1.8.2 Aims

A recent study by Hui *et al.*, 2018 [145], has shown that BPA can have significant effects on gene expression in SKOV3 & A2780 ovarian cancer cell lines. Although the study pinpointed to the regulatory interference of EDCs like BPA in gene expression, it also opened the floor to a number of unanswered questions. This project is structured around four key research questions:

1. Make use of online databases (i.e. TCGA and GTEX) to analyse and investigate gene changes in ovarian cancer patients and healthy controls following treatment with BPA. Transcriptomic analysis (RNAseq) became available at the beginning of the project demonstrating that 94 genes can be altered *in vitro* following BPA treatment on ovarian cancer cell line [145]. We will analyse the above-mentioned 94 differentially expressed genes (DEGs) to discover the biomarkers of OC and BPA exposure-associated OC.

2. Use the UK Biobank (and the 100,000 Genome Project), cBioPortal and TCGA repositories to study the frequency and consequences of accumulating mutations/variations/SNPs on biomarkers of OC and BPA exposure-associated OC in gynecological malignancies and identify potentially deleterious alterations at protein level.
3. Use the Next Generation Sequencing; RNAseq analysis to determine the differential expression profile and finding the possible underlying mechanisms by the exposure of environmental level of BPA (10 or 100 nM) on normal Human Epithelial Ovarian Cells (HOSEpiC).
4. Investigate whether there is a connection between severe COVID-19 and BPA exposure.

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Chapter 2

Identification of Potential Bisphenol A (BPA) Exposure Biomarkers in Ovarian Cancer

Statement of Contribution

In this manuscript I led and contributed the following parts:

- Methodology
- Formal analysis
- Writing—original draft
- Writing—review and editing

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Article

Identification of Potential Bisphenol A (BPA) Exposure Biomarkers in Ovarian Cancer

Aeman Zahra [1], Qiduo Dong [1], Marcia Hall [1,2], Jeyarooban Jeyaneethi [1], Elisabete Silva [1], Emmanouil Karteris [1,*] and Cristina Sisu [1,*]

[1] Biosciences, College of Health, Medicine and Life Sciences, Brunel University London, Uxbridge UB8 3PH, UK; aeman.zahra@brunel.ac.uk (A.Z.); 1706896@brunel.ac.uk (Q.D.); marcia.hall@nhs.net (M.H.); jeyarooban.jeyaneethi@brunel.ac.uk (J.J.); elisabete.silva@brunel.ac.uk (E.S.)
[2] Mount Vernon Cancer Centre, Northwood HA6 2RN, UK
* Correspondence: emmanouil.karteris@brunel.ac.uk (E.K.); cristina.sisu@brunel.ac.uk (C.S.)

Abstract: Endocrine-disrupting chemicals (EDCs) can exert multiple deleterious effects and have been implicated in carcinogenesis. The xenoestrogen Bisphenol A (BPA) that is found in various consumer products has been involved in the dysregulation of numerous signalling pathways. In this paper, we present the analysis of a set of 94 genes that have been shown to be dysregulated in presence of BPA in ovarian cancer cell lines since we hypothesised that these genes might be of biomarker potential. This study sought to identify biomarkers of disease and biomarkers of disease-associated exposure. In silico analyses took place using gene expression data extracted from The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) databases. Differential expression was further validated at protein level using immunohistochemistry on an ovarian cancer tissue microarray. We found that 14 out of 94 genes are solely dysregulated in the presence of BPA, while the remaining 80 genes are already dysregulated (p-value < 0.05) in their expression pattern as a consequence of the disease. We also found that seven genes have prognostic power for the overall survival in OC in relation to their expression levels. Out of these seven genes, Keratin 4 (KRT4) appears to be a biomarker of exposure-associated ovarian cancer, whereas Guanylate Binding Protein 5 (GBP5), long intergenic non-protein coding RNA 707 (LINC00707) and Solute Carrier Family 4 Member 11 (SLC4A11) are biomarkers of disease. BPA can exert a plethora of effects that can be tissue- or cancer-specific. Our in silico findings generate a hypothesis around biomarkers of disease and exposure that could potentially inform regulation and policy making.

Keywords: EDC; BPA; ovarian cancer; biomarker; bioinformatics

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1. Introduction

Endocrine-disrupting chemicals (EDCs) are exogenous substances that can disturb/compromise the normal functions of the endocrine system in both humans and animals and, subsequently, increase the risk of adverse health effects [1]. EDCs are widespread in the environment and can accumulate across the entire food chain, primarily due to their long half-life and the inability of the body to metabolize them [2]. Depending on their origin, EDCs can be subclassified as industrial, agricultural, residential and pharmaceutical [2].

Bisphenol A (BPA) is an EDC that is commonly used as a monomer to manufacture polycarbonate plastics [3]. The world production of BPA is estimated to reach over 7000 thousand tons annually by the end of 2023 [4], making it one of the highest volume chemicals. Its prevalence in numerous commercial products, ranging from food packaging and food contact materials to thermal paper, and medical materials and devices means that humans are exposed to BPA on a daily basis [5]. Previous studies have shown that ingestion of contaminated foods and beverages, as well as inhalation and skin absorption, are common routes of human exposure to this chemical [6]. Environmental factors such

as heat or pH can cause leaching of BPA into its surroundings, leading to potential environmental and human exposure, as well as risks to health. Infants aged 0–6 exclusively fed with canned liquid formula and using polycarbonate bottles have been estimated to have highest BPA exposures [7]. As a result, BPA has been found to accumulate in the body with various levels being detected in the adipose tissue [8], serum [9], maternal and fetal plasma [10], breast milk [11], placenta [12] and umbilical cord [9].

At the molecular level, BPA is a xenoestrogen (i.e., it has estrogen-like activity) and therefore can interfere with the estrogen signalling pathways [5,13,14]. The estrogen signalling pathway is regulated at genomic level by estrogen receptors (ERα and ERβ) that can bind to estrogen response elements in the nucleus upon activation and modulate transcriptional responses. In addition, the G protein-coupled receptor 30 (GPR30) mediates the non-genomic signalling of estrogen [15]. GPR30 plays a key role in the physiology of the reproductive system [16,17] and metabolism [18]. In the case of BPA, it has been shown to bind to multiple ERs including ERα, ERβ (cytoplasmic and membrane-bound), GPR30 and human nuclear receptor estrogen-related receptor gamma (ERRγ) [19–26].

There is growing evidence that BPA can affect both male and female reproductive systems resulting in infertility, precocious puberty, endometriosis [27] and even many hormone-dependent malignancies such as breast and prostate cancers [14,28]. Moreover, studies [29,30] have raised the possibility of a direct link between BPA and ovarian cancer, prompting precautionary actions against excess exposure to this EDC [31].

Ovarian cancer (OC) is the sixth most common cancer among females in the UK, accounting for 4% of all new cases of cancer [32]. Every year over 7300 women are diagnosed with ovarian cancer, and it is projected that 10,501 new cases will be diagnosed in the UK in 2035 [32,33]. The rise in cases, as well as the staggering costs of treatment, highlight the need for investigating all the potentially preventable causes for this disease. Earlier studies of the effects of BPA on ovaries have indicated a time-dependent relationship. In particular, the study by Susiarjo et al. [34] on pregnant mice exposed to BPA showed synaptic abnormalities, e.g., partial or complete synaptic failure of a single chromosome pair, end-to-end associations between non-homologous chromosomes and an increased risk of aneuploidy. Treatment of an ERα- and ERβ-positive ovarian cell line with estrogen or BPA altered expression of genes involved in apoptosis, cancer and cell cycle [35]. Further studies have also implicated BPA in ovarian cancer in vitro. Using OVCAR-3, an ovarian cancer cell line, BPA exerted an estrogenic effect stimulating cell migration and up-regulation of certain metalloproteinases and N-cadherin [36]. In the same cell line, BPA increased cell proliferation and decreased activity of the caspase-3 pathway [37].

In this paper, we present the analysis of a set of 94 genes that have been shown to be dysregulated in presence of BPA in OC cell lines [30]. We looked at comparing the expression landscape in ovarian normal tissue and OC under the influence of BPA. We found that 14 out of 94 genes are solely dysregulated in the presence of BPA, while the remaining 80 genes are already dysregulated (p-value < 0.05) in their expression pattern, presumably as a consequence of the disease. This study sought to identify biomarkers of disease and associated exposure that could potentially inform regulation and policy making.

2. Materials and Methods

2.1. Bioinformatics Analysis

2.1.1. Data Availability

The group of 94 genes shown to be dysregulated in the SKOV3 cell line in the presence of BPA was extracted from the published paper by Hui et al., 2018 [30]. SKOV3 cell line is a commonly used cellular model of high-grade serous ovarian cancer (HGSOC). The 94 genes were annotated using information regarding their genomic location, gene name, biotype and Ensembl ID from GeneCards/Ensembl v96.

Gene expression data and sample phenotype information (Table 1) were extracted from the data generated by The Cancer Genome Atlas (TCGA) research network (https://www.cancer.gov/tcga, last accessed on 20 November 2020) and the Genotype-Tissue

Expression (GTEx) project (https://www.gtexportal.org, last accessed on 20 November 2020) as published in the Xena repository hosted at the University of California Santa Cruz (UCSC) [38]. Specifically, we analysed data from the TCGA-TARGET-GTEX pan-dataset normalised cohort. The raw RNAseq data from TCGA and GTEx were processed and normalised by the UCSC using the TOIL pipeline, a computation framework that facilitates the quantification of gene expression as well as cross-dataset comparison without any computational batch effects [39]. The gene expression values are presented in units of $\log_2$(norm_count+ 1). In terms of histological grades, the National Cancer Institute grading system (National Institute of Health, Bethesda, Maryland, USA) was used (i.e., G1–G4) [40].

Table 1. Data summary for the normal ovarian tissue and ovarian cancer samples from TCGA and GTEx datasets. NOS: not otherwise specified; NA: not applicable; FNA: fine-needle aspiration; GB: grade borderline; GX: grade cannot be assessed.

Phenotype	TCGA	GTEx
Total Samples	427	88
Normal tissue	-	88 (100%)
Primary tumour	419 (98.13%)	-
Recurrent tumour	8 (1.87%)	-
Category		
Normal ovary	-	
Ovarian serous	427 (100%)	88 (100%)
Cystadenocarcinoma		NA
Primary diagnosis		NA
Serous cystadenocarcinoma, NOS	422 (98.83%)	
Papillary serous cystadenocarcinoma	4 (0.94%)	
Cystadenocarcinoma, NOS	1 (0.23%)	
Clinical stage		
Stage I	1 (0.23%)	
Stage II	26 (6.09%)	NA
Stage III	334 (78.22%)	
Stage IV	63 (14.75%)	
Overall survival (days)	Min 8 Max 5481	NA
Age range (years)	30–87	20–69
Age < 50	103 (24.12%)	39 (44.31%)
Age > 50	324 (75.88%)	49(55.68%)
Mortality		NA
Living	162 (37.94%)	
Deceased	265 (62.06%)	
Initial Diagnosis Methods		NA
Cytology (e.g., pleural fluid)	54 (12.65%)	
Excisional biopsy	5 (1.17%)	
FNA biopsy	9 (2.11%)	
Incisional biopsy	6 (1.41%)	
Tumour resection	347 (81.26%)	
Unspecified method	6 (1.41%)	
Neoplasm Histologic Grade		NA
G1	1 (0.23%)	
G2	52 (12.18%)	
G3	363 (85.01%)	
G4	1 (0.23%)	
GB	2 (0.47%)	
GX	6 (1.41%)	
Unspecified grade	2 (0.47%)	

2.1.2. Functional Analysis

The genes were functionally characterised using Gene Ontology (GO) database [41] as recorded in FunRich (version 3.1.3) software [42]. Seventy-seven (protein-coding genes) of the ninety-four analysed genes were matched in the FunRich, with the remainder 17 having no associated data. The enrichment of the GO terms related to biological processes, biological pathways, molecular functions and expression sites was computed. A threshold *p*-value of 0.05 was used to ascertain the statistical significance of the results.

2.1.3. Immunohistochemistry (IHC)

Immunohistochemistry was used to measure the gene expression at the protein level in tissue samples from ovarian cancer patients (all patient information is given in the Supplementary Table S1). Commercially available ovarian carcinoma tissue arrays, containing 90 cases of ovarian tumour with 10 adjacent normal ovary tissues, single core per case (Biomax, Derwood, MD, USA), were used to examine the expression of SLC4A11 and RARRES3. All tissues were collected under the highest ethical standards with the donor being informed completely and with their consent. Moreover, all human tissues were collected under Health Insurance Portability and Accountability Act (HIPAA) approved protocols. The slides were deparaffinized following a series of washes in Histo-Clear (National Diagnostics) and decreasing concentrations of ethanol. Slides were subsequently boiled in sodium citrate (Merck Life Science UK Ltd, Gillingham, UK) for 20 min using a microwave and cooled down using running tap water for 10 min. The slides were washed twice in phosphate-buffered saline (PBS) with 0.025% *v/v* Triton-X 100 (PBS-T) for 5 min each and further incubated with 3% *v/v* hydrogen peroxide in PBS for 15 min before 3 more washes in PBS-T. The slides were blocked using 5% BSA in PBS for 1 h within a humidity chamber (HC) at room temperature before the addition of primary antibodies to each slide: SLC4A11 (HPA018120—Merck Life Science UK Ltd, Gillingham, UK) and RARRES3 (HPA011219— Merck Life Science UK Ltd, Gillingham, UK) (1:100 dilution in 5% BSA in PBS)—and incubated overnight at 4 °C in the HC. After incubation, the slides were washed 3 times for 5 min each with PBS-T before the addition of anti-rabbit secondary (Zytochem Plus kit), 2BSCIENTIFIC Ltd, Upper Heyford, UK and left to incubate for 1 h at room temperature in the HC. The washes were repeated, and the slides were further incubated with streptavidin–HRP conjugate (Zytochem Plus kit) for 30 min in HC at room temperature. DAB (3,3′-diaminobenzidine) substrate solution (Vector Laboratories, Burlingame, CA, USA) containing hydrogen peroxide was loaded on the slides for 10 min after 3 washes with PBS-T. Slides were washed in H_2O for 5 min and then incubated with Harris' haematoxylin for 30 s followed by 0.1% *w/v* sodium bicarbonate for 60 s before dehydration in increasing ethanol concentrations and Histo-Clear. Images of the stained cores were captured using an EOS 1200D camera attached to a light microscope. The images were then analysed under a light microscope giving a score based on how well the cores on the slide were stained (0 = <10% stained, 1 = 10–25% stained, 2 = 25–50% stained, 3 = 50–75% stained and 4 = >75% stained). This was repeated 3 times, and an average was calculated based on the scores for each core.

2.1.4. Statistical Analysis

All data processing and statistical analyses were conducted using R (v. 3.5.2, The R Foundation for Statistical Computing, Vienna, Austria) under R Studio desktop application (version 1.1.463, RStudio, Boston, Massachusetts, USA). Student *t*-test was used to test the statistical significance in the change in expression between two given states (e.g., normal vs. tumour) with a significance threshold set at a *p*-value lower than 0.05. *t*-test was selected as the primary statistics test for normally distributed data. The Kaplan–Meier estimator was used to calculate and analyse the survival of ovarian cancer patients over time in regard to the stage of cancer or expression of genes. Survival analysis was conducted using R library "survminer". The Pearson correlation coefficient was calculated to estimate the

correlation between genes based on their expression pattern in both normal and cancerous ovary tissue.

3. Results

3.1. Transcriptional and Functional Characterisation

In order to gain a better understanding of the importance and magnitude of the differential expression pattern previously observed for 94 genes in the ovarian cancer cell line SKOV3 under exposure to BPA [30], we set out to analyse the transcriptional landscape of these genes in normal and cancerous ovarian tissues leveraging expression data from unmatched samples from TCGA and GTEx. We computed the *p*-value as a measure of statistical significance for the difference in gene expression levels in three cases: normal vs. primary tumour, normal vs. recurrent tumour and primary vs. recurrent tumour. We selected two thresholds, *p*-value < 0.05 and *p*-value < 0.00005 indicating significant and, respectively, highly significant change in expression, and further differentiated the genes based on whether they were up- or down-regulated. Using these criteria, we were able to distinguish seven gene groups (Figure 1).

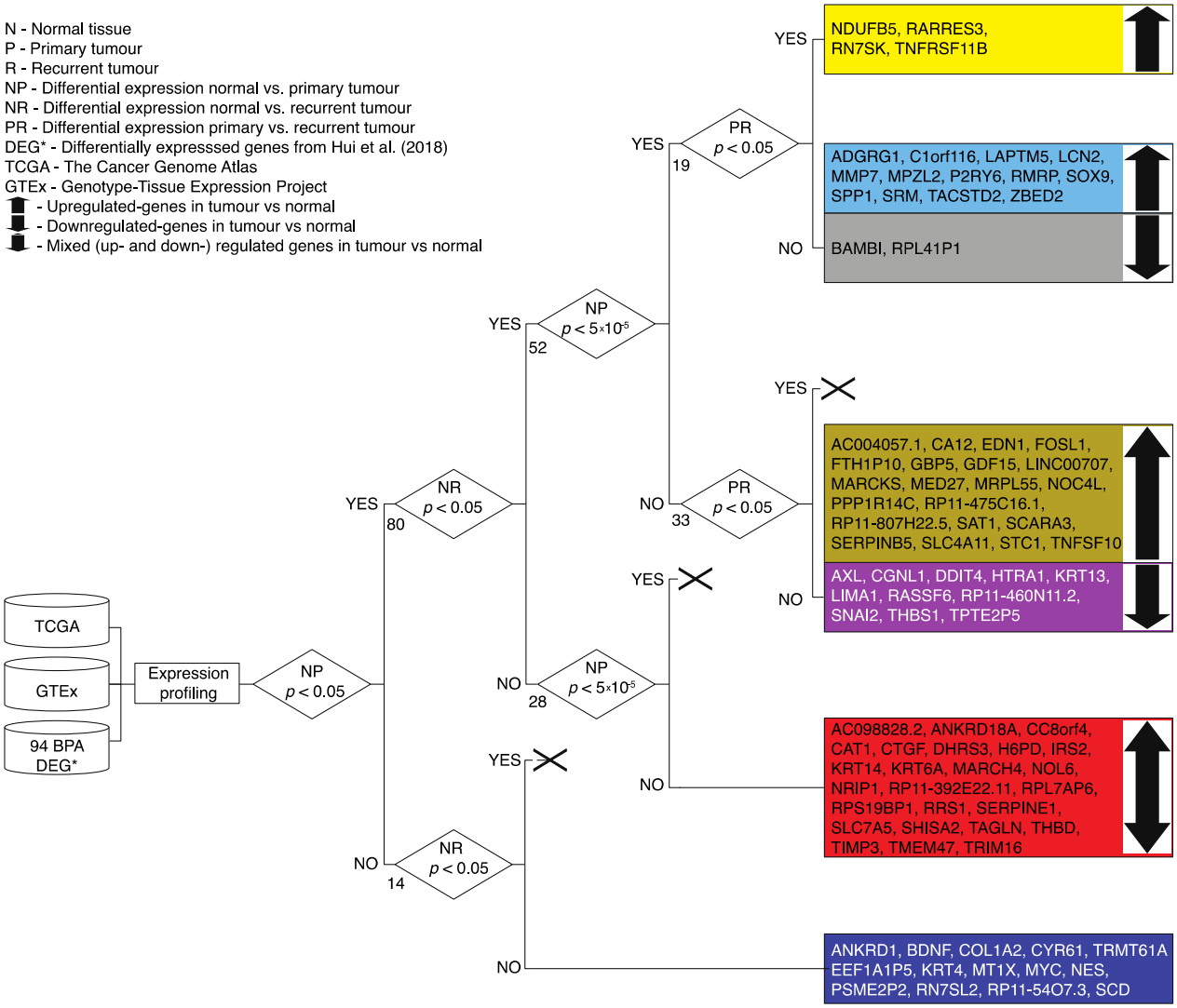


Figure 1. Workflow diagram presenting the data availability, expression analysis and gene selection criteria used in this project. Big black cross represents no gene passing the given condition. The numbers next to the "YES" and "NO" branches indicate the number of genes associated with that condition.

Overall, we found 14 genes that show no significant change in expression in tumour samples as compared to controls, hinting that the earlier reported effect of the BPA in ovarian cancer cell line can potentially be regarded as a key driver for some of the associated phenotypical changes (see Figure 1 navy block). At the other end of the spectrum, we identified four genes (yellow block), namely: RNA Component Of 7SK Nuclear Ribonucleoprotein (*RN7SK*), tumour necrosis factor receptor superfamily member 11B (*TNFRSF11B)*, NADH dehydrogenase 1 beta subcomplex 5 (*NDUFB5*) and the retinoic acid receptor responder protein 3 (*RARRES3*). Unsurprisingly, these genes have been previously associated with various malignancies [43–47] including breast and ovarian cancers. The remainder 76 genes were stratified into five groups based on the level of significance in the change of their expression patterns. Thirteen genes (light blue block) were significantly ($p < 0.05$) up-regulated in tumour compared to healthy ovarian tissue. Twenty-two genes (yellow-brown block) were found up-regulated with moderate significant difference ($p < 0.05$) compared to controls. Thirteen genes (grey and purple blocks) were down-regulated in cancer with moderate significant difference. While in the remaining 28 genes in the red block no overall trend was observed, they have statistically high significant difference in primary tumour vs. healthy tissue.

Next, we looked at functional terms enrichment in the groups of genes that show no change in their transcriptional landscape in cancer (14 genes) as compared to those that do (80 genes). The results are shown in Figure 2.

Gene Ontology analysis results show that majority of genes dysregulated in cancer are enriched in expression sites associated with the female reproductive system. Specifically, the majority of these genes are expressed in ovarian cancer, cervical cancer and normal ovarian tissue, while a small number of genes, namely high-temperature requirement factor A1 (*HTRA1*) and carbonic anhydrase 12 (*CA12*), are highly enriched in the germ cell layer and uterine epithelium. Earlier studies have shown a down-regulation of *HTRA1* in ovarian carcinoma [48] and an up-regulation of the *CA12* gene in breast carcinoma [49]. Cellular components ontology terms enrichment analysis showed that the majority of genes are associated with the cytoplasm and nucleus. Two genes Collagen type III alpha 1 chain (*COL3A1)* and metallothionein 2A (*MT2A*) show a significant fold enrichment in collagen type III and nuclei, respectively. *COL3A1* has been associated with gastric cancer diagnosis, prognosis and therapy [50]. At biological processes level, we see that the majority of genes are involved in signal transduction and cell communication. Significant fold enrichment was observed for transgelin (*TAGLN*) and myelin protein zero-like 2 (*MPZL2*) in relation to organogenesis and muscle development. *MPZL2* has been observed in cell growth, invasion and adhesion of breast cancer cells [51]. Finally, 18 genes, namely *MMP7*, *SPP1*, *SERPINB5*, *FOSL1*, *GDF15*, *EDN1*, *BAMBI*, *DDIT4*, *SNAI2*, *LIMA1*, *KRT14*, *CTGF*, *MT2A*, *NRIP1*, *THBD*, *IRS2*, *SERPINE1* and *TAGLN* are associated with the mTOR signalling and plasma membrane estrogen receptor signalling pathways.

Functional enrichment analysis of the 14 remaining genes revealed that expression sites are enriched for female reproductive systems. Specifically, the majority of these genes (60%) are expressed in the vagina and ovarian cancer, while a small fraction (10–20%) is enriched in terms related to umbilical cord and ovarian follicles. Biological processes terms enrichment analysis showed a third of the genes, namely *COL1A2*, *KRT4*, *NES*, *MYC*, *TRMT61A* and *ANKRD1*, is enriched in cell growth and regulation of nucleobase. From the biological pathway terms enrichment analysis, we observed that the majority of the genes (66.67%), namely *MYC*, *COL1A2*, *CYR61* and *BDNF* are associated with the mTOR pathway and plasma membrane estrogen receptor signalling.

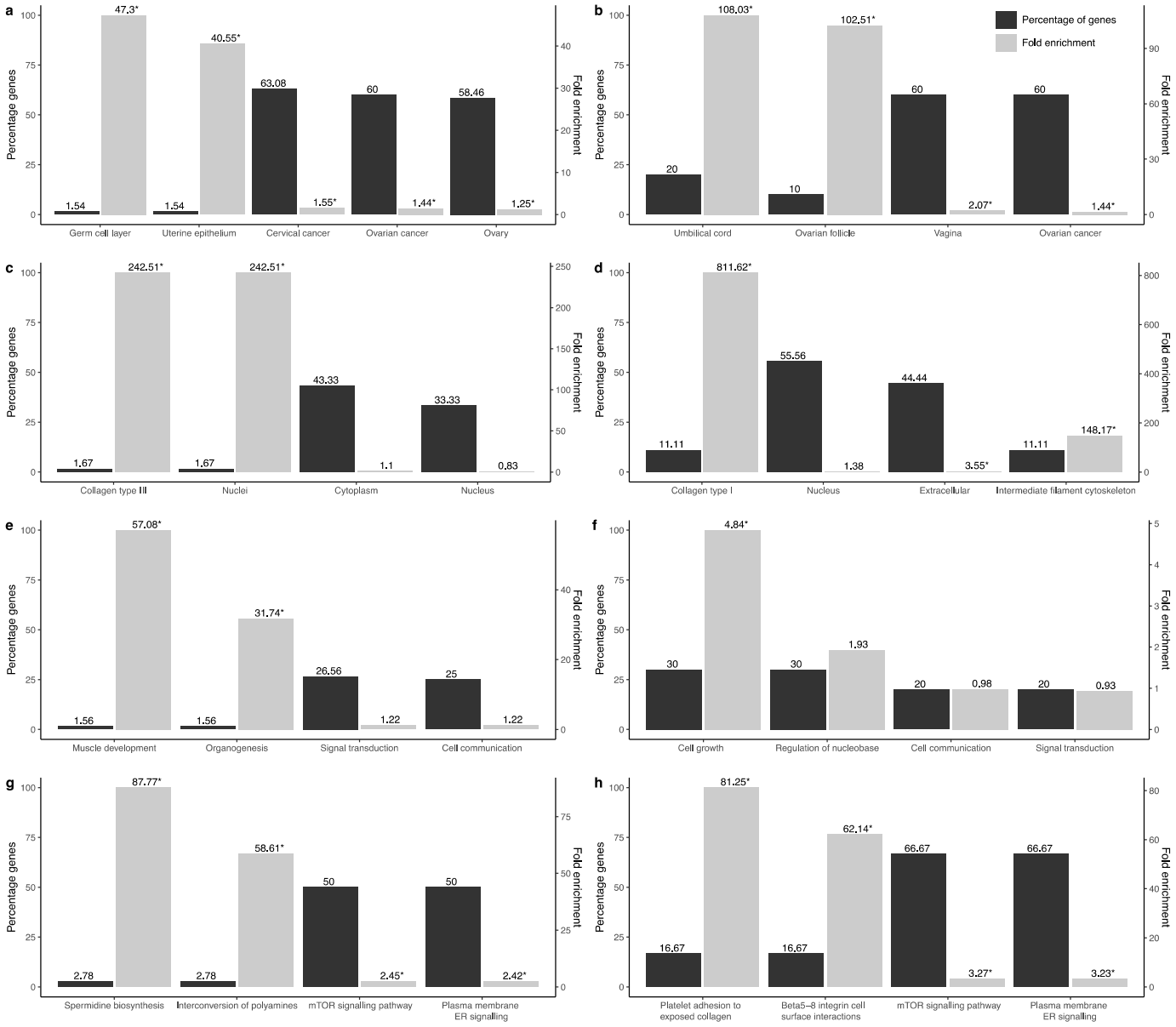


Figure 2. The functional enrichment in Gene Ontology terms in 80 genes (**a**,**c**,**e**,**g**) and 14 genes (**b**,**d**,**f**,**h**) in relation to site of expression (**a**,**b**), cellular components (**c**,**d**), biological processes (**e**,**f**) and biological pathways (**g**,**h**). * p-value < 0.05. Mirror figures highlighting the fold enrichment and the gene percentage separately are available in Figures S1 and S2. The genes associated with all the shown phenotypes are given in Table S2.

As the GO terms enrichment analysis suggested a couple of major trends, we investigated whether the similarities between genes are preserved at expression level. To this end, we computed the Pearson correlation coefficient for all possible gene pairs using their expression profiles in normal and tumour samples (Figure S3). Overall, we observed a weaker correlation in healthy tissue compared to cancer, suggesting a pervasive expression pattern in tumour mainly driven by the disease state.

We expanded further the functional analysis by leveraging data on biological pathways from the Kyoto Encyclopedia of Genes and Genome (KEGG), Comparative Toxicogenomics Database (CTD), and Reactome biological data repositories (Figure 3). We found that the 94 genes are mainly involved in pathways associated with human diseases, in particular cancer (Figure 3a) and various infectious diseases (viral, bacterial and parasitic), and environmental information processing (Figure 3b). Furthermore, 388 pathways have been previously described in literature as being impacted by BPA exposure (see Figure 3c).

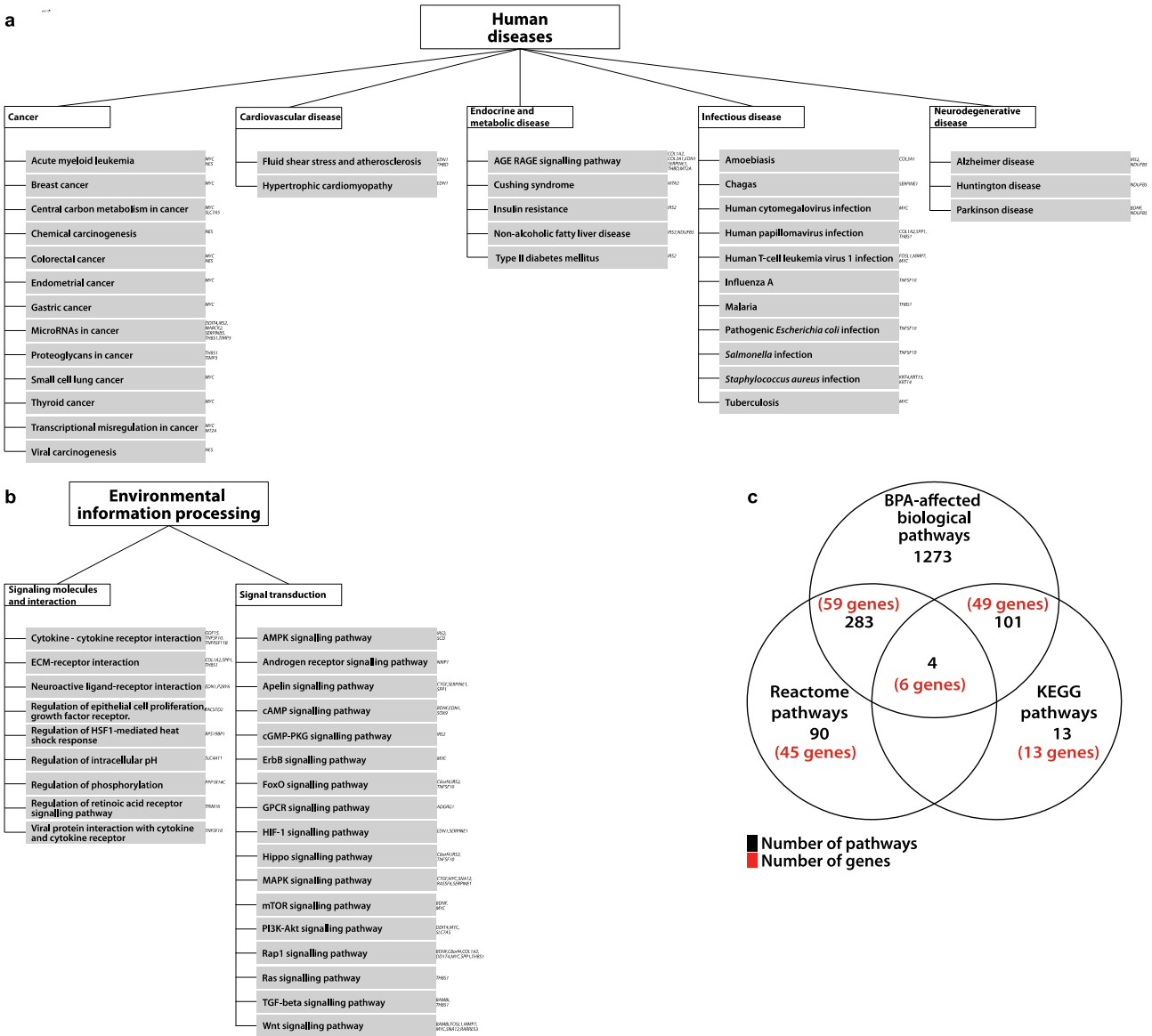


Figure 3. Biological pathways associated with 94 BPA dysregulated genes in humans. (**a**) Human-disease-associated pathways. (**b**) Environmental information processing pathways. (**c**) Venn diagram showing the common pathways in KEGG and Reactome and their intersection with BPA-impacted pathways reported in CTD. Genes that are affecting each pathway are shown on the left corner of each block.

