CHAPTER X Plastics additives and human health: a case study of bisphenol A (BPA)

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Abstract

Bisphenol A (BPA) is a chemical widely used in food and drinks packaging. We previously reported that the Estrogen Related Receptor α (*ESRRA*) gene is BPA-responsive. *ESRRA* encodes ERRα, a protein important in hormone signalling, cellular metabolism, immunity and energy sensing. We hypothesised that the *ESRRA* gene may undergo changes in isoform usage in response to BPA as has been noted for other estrogen responsive genes. We first examined the natural expression profile of *ESRRA* transcripts and showed that 3 transcripts are produced from 2 alternative promoters in multiple human tissues. We then treated the human T cell line Jurkat with physiologically-relevant low concentration (5nM) or high concentration (50nM) of BPA. Exposure to BPA caused isoform-switching, leading to increased *ESRRA* isoform 1&2 expression, and reduced *ESRRA* isoform 3 expression. Finally, we assessed the effect of changes in urinary BPA levels on *ESRRA* isoform usage in a cohort of student researcher participants who had provided blood samples following an intervention diet. Where participants had shown a decrease in urinary BPA concentration, change in BPA was positively associated with changes in the expression of the short isoform of the *ESRRA* gene alone (beta coefficient 0.49; $p = 0.02$). Our data support the hypothesis that exposure to BPA leads to changes in *ESRRA* isoform expression. Given the tissue specificity of isoform expression, altered expression has the potential to induce phenotypic effects in tissues reliant on particular isoforms for correct function.

Introduction

Plastics are extremely useful materials that provide many benefits to society. The combination of cost-effective production and versatility has led to plastics finding uses in all aspects of modern life, from food and drinks containers, to medical devices, consumer items and even building materials. The growing popularity of plastics has however come at a cost; there is widespread leakage of plastic waste into the environment, for example from single use items designed to be used once (with an average of 20 minutes of use for items such as plastic bags) and then discarded. Such leakage, amounting to some 25.8million tonnes per year in Europe alone (Plastics Europe, 2018) is economically detrimental and is increasingly being associated with adverse effects to the food chain, human health and the environment. In a recent general population survey conducted across Europe by the trade organisation Plastics Europe, 87% of respondents expressed concern about the impact on the environment of everyday products made of plastics, whilst 74% expressed concern over the potential for plastic products to damage their own health. (ref) Much of that concern lies in the potential for the continuous interactions with plastic items that most people experience in their daily life to lead to uptake of tiny pieces of plastic or plastics additives across the skin or airways or through ingestion of contaminated food and drink. Contamination of food may come from various sources, either from direct contact with packaging or processing materials during food manufacture or may be the consequence of leaching of additives and degradation of plastic litter into the environment and the food chain of plastics.

It is not an easy task to assess the overall risks of such interactions. It is estimated that around 14.5 million tonnes of the 300 million tonnes of plastic produced each year is used for food and drinks packaging. Migration from packaging directly into food is considered to be the main route of exposure for most people and there are rigorous standards in place to regulate what chemicals can be present in food packaging materials and to set standards for the rates of migration into food that are allowable (ref). Despite this, only a fraction of the thousands of chemicals in common use have been rigorously tested, in part because it is not practically

feasible to do so. What happens to most plastic polymers once they reach the wider environment and start to degrade remains largely unknown, making it extremely challenging to adequately assess any risks to human health.

Foodcontactarticles(FCAs)areusedinproduction,process- ing, transport,handling,andstorageoffood(e.g.,foodpackaging, storagetanks,andconveyorbelts).Variousfoodcontact materials (FCMs)areusedtomakeFCAssuchasplastics,paperandboard,metal,glass,adhesives,andprintinginks.Food packagingandotherFCAsareofhighsocietalimportance because theyprotectfoodfrom physical damage,soiling,and microbialspoilage,therebyreducingfoodwaste.However, chemicals canmigratefromfoodpackagingandotherFCAs into foodandtherebypotentiallyaffect humanhealth.Food safety canalsobecompromisedbychemicalcontaminantspres- ent intherawfood(e.g.,pesticidesandheavymetals),produced during processingandcooking(e.g.,acrylamideandpolycyclic-aromatic hydrocarbons),orintroducedduringimproperhandling (e.g., residualcleaningagents)(FigureS1).

Althoughfoodcon- tact articlesareessentialforthefoodsupplychain,theirbenefits need tobebalancedwiththepotentialforhuman-healthrisks associated withexposuretomigratingchemicals,someofwhich have beenclassified asendocrine-disruptingchemicals(EDCs) (Geueke etal.2014). Arecentexpertpanelestimatedtheeco- nomic burdenofhealthconditions(adultdiabetes,obesity,IQ loss andassociatedintellectualdisability,cryptorchidism,and male infertility)withareasonablyhighprobabilityofcausation by EDCs[includingbisphenolA(BPA),phthalates,andorgano- phosphate pesticides]tobe €157 billionannuallyintheEuropean Union (Trasande etal.2015). Therefore,addressingexposureto EDCs andotherhazardouschemicalsfromfoodpackagingand other FCAsisanopportunityforpublichealthinterventionand may contributesubstantiallytoreducinghealthcosts.