3.2. Evaluation of Prognosis and Diagnosis Potential

We evaluated the biomarker potential of the 94 genes by studying the overall survival rate in ovarian cancer patients using the TCGA data in Kaplan-Meyer analysis. We started by examining the baseline survival rate for patients with ovarian cancer by age, stage and disease recurrence observations (Figure S4). As expected, these phenotypes indicated that patients diagnosed at an earlier stage or younger age had a better overall prognosis. However, they provided no indication with respect to the effect of individual gene activity on the survival potential. To this end, we stratified the transcriptional profile of each gene into high and low expression levels using the mean expression value as a discriminant. Overall, we found five up-regulated genes, namely solute carrier family 4 member 11 (*SLC4A11*), guanylate binding protein 5 (*GBP5*), long intergenic non-protein coding RNA 707 (*LINC00707*), mitochondrial ribosomal protein L55 (*MRPL55*) and ribosome biogenesis regulator 1 homolog (*RRS1*), and two down-regulated genes in ovarian

cancer, insulin receptor substrate 2 (*IRS2*) and keratin 4 (*KRT4*) that show a statistically significant predictive power for the patient outcome (Figure 4). The seven genes, with the exception of *KRT4*, also show a statistically significant change in expression between the normal and primary tumour samples.

In summary, Kaplan-Meyer analysis showed that four genes (*GBP5*, *LINC00707*, *MRPL55*, *RRS1*) are associated with a positive patient outcome when over-expressed, while for the other three (*SLC4A11*, *KRT4* and *IRS2*), their up-regulation is related with a poorer prognosis. It should be noted that the above-mentioned genes are also dysregulated in other cancers, and therefore their prognostic potential might not be limited to ovarian cancer. Similar, the association of high-expression with positive patient outcome has been previously reported for *GBP5* in other cancer types such as skin [52], breast and colorectal cancer [53,54]. Pathway analysis of the five protein-coding genes from this group (Figure 5a) suggests a wide repertoire of roles. For example, GBP5 might play a role in immune responses, MRPL55 in energy production and SLC4A11 in signal transduction mechanisms. The most diverse effects on a variety of signalling pathways implicated in carcinogenesis were exhibited by IRS2. Finally, we looked at the association between the seven prognostic genes and BPA-affected pathways (Figure 5b). We found that earlier studies link four genes (*IRS2*, *KRT4*, *GBP5* and *MRPL55*) with BPA suggesting that exposure to this EDC agent can potentially affect their prognostic power.

Building on the differential expression analysis, we tested the ovarian cancer diagnostic power for the 94 gene set. To this end, we used t-distributed stochastic neighbour embedding (t-SNE) dimensionality reduction method to discriminate between the normal and tumour samples using the gene expression profiles (Figure 6).

We found that, overall, the 94 genes are an excellent collective ovarian cancer diagnosis biomarker. Given that the data are curated from the ovarian cancer genome sets from GTEx and TGCA, this diagnostic feature might be likely to be for all ovarian cancers, but further research is needed to include a wider repertoire of OC subgroups. Moreover, the seven genes with prognostic power seem to perform also very well in discriminating the healthy and cancerous samples.

Next, we investigated whether the 94 genes are able to distinguish potential risk groups in the human population. For this, we analysed the t-SNE stratification on a number of factors such as age, race and ethnicity (Figure S5). No statistically significant correlation between the gene expression pattern and the selected phenotypes was observed. Furthermore, the gene transcriptional landscape was also not correlated with the cancer stage.

We further performed a gene set enrichment analysis to evaluate the relative importance of the genes in the seven groups with respect to the differential expression pattern in tumour (primary and recurrent) compared to normal. We found that the set of 94 genes had a statically significant negative enrichment score, with the bulk of the genes (51) forming the core set of genes that account for the enrichment signal [55] (see Figure 7, Table S3). Furthermore, from the seven genes with biomarker potential, *LINC00707*, *GBP5* and *IRS2* were shown to be key contributors to the enrichment score suggesting a strong association with differential expression in ovarian cancer versus normal.

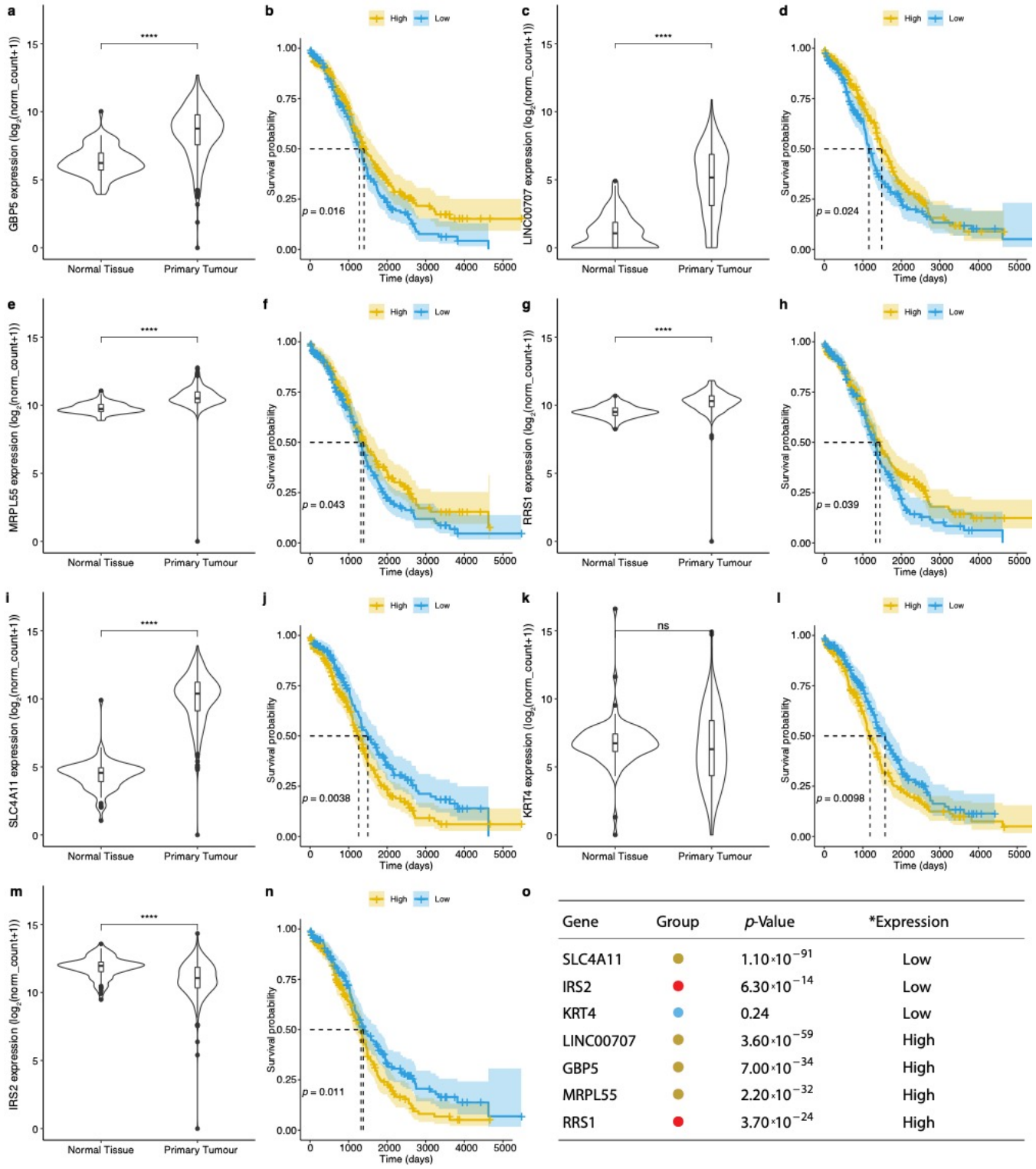


Figure 4. (**a**,**c**,**e**,**g**,**i**,**k**,**m**) Violin plots summarising the distribution of expression values of 7 genes, namely GBP5, LINC00707, MRPL55, RRS1, SLC4A11, KRT4 and IRS2, in normal, primary and recurrent tumour samples. (**b**,**d**,**f**,**h**,**j**,**l**,**n**) KM plots for the overall survival rate for samples stratified based on their expression value. *p*-value indicates the statistically significant difference between patients' survival stratification by high and low expression groups. **** indicates a significant change in expression with a *p*-value $< 5\times10^{-5}$, ns indicates that there is no significant change in the expression between the two states. Table (**o**) shows genes with a significant change in the OSR with the change in their expression. * Expression associated with higher survival rate. *p*-value indicates the statistically significant difference between OC and normal control. Coloured dots represent a group these genes belong to according to Figure 1.

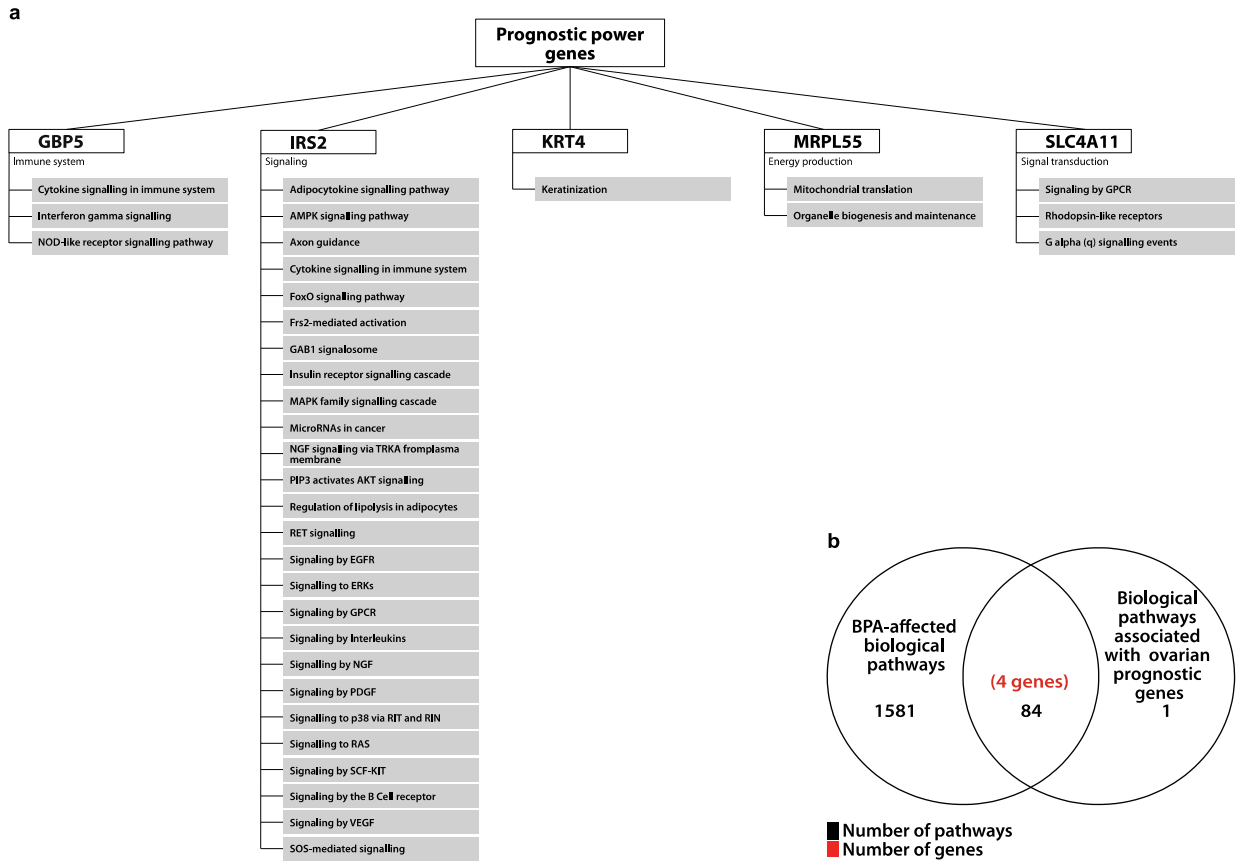


Figure 5. (**a**) represents all possible pathways affected by 5 potential predictive power genes in humans. (**b**) Venn diagram represents all possibly affected pathways upon the exposure of BPA to 7 genes.

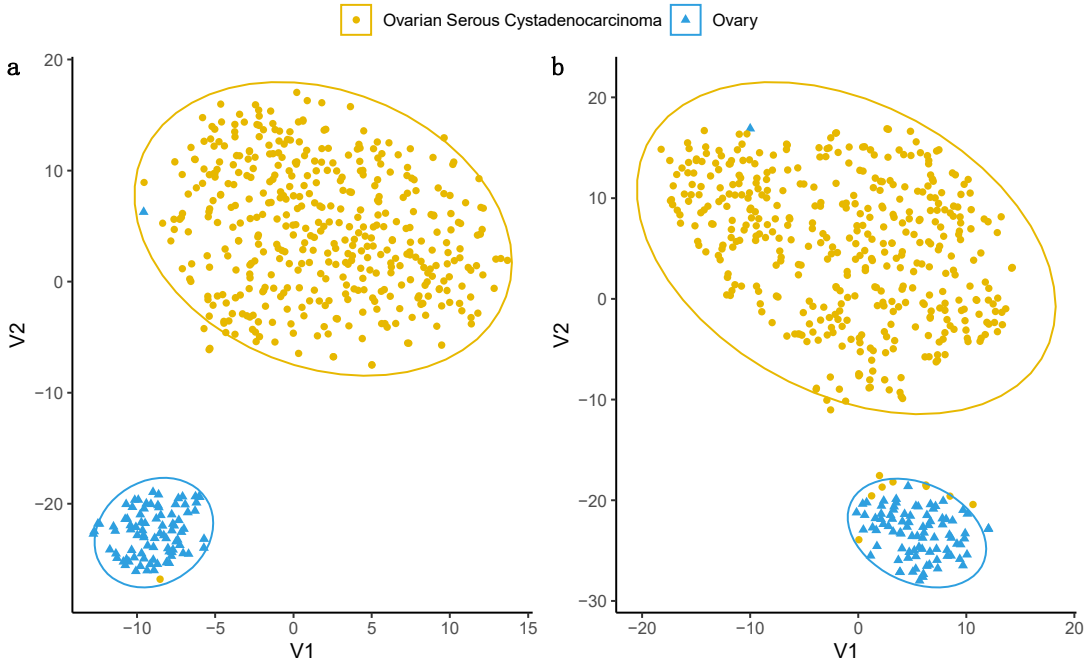


Figure 6. Tumour and normal tissue classification potential revealed by t-distributed stochastic neighbour embedding (t-SNE). Green points represent ovarian tumour samples (n = 427), and black points represent ovarian normal tissue samples (n = 88). The V1 and V2 are the t-SNE projection axis and do not have a biological meaning. (**a**) represents 94 genes' expression matrix in TCGA and GTEx embedded using t-SNE. (**b**) represents seven prognostic power gene expression matrix in TCGA and GTEx embedded using t-SNE.

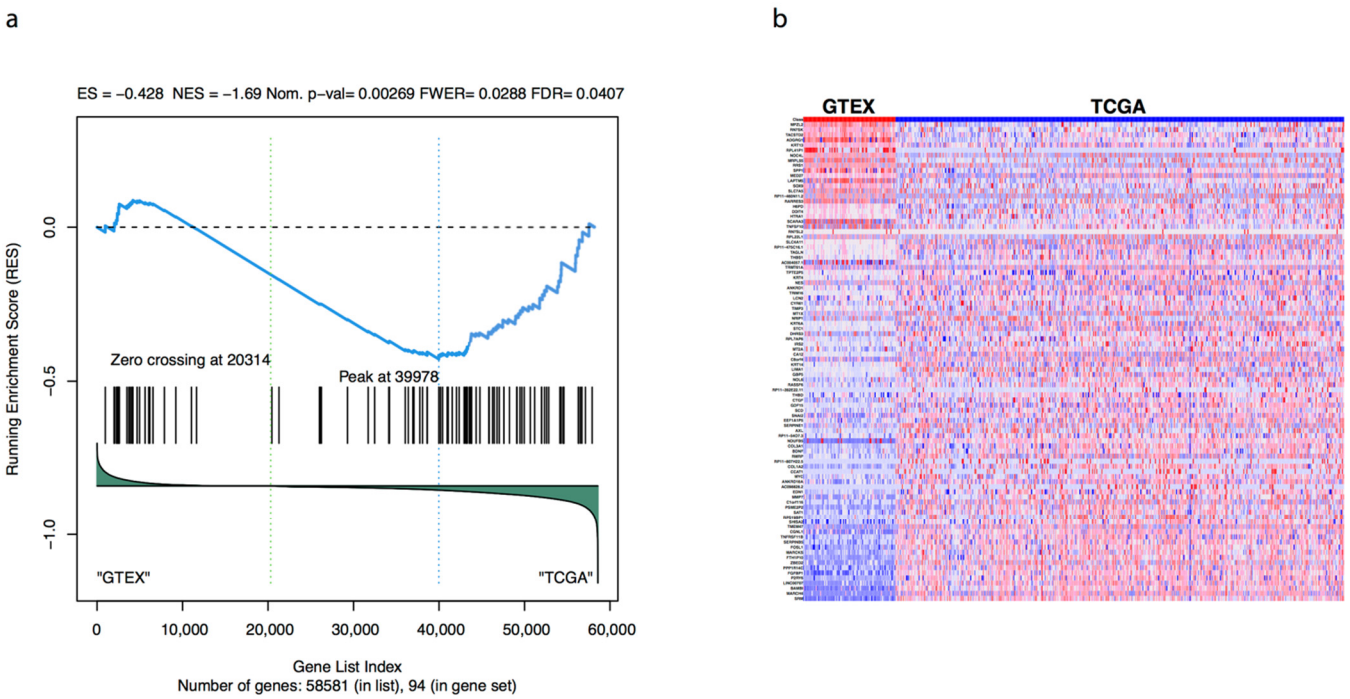


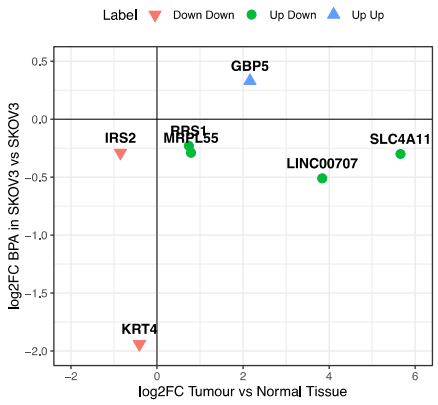
Figure 7. Gene set enrichment analysis for the 94 BPA dysregulated genes. (**a**) Running sum and relative ranks of genes against the human gene set background (58,581 genes). (**b**) Expression dataset sorted by correlation with the phenotype.

3.3. BPA Effect on Gene Function and Activity

The analysis of Hui et al. [30] showed that the environmental dose of BPA can significantly alter the expression of 94 genes in ovarian cancer cell lines. As some of these genes have diagnostic and prognostic power and can be potentially used as clinical biomarkers, it is important to evaluate the effect of low-level (10 nM) BPA exposure of the predictive characteristics. For this, we compared the observed fold change in gene expression between two states in the following two experiments: (1) normal ovarian tissue vs. ovarian cancer (data extracted from TCGA and GTEx) and (2) SKOV3 ovarian cancer cell line in presence and absence of BPA (data extracted from [29]) as shown in Figure 8.

Gene	Cancer vs. Control		SKOV3 + BPA vs. SKOV3	
	Log_2 FC *	Regulation	Regulation	Log_2 FC *
SLC4A11	5.66	Up	Down	−0.30
LINC00707	3.84	Up	Down	−0.51
GBP5	2.16	Up	Up	0.33
MRPL55	0.79	Up	Down	−0.29
RRS1	0.74	Up	Down	−0.23
KRT4	−0.41	Down	Down	−1.94
IRS2	−0.85	Down	Down	−0.29

(**a**)



(**b**)

Figure 8. Evaluation of BPA effect on genes with biomarker potential. (**a**) Table of expression changes for tumour tissue and cancer cell line experiments. * FC is the fold change ratio between the two states. (**b**) Scatter plot of the expression changes upon BPA exposure.

Overall, we found that for three genes, *GBP5*, *LINC00707* and *SLC4A11*, the BPA effect on the expression is substantially smaller compared to the effect observed as a consequence

of cancer. Moreover, their collective pattern of expression is a good discriminant between tumour and normal samples (see Figure S6). For *IRS2*, *RRS1* and *MRPL5*, we observed that the fold change in expression is comparable in cancer and under BPA treatment, suggesting that BPA presence can bias the predictive power of these genes. By contrast, we found that BPA exposure is the main driving force for the change in expression in *KRT4*, making it a potential exposure biomarker for BPA. This feature is unique to the keratin 4 among all 94 genes investigated in both its magnitude level and its statistical significance (see Figure S7).

One potential confounding factor is the lack of information regarding the BPA exposure in TCGA and GTEx samples. To address this, we investigated the potential BPA contamination in these datasets by looking at the gene expression rank, where top rank is given to the gene with the highest expression level and the lowest rank to the gene with the lowest expression level (Table S4). We worked under the premise that if a significant number of patients were exposed to BPA under similar levels as those described by Hui et al., when sorting the genes by their expression values, we would observe a similar order to that seen under the BPA influence. We found no significant correlation between the gene expression rank in presence of BPA and the tumour and normal ovarian samples from TCGA and GTEx, respectively. This result suggests that although we cannot establish with confidence whether some samples have been exposed to BPA, overall, the effects can be attributed to the specific genome biology in each case.

3.4. Ovarian Cancer Immunohistochemistry Analysis

In order to validate our in silico data and identify any changes in protein expression with respect to type or stage of the disease, we used an ovarian cancer tissue array to perform immunohistochemistry in a number of clinical samples (90 ovarian cancer patients' data and 10 normal adjacent controls). We validated the expression of *RARRES3* (in Figure 9) and *SLC4A11* (in Figure 10). These genes were selected as representatives of the highly significant up-regulated genes in the ovarian cancer and the biomarker groups, respectively.

RARRES3 was expressed in high-grade serous carcinoma, mucinous adenocarcinoma and metastatic serous carcinoma (Figure 9a–c). Statistical analysis on *RARRES3* revealed that despite the interpatient variation, OC patients expressed more *RARRES3* (p-value < 0.05) at protein level when compared to normal adjacent control tissue (NAT) as shown in (Figure 9e). We observed from Figure 9f that change in the expression of *RARRES3* is significantly up-regulated (** p-value < 0.001) in high-grade serous carcinoma compared to NAT and metastatic serous carcinoma (* p-value < 0.05). When OC patients were grouped in early stages (I and II) and late (III and IV), no apparent differences in the expression of RARRES3 protein were evident. However, RARRES3 was over-expressed in both groups compared to NAT (* p-value < 0.05) as shown in Figure 9g.

SLC4A11 was expressed in high-grade serous carcinoma, low-grade serous carcinoma, mucinous adenocarcinoma and metastatic serous carcinoma (as shown in Figure 10a–d). Here we may infer that high *SLC4A11* expression can be a potential predictor for poor overall survival in low-grade serous ovarian carcinoma. Scoring of immunostaining revealed an apparent difference in the *SLC4A11* expression compared to the normal control (Figure 10f–g), thus corroborating the gene expression reported through data analysis. We then measured *SLC4A11* expression in clinical samples of different stages: I, II, III and IV (Figure 10h). It is also evident that despite the interpatient variation, expression of *SLC4A11* is highly significant (** p-value = 0.0074) in OC patients at protein level when compared to NAT (see Figure 10f). However, no significant change was observed between different types and stages of ovarian cancer.

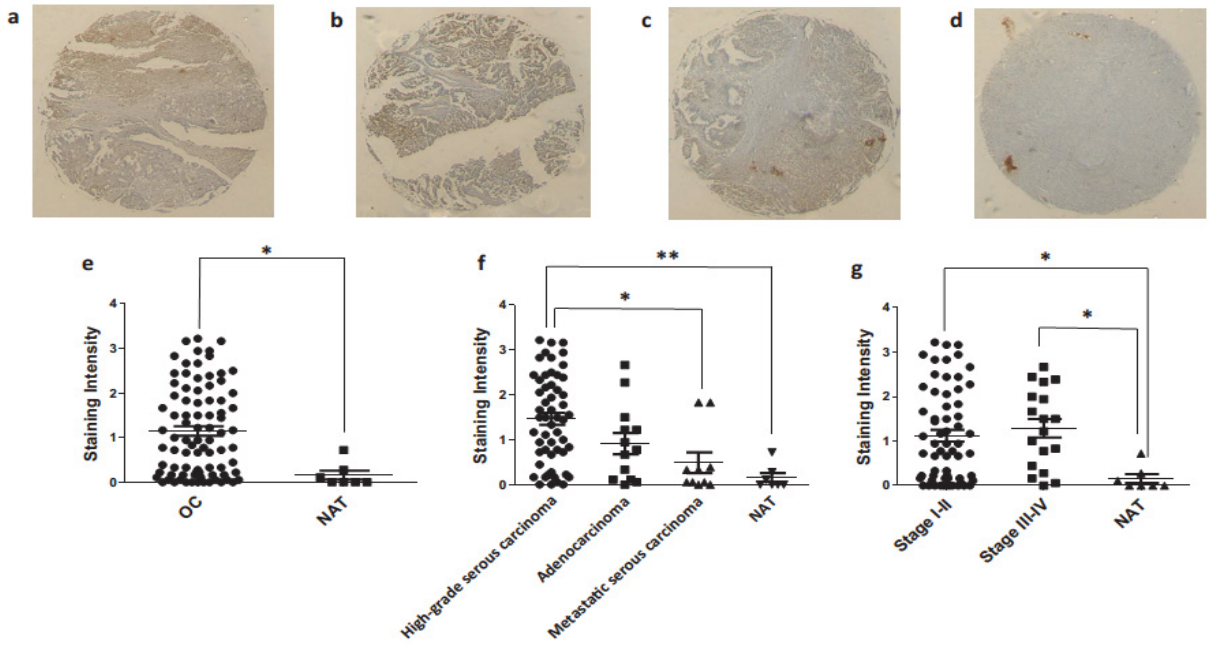


Figure 9. Immunohistochemistry for RARRES3 expression in different pathologies of ovarian tissue array clinical samples: high-grade serous carcinoma (**a**), mucinous adenocarcinoma (**b**), metastatic carcinoma (**c**), normal adjacent tissue (**d**), expression of RARRES3 in ovarian cancer (OC; including high- and low-grade serous carcinoma, mucinous adenocarcinoma, metastatic serous carcinoma) compared to the normal control (**e**), RARRES3 expression in different pathologies of ovarian cancer (**f**) and RARRES3 expression in clinical samples of different stages (**g**). NAT: normal adjacent tissue, * p-value < 0.05, ** p-value < 0.001.

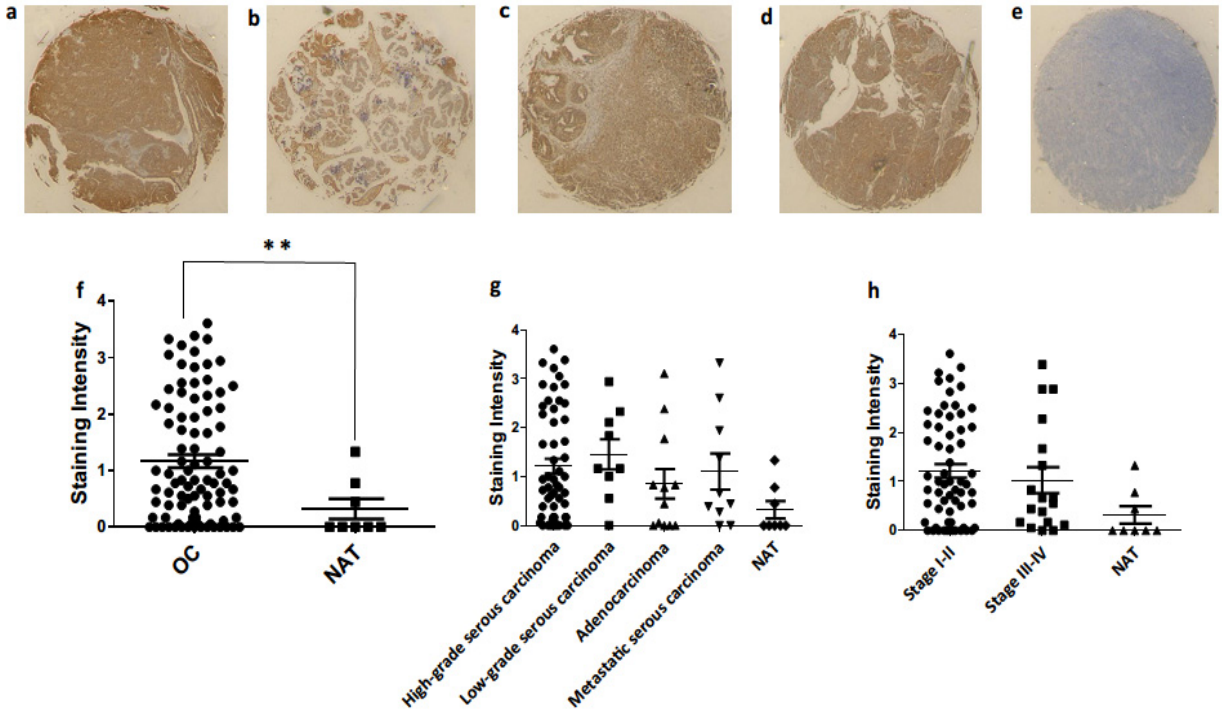


Figure 10. Immunohistochemistry for SLC4A11 expression in different pathologies of ovarian tissue array clinical samples: high-grade serous carcinoma (**a**), low-grade serous carcinoma (**b**), mucinous adenocarcinoma (**c**), metastatic serous carcinoma (**d**), normal adjacent tissue (**e**), expression of SLC4A11 compared to the normal control (**f**), SLC4A11 expression in different pathologies of ovarian cancer (**g**) and RARRES3 expression in clinical samples of different stages (**h**). OC: ovarian cancer (including high- and low-grade serous carcinoma, mucinous adenocarcinoma, metastatic serous carcinoma); NAT: normal adjacent tissue, ** p-value < 0.001.

4. Discussion

Here we provide a detailed analysis of the functional and activity landscape in ovarian cancer for a set of 94 genes that have been previously shown to be dysregulated under exposure to environmental levels of BPA in ovarian cancer cell lines. Apart from genetic influences on the development of malignancies, other environmental factors such as EDCs may also be an important determinant [56]. However, to date, availability of biomarkers of exposure specific to ovarian cancer is very limited.

We showed that 14 genes do not exhibit any significant changes in tumour compared to normal tissue, and thus the effects observed under BPA treatment can be regarded as the key driving forces for the associated phenotypes. The majority of the genes, however, showed a statistically significant differential expression pattern in cancer, hinting that a combined BPA tumour effect can play a key role in the future development of the disease. Specifically, four genes (*RN7SK*, *TNFRSF11B*, *NDUFB5* and *RARRES3*) were shown to be progressively up-regulated in primary and recurrent tumours compared to normal. These results are in accord with previous reports indicating these genes are highly dysregulated in a variety of diseases [43–45]. For example, *TNFRSF11B* exhibited a cancer-specific behaviour in ovarian cancer by contrast to breast, where it was found to be down-regulated and was proposed as a potential prognostic biomarker [57]. Our data suggest that while *TNFRSF11B* can potentially exhibit diagnostic potential, even differentiating between primary and recurrent tumours, it does not have any predictive power for the overall patient outcome.

Gene Ontology analysis of the 80 genes revealed interesting targets in relation to site of expression (e.g., ovarian cancer, cervical cancer and normal ovarian tissue), cellular components (primarily cytoplasm and nucleus), biological processes (e.g., signal transduction) and biological pathways (mainly mTOR and plasma membrane estrogen receptor signalling pathways). Both of these signalling pathways have been implicated in ovarian cancer. The mTOR pathway is a central regulator of cellular events such as proliferation, apoptosis and angiogenesis gauging external energy, growth factor and stress signals with the PI3K/AKT/mTOR pathway being a highly activated cellular signalling pathway in advanced ovarian cancer [58–60]. Similarly, there is evidence of involvement of the membrane-bound estrogen receptor GPR30 in cancer [61]. As mentioned, GPR30 can drive genomic and non-genomic events upon activation with estrogen or other estrogen-like compounds such as BPA [62,63].

On the other hand, functional enrichment analysis of the 14 genes revealed that expression sites are enriched for ovarian cancer, vagina and umbilical cord. Similarly, to the 80 genes in question, the genes including *MYC*, *COL1A2*, *CYR61* and *BDNF* are associated with the mTOR pathway and plasma membrane estrogen receptor signalling. Of note, extensive copy number alterations of MYC proto-oncogene BHLH transcription factor *(MYC)* have been observed in high-grade serous ovarian cancer [64], whereas *BDNF* appears to play a role in ovarian cancer, cell migration and angiogenesis [65] and cysteine-rich angiogenic inducer 61 (*CYR61*) is a potential biomarker for prognostic insinuations of ovarian carcinoma [66]. Kaplan–Meyer analysis enabled us to identify seven genes (*GBP5*, *LINC00707*, *MRPL55*, *RRS1*, *SLC4A11*, *KRT4* and *IRS2)* with overall prognostic biomarker potential. The majority of genes displayed a varied phenotype schema: up-regulated in cancer, with positive outcome on up-regulation; up-regulated in cancer, with negative outcome on up-regulation; and down-regulated in cancer, with negative outcome on up-regulation. Next, using the t-SNE dimensionality reduction analysis method, we showed that the combined predictive power of the seven genes results in a strong collective diagnostic marker, suggesting that the seven genes can be used clinically as a cancer panel for both diagnosis and prognosis. However, the selected seven genes could not provide any information regarding population at risk.

Given the fact that all these genes were previously highlighted as having a differential expression pattern under BPA treatment, we investigated further which genes can be suitable candidates for biomarkers of exposure and biomarkers of disease. By evaluating

the fold change in expression between normal and primary tumours and comparing it to the fold change between expression in SKOV3 cell line in presence and absence of low-dose BPA, we were able to further stratify the seven genes into three groups. We found that for *GBP5*, *LINC00707* and *SLC4A11*, the effect of BPA exposure is minimal with a potential positive bias in *GBP5* and negative bias in *LINC00707* and *SLC4A11.* By contrast, *KRT4* was shown to be strongly and negatively impacted by BPA exposure, suggesting that BPA can alter the predictive outcome of *KRT4*. Of note, KRT4 shows a particular behaviour exhibiting no significant change in expression between normal and primary tumours but showing a strong positive patient outlook upon down-regulation. Finally, for *IRS2*, *RRS2* and *MRPL5*, we found comparable effects on gene expression under tumour conditions or exposure to BPA. Collectively, these results suggest that a conservative functional cancer panel formed by *GBP5*, *LINC00707* and *SLC4A11* can provide useful insights regarding the diagnosis and overall survival prognosis regardless of the status of BPA exposure of the patient (i.e., biomarkers of disease), while *KRT4* can act as a marker for exposure-associated disease.

The finding that KRT4 can be a potential biomarker of BPA exposure-associated ovarian cancer is of increasing importance given that this gene appears to be under the influence of estrogenic responses. Indeed, estrogens play an important role in the development and growth of ovarian cancer as well as in its subsequent metastatic events. When ER-positive ovarian cancer cells were treated with E2, KRT4 expression was dramatically down-regulated [67,68]. Moreover, when estrogen receptor β (ERβ) was silenced in breast cancer MDA-MB-231 cells, KRT4 expression was significantly increased [69]. When p53 null mammary epithelial cells were treated with the selective estrogen receptor modulator Tamoxifen, it led to a significant up-regulation of KRT4 [70]. Nguyen et al. suggested a functional interplay between Zinc-finger protein 217 (ZNF217) and ERα exists in breast cancer [71]. Interestingly, when ZNF217 is silenced in ovarian cancer in vitro, the KRT4 gene was also significantly down-regulated [72]. A direct link between BPA and KRT4 comes from an in vivo study, where KRT4 promoter was hypomethylated in two-week mice following BPA treatment in utero [73].

In summary, leveraging the available RNAseq data from TCGA and GTEx, we were able to identify a number of new potential biomarkers of exposure-associated disease and biomarkers of diagnostic/prognostic potential for ovarian cancer. Future studies should concentrate on elucidating the impact of BPA on normal ovarian function and correlating the biomarker potential of the above-mentioned genes with clinical data. It would be of interest to measure circulating BPA levels in patients and correlate these concentrations with expression of certain genes, especially KRT4 in both tissue and liquid biopsies. Ultimately, these data can be used to put in place preventative measures to reduce exposure to BPA that consequently might impact disease progression.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcm10091979/s1, Figure S1. The functional enrichment in gene ontology terms in 14 genes in relation to site of expression (a,b), cellular components (c,d), biological processes (e,f) and biological pathways (g,h). *p-val < 0.05. Figure S2. The functional enrichment in gene ontology terms in 80 genes in relation to site of expression (a,b), cellular components (c,d), biological processes (e,f) and biological pathways (g,h). *p-val < 0.05. Figure S3. Heatmap of 94 genes in (a) normal ovarian tissue and (b) tumorous ovarian tissue showing correlation between these genes. Deep dark blue colour shows a strong correlation, while deep red colour shows no correlation. Figure S4. KM-plots for stratifying by (a) stage (late – III& IV vs early – I&II), (b) age (late – >60 vs early – <60), and (c) recurrent disease (yes vs no). Figure S5. tSNE discrimination between various phenotypes using the information from the 94 gene expression profiles. Figure S6. tSNE discrimination between tumour and normal samples using the information from the GBP5, SCL4A11 and LINC0070 gene expression profiles. Figure S7. Scatter plot of the expression changes upon BPA exposure as compared to the changes in expression driven by ovarian cancer alone. The labels indicate the pairing in the change in expression in cancer as in SKOV3 cell lines under BPA treatment as compared to their respective controls. The colours are indicative of the statistical significance of the change in expression in

ovarian tumor samples vs normal healthy tissue. Table S1. Details of the clinicopathological features of the tissues used for the microarray. Table S2. List of genes associated with the phenotypes in Figure 2. Table S3. Gene set enrichment analysis results for 94 BPA dysregulated genes. Table S4. Gene expression rank in Hui et al, TCGA, and GTEx datasets.

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Supplementary information

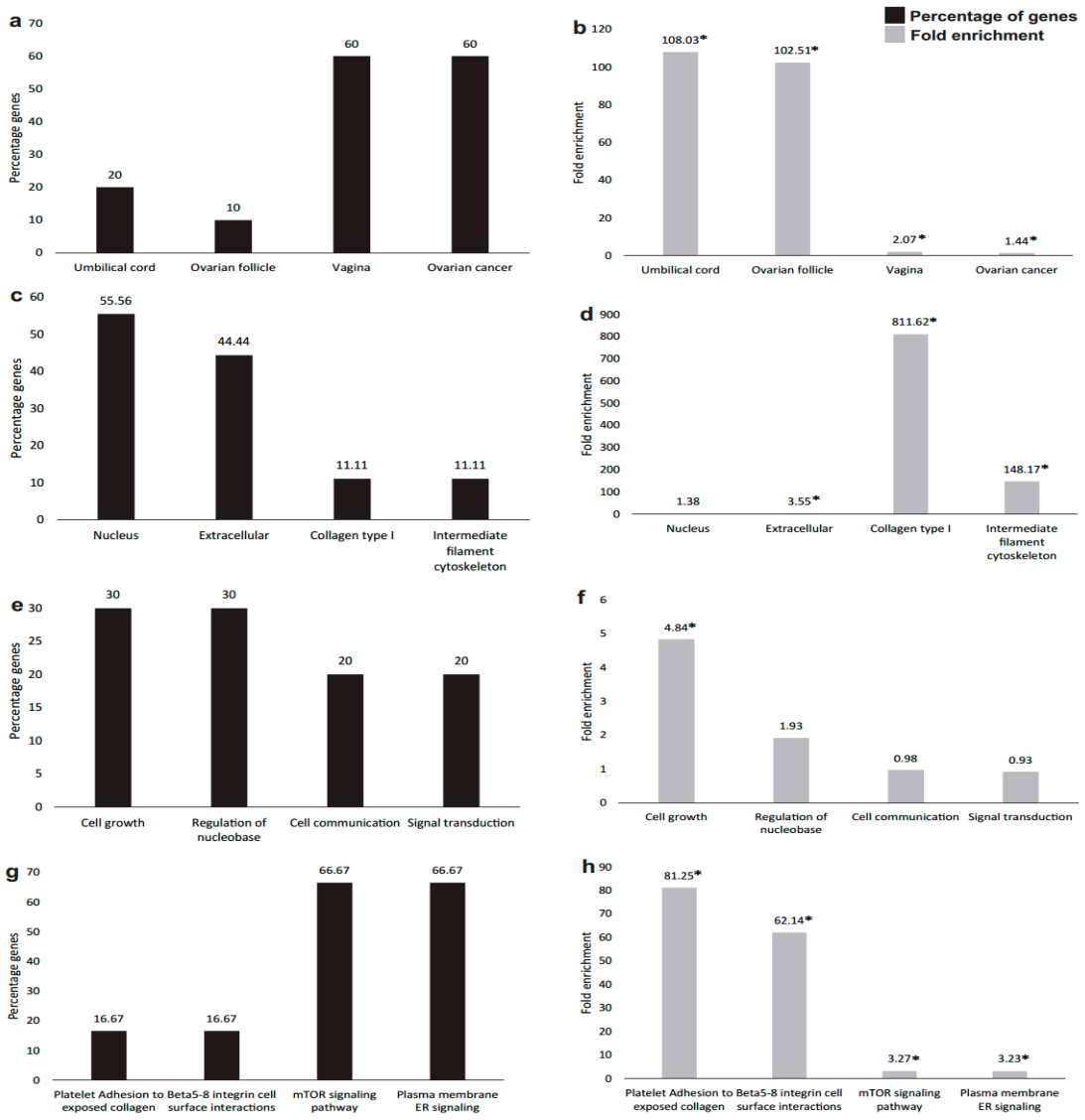

Figure S1. The functional enrichment in gene ontology terms in 14 genes in relation to site of expression (a,b), cellular components (c,d), biological processes (e,f) and biological pathways (g,h). *p-val<0.05.

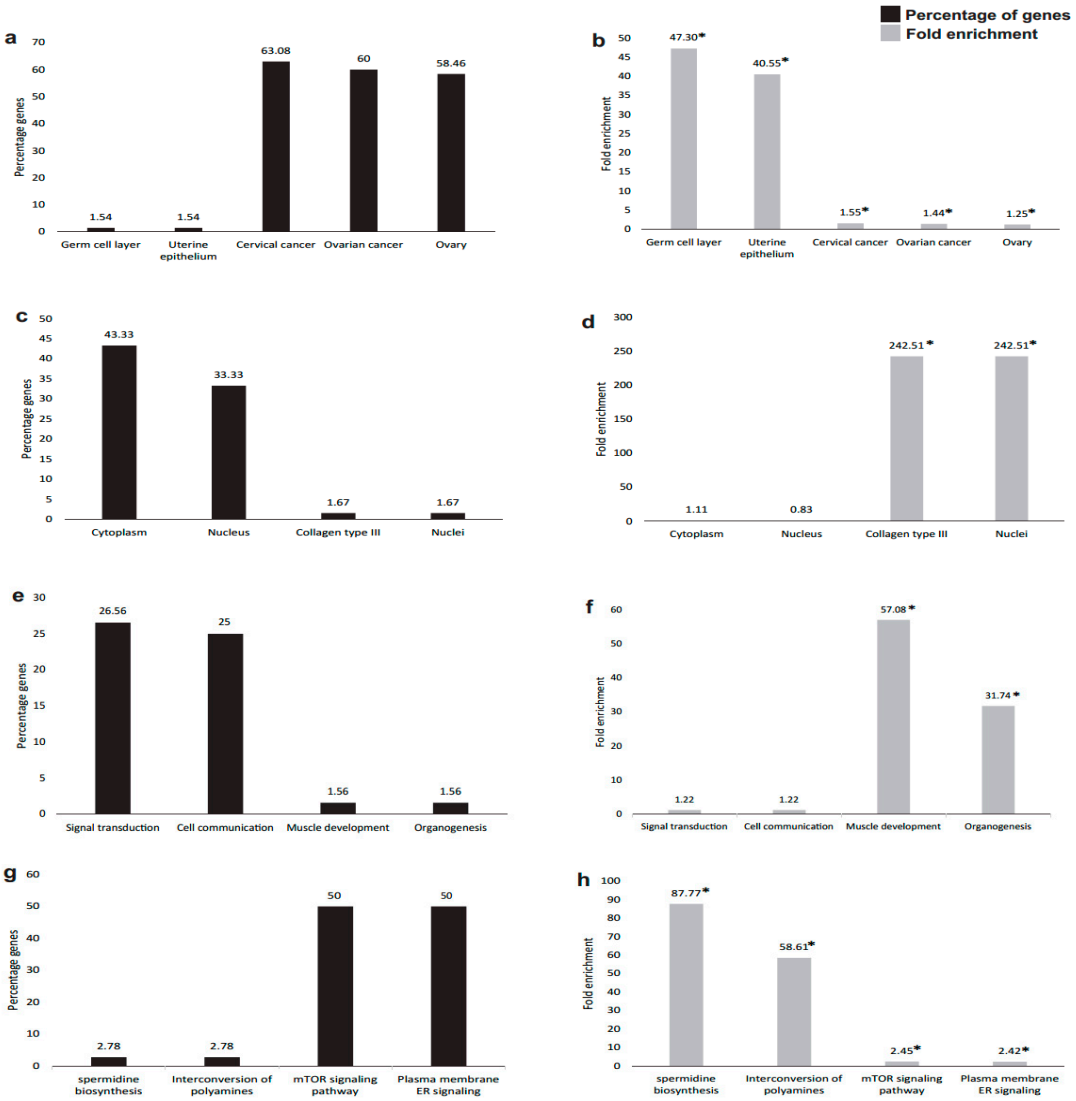

Figure S2. The functional enrichment in gene ontology terms in 80 genes in relation to site of expression (a,b), cellular components (c,d), biological processes (e,f) and biological pathways (g,h). *p-val<0.05.

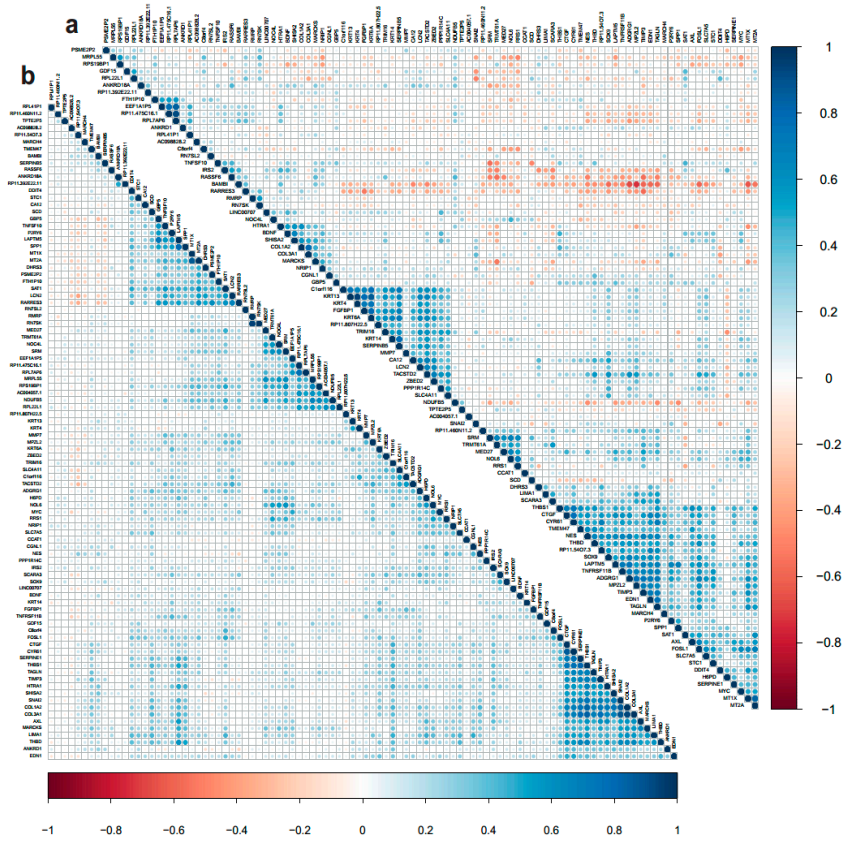

Figure S3. Heatmap of 94 genes in (a) normal ovarian tissue and (b) tumorous ovarian tissue showing correlation between these genes. Deep dark blue colour shows a strong correlation, while deep red colour shows no correlation.

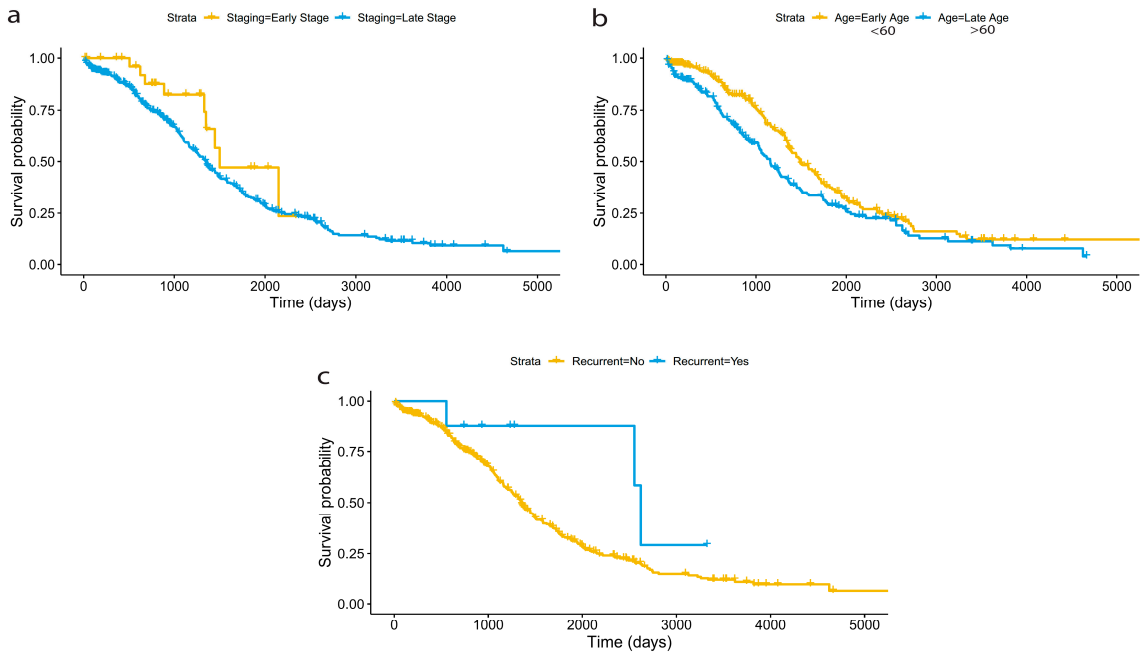

Figure S4. KM-plots for stratifying by (a) stage (late – III& IV vs early – I&II), (b) age (late – >60 vs early – <60), and (c) recurrent disease (yes vs no).

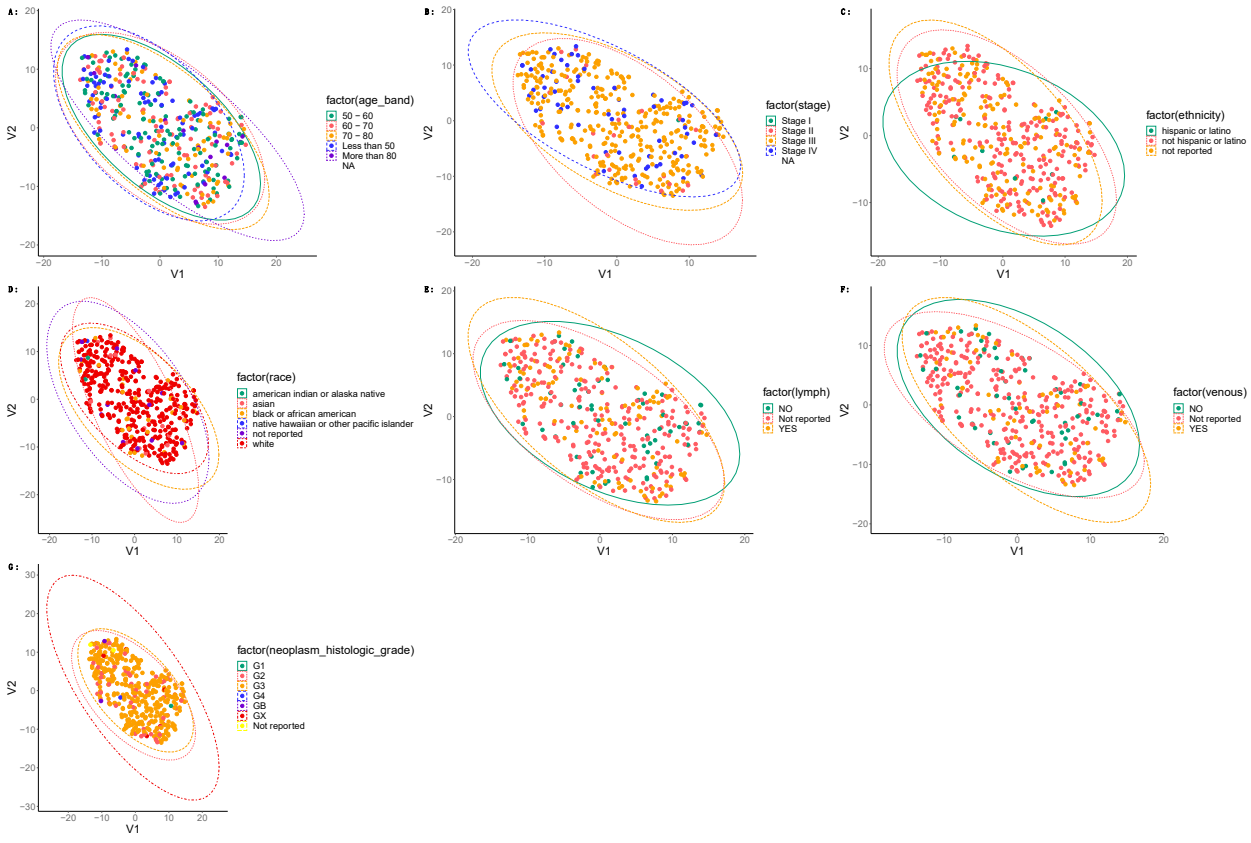

Figure S5. tSNE discrimination between various phenotypes using the information from the 94 gene expression profiles.

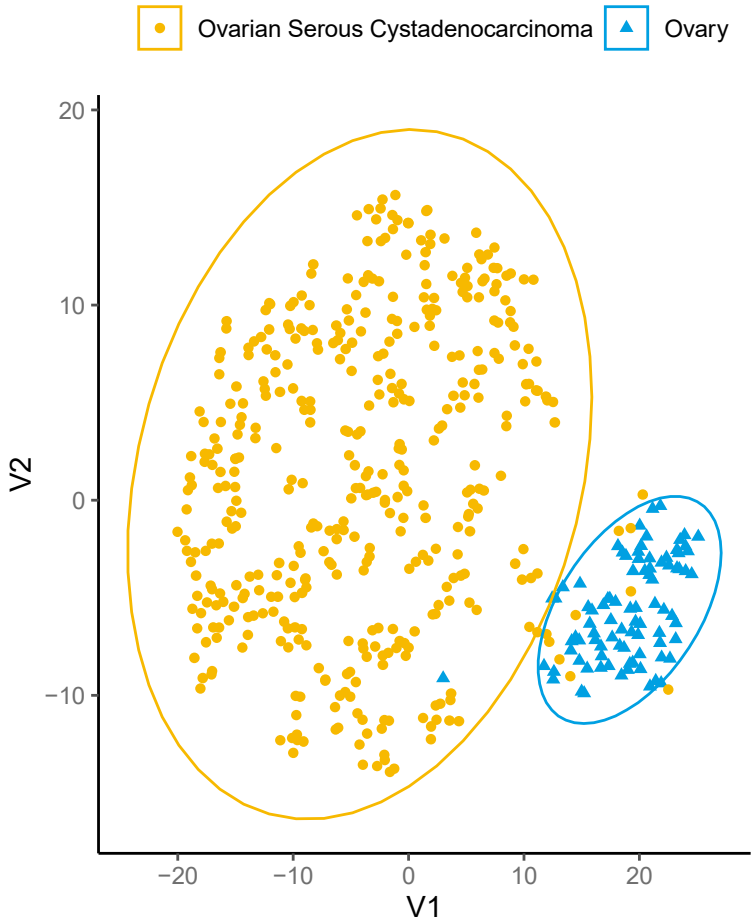

Figure S6. tSNE discrimination between tumour and normal samples using the information from the GBP5, SCL4A11 and LINC0070 gene expression profiles.

Figure S7. Scatter plot of the expression changes upon BPA exposure as compared to the changes in expression driven by ovarian cancer alone. The labels indicate the pairing in the change in expression in cancer as in SKOV3 cell lines under BPA treatment as compared to their respective controls. The colours are indicative of the statistical significance of the change in expression in ovarian tumour samples vs normal healthy tissue.

Table S1. Details of the clinicopathological features of the tissues used for the microarray.

#	Tissue type	STAGES
A1	Clear cell carcinoma	I
A2	Clear cell carcinoma	I
A3	Clear cell carcinoma	I
A4	Clear cell carcinoma	II
A5	Clear cell carcinoma (necrosis)	IIA
A6	Low grade serous carcinoma	IC
A7	Low grade serous carcinoma	IA
A8	Endometrioid adenocarcinoma	I
A9	Low grade serous carcinoma	IA
A10	Low grade serous carcinoma	IA
B1	Low grade serous carcinoma	I
B2	Low grade serous carcinoma	IA
B3	Low grade serous carcinoma	IB
B4	Low grade serous carcinoma	II
B5	High grade serous carcinoma	IIB
B6	High grade serous carcinoma	I
B7	High grade serous carcinoma	I
B8	High grade serous carcinoma	I
B9	High grade serous carcinoma	I
B10	High grade serous carcinoma	IA
C1	High grade serous carcinoma	IIB
C2	High grade serous carcinoma	I
C3	High grade serous carcinoma	III
C4	High grade serous carcinoma	I
C5	High grade serous carcinoma	IA
C6	High grade serous carcinoma	IV
C7	High grade serous carcinoma	IB
C8	High grade serous carcinoma	IIIC
C9	High grade serous carcinoma	I
C10	High grade serous carcinoma	IIIC
D1	High grade serous carcinoma	IA
D2	High grade serous carcinoma	IIIC
D3	High grade serous carcinoma	IC
D4	High grade serous carcinoma	IIIC
D5	High grade serous carcinoma	IA
D6	High grade serous carcinoma	I
D7	High grade serous carcinoma	IA
D8	High grade serous carcinoma	I
D9	High grade serous carcinoma	IA
D10	High grade serous carcinoma	I

E1	High grade serous carcinoma	IIIC
E2	High grade serous carcinoma	I
E3	High grade serous carcinoma with necrosis	II
E4	High grade serous carcinoma	IIIC
E5	High grade serous carcinoma	IC
E6	High grade serous carcinoma	IIIC
E7	High grade serous carcinoma	II
E8	High grade serous carcinoma	II
E9	High grade serous carcinoma	II
E10	High grade serous carcinoma	I
F1	High grade serous carcinoma with necrosis	IC
F2	High grade serous carcinoma (sparse)	IA
F3	High grade serous carcinoma	II
F4	High grade serous carcinoma	IIIC
F5	High grade serous carcinoma	IA
F6	High grade serous carcinoma with necrosis	IC
F7	High grade serous carcinoma	IIIC
F8	High grade serous carcinoma	IIIC
F9	High grade serous carcinoma	IIIC
F10	High grade serous carcinoma	IIIC
G1	High grade serous carcinoma	II
G2	High grade serous carcinoma	IA
G3	High grade serous carcinoma	III
G4	High grade serous carcinoma	I
G5	High grade serous carcinoma	IIIA
G6	High grade serous carcinoma	IIB
G7	High grade serous carcinoma	IA
G8	High grade serous carcinoma	IA
G9	Mucinous papillary adenocarcinoma (necrosis)	I
G10	Endometrioid adenocarcinoma	II
H1	Mucinous adenocarcinoma	IB
H2	Mucinous adenocarcinoma	IA
H3	Mucinous adenocarcinoma with necrosis	IIA
H4	Mucinous adenocarcinoma	IB
H5	Mucinous adenocarcinoma with necrosis	IIIC
H6	Mucinous adenocarcinoma	IB
H7	Mucinous adenocarcinoma	I
H8	Mucinous adenocarcinoma	III
H9	Mucinous adenocarcinoma	IA
H10	Endometrioid adenocarcinoma	IA
I1	Metastatic serous carcinoma from ovary	-
I2	Metastatic serous carcinoma from ovary	-

I3	Metastatic serous carcinoma from ovary	-
I4	Metastatic serous carcinoma from ovary	-
I5	Metastatic serous carcinoma from ovary	-
I6	Metastatic clear cell carcinoma from ovary	-
I7	Metastatic serous carcinoma of fibrofatty tissue from ovary of No.64	-
I8	Metastatic serous carcinoma from ovary	-
I9	Metastatic serous carcinoma from ovary	-
I10	Metastatic serous carcinoma of fibrofatty tissue from ovary	-
J1	Adjacent normal ovary tissue	-
J2	Adjacent normal ovary tissue	-
J3	Adjacent normal ovary tissue	-
J4	Adjacent normal ovary tissue	-
J5	Adjacent normal ovary tissue	-
J6	Adjacent normal ovary tissue	-
J7	Adjacent normal ovary tissue	-
J8	Adjacent normal ovary tissue	-
J9	Adjacent normal ovary tissue	-
J10	Adjacent normal ovary tissue	-

Table S2. List of genes associated with the phenotypes in **Figure 2**.

Phenotypes	Genes
Site of Expression (14 genes)	
Umbilical cord	*MT1X; NES*
Ovarian follicle	*BDNF*
Vagina	*MYC; SCD; CYR61; BDNF; KRT4; NES*
Ovarian cancer	*MYC; SCD; CYR61; BDNF; KRT4; NES*
Cellular Components (14 genes)	
Nucleus	*MYC; MT1X; SCD; ANKRD1; NES*
Extracellular	*MT1X; COL1A2; CYR61; BDNF*
Collagen type I	*COL1A2*
Intermediate filament cytoskeleton	*NES*
Biological Processes (14 genes)	
Cell growth	*COL1A2; KRT4; NES*
Regulation of nucleobase	*MYC; TRMT61A; ANKRD1*
Cell communication	*CYR61; BDNF*
Signal transduction	*CYR61; BDNF*
Biological Pathways (14 genes)	
Platelet Adhesion to exposed collagen	*COL1A2*
Beta5-8 integrin cell surface interactions	*CYR61*
mTOR signaling pathway	*MYC; COL1A2; CYR61; BDNF*
Plasma membrane ER signaling	*MYC; COL1A2; CYR61; BDNF*

Site of Expression (80 genes)	
Germ cell layer	*HTRA1*
Uterine epithelium	*CA12*
Cervical cancer	*TNFRSF11B; NDUFB5; RARRES3; MMP7; LCN2; SOX9; C1orf116; SPP1; P2RY6; SRM; SLC4A11; CA12; FGFBP1; SERPINB5; STC1; FOSL1; GDF15; GBP5; MED27; MRPL55; MARCKS; BAMBI; KRT13; DDIT4; SNAI2; CGNL1; LIMA1; KRT14; DHRS3; TRIM16; CTGF; COL3A1; TIMP3; THBD; IRS2; C8orf4; SERPINE1; H6PD; TAGLN; EDN1; ZBED2*
Ovarian cancer	*TNFRSF11B; NDUFB5; RARRES3; MMP7; LCN2; SOX9; C1orf116; SPP1; P2RY6; SRM; SLC4A11; CA12; FGFBP1; SERPINB5; STC1; FOSL1; GDF15; GBP5; TNFSF10; EDN1; MED27; MRPL55; MARCKS; BAMBI; DDIT4; SNAI2; LIMA1; KRT14; DHRS3; TRIM16; CTGF; COL3A1; TIMP3; IRS2; C8orf4; SERPINE1; H6PD; TAGLN ; ZBED2*
Ovary	*TNFRSF11B; NDUFB5; RARRES3; MMP7; LCN2; TACSTD2; C1orf116; SPP1; P2RY6; SRM; SLC4A11; CA12; SERPINB5; STC1; FOSL1; GDF15; GBP5;; MRPL55; MARCKS; KRT13; DDIT4; HTRA1; SNAI2; LIMA1; THBS1; KRT6A; KRT14; DHRS3; TRIM16; COL3A1; NOL6; TIMP3; IRS2; C8orf4; H6PD; TAGLN; MED27; CTGF*
Cellular Components (80 genes)	
Cytoplasm	*TACSTD2; C1orf116; SRM; PPP1R14C; FGFBP1; SERPINB5; SCARA3; STC1; EDN1; MED27; MARCKS; SAT1; BAMBI; KRT13; DDIT4; LIMA1; THBS1; KRT6A; KRT14; TRIM16; MT2A; NRIP1; IRS2; SERPINE1; H6PD; TAGLN*
Nucleus	*RARRES3; ZBED2; SOX9; STC1; FOSL1; TNFSF10; MED27; SAT1; KRT13; DDIT4; SNAI2; LIMA1; THBS1; KRT14; TRIM16; RRS1; MT2A; NOL6; NRIP1; IRS2*
Collagen type III	*COL3A1*
Nuclei	*MT2A*
Biological Processes (80 genes)	
Signal transduction	*TNFRSF11B; RARRES3; TACSTD2; P2RY6; ADGRG1; LAPTM5; PPP1R14C; FGFBP1; SCARA3; STC1; GDF15; GBP5; TNFSF10; EDN1; BAMBI; AXL; IRS2*
Cell communication	*TNFRSF11B; RARRES3; TACSTD2; P2RY6; LAPTM5; PPP1R14C; FGFBP1; SCARA3; STC1; GDF15; GBP5; TNFSF10; EDN1; BAMBI; AXL; IRS2*
Muscle development	*TAGLN*

Organogenesis	*MPZL2*
Biological Pathways (80 genes)	
spermidine biosynthesis	*SRM*
Interconversion of polyamines	*SAT1*
mTOR signaling pathway	*MMP7; SPP1; SERPINB5; FOSL1; GDF15; EDN1; BAMBI; DDIT4; SNAI2; LIMA1; KRT14; CTGF; MT2A; NRIP1; THBD; IRS2; SERPINE1; TAGLN*
Plasma membrane ER signaling	*MMP7; SPP1; SERPINB5; FOSL1; GDF15; EDN1; BAMBI; DDIT4; SNAI2; LIMA1; KRT14; CTGF; MT2A; NRIP1; THBD; IRS2; SERPINE1; TAGLN*

Table S3. Gene set enrichment analysis results for 94 BPA dysregulated genes.

GENE	Rank	Test	Res	Core enrichment
SRM	57914	-28.8	0.0114	YES
MARCH4	57125	-22.3	-0.0165	YES
BAMBI	56716	-20.5	-0.0415	YES
LINC00707	56537	-19.8	-0.0679	YES
P2RY6	56320	-19	-0.0926	YES
FGFBP1	56288	-18.9	-0.119	YES
PPP1R14C	54609	-15.3	-0.118	YES
ZBED2	54590	-15.3	-0.139	YES
FTH1P10	54514	-15.2	-0.16	YES
MARCKS	54288	-14.8	-0.178	YES
FOSL1	54141	-14.6	-0.197	YES
SERPINB5	52835	-12.9	-0.195	YES
TNFRSF11B	52587	-12.6	-0.21	YES
CGNL1	52335	-12.3	-0.223	YES
TMEM47	52005	-12	-0.235	YES
SHISA2	52000	-12	-0.253	YES
RPS19BP1	51190	-11.2	-0.256	YES
SAT1	50681	-10.7	-0.263	YES
PSME2P2	49970	-10.1	-0.266	YES
C1orf116	49696	-9.86	-0.276	YES
MMP7	49468	-9.69	-0.287	YES
EDN1	49133	-9.45	-0.295	YES
AC098828.2	49087	-9.42	-0.308	YES
ANKRD18A	48242	-8.88	-0.307	YES
MYC	47592	-8.46	-0.308	YES
CCAT1	47590	-8.46	-0.32	YES
COL1A2	47051	-8.12	-0.323	YES

RP11-807H22.5	46789	-7.94	-0.331	YES
RMRP	46472	-7.72	-0.337	YES
BDNF	46272	-7.61	-0.344	YES
COL3A1	45870	-7.37	-0.348	YES
NDUFB5	45825	-7.34	-0.358	YES
RP11-54O7.3	44782	-6.73	-0.351	YES
AXL	44343	-6.49	-0.353	YES
SERPINE1	43816	-6.21	-0.353	YES
EEF1A1P5	43789	-6.2	-0.362	YES
SNAI2	43737	-6.17	-0.37	YES
SCD	43577	-6.08	-0.376	YES
GDF15	43530	-6.05	-0.384	YES
CTGF	43302	-5.94	-0.389	YES
THBD	43226	-5.9	-0.396	YES
RP11-392E22.11	43093	-5.84	-0.402	YES
RASSF6	42928	-5.76	-0.408	YES
NOL6	42355	-5.48	-0.406	YES
GBP5	42043	-5.34	-0.409	YES
LIMA1	41545	-5.1	-0.408	YES
KRT14	41076	-4.89	-0.407	YES
C8orf4	40959	-4.84	-0.412	YES
CA12	40417	-4.62	-0.41	YES
MT2A	40189	-4.51	-0.413	YES
IRS2	39985	-4.42	-0.416	YES
RPL7AP6	39979	-4.41	-0.422	YES
DHRS3	38626	-3.9	-0.405	NO
STC1	38617	-3.9	-0.411	NO
KRT6A	38074	-3.7	-0.407	NO
NRIP1	37754	-3.58	-0.407	NO
MT1X	37072	-3.34	-0.4	NO
TIMP3	36940	-3.3	-0.403	NO
CYR61	36407	-3.15	-0.399	NO
LCN2	36049	-3.04	-0.397	NO
TRIM16	34201	-2.55	-0.37	NO
ANKRD1	34126	-2.54	-0.372	NO
NES	32473	-2.18	-0.348	NO
KRT4	31712	-2.02	-0.338	NO
TPTE2P5	29299	-1.63	-0.299	NO
TRMT61A	26245	-1.21	-0.25	NO
AC004057.1	26108	-1.19	-0.249	NO
THBS1	26012	-1.17	-0.249	NO
TAGLN	21281	-0.334	-0.17	NO
RP11-475C16.1	20450	-0.0528	-0.156	NO

SLC4A11	11635	0.516	-0.00559	NO
RPL22L1	11041	0.69	0.00381	NO
RN7SL2	9215	1.14	0.034	NO
TNFSF10	7879	1.68	0.0552	NO
SCARA3	6547	2.48	0.0756	NO
HTRA1	6181	2.74	0.0782	NO
DDIT4	6036	2.88	0.0768	NO
H6PD	5620	3.29	0.0797	NO
RARRES3	4970	3.98	0.0861	NO
RP11-460N11.2	4690	4.31	0.0851	NO
SLC7A5	4194	4.93	0.0874	NO
SOX9	4073	5.13	0.0824	NO
LAPTM5	3889	5.41	0.0782	NO
MED27	3720	5.71	0.0733	NO
SPP1	3495	6.07	0.0689	NO
RRS1	2615	7.9	0.0752	NO
MRPL55	2537	8.03	0.0652	NO
NOC4L	2531	8.04	0.0537	NO
RPL41P1	2378	8.42	0.0448	NO
KRT13	2366	8.45	0.0329	NO
ADGRG1	2255	8.79	0.0226	NO
TACSTD2	2048	9.43	0.0135	NO
RN7SK	1989	9.61	0.000983	NO
MPZL2	964	14.7	0.00469	NO

Table S4. Gene expression rank in Hui et al, TCGA, and GTEx datasets.

Gene Name	Gene ID	TCGA	GTEx	SKOV3 w BPA	SKOV3
AC004057.1	ENSG00000196656	3	16	70	74
AC098828.2	ENSG00000234378	76	77	76	75
ANKRD1	ENSG00000148677	75	64	64	67
ANKRD18A	ENSG00000180071	73	71	57	59
AXL	ENSG00000167601	33	19	2	2
BAMBI	ENSG00000095739	64	20	54	53
BDNF	ENSG00000176697	71	59	38	35
CA12	ENSG00000074410	45	60	19	24
CGNL1	ENSG00000128849	50	30	20	30
COL1A2	ENSG00000164692	1	1	71	66
COL3A1	ENSG00000168542	2	4	75	64
DDIT4	ENSG00000168209	25	3	32	42
DHRS3	ENSG00000162496	24	23	62	73

EDN1	ENSG00000078401	60	51	45	40
EEF1A1P5	ENSG00000196205	12	10	27	22
FGFBP1	ENSG00000137440	70	74	7	5
FOSL1	ENSG00000175592	63	61	9	9
FTH1P10	ENSG00000223361	62	58	74	76
GBP5	ENSG00000154451	52	52	47	51
GDF15	ENSG00000130513	51	53	56	61
H6PD	ENSG00000049239	18	7	17	21
HTRA1	ENSG00000166033	22	5	24	27
IRS2	ENSG00000185950	29	14	50	47
KRT13	ENSG00000171401	74	45	78	70
KRT14	ENSG00000186847	61	57	65	65
KRT4	ENSG00000170477	65	49	69	60
KRT6A	ENSG00000205420	53	54	77	71
LAPTM5	ENSG00000162511	10	40	60	68
LCN2	ENSG00000148346	9	68	44	50
LIMA1	ENSG00000050405	35	15	22	14
LINC00707	ENSG00000238266	72	76	63	62
MARCKS	ENSG00000277443	19	18	13	19
MED27	ENSG00000160563	43	42	43	41
MMP7	ENSG00000137673	23	73	6	7
MPZL2	ENSG00000149573	27	47	28	34
MRPL55	ENSG00000162910	31	36	48	46
MT1X	ENSG00000187193	40	26	55	52
MT2A	ENSG00000125148	11	13	3	3
MYC	ENSG00000136997	16	11	21	12
NDUFB5	ENSG00000136521	17	28	18	17
NES	ENSG00000132688	42	29	26	25
NOC4L	ENSG00000184967	39	37	41	36
NOL6	ENSG00000165271	28	22	8	8
NRIP1	ENSG00000180530	32	27	12	16
P2RY6	ENSG00000171631	55	66	58	63
PPP1R14C	ENSG00000198729	58	69	39	44
PSME2P2	ENSG00000225131	66	56	73	78
RASSF6	ENSG00000169435	69	50	29	37
RN7SL2	ENSG00000274012	78	78	67	58
RPL22L1	ENSG00000163584	34	35	51	49
RPL41P1	ENSG00000227063	77	72	72	77
RPL7AP6	ENSG00000242071	56	43	59	57
RPS19BP1	ENSG00000187051	36	24	31	28
RRS1	ENSG00000179041	37	39	34	32
SAT1	ENSG00000130066	6	9	40	45

SCARA3	ENSG00000168077	8	31	25	15
SCD	ENSG00000099194	20	12	11	13
SERPINB5	ENSG00000206075	67	70	30	23
SERPINE1	ENSG00000106366	38	17	46	39
SHISA2	ENSG00000180730	68	67	61	56
SLC4A11	ENSG00000088836	41	62	33	31
SLC7A5	ENSG00000103257	30	38	1	1
SNAI2	ENSG00000019549	57	33	42	38
SOX9	ENSG00000125398	26	65	66	72
SPP1	ENSG00000118785	4	46	5	6
SRM	ENSG00000116649	15	21	16	11
STC1	ENSG00000159167	49	48	53	54
TACSTD2	ENSG00000184292	5	55	14	20
TAGLN	ENSG00000149591	7	2	36	29
THBD	ENSG00000178726	54	41	23	26
THBS1	ENSG00000137801	14	6	4	4
TIMP3	ENSG00000100234	13	8	68	69
TMEM47	ENSG00000147027	46	25	15	18
TNFRSF11B	ENSG00000164761	59	63	35	43
TNFSF10	ENSG00000121858	21	32	49	55
TRIM16	ENSG00000221926	48	44	52	48
TRMT61A	ENSG00000166166	44	34	37	33
ZBED2	ENSG00000177494	47	75	10	10

Chapter 3

In Silico Study to Predict the Structural and Functional Consequences of SNPs on Biomarkers of Ovarian Cancer (OC) and BPA Exposure-Associated OC

Statement of Contribution

In this manuscript I led and contributed the following parts:

- Data curation
- Methodology
- Formal analysis
- Writing—original draft preparation
- Writing—review and editing
- Referencing
- Funding acquisition

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Article

In Silico Study to Predict the Structural and Functional Consequences of SNPs on Biomarkers of Ovarian Cancer (OC) and BPA Exposure-Associated OC

Aeman Zahra [1], Marcia Hall [1,2], Jayanta Chatterjee [1,3], Cristina Sisu [1,*] and Emmanouil Karteris [1,*]

[1] Biosciences, College of Health, Medicine and Life Sciences, Brunel University London, Uxbridge UB8 3PH, UK; aeman.zahra@brunel.ac.uk (A.Z.); marcia.hall@nhs.net (M.H.); jayanta.chatterjee1@nhs.net (J.C.)
[2] Mount Vernon Cancer Centre, Northwood HA6 2RN, UK
[3] Faculty of Health and Medical Sciences, School of Biosciences and Medicine, University of Surrey, Guildford GU2 7XH, UK
* Correspondence: cristina.sisu@brunel.ac.uk (C.S.); emmanouil.karteris@brunel.ac.uk (E.K.)

Abstract: Background: Recently, we have shown that seven genes, namely *GBP5*, *IRS2*, *KRT4*, *LINCOO707*, *MRPL55*, *RRS1* and *SLC4A11*, have prognostic power for the overall survival in ovarian cancer (OC). Methods: We present an analysis on the association of these genes with any phenotypes and mutations indicative of involvement in female cancers and predict the structural and functional consequences of those SNPS using in silico tools. Results: These seven genes present with 976 SNPs/mutations that are associated with human cancers, out of which 284 related to female cancers. We have then analysed the mutation impact on amino acid polarity, charge and water affinity, leading to the identification of 30 mutations in gynaecological cancers where amino acid (aa) changes lead to opposite polarity, charges and water affinity. Out of these 30 mutations identified, only a missense mutation (i.e., R831C/R804C in uterine corpus endometrial carcinomas, UCEC) was suggestive of structural damage on the *SLC4A11* protein. Conclusions: We demonstrate that the R831C/R804C mutation is deleterious and the predicted ΔΔG values suggest that the mutation reduces the stability of the protein. Future in vitro studies should provide further insight into the role of this transporter protein in UCEC.

Keywords: missense mutations; protein modelling; *SLC4A11*; uterine corpus endometrial carcinoma

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1. Introduction

Ovarian carcinoma (OC) is the most fatal gynaecologic malignancy, accounting for more than 200,000 deaths annually (WHO; Cancer Today). Over 80% of patients with advanced OC will relapse, and despite further good remissions from additional chemotherapy and surgery, they will usually die from their disease [1]. The median progression-free survival (PFS) for relapsed ovarian cancer (ROC) patients who last had treatment within 3–12 months previously is 4–9 months, with overall survival (OS) of ~12–20 months [2]. It should be noted that there is a genetic variation of response to chemotherapy and subsequently to tumour progression [3].

A plethora of studies—primarily via genome-wide association studies—have conclusively demonstrated an association between single-nucleotide polymorphisms (SNPs) and cancer risk [4]. There is a high frequency of SNPs occurrence in the human genome. In particular, amino acid point mutations or non-synonymous single-nucleotide polymorphisms (nsSNPs) may alter the structure and subsequently affect the function of the mutated protein [5]. More than 13,000 known SNPs are in exon regions, of which 58% are nsSNPs [6]. Indeed, a number of nsSNPs are associated with an increased cancer risk [7]. For example, nsSNPs in codon 31 of the *p21* gene are associated with an increased risk of cervical cancer development [8].

Apart from genetic changes, exposure to endocrine-disrupting chemicals (EDCs) can disturb the normal functions of the endocrine system in humans and increase the risk of adverse health effects [1]. Bisphenol A (BPA) (an EDC) has a pro-carcinogenic impact in hormone-dependent and hormone-independent cancers [9–11]. BPA exposure is reported to alter the cancer cells' biological behaviours, particularly, proliferation, invasion, growth, survival, migration and apoptosis [9,12–16]. Recently, we have identified seven genes that have prognostic power for the overall survival in OC, namely Guanylate Binding Protein 5 (*GBP5*), Insulin Receptor Substrate 2 (*IRS2*), Keratin 4 (*KRT4*), long intergenic non-protein coding RNA 707 (*LINC00707*), Mitochondrial Ribosomal Protein L55 (*MRPL55*), Ribosome Biogenesis Regulator 1 Homolog (*RRS1*) and Solute Carrier Family 4 Member 11 (*SLC4A11*). Out of these seven genes, *KRT4* appears to be a biomarker of BPA exposure-associated OC, whereas *GBP5*, *LINC00707* and *SLC4A11* appear to be biomarkers of disease [17].

In this study, we aimed to predict the structural and functional consequences of SNPs mapped in genetic variants of these seven biomarkers in gynaecological malignancies.

2. Results

2.1. Landscape of Mutations in Seven Biomarker Genes Based on TCGA, cBioPortal and UK Biobank

We have previously identified seven biomarkers of OC and exposure-associated OC, as discussed [17]. We found that these 7 biomarkers represent 976 and 284 SNPs/mutations associated with human cancers and female cancers, respectively. It should be noted that in Figure 1, we did not illustrate UK BioBank (PhenoScanner)-associated mutations (Table 1) as it has no overlapping/intersection with any other database (cBioPortal or TCGA).

Table 1. Data summary for the mutation samples from TCGA, UK BioBank and cBioPortal datasets. The "Total Samples" is with respect to the samples associated with the genes of interest.

Gene	Samples	TCGA	UK BioBank	cBioPortal
	Total Samples All cancers	713 713 (100%)	950 48 (100%)	647 647 (100%)
	Female cancers *	145 (20.33%)	7 (14.58%)	208 (32.14%)
GBP5	All cancers Female cancers	145 (20.33%) 27 (3.78%)	3 (6.25%) 1 (2.08%)	150 (23.18%) 54 (8.34%)
IRS2	All cancers Female cancers	114 (15.98%) 30 (4.20%)	8 (16.66%) -	82 (12.67%) 18 (2.78%)
KRT4	All cancers Female cancers	154 (21.59%) 22 (3.08%)	7 (14.58%) 2 (4.16%)	158 (24.42%) 50 (7.72%)
LINC00707	All cancers Female cancers	- -	24 (50%) 2 (4.16%)	- -
MRPL55	All cancers Female cancers	35 (4.90%) 10 (1.40%)	1 (2.08%) 1 (2.08%)	24 (3.70%) 9 (1.39%)
RRS1	All cancers Female cancers	57 (7.99%) 16 (2.24%)	1 (2.08%)-	38 (5.87%) 11 (1.70%)
SLC4A11	All cancers Female cancers	208 (29.17%) 40 (5.61%)	4 (8.33%) 1 (2.08%)	195 (30.13%) 67 (10.35%)

* Female cancers: ovarian, cervical/endocervical, uterine, breast and endometrial/uterine corpus endometrioid carcinoma.

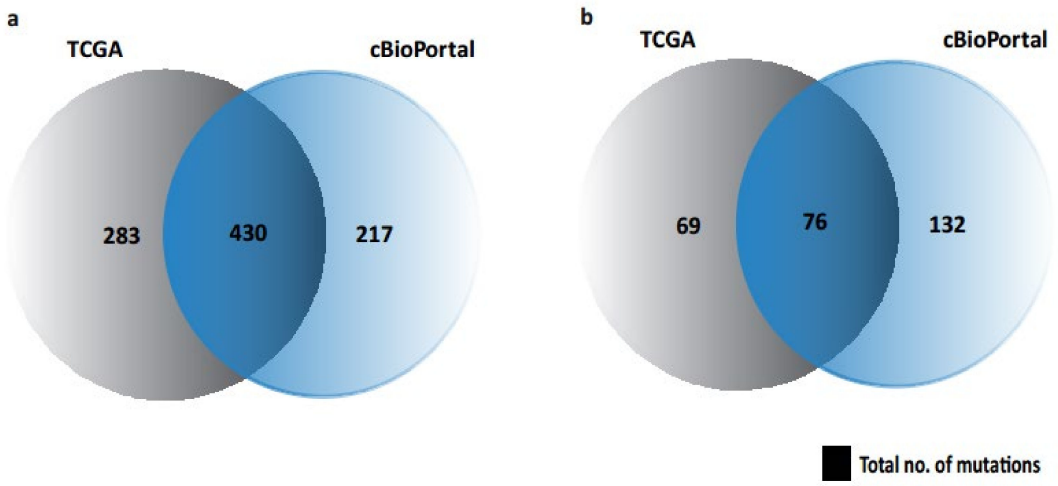

Figure 1. Venn diagram showing the possible mutations/SNPs associated with seven biomarkers in cBioPortal and UCSC Xena repository. (**a**) Mutations in human cancers. (**b**) Mutations in female cancers.

These SNPs were further analysed according to the number and percentage of mutations associated with seven biomarkers of interest in human cancers (Figure 2) and female cancers (Figure 3), along with mutation types.

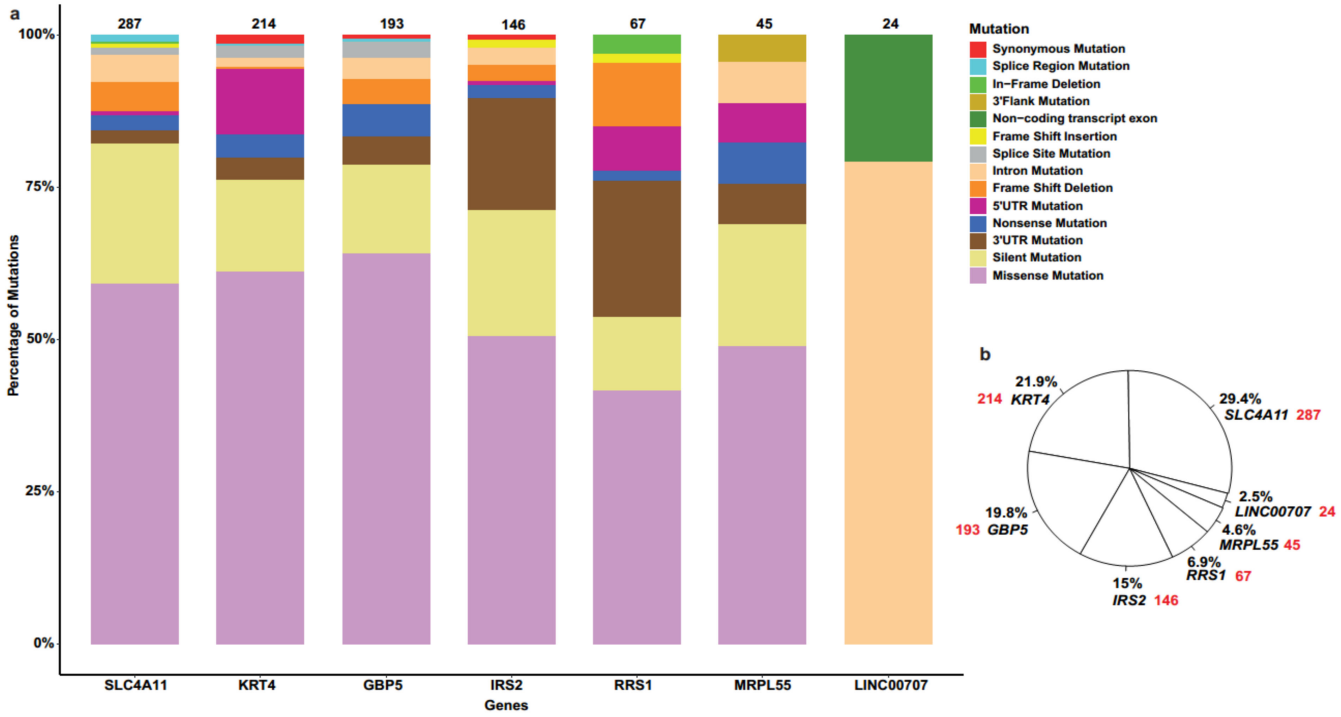

Figure 2. (**a**) Bar plot representing types of SNPs/mutations associated with seven biomarkers in human cancers. (**b**) Pie chart demonstrating the percentage distribution of 976 SNPs for 7 biomarkers in human cancers, where red colour represents the number of mutations in each gene.

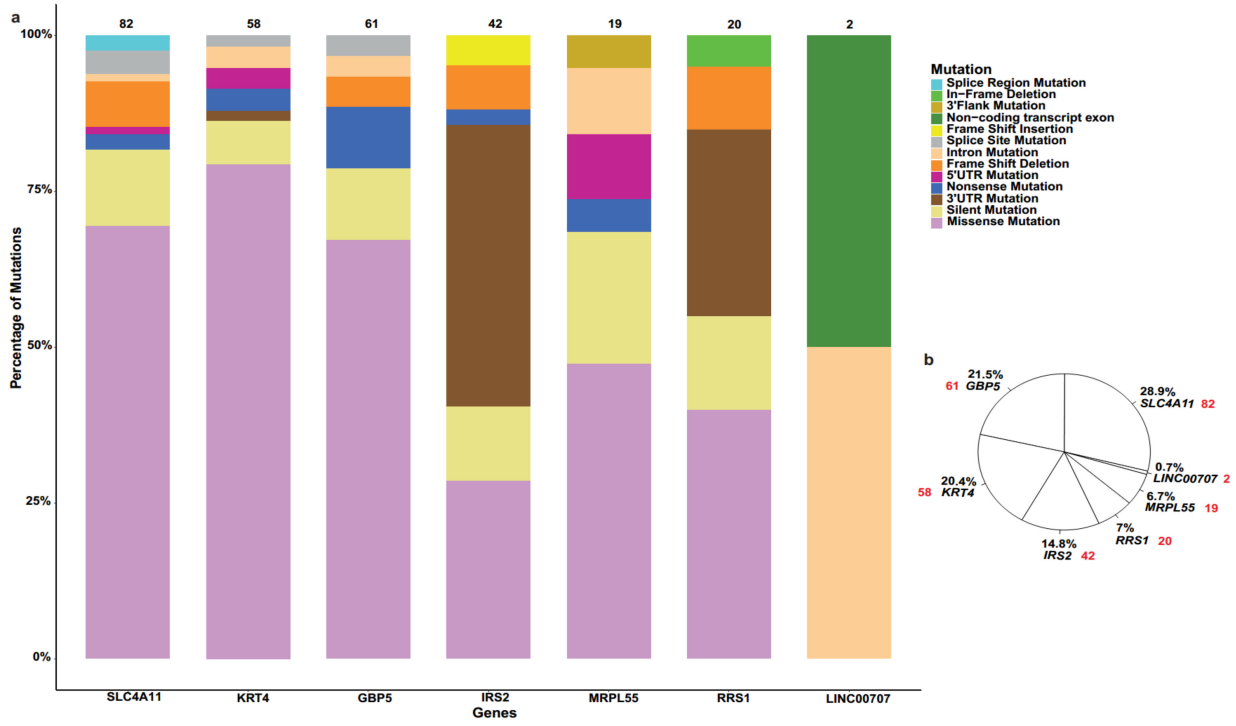

Figure 3. (**a**) Bar plot indicating different types of mutations associated with seven biomarkers in female cancers. (**b**) Pie chart specifying the percentage distribution of 284 SNPs for 7 biomarkers in female cancers, where red colour represents the number of mutations in each gene.

Further, we analysed the percentage of mutation and sample size in all related human cancers (Figure 4a) and female cancers (Figure 4b), along with associated biomarkers (highlighted in seven colours). Table 2 summarises the mutation impact on protein structure and function, including amino acid (aa) polarity, charges and water affinity.

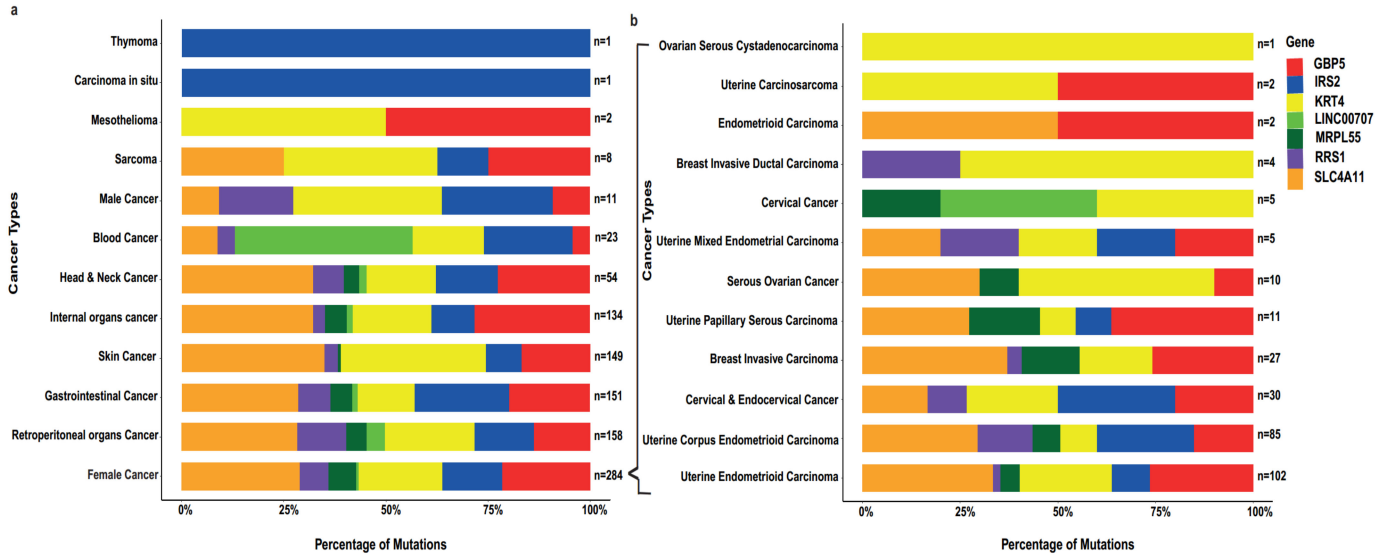

Figure 4. (**a**) Bar plot showing the sample size and percentage of mutation in seven biomarkers in each human cancer type, (**b**) with emphasis on female cancers.

Table 2. Data summary for the exon mutation samples used in this study from TCGA, UK BioBank and cBioPortal datasets to analyse the mutation impact at protein structure and function. Including amino acid polarity, charges and water affinity.

Feature	Count
Exon Mutation	**807 (100%)**
Non silent mutation	560 (69.39%)
Silent mutation	173 (21.43%)
Stop codon mutation	74 (9.16%)
Amino Acid Polarity	**560 (100%)**
Polar to Non-polar	104 (18.57%)
Non-polar to Polar	123 (21.96%)
No charge	333 (59.46%)
Amino Acid Charge	**560 (100%)**
Positive to Negative	1 (0.17%)
Positive to No charge	93 (16.60%)
No charge to Positive	37 (6.60%)
Negative to Positive	16 (2.85%)
Negative to No charge	31 (5.53%)
No charge to Negative	27 (4.82%)
No charge	355 (63.39%)
Amino Acid Water Affinity	**560 (100%)**
Hydrophobic to Hydrophilic	8 (1.42%)
Hydrophobic to Neutral	65 (11.60%)
Neutral to Hydrophobic	84 (15%)
Hydrophilic to Hydrophobic	47 (8.39%)
Hydrophilic to Neutral	76 (13.57%)
Neutral to Hydrophilic	46 (8.21%)
No charge	234 (41.78%)

We extracted the gynaecological cancer amino acid changes (n = 30) (Table 3) according to the selection criteria in Figure 5.

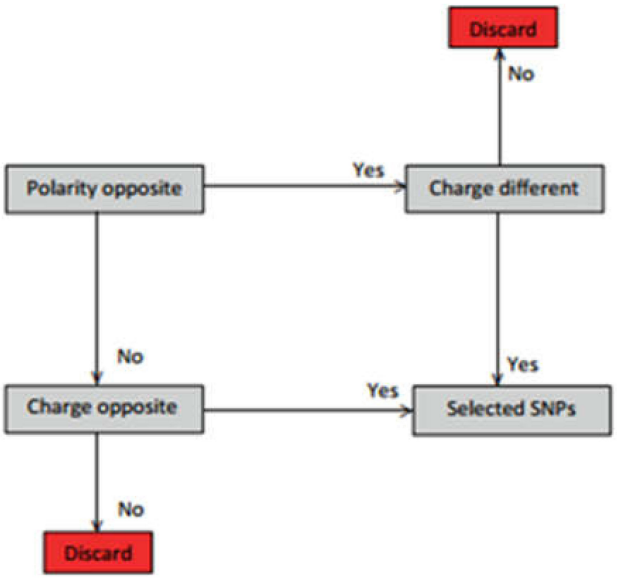

Figure 5. Amino acid change/SNP selection criteria according to the change in amino acid polarity and charge.

Table 3. Data summary of the gynaecological cancer amino acid changes, where n = 30, showing opposite polarity, charges and water affinity. 1—USCS Xena and 2—cBioPortal.

Database	Gene	Cancer Type	Amino Acid Change	Mutation
1/2	*GBP5*	Cervical and Endocervical Cancer	R520I	Missense
1/2	*GBP5*	Uterine Corpus Endometrioid Carcinoma	R450W	Missense
1/2	*GBP5*	Uterine Corpus Endometrioid Carcinoma	R290C	Missense
1/2	*GBP5*	Uterine Corpus Endometrioid Carcinoma	P415H	Missense
2	*GBP5*	Uterine Endometrioid Carcinoma	R396W	Missense
2	*GBP5*	Uterine Endometrioid Carcinoma	F267C	Missense
2	*IRS2*	Uterine Endometrioid Carcinoma	E1150K	Missense
1/2	*KRT4*	Ovarian Serous Cystadenocarcinoma	R49P	5′UTR
1/2	*KRT4*	Cervical and Endocervical Cancer	E238K/E312K	Missense
1/2	*KRT4*	Uterine Corpus Endometrioid Carcinoma	R196M/R270M	Missense
1/2	*KRT4*	Cervical and Endocervical Cancer	R9P/R83P	Missense
1/2	*KRT4*	Uterine Corpus Endometrioid Carcinoma	R27I/R101I	Missense
2	*KRT4*	Uterine Endometrioid Carcinoma	E509K	Missense
2	*KRT4*	Uterine Endometrioid Carcinoma	G84D	Missense
2	*KRT4*	Uterine Endometrioid Carcinoma	D507V	Missense
2	*KRT4*	Uterine Endometrioid Carcinoma	R270M	Missense
2	*KRT4*	Uterine Endometrioid Carcinoma	G578D	Missense
2	*MRPL55*	Uterine Endometrioid Carcinoma	G20R	Missense
2	*MRPL55*	Uterine Endometrioid Carcinoma	R96C	Missense
2	*MRPL55*	Uterine Endometrioid Carcinoma	P86H	Missense
1/2	*RRS1*	Uterine Corpus Endometrioid Carcinoma	R83C	Missense
1/2	*RRS1*	Uterine Corpus Endometrioid Carcinoma	L157R	Missense
1/2	*SLC4A11*	Uterine Corpus Endometrioid Carcinoma	R831C/R804C	Missense
1/2	*SLC4A11*	Cervical and Endocervical Cancer	R309C/R282C	Missense
1	*SLC4A11*	Uterine Corpus Endometrioid Carcinoma	R50M	Missense
2	*SLC4A11*	Serous Ovarian Cancer	R488M	Missense
2	*SLC4A11*	Uterine Endometrioid Carcinoma	R629W	Missense
2	*SLC4A11*	Uterine Endometrioid Carcinoma	D149V	Missense
2	*SLC4A11*	Uterine Endometrioid Carcinoma	E562K	Missense
2	*SLC4A11*	Uterine Endometrioid Carcinoma	R157C	Missense

2.2. Prediction of the Effects of R804C/R831C on SLC4A11 Protein Stability, Function and Physiochemical Properties

Out of 30 gynaecological cancer amino acid changes, only 1 amino acid change, at R831C/R804C, has detected the structural damage of the protein *SLC4A11*, therefore, we modelled this protein (SLC4A11) with SNP at R831C/R804C in uterine corpus endometrioid carcinoma (Figure 6). The reason for the 2 different positions is due to the presence of 3 distinct N-terminal variants of human *SLC4A11*: 918 amino acid splice form 1 (where the mutation is at position 831), 891 amino acid splice form 2 (where the mutation is at position 804) and 875 amino acid splice form 3 (where the mutation is at position 788) [18,19].

For the 918 amino acid variant, the R831C substitution does not alter the secondary structure, but this substitution leads to the expansion of cavity volume by 97.2 Å^3. Cavity also refers to a pocket on the surface (Figure 6). This substitution also results in a change between the buried and exposed state of the target variant residue. ARG is buried (RSA 7.6%) and CYS is exposed (RSA 20.7%). In the same protein, an increased z-score from −3.23 to −1.19 was noted, whereas for the mutant-type protein, the z-score changed from −3.24 to −1.16.

For the 891 amino acid variant, the R804C substitution does not alter the secondary structure, but this substitution leads to the expansion of cavity volume by 99.792 Å^3. Cavity also refers to a pocket on the surface (Figure 7). This substitution also results in a change between the buried and exposed state of the target variant residue. ARG is buried (RSA 6.8%) and CYS is exposed (RSA 20.0%). Similarly, an increased z-score from −3.22 to −1.09 was also recorded for the wildtype protein and a similar change (from −3.22 to −1.11) for the mutant.

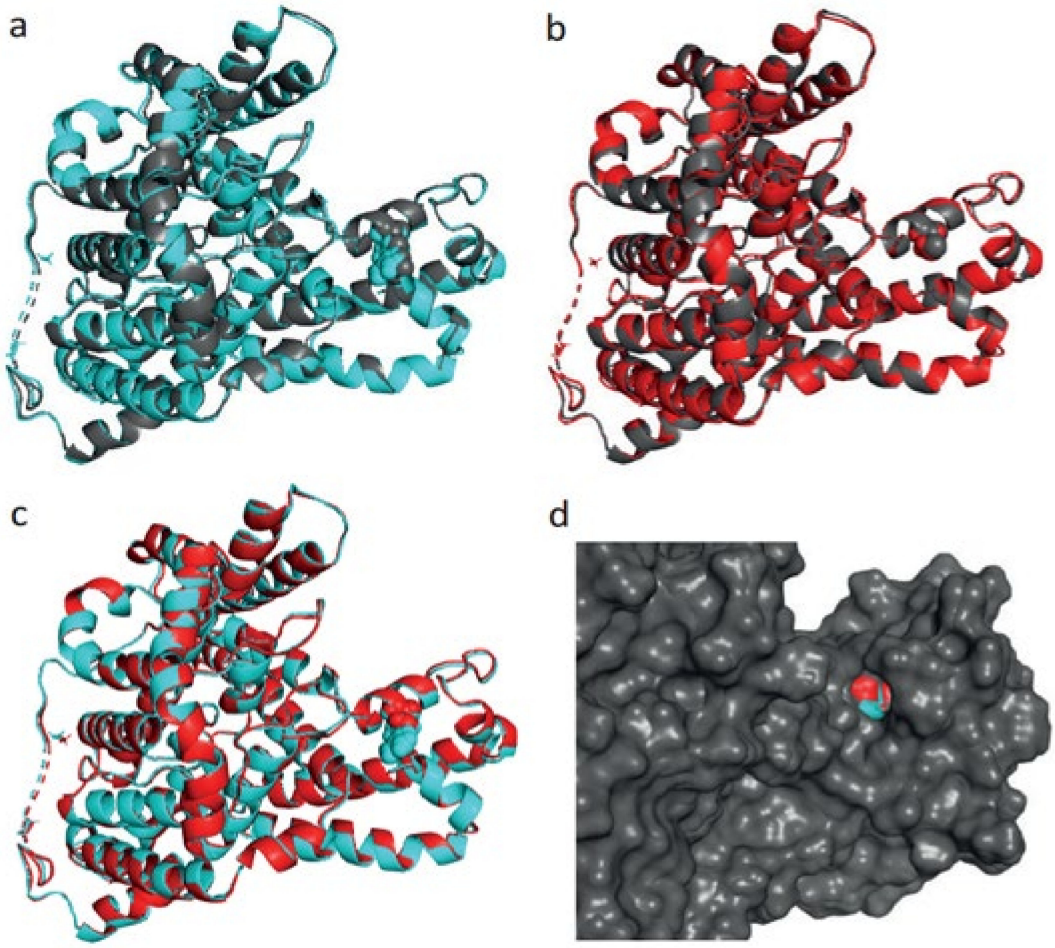

Figure 6. (**a**) Aligned structure of solute carrier family 4, sodium borate transporter, member 11 protein wildtype (918 aa, grey colour) and energy-minimised wildtype (cyan colour). (**b**) Aligned structure of SLC4A11 protein mutant (grey colour) and energy-minimised mutant (red colour). (**c**) Aligned structure of energy-minimised solute carrier family 4, sodium borate transporter, member 11 protein wildtype (cyan) and energy-minimised mutant (red). (**d**) Surface view of aligned structure of energy-minimised solute carrier family 4, sodium borate transporter, member 11 protein wildtype (cyan) and energy-minimised mutant (red).

Moreover, we created an electrostatic potential surface for solute carrier family 4, sodium borate transporter, member 11 protein (Figure 8). As the colour legend indicates, the red colour (negative potential) arises from an excess of negative charges near the surface and the blue colour (positive potential) occurs when the surface is positively charged. The white regions correspond to fairly neutral potentials.

Arginine (R) is a positively charged, polar and hydrophilic amino acid in proteins that has a profound role in protein structure and function that involves electrostatic interactions and protein solvation [20]. Alternatively, cysteine (C) is a non-polar, uncharged and hydrophobic amino acid, and the substitution from R to C may have a deleterious impact on the protein hydration and electrostatic interactions of the protein. When we used PROVEAN (Protein Variation Effect Analyzer), a software tool which predicts whether an amino acid substitution has an impact on the biological function of a protein, it provided a score of −7.292 with the annotation "Deleterious" for both R831C and R804C. The default score threshold is currently set at −2.5 for binary classification (i.e., deleterious vs. neutral).

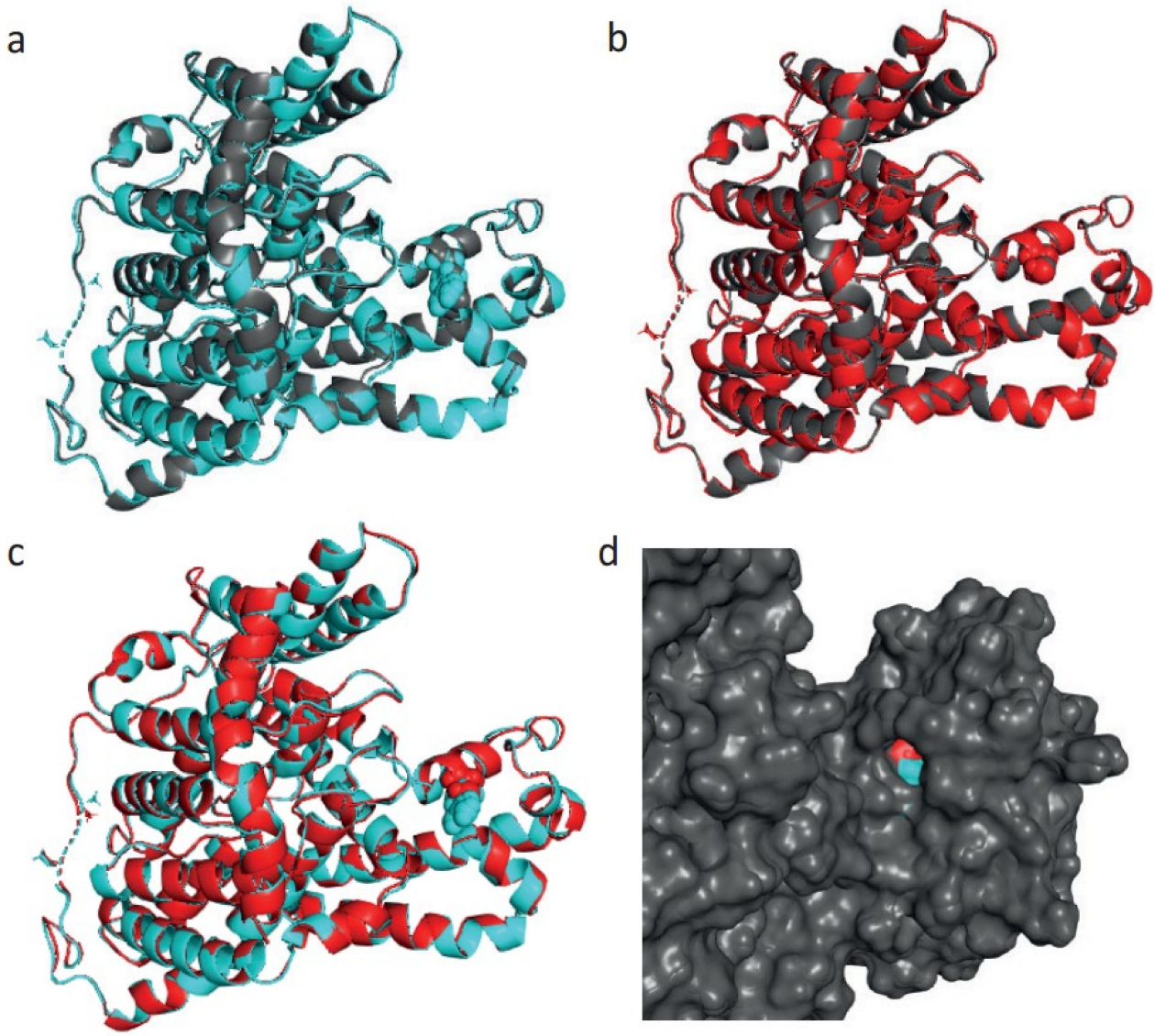

Figure 7. (**a**) Aligned structure of solute carrier family 4, sodium borate transporter, member 11 protein wildtype (891 aa, grey colour) and energy-minimised wildtype (cyan colour). (**b**) Aligned structure of SLC4A11 protein mutant type (grey colour) and energy-minimised mutant type (red colour). (**c**) Aligned structure of energy-minimised solute carrier family 4, sodium borate transporter, member 11 protein wildtype (cyan) and energy-minimised mutant type (red). (**d**) Surface view of aligned structure of energy-minimised SLC4A11 protein wildtype (cyan) and energy-minimised mutant type (red).

We have further evaluated changes in protein stability using MUpro: Prediction of Protein Stability Changes for Single-Site Mutations from Sequences [21,22], where Delta Delta G (DDG), a metric for predicting how a single point mutation will affect protein stability, was measured. In both variants, the predicted DDG was −0.704, suggesting a decrease in protein stability. Similar data were obtained from the BIOCOMP.UNIBO prediction server [23], with a DDG of −0.67 and a prediction of a disease-related mutation. Finally, we have used the DeepDDG server [24] that predicts the stability change of protein point mutations using neural networks and calculated a DDG value of −1.802 (kcal/mol).

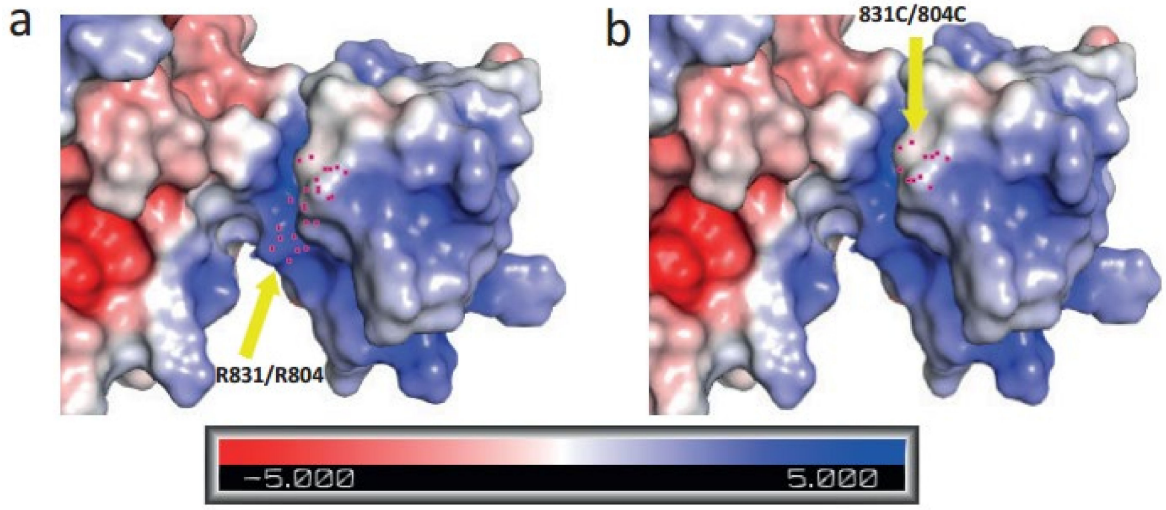

Figure 8. (**a**) An electrostatic potential surface of wildtype solute carrier family 4, sodium borate transporter, member 11 protein indicating amino acid residue ARG at position 831/804. (**b**) An electrostatic potential surface of mutant-type protein indicating amino acid residue CYS at position 831/804. In the colour legend, the red colour indicates negative potential, the blue colour indicates positive potential of the protein surface and the white regions correspond to fairly neutral potentials. Yellow arrow indicates towards the mutation site at position 831/804.

3. Discussion

In this study, we provided a comprehensive overview of a wide repertoire of mutations of seven recently predicted biomarkers for OC that can be acquired using a number of in silico tools. These 7 genes present with 976 SNPs/mutations that are associated with human cancers, out of which 284 are related to female cancers that include ovarian, cervical, endometrial cancer, as well as endometrioid and uterine carcinomas. The most prevalent type of mutation occurring on six (i.e., *GBP5*, *IRS2*, *KRT4*, *MRPL55*, *RRS1* and *SLC4A11*) out of seven genes was missense mutation, followed by silent and 3′untranslated region (3′UTR) mutations. In the case of *LINC00707*, being a long non-coding RNA (lncRNA), non-coding transcript exon and intron mutations were the only two types identified in both all cancers and female ones. In both cases, *SLC4A11* had the largest percentage of mutations out of all 7 genes at 29.4% and 28.9%, respectively.

In missense mutations, there is a change of a single nucleotide, resulting in a codon that can produce a different amino acid. Using the Human Genome Database as a paradigm, it is evident that several missense mutations are linked with inherited predispositions to malignancies [25]. For example, in a recent analysis of more than 113,000 women, missense variants for *BRCA1*, *BRCA2* and *TP53* were associated with a risk of breast cancer [26]. Equally, a number of studies have indicated that mutations at the 3′UTR can drive oncogene activation or inactivation of tumour suppressors by altering the binding efficiency of microRNAs [27,28]. For example, a *GAPDH* mutation in the 3′UTR creates a miR-125b binding site, and as a result facilitates the development of OC [27].

On the other hand, the mutational landscape for the lncRNA *LINC00707* is quite different. We know that lncRNAs exhibit a complex biology and are involved in a number of processes, including gene transcription or gene silencing [29]. Although there is no published data on intronic mutations and their impact on *LINC00707*, a recent study highlighted their importance in cancer, since 64 tumour suppressors were affected by intronic mutations, and blood cancers showed higher proportions of deep intronic mutations [30].

We have then provided a deeper insight into the percentage of mutation of each of the seven genes of interest in all cancers and in female cancers. For the latter, the largest percentage (28.9%) was attributed to *SLC4A11*, with *GBP5* and *KRT4* exhibiting a high percentage as well (21.5% and 20.4%, respectively). In this cohort of cancers, the largest datasets were of uterine endometrioid carcinoma (n = 102) and uterine corpus endometrioid

carcinoma (UCEC; n = 85). UCEC is the most common female pelvic malignancy, and the sixth most common gynaecological malignancy in females, with an estimated 417,367 new cases and 97,370 deaths worldwide in 2020 [31]. Despite the wide repertoire of therapeutic options for UCEC, there is an increase in the incidence of endometrial cancer. Of note, numerous shared and cancer type-specific mutation signatures have been identified, with UCEC depicting a number of clusters with distinct mutation frequencies [32]. Out of the seven genes in question, only one study associates the IRS2 polymorphism G1057D with endometrial cancer [33].

We then analysed the mutation impact on amino acid polarity, charge and water affinity, leading to the identification of 30 mutations in gynaecological cancers where amino acid changes lead to opposite polarity, charges and water affinity. Out of 30 gynaecological cancer amino acid changes, only missense mutation (i.e., R831C/R804C in UCEC) was suggestive of structural damage on the solute carrier family 4, sodium borate transporter, member 11 protein. Therefore, we modelled this protein and provided in silico evidence of how a change from arginine (R) to cysteine (C) can exert potential deleterious consequences.

SLC4A11 is a member of the SLC4 family of bicarbonate transporters that is primarily expressed as an integral membrane protein, with aberrant expression in the cornea, thyroid, salivary gland and kidney. This transporter is also involved in sodium-mediated fluid transport in different tissues. The human *SLC4A11* gene encodes three splice variants at the NH2 terminus. These include the 918 variant A, the 891 amino acid variant B and the 875 amino acid variant C [18,19]. Of these, according to UniProt, SLC4A11-B is the canonical sequence. To date, most of the work on *SLC4A11* is concentrated on corneal dystrophies. Indeed, mutations of *SLC4A11* are the cause of congenital hereditary endothelial dystrophy (CHED) and some cases of late-onset Fuchs endothelial corneal dystrophy (FECD) [18]. Interestingly, one the mutations found in families with autosomal recessive corneal endothelial dystrophy (CHED2) was on arginine 804 (G804A). The authors of the study argued that the mutation can alter the hydrophobic interaction of methyl groups located in the arginine stem, thus impacting on the loop stability [34].

In this study, we have shown that (1) the R831C/R804C mutation is deleterious and (2) predicted ΔΔG values suggest that the mutation reduces the stability of the protein. As mentioned, DDG is the change in Gibbs free energy (Gibbs free energy (G) = Enthalpy (H) − Temperature (T) × Entropy (S)) [24]. There is also a strong structural explanation for the change in stability: Arg-831 is in a salt bridge with nearby Glu-519, so R831C will have a large enthalpic impact. However, we acknowledge that it is difficult to further dissect the functional impact of this change in stability without embarking on in vitro studies, mutating the protein in cellular models of UCEC. We also acknowledge that the cavity hypothesis is limited by the neglect of protein–membrane interactions in YASARA. Very recently, a new artificial intelligence system (AI) that predicts 3D protein structures with high accuracy has emerged, termed AlphaFold [35]. Subsequently, we have modelled our predicted structures of the two SLC4A11 protein variants with that of AlphaFold and there is 100% alignment in the R804 transmembrane region (Supplementary Figure S1), suggesting a conserved 3D configuration irrespective of the modelling software.

In terms of its role in female reproductive organs, the only data available come from a study in OC, where high expression of *SLC4A11* is a predictor for poor overall survival in serous OC (grade 3/4) [36]. Leveraging data from TCGA and GTEX, we also demonstrated significant upregulation of *SLC4A11* in UCEC (Supplementary Figure S2). Future studies should concentrate on gaining a deeper understanding of the actual role of this transporter protein in UCEC and how this deleterious mutation might affect its function, as the normal function(s) of *SLC4A11* in gynaecological malignancies still remains unclear.

4. Materials and Methods

4.1. Data Availability

Xena Repository: Somatic mutation data and sample phenotype information were extracted from the data generated by The Cancer Genome Atlas (TCGA) research network

and TCGA somatic mutations (Pan-cancer Atlas), as published in the Xena repository hosted at the University of California Santa Cruz (UCSC) [37].

UK BioBank: Genetic variation/mutation data were extracted from PhenoScanner (version 2), which is a curated database holding publicly available results from large-scale genome-wide association studies (GWAS) for the UK Biobank data. This tool helps to facilitate "phenome scans", the cross-referencing of genetic variants with a broad range of phenotypes, to help aid the understanding of disease pathways and biology.

cBioPortal: Genomic alterations across a set of patients were quarried from cBioPortal (for cancer genomics), an exploratory analysis tool for exploring large-scale cancer genomic datasets that hosts data from large consortium efforts, such as TCGA and TARGET, as well as publications from individual labs. The cBioPortal assists to explore specific genes or a pathway of interest in one or more cancer types.

Statistical Analysis: All unstructured data gathering, processing, modelling and statistical analyses were conducted using R (v. 4.1.0, The R Foundation for Statistical Computing, Vienna, Austria) under the R Studio desktop application (version 1.4.1717, RStudio, Boston, MA, USA).

4.2. Protein Structure Prediction Tools

UniProt Knowledgebase: The amino acid sequence of the protein of interest was extracted from the UniProt Knowledgebase (UniProtKB) (https://www.uniprot.org (accessed on 10 November 2021)), which is the central hub for the collection of functional information on proteins, with accurate, consistent and rich annotation. It records the information extracted from the literature and curator-evaluated computational analysis.

Protein Data Bank (RCSB PDB): We used the Protein Data Bank (PDB) (https://www.rcsb.org (accessed on 10 November 2021)) to gather the known protein structure information of our genes of interest. It is the single worldwide archive of structural data of biological macromolecules. It includes data obtained by X-ray crystallography and nuclear magnetic resonance (NMR) spectrometry submitted by biologists and biochemists from all over the world.

Phyre2: In order to predict the three-dimensional (3D) structure of our desired protein sequence/gene, we used Phyre2 (v. 2.0). The software assists with the construction of 3D models of our protein of interest based on the alignments between the hidden Markov model (HMM) of the desired sequence and the HMMs of known structure.

SWISS-MODEL: We also used a fully automated 3D protein structure homology-modelling server, SWISS-MODEL (https://swissmodel.expasy.org/ (accessed on 10 November 2021)), to predict the 3D structure of our desired protein sequence. Homology modelling is currently the most accurate method to generate reliable 3D protein structure models, as it makes use of experimental protein structures ("templates") to build models for evolutionary-related proteins ("targets").

AlphaFold: The Protein Structure Database (https://alphafold.ebi.ac.uk/ (accessed on 10 November 2021)), an AI system which is able to computationally predict protein structures with unprecedented accuracy and speed, was also used to predict the 3D structure.

Missense3D: Structural changes introduced by an amino acid substitution/SNP were measured and predicted by the Missense3D tool (http://missense3d.bc.ic.ac.uk/missense3d (accessed on 10 November 2021)).

YASARA Energy Minimisation Server: Energy minimisation of the protein was performed using the YASARA server (http://www.yasara.org/minimizationserver.htm (accessed on 10 November 2021)), and the YASARA application (v. 21.8.26) was used to view and save the 3D energy-minimised structure in PDB format.

PyMOL: Electrostatic potential surfaces, electron densities and three-dimensional (3D) visualisation of proteins were analysed by PyMOL (v. 2.4.1), which is an open-source molecular visualisation platform.

PROVEAN: Impacts on the biological function of protein sequence variations including single or multiple amino acid substitutions were predicted by the PROVEAN (Protein

Variation Effect Analyzer) (v. 1.1) tool (http://provean.jcvi.org/ (accessed on 10 November 2021)) [38].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23031725/s1.

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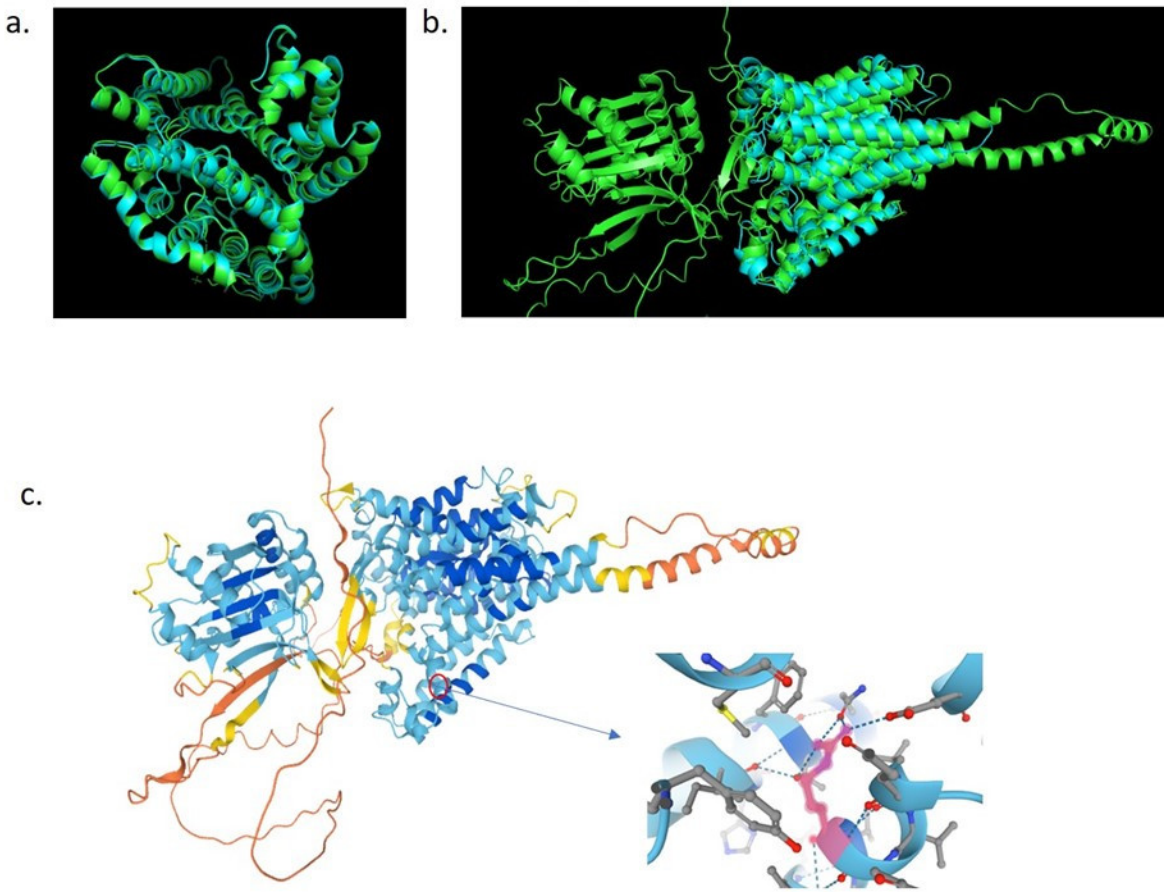
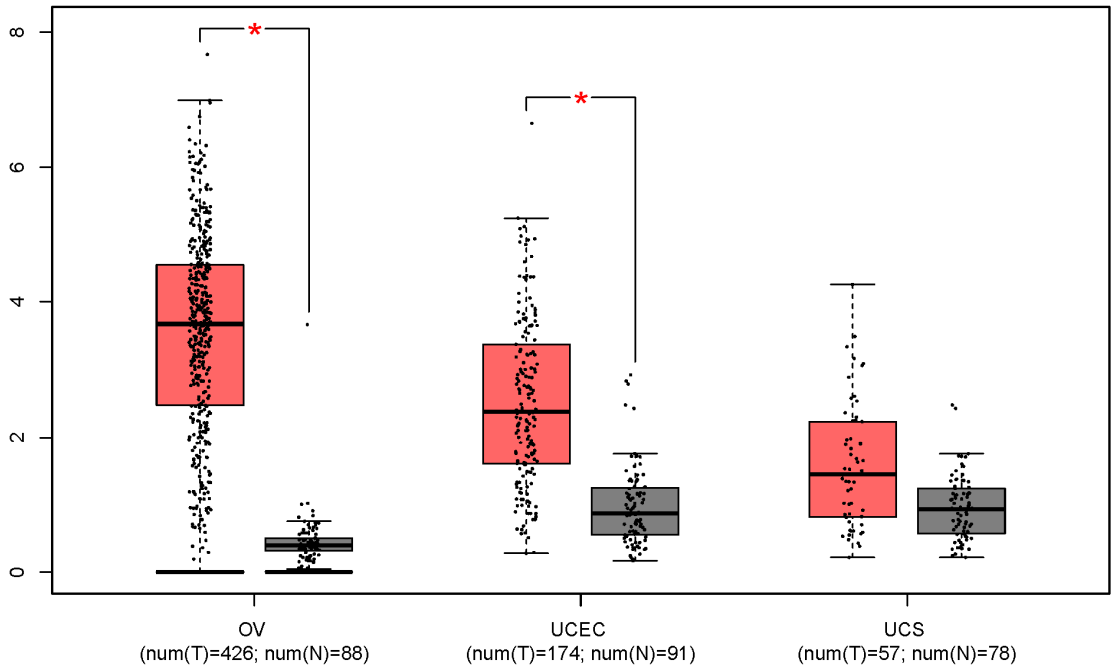
Supplementary Materials



Supplementary Figure S1. Panel (**a**): Alignment of the 891 and 918 amino acid (aa) variants of SLC4A11. Panel (**b**): Alignment of 891aa based on Swiss Model (blue) with AlphaFold (green). Panel (**c**): Predicted structure of SLC4A11 from AlphaFold, with R804C (red circle; insert confidence score 89.89) demonstrating full alignment with previous predictions.



Supplementary Figure S2. Expression of SLC4A11 in ovarian cancer (OV), uterine corpus endometrial carcinoma (UCEC) and uterine carcinosarcoma (UCS). * p <0.05. T: tumour, N: normal, num: number of patients.

Chapter 4

Impact of Environmentally Relevant Concentrations of Bisphenol A (BPA) on the Gene Expression Profile in an In Vitro Model of the Normal Human Ovary

Statement of Contribution

In this manuscript I led and contributed the following parts:

- Conceptualization.
- Methodology
- Formal analysis
- Data curation
- Writing—original draft preparation
- Writing—review and editing
- Referencing
- Funding acquisition

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Impact of Environmentally Relevant Concentrations of Bisphenol A (BPA) on the Gene Expression Profile in an In Vitro Model of the Normal Human Ovary

Aeman Zahra [1], Rachel Kerslake [1], Ioannis Kyrou [2,3,4,5,6], Harpal S. Randeva [2,3,4], Cristina Sisu [1,*] and Emmanouil Karteris [1,*]

1 Department of Life Sciences, Division of Biosciences, College of Health, Medicine and Life Sciences, Brunel University London, Uxbridge UB8 3PH, UK; aeman.zahra@brunel.ac.uk (A.Z.); Rachel.Kerslake3@brunel.ac.uk (R.K.)
2 Warwickshire Institute for the Study of Diabetes, Endocrinology and Metabolism (WISDEM), University Hospitals Coventry and Warwickshire NHS Trust, Coventry CV2 2DX, UK; kyrouj@gmail.com (I.K.); harpal.randeva@uhcw.nhs.uk (H.S.R.)
3 Warwick Medical School, University of Warwick, Coventry CV4 7AL, UK
4 Centre for Sport, Exercise and Life Sciences, Research Institute for Health & Wellbeing, Coventry University, Coventry CV1 5FB, UK
5 Aston Medical Research Institute, Aston Medical School, College of Health and Life Sciences, Aston University, Birmingham B4 7ET, UK
6 Laboratory of Dietetics and Quality of Life, Department of Food Science and Human Nutrition, School of Food and Nutritional Sciences, Agricultural University of Athens, 11855 Athens, Greece
* Correspondence: cristina.sisu@brunel.ac.uk (C.S.); emmanouil.karteris@brunel.ac.uk (E.K.)

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Abstract: Endocrine-disrupting chemicals (EDCs), including the xenoestrogen Bisphenol A (BPA), can interfere with hormonal signalling. Despite increasing reports of adverse health effects associated with exposure to EDCs, there are limited data on the effect of BPA in normal human ovaries. In this paper, we present a detailed analysis of the transcriptomic landscape in normal Human Epithelial Ovarian Cells (HOSEpiC) treated with BPA (10 and 100 nM). Gene expression profiles were determined using high-throughput RNA sequencing, followed by functional analyses using bioinformatics tools. In total, 272 and 454 differentially expressed genes (DEGs) were identified in 10 and 100 nM BPA-treated HOSEpiCs, respectively, compared to untreated controls. Biological pathways included mRNA surveillance pathways, oocyte meiosis, cellular senescence, and transcriptional misregulation in cancer. BPA exposure has a considerable impact on 10 genes: *ANAPC2*, *AURKA*, *CDK1*, *CCNA2*, *CCNB1*, *PLK1*, *BUB1*, *KIF22*, *PDE3B*, and *CCNB3*, which are also associated with progesterone-mediated oocyte maturation pathways. Future studies should further explore the effects of BPA and its metabolites in the ovaries in health and disease, making use of validated in vitro and in vivo models to generate data that will address existing knowledge gaps in basic biology, hazard characterisation, and risk assessment associated with the use of xenoestrogens such as BPA.

Keywords: endocrine-disrupting chemicals; EDC; Bisphenol A; BPA; ovary; ovarian cancer

1. Introduction

Endocrine-disrupting chemicals (EDCs) are widespread in the environment, from manufacturing to packaging and waste materials. Once in the environment, EDCs can accumulate throughout food chains and have the potential to disturb the normal endocrine functions of organisms [1,2]. Notably, EDCs are not readily metabolised by the body and accumulate within tissues due to their lipophilic properties, whilst this accumulation appears to be associated with a diverse spectrum of health issues [1,3].

Bisphenol A (BPA) is one of the most common and thoroughly studied EDCs, representing one of the highest manufactured chemicals globally [4,5]. The world production of

BPA is estimated to reach over 7348 K tonnes annually by the end of 2023 [6]. BPA is widely used as a monomer to manufacture polycarbonate plastics and metal tins [7]. Accordingly, due to its presence in numerous commercial products—ranging from food packaging and food contact materials to thermal paper, cosmetics, dust and medical materials—humans are exposed to BPA on a daily basis [8]. The most common routes of human BPA exposure are inhalation, ingestion, and transdermal contact [9,10]. Of note, studies have shown that the levels of accumulated BPA within human adipose tissue lie between 8 nM and 80 nM [11]. Interestingly, infants aged 0–6 months that are exclusively fed with canned formula milk and using polycarbonate bottles have been estimated to have the highest BPA exposure [12,13]. Such exposure during the developmental stages makes humans particularly vulnerable to harmful effects of BPA and other EDCs since their effects occur during crucial stages of organogenesis and tissue development that are normally mediated/controlled by finely regulated molecular and biochemical processes [14].

At a molecular level, BPA mimics the hormone estrogen and can therefore interfere with estrogen signalling pathways [8,15,16]. The estrogen signalling pathway is controlled at the genomic level by estrogen receptors ERα and ERβ; the non-genomic level by G protein-coupled receptor 30, GPR30; or GPER [17]. Particularly, GPR30 plays a role in reproductive physiology [18] and in the stimulation of female reproductive neoplasms, specifically breast, endometrial, ovarian, and cervical [19]. Accordingly, several studies have raised the possibility of a direct link between BPA and hormone-dependent cancers (e.g., ovarian, breast, and prostate cancer) [20,21].

Over the past decade, there have been a number of studies pointing toward the adverse effects of BPA on female reproductive tissues in both human and animal studies. For example, BPA was found to exert effects on normal ovaries, with oocyte abnormalities noted in adult mice exposed to BPA [22], whereas rats exposed to BPA (10 $mg \cdot kg^{-1} \cdot day^{-1}$) accelerated pubertal development [23]. BPA also disrupts and increases oocyte degeneration in human oocytes and meiotic maturation [24]. In a recent study of 106 women undergoing in vitro fertilisation–embryo transfer (IVF-ET), a significant decrease in embryo implantation rate was observed in the group with elevated BPA levels [25]. In the same study, BPA induced autophagy in human granulosa cells, involving the mTOR pathway. In a zebrafish model, low-dose exposure to BPA caused changes in oxidative stress response and metabolic fluxes that can potentially induce the premature maturation of oocytes [26]. Alterations in other reproductive tissues were also noted upon treatment with BPA. For example, prenatal BPA exposure in rhesus macaque altered the percentage of different cells in the fetal oviduct [27], and exposure of albino rats to BPA led to the degeneration of the vaginal epithelium [28]. In addition, CD1 mice treated with BPA exhibited uterine polyps and sarcoma of the uterine cervix [29]. In a recent meta-analysis and systematic review, an association was shown between higher BPA exposures and a higher risk of preterm birth [30]. Moreover, our group showed that BPA can drive post-translational modifications, alter cell proliferation, and induce gene changes in a placental in vitro model [31]. In terms of large-scale human epidemiological data on the effects of BPA, they are limited (source: epa.gov, accessed on 27 March 2022).

In this paper, we present an analysis of the genomic activity landscape in normal Human Epithelial Ovarian Cells (HOSEpiC) under the influence of BPA. We found that 76 genes are solely dysregulated ($p < 0.05$) in the presence of the environmental doses of BPA, and we proceeded to functionally annotate them and evaluate their potential as disease drivers.

2. Results

2.1. Identification of Differentially Expressed Genes (DEGs)

HOSEpiC cells were treated with 10 nM and 100 nM BPA treatments for 24 h (3 biological replicates), and DEGs were identified using the multiple-testing module from Cuffdiff, with significant changes defined based on a p-value < 0.05. To visualise the gene-expression profiles across all doses and replicates, volcano plots were generated using information

from the statistical significance data (*p*-value) and the magnitude of change (fold change) between two conditions: BPA 10 nM vs. control (Figure 1) and BPA 100 nM vs. control (Figure 2).

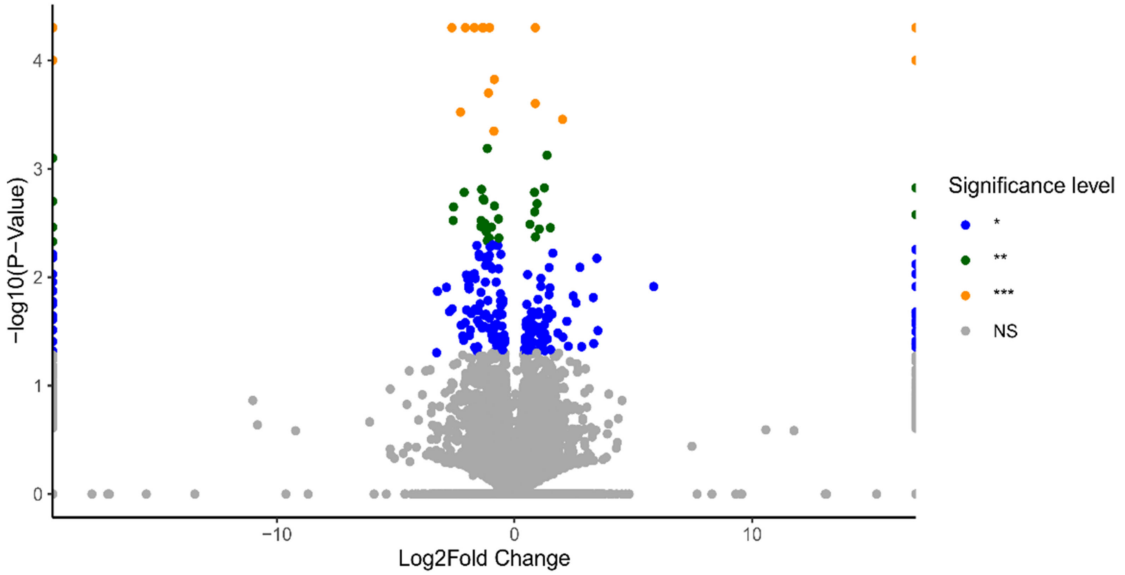

Figure 1. Volcano plot presenting all the differentially expressed genes (DEGs) upon the treatment of BPA 10 nM. Significance level for these gene was set as (blue dots * *p*-value < 0.05, green dots ** *p*-value < 0.005, orange dots *** *p*-value < 0.0005, and grey dots for no significant change (NS).

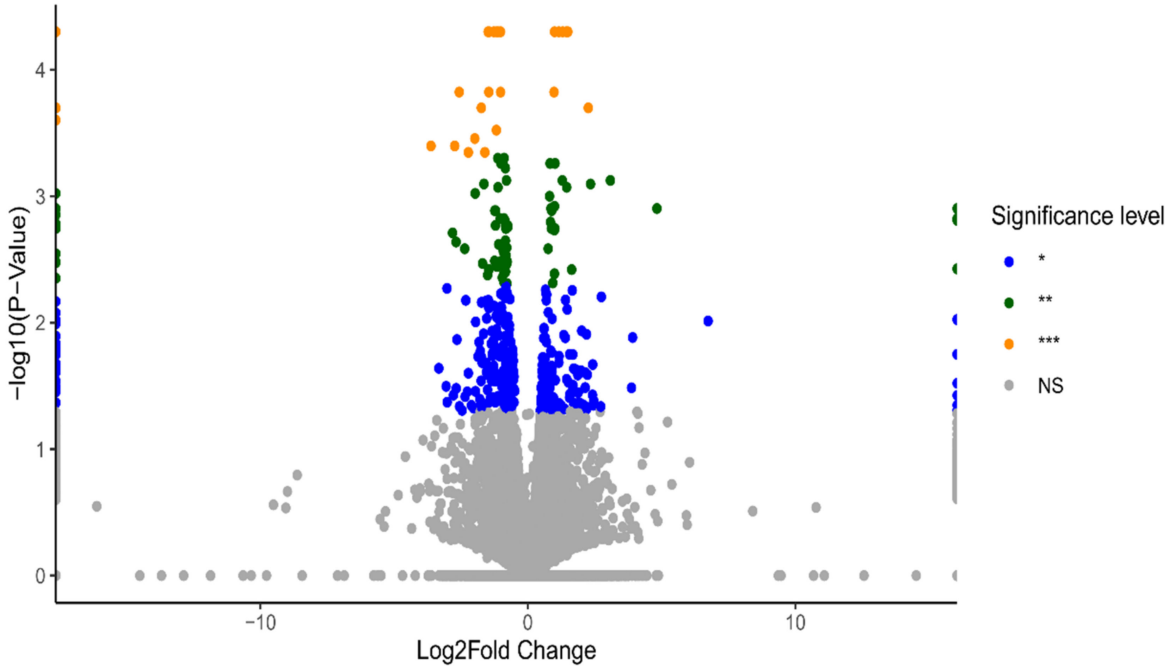

Figure 2. Volcano plot presenting the differentially expressed genes (DEGs) upon the treatment of BPA at 100 nM. Significance level for these gene was set as: blue dots * *p*-value < 0.05, green dots ** *p*-value < 0.005, orange dots *** *p*-value < 0.0005, and grey dots for no significant change (NS).

In total, 272 DEGs were identified in 10 nM BPA-treated HOSEpiC samples and 454 DEGs in the 100 nM BPA-treated ones compared to the control group. Among the DEGs identified in both groups, 76 genes were found to be commonly dysregulated irrespective of the level of BPA exposure (Figure 3).

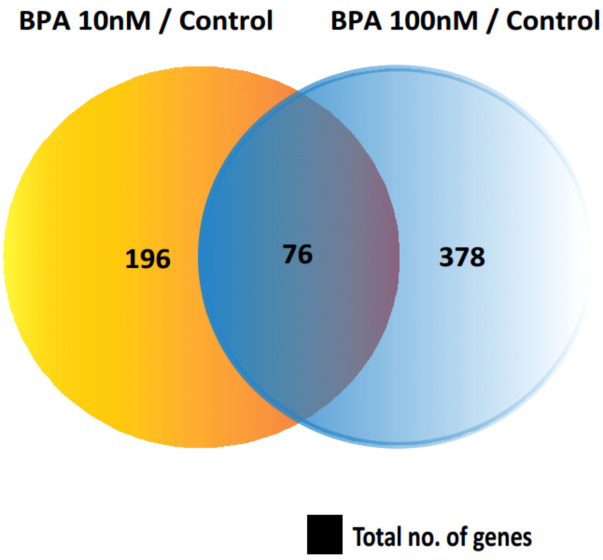

Figure 3. Venn diagram indicates the overlap of differentially expressed genes (DEGs) in cells treated with 10 nM and 100 nM BPA compared with the control group.

Furthermore, hierarchical clustering in the 76 differential gene-expression profiles for 10 nM and 100 nM BPA treatment demonstrated similarities in both upregulated (n = 10) and downregulated (n = 66) DEGs compared to non-treated (control group) HOSEpiC cells (Figure 4). The heatmap depicts the expression of each gene in all the samples from the different groups in the experiment (BPA 10 nM, BPA 100 nM, and untreated (control) groups).

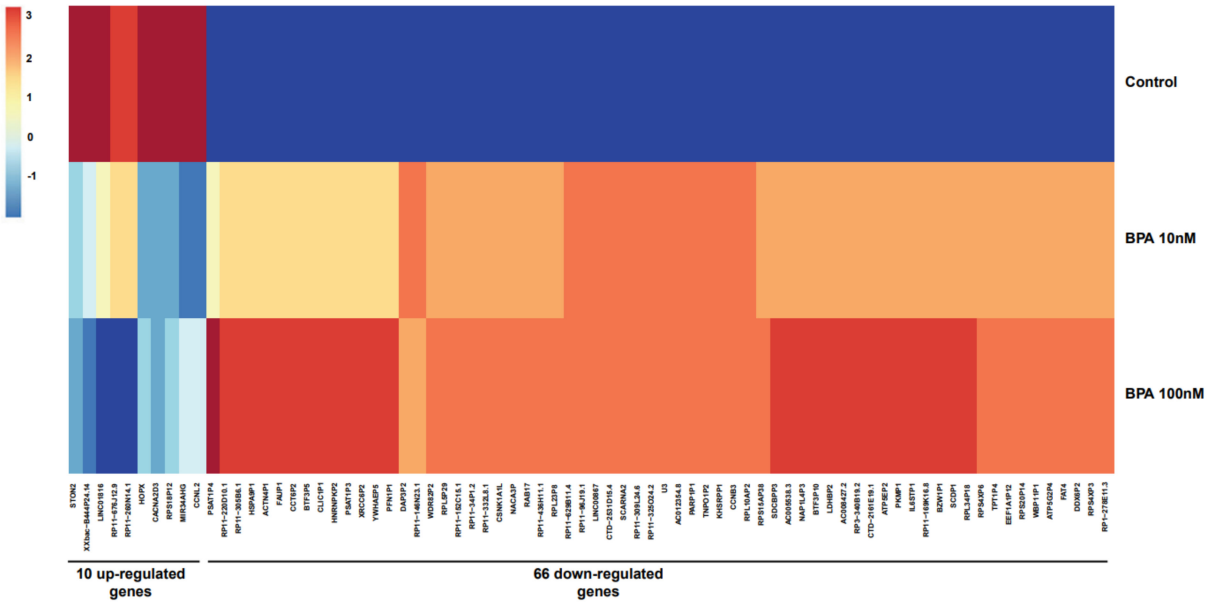

Figure 4. Heatmap reproduced expression profile for genes differently regulated ($p < 0.05$) over two used BPA doses (10 nM and 100 nM) and control group. Dark blue indicates low expression, and deep red indicates high expression.

2.2. Functional Annotation Analysis of the DEGs

Next, DEGs with cut-off criteria of $p < 0.05$ and [Log2FC] > 1 were selected for subsequent functional analysis (Figure 5). In total, 70 out of 196 DEGs by BPA 10 nM exposure were previously described in the literature and were identified by the functional

annotation FunRich database. An additional 286 out of 378 DEGs were recognised by the functional annotation FunRich database for the 100 nM BPA exposure.

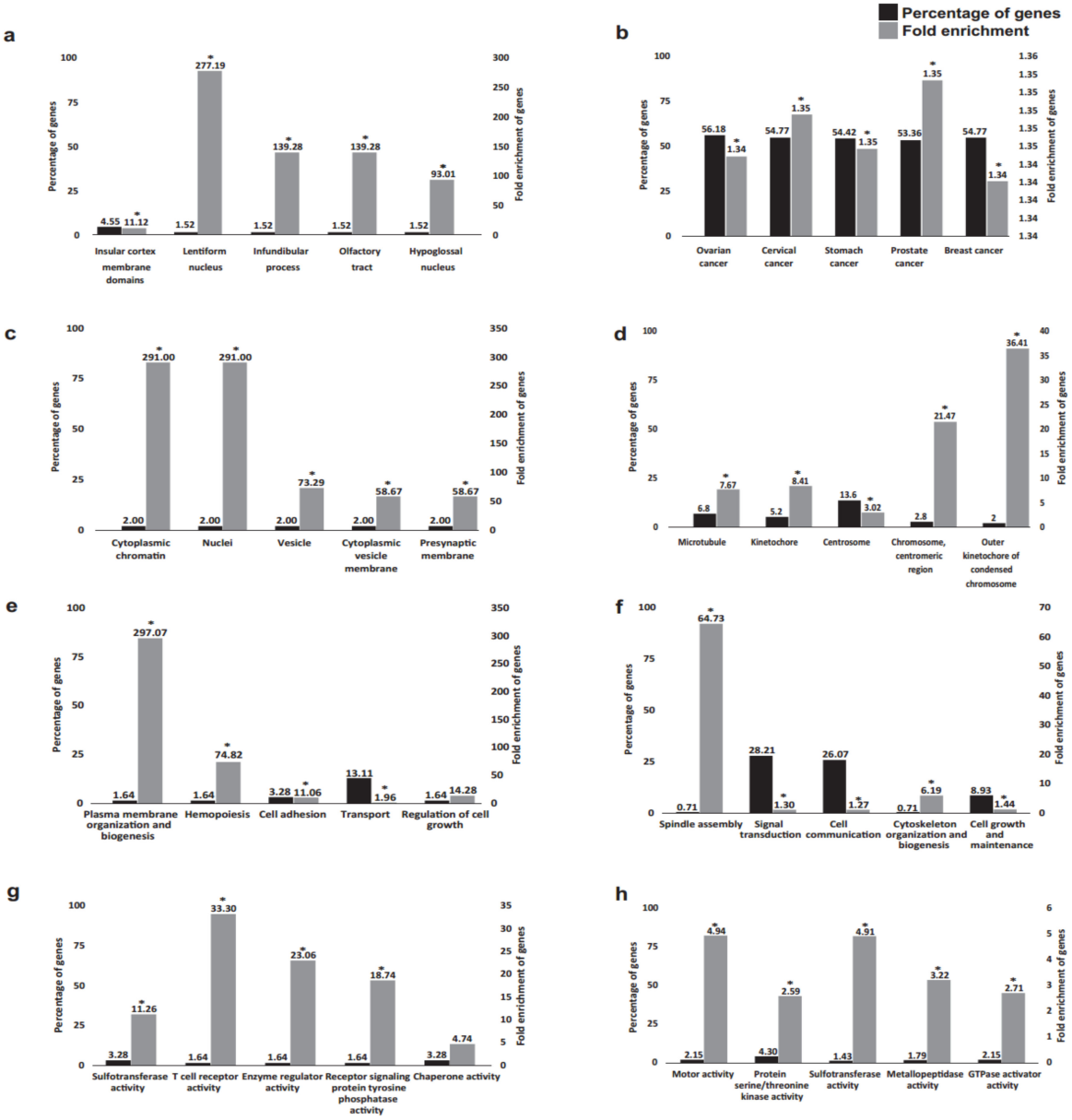

Figure 5. The functional enrichment in Gene Ontology terms in BPA 10 nM exposure DEGs (**a**,**c**,**e**,**g**) and BPA 100 nM exposure DEGs (**b**,**d**,**f**,**h**) in relation to site of expression (**a**,**b**), cellular components (**c**,**d**), biological processes (**e**,**f**), and molecular functions (**g**,**h**). * $p < 0.05$.

Gene Ontology (GO) analysis indicated that the majority of genes affected by exposure to 100 nM BPA are also dysregulated in various female cancers (specifically, 159 genes in ovarian cancer and 155 genes in cervical and breast cancer). Notably, the current literature describes the impact of BPA exposure for only 2 genes out of the 76 identified by our study (Supplementary Figure S1).

Furthermore, we looked at identifying the biological pathways associated with the three sets of DEGs: 10 nM BPA ($n = 78$)-specific, 100 nM BPA ($n = 289$)-specific, and common DEGs over these two doses ($n = 13$) (Figure 6a–c).

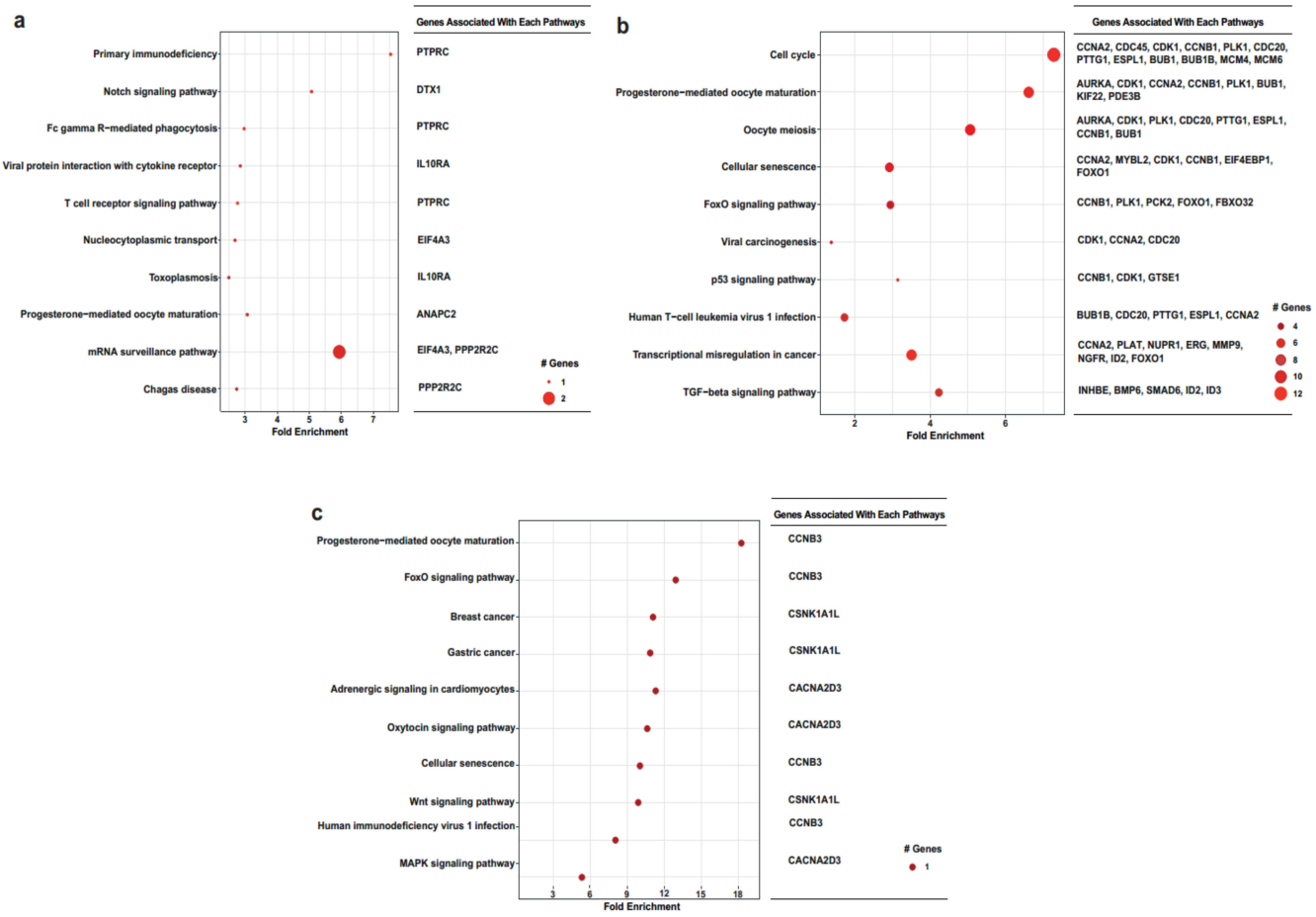

Figure 6. Biological pathways associated with the exposure of the different environmental doses of BPA (10 nM (**a**) and 100 nM (**b**)) dysregulated genes, along with shared common DEGs of these two doses (**c**).

The results show that BPA exposure has a considerable impact on 10 genes: *ANAPC2*, *AURKA*, *CDK1*, *CCNA2*, *CCNB1*, *PLK1*, *BUB1*, *KIF22*, *PDE3B*, and *CCNB3*, which are also associated with progesterone-mediated oocyte maturation pathways. Studies have suggested that exposure to BPA may cause an increase in meiotic disturbances in mice, such as aneuploidy in oocytes [32,33]. It is well documented that exposure to BPA in the prenatal period is associated with cystic endometrial hyperplasia, ovarian cysts, aneuploidy in oocytes, and a reduction in the primordial pool of follicles in mouse ovaries, indicating an association between BPA and the increased proliferation of ovarian cells mediated by estrogenic pathway [33–35].

Finally, we investigated biological pathways from the Kyoto Encyclopedia of Genes and Genome (KEGG) and Comparative Toxicogenomics Database (CTD) using the shared DEGs in the two used BPA doses (Figure 7). Accordingly, we found that the DEGs are mainly involved in pathways associated with human diseases, particularly cancer (Figure 7a) and various infectious diseases (viral, bacterial, and parasitic); environmental information processing (Figure 7b); cellular processes, including cell growth and death (Figure 7c); and organismal systems, i.e., the endocrine system (Figure 7d). Furthermore, 30 pathways have been previously described in the literature as being impacted by BPA exposure (Figure 7e). Out of those 30 pathways, 13 pathways (Table 1) were common between the 2 databases.

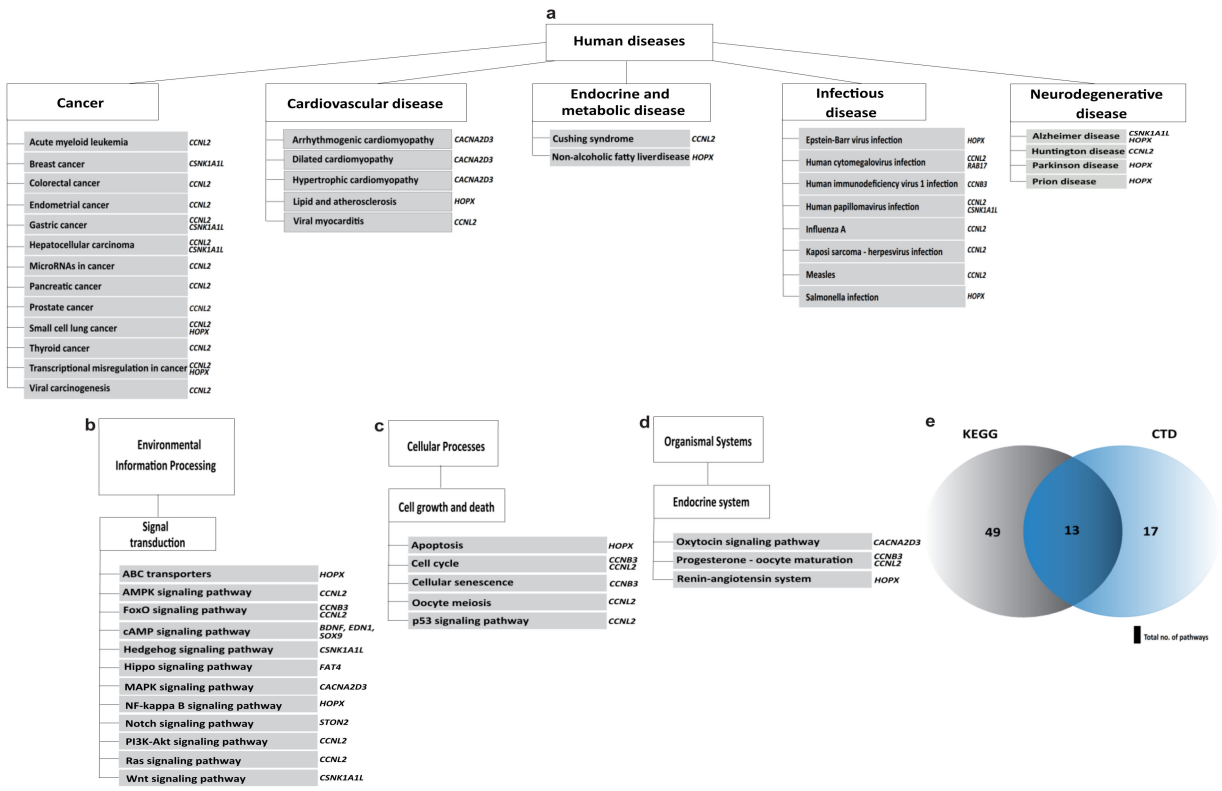

Figure 7. Biological pathways associated with BPA-dysregulated genes in humans. (**a**) Human-disease-associated pathways. (**b**) Environmental information processing pathways. (**c**) Cellular-processes-associated pathways. (**d**) Endocrine-system-associated pathways. (**e**) Venn diagram presenting the common pathways in KEGG- and BPA-impacted pathways reported in CTD. Genes that affect each pathway are shown on the right corner of each block.

Table 1. In existing literature, 13 common pathways have been previously described as being impacted by BPA exposure with associated DEGs from this study.

Pathways	Associated Genes
Arrhythmogenic right ventricular cardiomyopathy	CACNA2D3
Breast cancer	CSNK1A1L
Cell cycle	CCNB3, CCNL2
Dilated cardiomyopathy	CACNA2D3
FoxO signalling pathway	CCNB3, CCNL2
Hedgehog signalling pathway	CSNK1A1L
Hippo signalling pathway	FAT4
Hypertrophic cardiomyopathy (HCM)	CACNA2D3
MAPK signalling pathway	CACNA2D3
Oxytocin signalling pathway	CACNA2D3
Progesterone-mediated oocyte maturation	CCNB3, CCNL2
p53 signalling pathway	CCNB3, CCNL2
Wnt signalling pathway	CSNK1A1L

3. Discussion

In the present paper, we provide evidence of the impact that BPA can have across the ovarian transcriptome using a primary ovarian cell line (HOSEpiC) as an experimental model. In total, 272 DEGs were identified when cells were treated with 10 nM BPA, whereas at 100 nM, 454 DEGs were identified, out of which 76 were commonly regulated.

In accordance with differences in DEGs, functional analysis of expression site, cellular components, biological processes, and molecular function revealed dose-specific effects. For

example, a much higher percentage of genes was identified in cells treated with 100 nM BPA with enrichment primarily around gynaecological malignancies, including ovarian cancer, in terms of site of expression. Indeed, we and others have recently discussed the potential involvement of BPA in ovarian cancer aetiopathogenesis [21,33,36]. In terms of cellular components, both BPA concentrations used appear to modulate a wide repertoire, ranging from cytoplasmic chromatin and nuclei at 10 nM and chromosomal regions at 100 nM. Previous studies in mouse spermatozoa revealed that exposure to BPA led to incomplete chromatin condensation, as well as abnormalities in acrosome formation [37]. Similarly, in male zebrafish, when exposed to BPA (100 μg/L), sperm chromatin fragmentation was increased; hence, the authors suggested that "BPA male exposure jeopardises embryonic survival and development" [38]. Moreover, when rat ovaries were treated with BPA in vitro, this led to a reduction in primary and secondary follicle numbers with evident DNA damage (ovotoxicity) [39]. In line with such data, our data are also suggestive of BPA exerting similar deleterious effects in human ovaries, affecting chromatin reorganisation.

Furthermore, there were also non-overlapping modalities in biological processes. For example, previous studies have shown that the plasma membrane organisation and biogenesis were enriched at 10 nM BPA, whereas spindle assembly demonstrated the highest fold enrichment at 100 nM of BPA treatment. Notably, the speed assembly checkpoint is vital for the safeguarding of the transmission of sister chromatids to two daughter cells, monitoring chromosomal segregation [40]. In addition, Kim et al. showed that BPA interferes with spindle microtubule attachment to kinetochores during the process of mitosis, ultimately driving tumorigenesis by enhancing chromosome instability in vitro [41]. Of note, there is a correlation between spindle assembly checkpoint protein expression and a shorter time of ovarian cancer recurrence [42]. Molecular functions depicted a similar diversity, with T-cell-receptor activity being the most enriched function at 10 nM BPA and motor and sulfotransferase activity at 100 nM of treatment. Dysregulation of T-cell receptors can give rise to a number of diseases, given that adaptive immunity will be compromised [43]. Previous studies have also shown that prenatal exposure to BPA in mice resulted in altered immune response involving T-helper 1 (Th1) cells [44]. On the other hand, a number of sulfotransferases (SULTs) are highly expressed in the human ovary [45] and can be a potential therapeutic target for ovarian cancer.

We then took a "deep dive" into the biological pathways for all three sets of DEGs, where we showed that the most enriched pathway at 10 nM of BPA treatment was that of mRNA surveillance, a pathway crucial for the quality of mRNA by degrading harmful RNAs [46]. Mutations or dysregulation of this pathway can give rise to various diseases. Here, we found that the genes involved include EIF4A3 and PPP2R2C. To the best of our knowledge, this is the first time that it has been shown that these two genes are dysregulated by BPA at the normal ovarian level. In ovarian cancer, there is upregulation of EIF4A3 [47], whereas suppression of PPP2R2C leads to ovarian cancer cell proliferation [48]. In cells treated with 100 nM of BPA, the cell cycle was the most enriched modality, with some of the identified genes playing a crucial role in the ovaries. For example, when CDK1 activity is inhibited by phosphorylation, it leads to the prolonged arrest of prophase-I in female germ cells, thus underpinning its importance for the female reproductive lifespan [49]. BUB1 (a mitotic checkpoint serine/threonine kinase) is another identified gene within our data that is involved in the cell cycle. Of note, Leland et al. showed that there is a link between inherited aneuploidy in female germ cells and dysfunction of BUB1, which can ultimately lead to loss of pregnancy [50].

Interestingly, a common pathway that was enriched by both concentrations of BPA was that of progesterone-mediated oocyte maturation. Oocyte maturation, along with embryo development, is controlled by steroid hormones, including progesterone [51]. CCNA2 and CCNB3 are two DEGs affected by BPA. CCNA2, in particular, is of importance since when conditional knockout mice for CCNA2 were generated, the female mice were infertile [52,53]. Similarly, CCNB3-deficient female mice are also sterile [54]. In another

study, a CCNB3 mutation affected the metaphase–anaphase transition in oocyte meiosis I, again leading to infertility [55].

We acknowledge certain limitations of our study, including utilising a singular primary ovarian cell line as a relevant in vitro model and choosing to assess only two concentrations of BPA. However, the utilised doses reflect the range of BPA environmental doses. Future studies should concentrate on expanding the use of both in vitro and ex vivo models (including 3D cultures and ovarian explants), as well as discerning whether BPA effects are mediated via canonical nuclear estrogen receptors or membrane-bound GPR30. Finally, our RNA sequencing data can be further validated by using RT-qPCR in addition to Western blot analysis to measure gene and protein level changes exerted by the identified DEGs.

Ten years ago, in a foetal rhesus monkey model, BPA exposure was shown to alter oogenesis and follicle formation [56]. Since then, a number of studies have argued that the human ovary can also be a target for endocrine disruption [57]. Our study provides a novel insight into the transcriptome changes at the ovarian level upon exposure to BPA. We hope these data will be used as a starting point for future in vitro and in vivo studies assessing the impacts of EDCs on health and disease. It should be noted that the primary route of human exposure to BPA for most is through the diet, as this EDC leaches from drink and food containers, particularly when they are heated. Alternative—but minor—routes of exposure include dental sealants, inhalation, dermal absorption, and maternal exposure [58–61]. These diverse routes of exposure present certain challenges in how to assess effects in vitro, ex vivo, and in vivo. For example, 3D ovarian cultures might be a more physiologically relevant system than 2D, where the effect of BPA can be studied on spheroids of primary ovarian cells as well as in different ovarian cancer cells in an attempt to understand the implications of EDCs in the tumour microenvironment [62]. Alternatively, ovarian tissue explants can be used as preclinical models [63]. This approach might give a better representation of the multicellular environment, and a number of readouts can be performed, including spatial transcriptomics and X-ray microtomography, which will provide even more information on the role of BPA. Alternatively, in vivo models of exposure can also be used, but for those to take place, research groups must adhere to the principles of the 3Rs (Replacement, Reduction, and Refinement). Over the past decade (2012–2022), 2101 manuscripts have been published on "BPA treatment" in animal models (source: PubMed). However, the key question is how relevant are these models to ovarian physiology in the context of EDC exposure? Therefore, a number of considerations must be made in order to identify the right model that will mimic EDC exposure in humans [64]. Finally, when designing such experiments, the effects of multiple xenoestrogens should be taken into consideration since they can have a tremendous additive impact, altering hormonal actions [65].

To summarise, with the current study, we have added to the existing literature by providing a novel insight into the effects of BPA in the human ovary, which can potentially compromise specific signalling pathways, leading to alterations in reproductive physiology. Future studies using 3D cell cultures/spheroids and ex vivo and in vivo models will further address gaps in knowledge of the effect of BPA (and other EDCs or their mixtures) at the ovarian level. Collectively, emerging studies will play a pivotal role in the legislation around EDCs. For example, the European Food Safety Authority (EFSA) re-evaluated the risks associated with BPA and proposed to considerably lower the tolerable daily intake (TDI) compared to its previous assessment in 2015, from 4 μg/kg bw/day to 0.04 4 μg/kg bw/day (source: efsa.europa.eu, accessed on 27 March 2022). Therefore, particular emphasis should be given to future studies that will elucidate the precise signalling mechanisms involved in endocrine disruption in reproductive organs. Moreover, consideration should also be given to the role of analogues to BPA (e.g., BPS) and their mixtures in health and disease.

4. Materials and Methods

4.1. Cell Culture

Primary normal ovarian epithelial cells, HOSEpiC (#7310), acquired at passage 1 from ScienCell Ltd., were cultured with Ovarian Epithelial Cell Medium (OEpICM), supplemented with 1% Ovarian Cell Growth Supplement (ScienCell Ltd., Carlsbad, CA, USA), 1% penicillin–streptomycin, and 10% FBS (Thermo Fisher Scientific, Loughborough, UK) in Poly-L-Lysine (ScienCell Ltd., Carlsbad, CA, USA)-coated T25 flasks. Prior to cell seeding, all flasks and plates were treated with 5 ug/mL Poly-L-Lysine in sterile de-ionised water for 1 h at 37 °C, washed with de-ionised water, and returned to the incubator for an additional hour to dry. Cell count and viability were carried out manually using a Neubauer chamber and Trypan blue (Invitrogen; Thermo Fisher Scientific, Loughborough, UK) exclusion method. Adherent cells were detached using TrypLE express (Thermo Fisher Scientific, Loughborough, UK). At passage 2, cells were transferred to a T75 flask before seeding in 6-well plates at a density of 0.3×10^6. At a confluence of 80%, media was replenished, and cells were treated with 10 nM and 100 nM of BPA (Sigma-Aldrich, St. Louis, MO, USA) in triplicate (detail is given below).

4.2. RNA Extraction

Samples were extracted, and the experiments were arrested at 24 h. Media were removed, and cells were washed with 500 µL of cold sterile PBS (Thermo Fisher Scientific, Loughborough, UK). RNA isolation was achieved using Qiagen RNeasy extraction kit (Qiagen, Manchester, UK); following the manufacturer's instruction, 40 µL of RNA was eluted. Samples were then stored at −80 °C prior to shipment for sequencing.

4.3. RNA-Sequencing (RNA-Seq), Data Generation

The samples were sequenced using Illumina sequencing, which resulted in taking the average of reads for each experimental replicate of the three experiments (Table 2).

Table 2. Total number of reads. For paired-end sequencing, these values refer to the sum of read 1 and read 2.

Samples	Total Reads
Control	75,835,336
BPA 10 nM	82,440,001
BPA 100 nM	65,361,410

RNA-seq processing pipeline was designed using TopHat2 (v.2.1.1) tool to align RNA-Seq reads to the human reference genome GRCH38 (hg19) using the ultra high-throughput short read aligner Bowtie2 (v.2.2.6). Next, Samtools (v.0.1.19) was used to merge all experimental replicates and to view and select high-quality mapped reads (minimum quality threshold was set at 30). Transcript assembly and expression quantification in each sample was conducted using Cufflinks (v.2.2.1). Finally, a differential expression profile between two experiments was obtained using Cuffdiff.

4.4. Statistical RNA-Sequencing Analysis

All RNA-seq data processing, modelling, cleaning, visualising, and statistical analysis were conducted using R (v. 4.1.0, The R Foundation for Statistical Computing, Vienna, Austria) under R Studio desktop application (version 1.4.1717, RStudio, Boston, MA, USA). The Pearson correlation coefficient was calculated to estimate the correlation between genes based on their expression pattern in all the experiments. Student's *t*-test was used to assess the statistical significance of the change of expression between two given states (e.g., BPA 10 nM vs. BPA 100 nM) with a significance threshold set at a *p*-value lower than 0.05. Volcano plots, heatmap, and Venn diagram were also generated using R. R package pathfindR was used for comprehensive identification of enriched pathways in omics data.

4.5. Functional Annotation

The shared differentially expressed genes (DEGs) from HOSEpiC samples treated with 10 nM BPA and 100 nM BPA in comparison with the control ethanol-treated samples were used for further functional annotation, as outlined below.

4.5.1. KEGG Pathway Database

Pathway analysis of the DEGs was performed by quarrying the KEGG database (https://www.kegg.jp/kegg/pathway.html (accessed on 8 February 2022)). KEGG is a collection of manually drawn pathway maps representing the current knowledge base of the molecular interaction, reaction, and regulation networks for human diseases, environmental information processing, organismal systems, and drug development.

4.5.2. Comparative Toxicogenomics Database (CTD)

In order to understand how environmental exposures affect human health, the CTD (http://ctdbase.org/; accessed on 8 February 2022) was used since it provides manually curated information about small molecule chemicals–gene and small molecule chemicals–disease interactions, and gene–disease pathway relationships.

4.5.3. Functional Analysis

The genes were functionally characterised using the Gene Ontology (GO) database [66], as recorded in FunRich (version 3.1.3) software [67]. The enrichment of the GO terms related to biological processes, biological pathways, molecular functions, and expression sites was computed. A threshold p-value of 0.05 was used to ascertain the statistical significance of the results.

4.5.4. The Gene Ontology Consortium

GO Consortium resource (http://geneontology.org/ accessed on 5 March 2022) was used to develop a comprehensive, computational model of biological systems, ranging from the molecular to the organism level. The statistical significance of the results was obtained by threshold p-value of 0.05. Currently, the GO includes experimental findings from over 150,000 published papers, represented as over 700,000 experimentally supported annotations.

5. Conclusions

In the present paper, we provide evidence of the impact that BPA can have across the ovarian transcriptome using a primary ovarian cell line (HOSEpiC) as an experimental model. Future studies should further explore the changes that BPA and other common EDCs can elicit within the ovaries at gene, protein, and metabolic levels, subsequently addressing existing knowledge gaps in basic biology, hazard characterisation, and risk assessment associated with the use of xenoestrogens such as BPA at the ovarian level.

6. Patents

No patents resulted from the work reported in this manuscript.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23105334/s1.

Author Contributions: Conceptualisation, A.Z., C.S., and E.K.; methodology, A.Z, R.K., and C.S.; formal analysis, A.Z., R.K., C.S., and E.K.; investigation, I.K., H.S.R., C.S., and E.K.; data curation, A.Z., R.K., and C.S.; writing—original draft preparation, A.Z., C.S., and E.K.; writing—review and editing, A.Z., R.K., I.K., H.S.R., C.S., and E.K.; supervision, C.S. and E.K.; project administration, C.S. and E.K.; funding acquisition, E.K., I.K. and H.S.R.; E.K. and C.S. should be considered joint last authors. All authors have read and agreed to the published version of the manuscript.

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Supplementary Materials

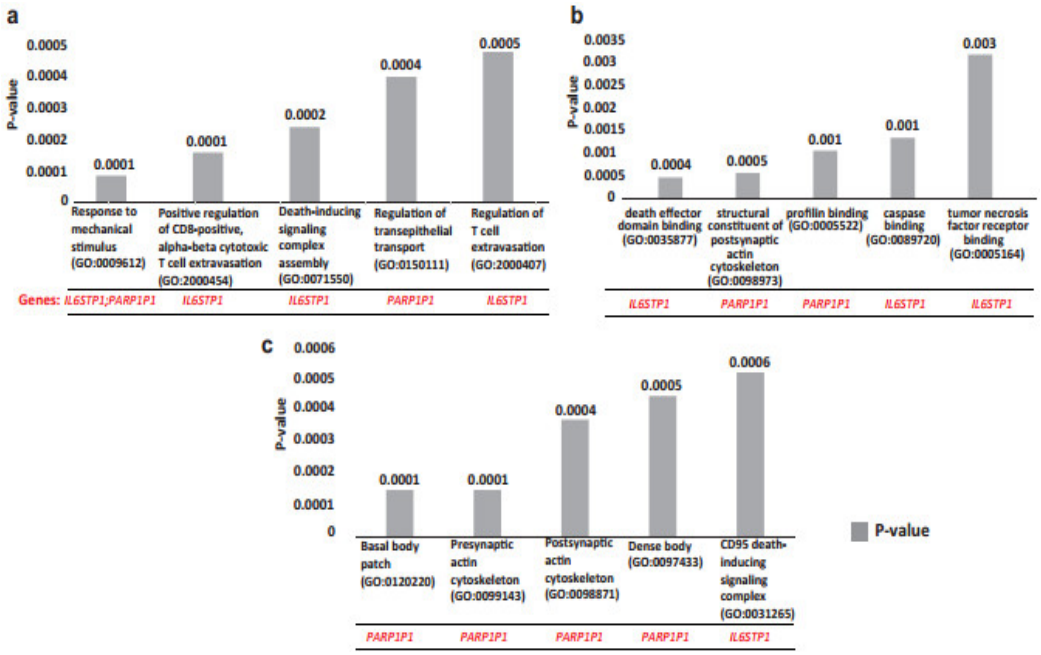

Figure S1. The functional enrichment in Gene Ontology terms in shared differentially expressed genes (DEGs) over the two used doses of BPA (10 nM and 100 nM) in relation to Biological processes (**a**), Molecular functions (**b**), and Cellular components (**c**).

Chapter 5

Is There a Link between Bisphenol A (BPA), a Key Endocrine Disruptor, and the Risk for SARS-CoV-2 Infection and Severe COVID-19?

Statement of Contribution

In this manuscript I led and contributed the following parts:

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- Writing—original draft preparation
- Writing—review and editing
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Review

Is There a Link between Bisphenol A (BPA), a Key Endocrine Disruptor, and the Risk for SARS-CoV-2 Infection and Severe COVID-19?

Aeman Zahra [1], Cristina Sisu [1], Elisabete Silva [1], Sophie-Christine De Aguiar Greca [1], Harpal S. Randeva [2,3,4], Kamaljit Chatha [4,5], Ioannis Kyrou [2,3,4,†] and Emmanouil Karteris [1,*,†]

1 Biosciences, College of Health, Medicine and Life Sciences, Brunel University London, Uxbridge UB8 3PH, UK; aeman.zahra@brunel.ac.uk (A.Z.); cristina.sisu@brunel.ac.uk (C.S.); elisabete.silva@brunel.ac.uk (E.S.); sophieja3@gmail.com (S.-C.D.A.G.)
2 Warwickshire Institute for the Study of Diabetes, Endocrinology and Metabolism (WISDEM), University Hospitals Coventry and Warwickshire NHS Trust, Coventry CV2 2DX, UK; harpal.randeva@uhcw.nhs.uk (H.S.R.); i.kyrou@aston.ac.uk (I.K.)
3 Aston Medical Research Institute, Aston Medical School, Aston University, Birmingham B4 7ET, UK
4 Warwick Medical School, University of Warwick, Coventry CV4 7AL, UK; kamaljit.chatha@uhcw.nhs.uk
5 Department of Biochemistry and Immunology, University Hospitals Coventry and Warwickshire NHS Trust, Coventry CV2 2DX, UK
* Correspondence: emmanouil.karteris@brunel.ac.uk
† Ioannis Kyrou and Emmanouil Karteris are joint senior co-authors.

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Abstract: Infection by the severe acute respiratory syndrome (SARS) coronavirus-2 (SARS-CoV-2) is the causative agent of a new disease (COVID-19). The risk of severe COVID-19 is increased by certain underlying comorbidities, including asthma, cancer, cardiovascular disease, hypertension, diabetes, and obesity. Notably, exposure to hormonally active chemicals called endocrine-disrupting chemicals (EDCs) can promote such cardio-metabolic diseases, endocrine-related cancers, and immune system dysregulation and thus, may also be linked to higher risk of severe COVID-19. Bisphenol A (BPA) is among the most common EDCs and exerts its effects via receptors which are widely distributed in human tissues, including nuclear oestrogen receptors (ERα and ERβ), membrane-bound oestrogen receptor (G protein-coupled receptor 30; GPR30), and human nuclear receptor oestrogen-related receptor gamma. As such, this paper focuses on the potential role of BPA in promoting comorbidities associated with severe COVID-19, as well as on potential BPA-induced effects on key SARS-CoV-2 infection mediators, such as angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2). Interestingly, GPR30 appears to exhibit greater co-localisation with TMPRSS2 in key tissues like lung and prostate, suggesting that BPA exposure may impact on the local expression of these SARS-CoV-2 infection mediators. Overall, the potential role of BPA on the risk and severity of COVID-19 merits further investigation.

Keywords: SARS-CoV-2; COVID-19; BPA; oestrogen receptors; ACE2; TMPRSS2; endocrine disruptors

1. Introduction

Infection by the novel severe acute respiratory syndrome (SARS) coronavirus-2 (SARS-CoV-2) causes a severe new disease, i.e., COVID-19. Following the initial outbreak of COVID-19 cases at the end of 2019, COVID-19 reached pandemic status within months [1]. Growing data indicate that certain underlying diseases/conditions exhibit a direct association with significantly higher risk for adverse clinical outcomes of COVID-19 [1]. Indeed, chronic respiratory diseases (e.g., asthma

and chronic obstructive pulmonary disease), cardiovascular disease (CVD), hypertension, diabetes, immunosuppression, and cancer are among the identified comorbidities which predispose individuals to severe COVID-19 [1].

Endocrine-disrupting chemicals (EDCs) are exogenous substances which can disrupt normal functions of the endocrine system in animals and humans, increasing the risk of adverse health effects [2]. Common EDCs include industrial solvents or lubricants and their by-products, pesticides, fungicides, plasticisers (e.g., bisphenol A (BPA) and phthalates), and pharmaceuticals [3]. EDCs are widespread in the environment and can accumulate across the entire food chain due to the long half-lives which commonly characterize these lipophilic chemicals, as well as the inability of the body to metabolize them [4]. Data from the US Centers for Disease Control and Prevention (CDC) suggest that humans can be exposed to hundreds of chemicals including EDCs [3]. Of note, research has suggested that increased and/or prolonged exposure of humans to EDCs can cause cardio-metabolic dysfunction, disorders of the reproductive system, endocrine-related cancers, and immune system dysregulation [5].

As more data on COVID-19 become available, the identified number of relevant predisposing risk factors is increasing, including factors such as obesity [6] and low socioeconomic and/or Black, Asian, and minority ethnic (BAME) background [7], which may be also linked to higher exposure to EDCs [8,9]. Indeed, a recent review has further proposed that long-term exposure to chemicals in mixtures, as well as lifestyle habits, may be linked to compromised immunity and predispose to the complications observed in patients with severe COVID-19 [10]. Moreover, a computational systems biology approach revealed that a number of signalling pathways which are dysregulated by EDCs (e.g., Th17 and advanced glycation end-products (AGE)/receptor for AGE (RAGE), AGE/RAGE, pathways) might also be related to the severity of COVID-19 [11]. As these detrimental effects of EDCs overlap with key risk factors for severe COVID-19, the hypothesis that exposure to EDCs may be also linked to the severity of COVID-19 merits further investigation [12].

Among the various EDCs, BPA is extensively used in a variety of products, including plastics, thermal receipts, and the lining of aluminium cans [13]. Accordingly, BPA is now one of the most frequently detected pollutants in the environment [14]. As such, in the present paper, we focus on the potential role of BPA in promoting the development of comorbidities which increase the risk of severe COVID-19, as well as on potential BPA-induced effects on key molecular targets which mediate the infection by SARS-CoV-2.

2. BPA and Comorbidities Predisposing to Severe COVID-19

2.1. BPA and Cardiometabolic Diseases

BPA is now recognized as a potential additional factor implicated in the development of cardio-metabolic diseases [15]. Indeed, BPA accumulates in adipose tissue and increases the number and size of adipocytes, thus contributing to increased adiposity and weight gain [16]. Moreover, a recent systematic review with a meta-analysis of the relevant epidemiological evidence reported that BPA exposure shows a significant positive association with indices of both generalized and central/abdominal obesity [17,18]. Similarly, systematic review data also support a relationship between BPA exposure and type 2 diabetes (T2DM) [19]. BPA exposure might be also associated with adiposity both in childhood and later in life [20]. Furthermore, a positive association has also been documented between low-dose BPA exposure during critical developmental periods (e.g., during foetal development) and metabolic diseases, such as T2DM [21].

Data from epidemiological and mechanistic studies also suggest a link between increased BPA exposure and hypertension [22], which is a key component of the metabolic syndrome and a leading CVD risk factor globally [23,24]. Of note, this positive association was documented in a multi-ethnic sample of US adults, independently of confounding factors such as age, gender, smoking, body mass index (BMI), diabetes, and cholesterol levels [25]. A positive association was noted between urinary

BPA levels and hypertension in 1380 subjects from the National Health and Nutritional Examination Survey (NHANES), independent of confounding factors such as age, gender, race/ethnicity, diabetes, smoking, BMI, and total serum cholesterol levels [25]. This was further corroborated by another study of 2588 sera samples from the Thai NHANES, where BPA exhibited a positive association with hypertension which was also independent of age, sex, BMI, diabetes, and oestrogen levels [26]. Finally, in a more recent study in Seoul where 560 elderly participants were recruited, BPA exposure was associated with increased blood pressure and decreased heart rate variability, which are both risk factors of CVD [27]. Moreover, in terms of underlying mechanisms, a number of studies point towards an involvement of BPA in vascular dysfunction. For example, in the population-based Prospective Investigation of the Vasculature in Uppsala Seniors study, BPA was related to the echogenicity of atherosclerotic plaques of the carotid arteries, suggesting a role for plaque-associated chemicals in atherosclerosis [28]. In addition, high BPA serum levels were also associated with increased carotid intima-media thickness in a cross-sectional study of adolescents and young adults [29]. In line with these findings, in an in vivo study where BPA was administered in male rats, BPA was shown to exert a cardiotoxic effect, inducing a state of oxidative stress and leading to the overproduction of free radicals [30]. Furthermore, in a more recent study using cardiomyoblasts in vitro, BPA induced pro-inflammatory interleukins (IL) involved in CVD (i.e., IL-8, IL-6, and IL-1β), whilst also enhanced doxorubicin-induced cardiotoxicity phenomena [31].

Finally, a strong relationship between BPA and circulating androgen levels has been shown, suggesting a link to ovarian dysfunction and polycystic ovary syndrome (PCOS) [32]. The latter is also strongly linked to the metabolic syndrome in women [33,34], with systematic review data suggesting that BPA is involved in both hyperandrogenism and insulin resistance of PCOS [35,36].

Overall, it is noteworthy that CVD and all these chronic diseases which commonly cluster within the metabolic syndrome spectrum (e.g., obesity, T2DM, and hypertension) are now consistently recognized as key factors that predispose to severe COVID-19 [37–42]. Thus, BPA exposure by promoting the development of these cardio-metabolic diseases over time may be also indirectly linked to higher risk of severe COVID-19, particularly in older individuals that are at a high risk group for severe COVID-19 [43].

2.2. BPA and Cancer

BPA exposure has been linked to carcinogenicity, especially of hormone-dependent tumours, such as prostate, breast, and ovarian cancers [44]. As such, prenatal BPA exposure may influence the development of prostate cancer in later life, and also increase the frequency of breast tumours through either alteration of foetal glands or by mediating oestrogen-dependent growth of tumour cells [16]. Interestingly, pregnant mice which were exposed to BPA levels within the range of human exposure showed increased prostate volume and decreased sperm production in the adult male offspring [45–47]. Furthermore, increasing evidence from both in vitro and animal studies suggest that BPA exposure, even at low doses, may have carcinogenic effects on breast cancer [48]. Moreover, BPA appears to increase the risk of endometriosis which, in turn, increases the risk of both coronary heart disease and ovarian cancer [49,50]. Finally, BPA exposure may induce endometrial stromal cell invasion and has a positive association with peritoneal endometriosis [51].

To date, an increasing body of evidence, including meta-analysis data, indicate that cancer comorbidity exhibits an association with both the risk and severity of COVID-19 [52]. In a recent UK study of 156 cancer patients with confirmed COVID-19 diagnosis it was shown that patients who live longer with cancer are at greater risk of infection as well as of COVID-19 related death [53]. Of note, cancer patients with urological/gynaecological, breast, and lung cancers, as well as haematological malignancies, were presented with severe COVID-19 [53]. As aforementioned before, BPA has been involved in the development of certain cancers and a number of mechanisms have been proposed. For example, exposure of mouse mammary tumor virus (MMTV)-erbB2 mice to low BPA doses in utero has been shown to lead in mammary tumourigenesis and mammary epithelial reprogramming involving the oestrogen receptor (ER)-erbB2 pathway [54]. Similarly, perinatal exposure of adult

CD-1 mice to BPA resulted in induction of mammary intraductal hyperplasia [55]. Furthermore, in an in vitro study, BPA increased the migration and invasion of triple-negative breast cancer cells, while it also induced protein expression of matrix metalloproteinase-2 (MMP-2) and MMP-9 [56].

However, a systematic review reporting on the effects of cancer—among other comorbidities—on COVID-19 severity concluded that this association must be interpreted with caution due to a number of confounding factors, including old age, smoking history, and co-existing comorbidities of the involved study participants, as well as the sample size of these studies [42]. Accordingly, additional research should also be focused on the potential links between endocrine-dependent tumours with known associations to BPA exposure (e.g., prostate, breast, and ovarian cancers) and COVID-19, including exploring the potential underlying molecular mechanisms using in vitro and in vivo models, as well as clinicopathological data.

2.3. BPA and Modulation of Immune System Responses

An increasing number of studies have also drawn attention to the potential involvement of BPA in modulating immune system responses, and, particularly, to its potential ability to facilitate airway inflammation and respiratory allergies, as well as impair immunotolerance to dietary proteins [57–60]. Multiple mechanisms have been suggested to mediate the potential effects of BPA on the immune system, such as direct effects on relevant receptors (e.g., oestrogen receptors) and cellular signalling pathways, as well as epigenetic effects and changes of the gut microbiome [57]. Overall, BPA exposure may impact on both the sub-type and function of the adaptive and innate immune system cells, leading to changes in produced cytokines and chemokines (e.g., upregulation of pro-inflammatory cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), IL-10, and IL-4) and decreased T regulatory (Treg) cells [57,58]. Interestingly, oral BPA exposure of ovariectomized rats has been shown to induce a pro-inflammatory response in their adult female offspring, suggesting potential long-term effects of BPA on the immune system of the progeny [61].

In this context, it should be highlighted that COVID-19 severity also appears to be linked to increased local and systemic levels of an array of pro-inflammatory cytokines and chemokines (e.g., TNF-α, IL-1β, IL-6, IL-8, and IL-2) [62–64]. This may further induce a vicious cycle of hyperinflammatory reactions in certain patients with severe COVID-19, resulting in an underlying cytokine storm with adverse clinical outcomes [62–64]. As these pro-inflammatory pathways may be also triggered by increased and/or prolonged exposure to BPA, this may represent an additional indirect pathophysiologic mechanism via which BPA could potentially increase the risk of severe COVID-19 in vulnerable individuals, particularly those with T2DM, obesity, hypertension, and CVD who already exhibit various degrees of underlying low-grade chronic inflammation [62]. However, recently it was shown that critically ill patients with COVID-19 suffering with acute respiratory distress syndrome (ARDS) had lower circulating cytokine levels when compared with sepsis or other critically ill patients [65]. This was further corroborated by data demonstrating that although COVID-19 patients exhibited increased pro-inflammatory cytokine levels (e.g., IL-16, IL-10, and monocyte chemoattractant protein-1, MCP-1), these levels were not as high as in other non-COVID-19 patients suffering from cytokine-release syndrome [66]. Therefore, it appears that there might be a higher order of complexity regarding the role and potential implications of an underlying "cytokine storm" in COVID-19 that also merits further investigation. In this context, the role of BPA on immunity should be further investigated as this may be further implicated in the potential mechanisms linking BPA with higher risk for COVID-19 [57].

2.4. BPA and Links to Pregnancy and Placentation Complications

A growing body of evidence has further shown that BPA exposure, even at low doses, may have adverse effects on the outcomes of pregnancy in humans, resulting in potentially harmful conditions for both the mother and the offspring (e.g., affecting the normal development of the foetus and/or causing problems later in life) [67–73]. There is also a correlation between BPA exposure and preeclampsia during pregnancy [74,75], which is characterized by newly diagnosed hypertension and proteinuria [76]

and is associated with increased risk of both maternal mortality and health problems for the offspring later in life (e.g., obesity and T2DM) [76,77].

Although more data are necessary to prove a direct association between BPA exposure and preeclampsia or placental alterations, the potential link between BPA and preeclampsia is of particular interest in relation to COVID-19, given that pregnant women with severe COVID-19 can develop a preeclampsia-like syndrome [78]. So far, single cases of COVID-19 causing preeclampsia or pregnancy-induced hypertension have been described [79,80]. Moreover, Shanes et al. found that third trimester placentas from women with COVID-19 had significantly higher probability of vascular malperfusion, showing features such as abnormal or injured maternal vessels or intervillous thrombi [81]. Similarly, Baergen et al. found that half of the studied placentas in a cohort of 20 mothers with COVID-19 showed evidence of foetal vascular thrombosis or foetal vascular malperfusion [82]. In another study, in five pregnant women with COVID-19 who delivered at term without complications, all five placentas showed focal avascular villi and thrombi in larger vessels [83], although no direct SARS-CoV-2 infection of the placenta was noted and the placental changes were attributed to systemic rather than local infection [83]. Given that, in addition to the pro-thrombotic nature of pregnancy, COVID-19 appears to be associated with pro-thrombotic effects on both the placenta [83] and systemic infection [84], importance has been given to continuing prophylactic aspirin in women with COVID-19 at risk for preeclampsia, although some studies have questioned whether non-steroidal anti-inflammatory drugs can exacerbate COVID-19 symptoms [85].

Overall, whether COVID-19 symptoms could be exacerbated in pregnant women and whether BPA exposure may further increase the relevant risk need further investigation, particularly since the immune system during pregnancy is in a state of constant adaptation with pregnant women being more susceptible to respiratory infections [79]. Notably, a study from Spain on the clinical outcomes of 60 pregnant women with confirmed COVID-19 has reported that most of these patients had a good clinical outcome, with one-third developing pneumonia and 5% classified as being in critical condition [86]. Similar findings were reported by another recent study showing that there were no severe cases of pneumonia and no maternal deaths in pregnant women with COVID-19 [87]. So far, there is very limited evidence on the potential vertical transmission of COVID-19 from a mother to a child, with a recent review of the relevant existing literature reporting little evidence for such transmission [88]. However, there are rare reported cases of vertical transmission of COVID-19 from mothers to neonates. For example, two cases of COVID-19 (one delivered vaginally after spontaneous labour and one via caesarean section) were found in the neonates of a cohort of 22 women who were affected by COVID-19 during the third trimester of pregnancy [89]. Although such research studies on pregnancy and COVID-19 are increasing, currently there are no reported studies on BPA blood/urine levels in pregnant women diagnosed with COVID-19 and their offspring.

3. BPA and Key Molecular Targets of SARS-CoV-2

SARS-CoV-2 infection of target/host cells is mediated by a number of cellular receptors and proteases. As such, SARS-CoV-2 binds with high affinity to angiotensin-converting enzyme 2 (ACE2) on the cell membrane, which facilitates viral entry into host cells [90]. Moreover, transmembrane serine protease 2 (TMPRSS2) is co-expressed with ACE2 on the cell membrane and it can prime the viral spike proteins, thus mediating the fusion of the virus with the membrane lipid layer and its uptake into host cells [91]. In addition, cathepsin L (CTSL), a lysosomal protease which is known to mediate the cellular entry of the SARS virus via endosomes by priming the viral spike proteins for membrane fusion [92], appears to also facilitate the infection of host cells by SARS-CoV-2 [91]. Similarly, furin is a protease known for cleaving inactive precursor proteins into their biologically active products [93], while furin inhibitors have been investigated in the search for novel SARS-CoV-2 treatments since a relevant site has been discovered in the protein sequence of the SARS-CoV-2 spike protein [94,95].

As more research is now focused on the role of cellular mediators in SARS-CoV-2 infection and potential factors affecting their expression/functions, we also present data on the potential effects of BPA on these key SARS-CoV-2 infection mediators in this review.

3.1. BPA and Expression of TMPRSS2

Evidence from animal studies indicate that BPA can affect TMPRSS2 expression. Indeed, when BPA was administered subcutaneously to male rats from days 1 to 3, the expression of TMPRSS2 was upregulated in their medial amygdala [96]. This BPA-induced increase in the density of TMPRSS2 immunoreactive cells in the medial amygdala of neonatal male rats suggests that BPA has the potential to disturb central nervous system (CNS) and neurodevelopmental processes [96]. Interestingly, increasing attention is now placed on the neurotropism of coronaviruses, such as SARS-CoV-2, and their potential effects on neuropathogenesis and the CNS [97,98].

On the other hand, in vitro studies in Ishikawa cells, i.e., a well-characterized human endometrial cell line which can be used as an in vitro model for testing potential estrogenic effects of various chemicals, showed that BPA treatment can induce the downregulation of TMPRSS2 [99]. Moreover, we have recently published our research findings on the effects of physiologically relevant doses of BPA on the human placenta using non-syncytialised and syncytialised BeWo cells as in vitro models [100]. In the context of COVID-19, we revisited the microarray data from these experiments and we found that the applied BPA treatment induced a modest increase of TMPRSS2 expression in non-syncytialised and syncytialised BeWo cells, with no effect on ACE2 and CTSL expression (unpublished data). Interestingly, one of the significantly enriched processes in non-syncytialised BeWo cells treated with BPA (3 nM) in our experiments appears to be implicated in the regulation of viral life cell cycle [100].

Considering the available evidence which suggests that BPA can variably impact on the expression of TMPRSS2, further research is needed in order to explore whether any such BPA-induced effects on this key SARS-CoV-2 infection mediator may have a clinically relevant impact on the risk of developing COVID-19 and its subsequent severity.

3.2. BPA and Expression of ACE2 and Furin

Limited data on the potential effects of BPA on the expression of ACE2 and furin exist so far. As BPA is suspected to promote male reproductive impairments, an ex vivo toxicogenomic study using a rat seminiferous tubule culture model to investigate BPA effects on spermatogenesis showed that exposure to low-dose BPA (1 nM) can downregulate ACE2 and furin after 21 and 14 days of exposure, respectively [101]. Furthermore, a study with RNA-seq analyses of the testicular mRNA libraries of adult male rare minnows (*Gobiocypris rarus*; a small cyprinid fish used as a model for aquatic toxicology research) which were exposed to different BPA concentrations (1, 15, and 225 µg/L for 7 days) showed that ACE2, which is expressed in Leydig cells and may serve as a regulator of testicular steroidogenesis, was one of the most significantly increased genes of the renin-angiotensin system following BPA exposure (1 µg/L for 7 days) [102]. On the other hand, another study investigating the potential adverse impact of BPA exposure (50 mg/kg of body weight for 6 weeks) during puberty in male mice showed significantly decreased ACE2 protein expression in the cauda epididymis of BPA-exposed mice [103]. As men are consistently at higher age-adjusted risk for severe COVID-19 compared with women [104], and there is currently ongoing research regarding whether the human reproductive system constitutes an additional target for SARS-CoV-2 infection [105–108], future research studies should also investigate whether BPA may play a role in such COVID-19-related complications by modulating the local expression of key SARS-CoV-2 infection mediators, such as ACE2.

3.3. Co-expression of Receptors Mediating BPA Effects with SARS-CoV-2 Infection Mediators

BPA exerts its effects by acting on receptors which, based on available data from the Genotype-Tissue Expression (GTEx) project, are widely distributed in human tissues, including nuclear oestrogen

receptors (ERα and ERβ), membrane-bound oestrogen receptor (G protein-coupled receptor 30; GPR30), and human nuclear receptor oestrogen-related receptor gamma (Figure 1) [100,109–111].

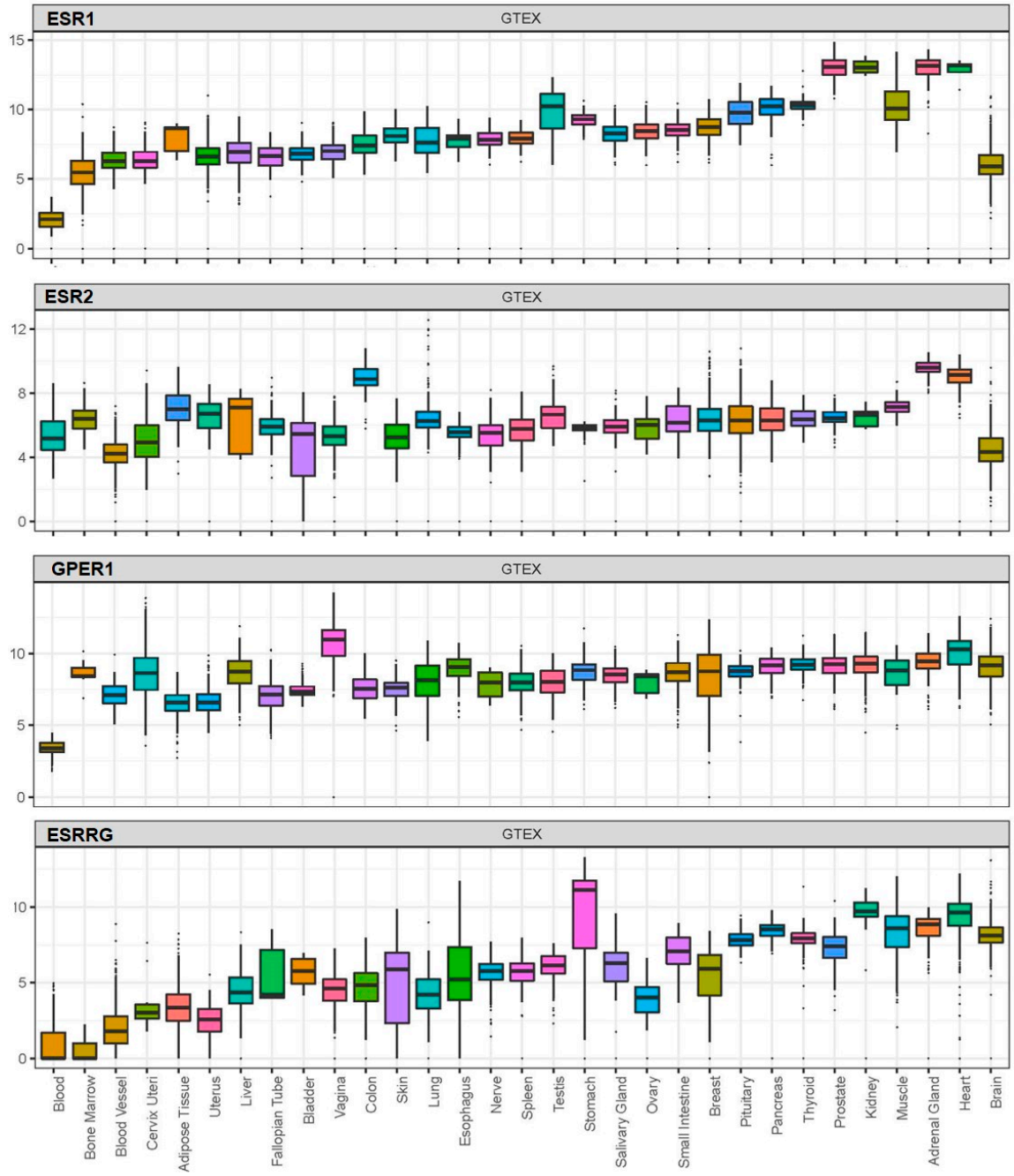

Figure 1. Expression (log2(norm_count+1)) of the nuclear oestrogen receptors ERα (ESR1) and ERβ (ESR2), G protein-coupled membrane-bound oestrogen receptor (GPR30 or GPER1), and oestrogen-related receptor gamma (ESRRG) across human tissues based on available data from the Genotype-Tissue Expression (GTEx) project.

Here, we expanded on these in silico observations by assessing the co-expression of receptors mediating BPA effects with SARS-CoV-2 infection mediators. As such, among these receptors which mediate BPA effects, the membrane-bound oestrogen receptor GPR30 appeared to co-localise with TMPRSS2 in the lung, colon, stomach, small intestine, thyroid, kidney, liver, and prostate (Figure 2A). This finding suggests that BPA exposure may impact via GPR30 on these SARS-CoV-2 infection mediators in these tissues and, thus, have potential implications on the severity of COVID-19 (e.g.,

on the consequences of SARS-CoV-2 infection in the lungs). We have dissected these data further, using available data from the GTEx project, to investigate any potential correlation among the expression patterns of these genes. For this, we computed the Pearson correlation coefficient between the genes' expression levels in healthy tissue samples. A high degree of correlation was noted between ACE2 with ERβ (0.37) and TMPRSS2 (0.38), whereas moderate correlation was noted between ACE2 with ERα (0.28) and oestrogen-related receptor gamma (0.23) (Figure 2B). The results suggest that these genes have a correlated expression pattern.

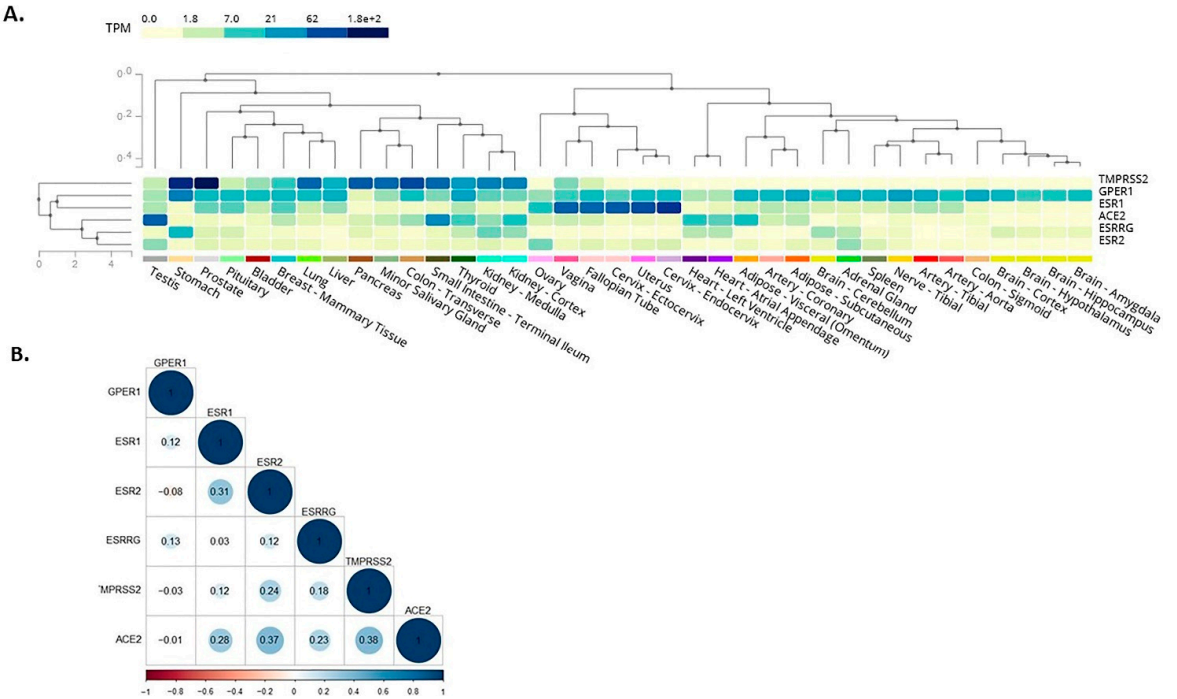

Figure 2. Co-expression (**A**) and correlation (**B**) of the main known receptors, i.e., nuclear oestrogen receptors ERα (ESR1) and ERβ (ESR2), membrane-bound oestrogen receptor (G protein-coupled receptor 30; GPR30 or GPER1), and oestrogen-related receptor gamma (ESRRG) which mediate the effects of bisphenol A (BPA) with key SARS-CoV-2 infection mediators, i.e., angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2), based on available data from the Genotype-Tissue Expression (GTEx) project.

4. Conclusions

Exposure to BPA, one of the most common EDCs, can promote the development of cardio-metabolic diseases, endocrine-related cancers, and immune system dysregulation and, through that, may be indirectly linked to higher risk of severe COVID-19 (Figure 3). Moreover, receptors which directly mediate BPA effects, such as the membrane-bound oestrogen receptor GPR30, are widely distributed in human tissues and may co-localise with SARS-CoV-2 infection mediators (e.g., co-localisation of GPR30 with TMPRSS2 and CTSL in the lung), potentially affecting their local tissue expression. Therefore, it becomes evident that there might be potential implications of exposure to BPA and other common EDCs on the risk of SARS-CoV-2 infection and the severity of COVID-19 [11,12]. This is a developing topic and clearly further in vitro, computational, preclinical, and in vivo studies are needed to elucidate any such direct links between BPA and COVID-19 and clarify the molecular mechanisms that may be involved. Ultimately, this can lead to a new framework and guidelines for reducing relevant EDC exposure(s) in the context of COVID-19, particularly in high COVID-19 risk groups (e.g., men and older individuals, as well as patients with comorbidities such as T2DM, hypertension, obesity, and CVD).

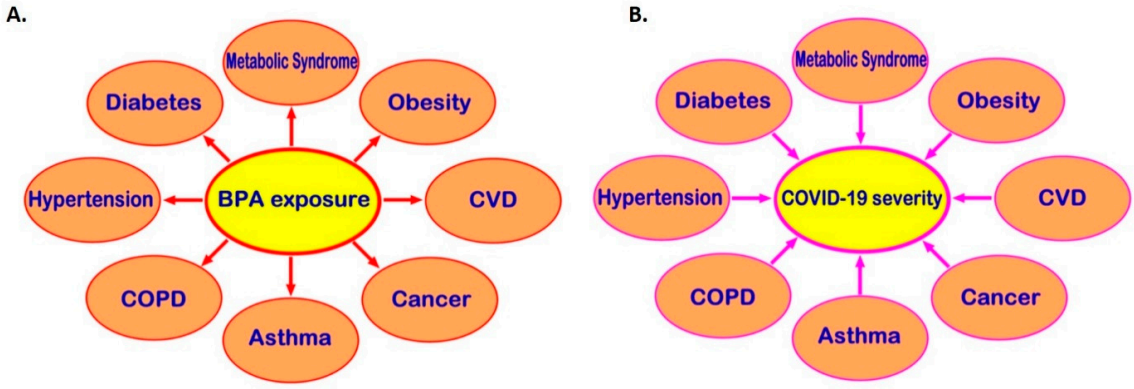

Figure 3. Potential links via which bisphenol A (BPA) could indirectly increase the risk for severe COVID-19. Exposure to BPA can promote the development of multiple cardio-metabolic diseases and endocrine-related cancers (**A**). These comorbidities predispose to worse COVID-19 clinical outcomes (**B**); hence, BPA exposure may be indirectly linked to higher risk of severe COVID-19. CVD: cardiovascular disease; COPD: chronic obstructive pulmonary disease; COVID-19: coronavirus disease 2019.

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Chapter 6

Discussion

6.1 General Remarks

EDCs have been reported to exert a diverse range of health problems, as they mimic, interfere and subsequently alter endocrine signalling pathways [1][2][3][4][5][6]. EDCs are linked with deleterious effects on both male and female reproductive systems e.g. infertility [7], PCOS [8], endometriosis [9], precocious puberty [10], and spermatogenesis [11].

BPA is also associated with cardiovascular disease [3], metabolic disorders [12], diabetes [13], thyroid homeostasis [12], and increases the risk of hormone-sensitive cancers [14]. The important message outlined in the recent study [15] is the importance of windows of exposure, as the developing fetus might be more sensitive to EDCs than the adult. This is due to the human placenta not being an effective barrier against certain chemicals, thus EDCs may enter the fetal circulating system easily [15]. Indeed, in recent years, several evidence obtained on in vitro and animal studies suggest that infants and children may be the most vulnerable to the effects of BPA [16][17][18].

The 2013-2014 National Health and National Examination Survey (NHANES) in U.S. found the detectable level of BPA (95.7%) in randomly selected urine samples (adults number of sample = 1808 and children number of sample = 868) [19]. In 2021 a multi-agency research program developed by the National Toxicology Program (NTP) is known as the Consortium Linking Academic and Regulatory Insights on Bisphenol A Toxicity (CLARITY-BPA), the main aim to design this program was to use the regulatory expertise and academic research approaches on BPA to provide

awareness, fill knowledge gaps, improve quality control methods, notify chemical risk assessment, and identify novel methods for regulatory hazard assessments [20]. CLARITY-BPA program was supported and participated by U.S. Food and Drug Administration (FDA) and the National Institute of Environmental Health Sciences (NIEHS) [20]. NIH has also suggested to reduce the exposure by avoiding polycarbonate plastic food containers in microwave, reducing tin foods, and use BPA free baby bottles [21]. The Endocrine Society; the world's oldest organization founded in 1916, is the international medical organization in the field of endocrinology and metabolism [22]. The Endocrine Society urge the European Food Safety Agency (EFSA) to recognize and determine the serious need to reduce the exposure to hazardous chemical BPA to achieve health-protective objectives [23]. EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP) noted that a tolerable daily intake (TDI) of BPA (4 μg/kg of body weight per day) exceeded the ovarian follicle counts by two to three orders of magnitude via dietary exposure, and concluded that there is a health concern from even the TDI dietary BPA exposure for all age groups [24]. In 2013, FDA revised its regulations to no longer provide the BPA-based epoxy resins as coatings in packaging for infant formula and BPA-based polycarbonate resins in baby bottles and sippy cups [25]. In 2017, the European countries decided to categorise BPA as a highly concerning substance due to the potential serious effects to human health [26]. EU has banned the use of BPA in thermal paper since January 2020, as BPA dermal penetration studies propose that you can pick up quite a lot of BPA via skin by just touching receipts [26][27]. Therefore, BPA has been controlled and banned in many countries especially in baby bottles, in Canada it was banned in 2008, in France in 2010, and in the European Union in 2011 [28].

The European Chemicals Agency (ECHA), survey confirms that paper manufacturing industries can continue to replace BPA with bisphenol S (BPS) [29]. However, new concerns are rising that BPS may also has potential endocrine disrupting properties [29]. BPS is currently being studied by Belgian authorities to evaluate whether the use of BPS will have hazardous effects on human health or the environment [30]. Apart from BPS, another bisphenol called BPF has been currently used as an alternative to BPA [28]. A recent study revealed that BPS potentially stays in the body for much longer period and at much higher concentration compare to BPA [31]. Emerging data suggest an association between BPA, BPS and BPF and consequences of

obesity in children aged 6 to 19 years [31]. Moreover, BPS also has shown similar effects to that of oestradiol in membrane-mediated pathways, which are essential for cell proliferation, differentiation and death [32]. BPS and BPF may also cause chromosomal abnormalities that resemble those seen with BPA [33]. Currently, there are many BPA replacements that manufacturers are using by just changing small parts of the chemical structure of BPA. that can exert similar deleterious effects in human and animal life[34]. Therefore, there is need for further research to provide a better insight into the role of these new compounds. As the WHO's Director for Public Health and Environment said: "*We urgently need more research to obtain a fuller picture of the health and environment impacts of EDCs*".

Our research has mainly focused on female cancers (hormone related cancers) associated with EDCs specifically BPA. To date, numerous studies provided a better understanding the possible mechanisms underlying the effects of BPA like in the case of breast cancer, however, data on the effect of BPA on normal ovaries is very limited. This study was designed to address this scientific gap of knowledge by assessing the effects of BPA in normal ovaries as well as in ovarian cancer (OC).

As I was about to start my PhD, a study provided some preliminary data on the effect of BPA in OC cells in vitro, identifying 94 differentially expressed genes (DEGs), between treated cells and controls [35]. But this study raises the question, if these 94 genes were differentially expressed because of BPA or the disease (OC) itself? From this point we carried our first paper [36], in which we performed an in-depth investigation on those 94 DEGs, to find the functional and activity landscape in OC as well as normal ovaries by leveraging the available RNAseq data from TCGA and GTEx. In this paper [36], we were also successful in identifying seven potential biomarkers of BPA exposure-associated disease (OC) and biomarkers of prognostic potential for OC. We also performed t-distributed stochastic neighbour embedding (t-SNE) dimensionality reduction analysis method, to predict the collective diagnostic power of the seven genes. Our data strongly suggested that the seven genes (namely GBP5, IRS2, KRT4, LINCOO707, MRPL55, RRS1 and SLC4A11) can be of a clinical utility as diagnostic biomarkers. Out of these seven biomarkers, only KRT4 appears to be a marker for exposure-associated disease. This finding increases the importance of KRT4 by suggesting that might be under the influence of oestrogenic responses. Indeed, KRT4 expression was intensely down-regulated when ER-positive ovarian cancer cells were treated with oestrogen [37]. A recent study has found KRT4 to be

2.7-fold downregulated after exposure to 150 μg BPA in novel ERE transgenic (ERE-TG) zebrafish, compared to controls [38]. Further studies are needed to measure the circulating BPA levels in OC patients and correlate these concentrations with expression of exposure-associated OC biomarker (KRT4) in both tissue and liquid biopsies.

Due to COVID pandemic situation in 2020 – 2021, we were restricted to work from home. Utilising that time, we provided an extensive overview of a wide range of mutations on those seven recently predicted biomarkers for OC (in our first paper [36]) by using a number of in silico tools and databases (UK BioBank, TCGA Xena repository, and cBioPortal). We were interested to find the mutational impact on tissue targets, molecular mechanisms and health effects of bisphenolic chemicals on these genes. We also assessed the mutation impact on amino acid polarity, amino acid charge and amino acid water affinity, leading to the identification of only one missense mutation (i.e., R831C/R804C in uterine corpus endometrioid carcinoma - UCEC) that was indicative of structural damage on the solute carrier family 4, sodium borate transporter, member 11 (SLC4A11) protein in gynecological cancers. Furthermore, we modelled two variants of this protein (918 amino acid variant and 891 amino acid variant) and provided in silico evidence of how a variation from arginine (R) to cysteine (C) can cause potential deleterious consequences on biological functions and processes by using an array of protein modelling tools and databases (e.g. Missense3D, PyMOL, YASARA Energy Minimization Server, PROVEAN, SWISS-MODEL, Phyre2, PDB and UniProt). Finally, we aligned our modelled of predicted SLC4A11 protein variants structures with AlphaFold and found 100% alignment score in the R804 region. In this study, we were successful in finding the impact of mutation on the biological function of a protein, gave a score of -7.292 with the annotation "Deleterious" for both R831C and R804C. However, further studies are needed to gain in depth knowledge of the actual role of this transporter protein SLC4A11 in UCEC and how this deleterious mutation lead towards the gynaecological malignancies. For example, an association between high SLC4A11 expression and poor overall survival rate in grade 3/4 serous OC patients has been noted [39]. Apart from providing a novel insight into a deleterious mutation for SLC4A11, this paper will also constitute the very basis of a methodological platform that scientists can use as a step-by-step approach to interrogate functional consequences of SNPs.

As mentioned, little is known about the impact of BPA at normal ovarian level in humans. Therefore, to understand the possible mechanisms underlying we exposed normal primary Human Epithelial Ovarian Cells (HOSEpiC) to environmental levels of BPA (10 and 100 nM), to determine changes in transcriptomics by using high-throughput RNA sequencing. Transcriptomic analysis revealed 76 DEGs (10 up-regulated and 66 down-regulated genes) that were common between two BPA doses (i.e., 10nM and 100nM). Enrichment analysis was carried out to find out relevant functions and pathways within which differentially expressed genes were significantly enriched. In terms of site of expression, we identified the highest percentage of genes involved in female malignancies including ovarian cancer when exposed to 100nM BPA. We also found the extremely effected pathway, common upon the exposure of two different doses of BPA was that of progesterone-mediated oocyte maturation. This pathway mainly involve in the regulation of mammalian ovulation and fertilisation [40]. Therefore, our data shows a direct link between BPA and fertility. Building on the present novel findings, future studies should further explore the changes that BPA and other endocrine disruptors (e.g. BPS and BPF) can elicit within the ovaries at gene, protein and metabolic level, subsequently addressing relevant gaps in our current knowledge on basic biology, hazard characterisation and risk assessment associated with the use of xenoestrogens at the ovarian level in health and disease.

We also found that 10 genes namely: *MT2A, RN7SK, LCN2, SPP1, ADGRG1, CA12, SAT1, CGNL1, SLC7A5* and *MT1X* were common between two experiments (experiment 1: control and BPA-treated normal HOSEpiC cells, experiment 2: control and BPA-treated epithelial cancer SKOV3 cells). Unsurprisingly, seven out of ten genes namely: *RN7SK, CA12, LCN2, SPP1, SLC7A5, ADGRG1* and *CGNL1* have been previously associated with various female malignancies including cervical, breast and ovarian cancers [36][41]. Two genes *MT2A* and *SLC7A5* are also associated with the mTOR signalling and plasma membrane oestrogen receptor signalling pathways [36][41][42]. The mTOR pathway is a central regulator of cellular events such as proliferation, apoptosis and angiogenesis estimating external energy, growth factor and stress signals with the PI3K/AKT/mTOR pathway being a highly activated cellular signalling pathway in advanced ovarian cancer [43][44]. While studies have shown the association of *SAT1* and *MT1X* with other tumours e.g. glioblastoma [45][46].

Furthermore, during the peak of COVID pandemic in 2020, I also contributed in

the review article demonstrating the link between one of the most abundant EDC; BPA and the severe COVID-19 [41]. We discussed the Potential links via which BPA could be indirectly increasing the risk for severe COVID-19. Environmental doses of BPA exposure can contribute in cardio-metabolic diseases, endocrine-related cancers, and immune system dysregulation and, concluded that, may be indirectly linked to higher risk of severe COVID-19 (Introduction Figure 1.15). Moreover, as BPA mimics oestrogen, oestrogen receptors which directly facilitate BPA effects, such as the membrane-bound oestrogen receptor (GPR30), are extensively distributed in human tissues [42], and may co-localise with SARS-CoV-2 infection mediators (e.g. co-localisation of GPR30 with TMPRSS2 in the lung), and possibly affecting the tissue expression. Therefore, there might be potential association between BPA exposure and the risk of SARS-CoV-2 infection and the severity of COVID-19 [43][44][45].

6.2 Animal and Preclinical Models

In order to gain a deeper understanding of EDCs in ovarian cancer, the correct preclinical or in vivo model needs to be chosen. What makes it harder is the fact that OC is a diverse disease in its histology, as it includes HGSC, LGSC, CCC and MC as described previously. Moreover, approximately 20% of OC patients also have mutations on BRCA1 and BRAC2 genes, so again the mutational landscape will also dictate use of appropriate models to interrogate a specific research objective. Moreover, ovary is an endocrine organ, and its role under normal conditions should not go unnoticed when experiments are planned.

Largely, a classification can be made based on whether the model is in vivo or in vitro [52]. There are a number of in vivo OC models described in the literature. These include genetically engineered mouse models with similar ovarian physiology and anatomy [53]. For example, female TgMISIIR-TAg-DR26 transgenic mice have been used to study the effect of mTOR inhibitors [54]. In the design of OC mouse models, careful consideration should be given on the use of human cell line or patient-derived xenograft (PDX), the location of the transplanted tumour, as well as the immunity of the model [55]. For example, in orthotopic models (i.e. where there is surgery intervention), OC cells can be transplanted in anatomically-related

areas (i.e., where tumour cells were originated) [55]. During the past decade (2012-2022), 4724 manuscripts were published using animal models for OC (including mice, rats and hen; source: PubMed.gov). To date, very few studies have been published on the use of OC animal models to study EDCs in the past decade. An example is that of BALB/c nu/nu mice, xenografted with BG-1 OC cell line, to study the cross-talk between genistein, estradiol and BPA [56]. The lack of in vivo studies might highlight the fact that in the cancer field, approximately 5% of anti-cancer therapies tested in mice make their way to phase III clinical trials [55]. This poses a serious limitation and given the global drive of 3Rs in animal experimentation, highlights the need of reliable alternative preclinical models.

An alternative to animal models is the use of human ovarian cancer explants. During the past decade (2012-2022) 39 studies were published using OC explants (source: PubMed.gov). Indeed, ex vivo models (including explants or tissue slices) have been shown to "maintain the spatial conformation of the tissue, heterogeneity and tumour stage" [57]. In a recent study by Abreu et al., they have shown that it is possible to establish OC patient-derived explants (PDE) and keep them alive for 30 days, while preserving the tumour characteristics [57]. The clinical utility of these ex vivo models in EDC research should intensify in the near future. However, one obstacle that we need to overcome is the patient variation when it comes to these models.

One way to circumvent this, is to use well-characterised OC cell lines as we and a plethora of laboratories worldwide have used [58]. During the past years, there has been a move from the classical 2D to the more complex 3D in vitro systems, as extracellular parameters are more physiologically relevant in 3D, allowing cancer cells to grow into organoid-like structures. We found 341 reports of 3D OC models since 2012 (source: PubMed). These models have enabled scientists to study in more detail changes in the tumour microenvironment (TME) and effects of therapeutic agents and other peptides. However, no studies on EDCs (including BPA) have been published making use of these models.

It is evident therefore, that there is an increase need for reliable models to study EDCs. There should be a fine balance between animal models and their excess use and the reproducibility of data from ex vivo and 3D models. Only then, findings can be of use to drive global policies on chemicals and their impact on endocrine

disruption. It should be noted however, that regulatory policies vary between the EU, USA, and other developed and industrialising nations [59].

6.3 References

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