Human biomonitoring

Central to assessing any risks to human health is to know exactly what chemicals and plastics are actually getting into people. Most people are exposed to complex and variable mixtures of chemicals and other substances throughout their normal daily activities, such as from consuming food and drink, handling and using consumer products and through interactions with the wider environment; inhaling chemicals through air, or ingesting household or roadside dust. For most chemicals, the impacts on health associated with

aggregated exposures over a lifetime remain uncertain, as do the added complexities of exposure to mixtures of different substances. Human biomonitoring can be helpful in this regard because it involves determining an individual's exposure to chemicals and other substances by measuring either the chemicals themselves or their metabolites or degradation products in body fluids or tissues. Biomonitoring is considered a gold standard because it provides an integrated measure of exposure from varied sources (Sexton, 2004) that can be used to establish exposure-response relationships and to inform epidemiological studies and identify sources or routes of exposure. Samples can be obtained from tissues or from body fluids including urine, blood or serum, breast milk, saliva and even hair, allowing for non-invasive and repeated sampling.

A number of large scale population relevant biomonitoring programmes have been established over the last decades, such as the United States National Health and Nutrition Examination Survey (NHANES), a program of studies designed to allow the assessment of the health and nutritional status of adults and children (http://www.cdc.gov/nchs/nhanes.htm). Of relevance to this article, NHANES includes the measurement in population representative samples of numerous chemicals associated with the use or manufacture of plastics, polymers and resins including bisphenol A, styrene, phthalates, triclosan, acrylamide, and brominated flame retardants. In Europe, the European Human Biomonitoring Initiative, (HBM4EU) was set up to aid in assessing and minimising risks to the environment and human health associated with the use of hazardous substances. It is a large scale programme involving 26 countries, the European Environment Agency and the European Commission. The current priority list for HBM4EU, whilst still relatively modest in comparison with NHANES includes phthalates, bisphenols, and perfluorinated compounds amongst others (https://www.hbm4eu.eu/wp-content/uploads/2017/03/scoping-documents-for-2018).

These approaches have shown that certain chemicals associated with the production or use of plastics are detectable in a significant percentage of the human population. A key feature of programmes such as these is the open access of the data to scientists to enable and encourage studies of potential health effects and . For some of these chemicals, their widespread presence in the general population at concentrations capable of causing harm in animal models has raised public health concerns (Talsness et al.,2009 , Melzer and Galloway 2011).

Bisphenol A (BPA) is a synthetic compound with estrogenic properties that is widely used in the plastics industry and is predominantly found in food packaging (polycarbonate plastics, epoxy can linings), thermal paper and dental sealants (1). It is labile within plastics, particularly when in contact with lipid-rich foods or during heating (2), and can readily leach into the contents of the packaging. In the Western world, there is near ubiquitous exposure, with greater than 95% of people showing measureable levels of BPA metabolites in their urine (3, 4). Although not definitive proof of causality, exposure to BPA has been associated with adverse human health outcomes, including type 2 diabetes, cardiovascular disease, obesity and abnormalities of sex hormone levels in cross-sectional studies (4-7) and prospective studies; reviewed in (8). We have previously identified the Estrogen Related Receptor α (*ESRRA*) gene as a molecular target of BPA in vivo and in vitro (9, 10). This gene has a key role in cardiac function, immune response and energy sensing (11-13). Sequences corresponding to alternative *ESRRA* transcripts have been identified in cDNA libraries along with histone marks indicative of dual promoters. To date, the expression of these alternative transcripts has not been demonstrated in multiple human primary tissues and their responses to estrogenic stimuli are unknown.

Estrogen and estrogen-like chemicals are known to alter patterns of isoform usage in estrogen responsive genes. A targeted cloning approach in zebrafish revealed that the estrogen receptor alpha (*ESR1*) gene produces six isoforms, and that the expression of these was sensitive to estrogen exposure. The authors of this study proposed that the estrogen-responsive changes in promoter choice and isoform usage form part of an auto-regulatory mechanism by which estrogen may modulate the expression of its receptors (14). In accordance with its estrogenic activity, BPA has also been shown to modulate the expression of specific *ESR1* isoforms in prepubertal female rats exposed to BPA in the neonatal period (15). BPA has also been shown to alter the splicing patterns of other target genes such as *VEGF,* in the reproductive tissues of both Fisher and Sprague Dawley rats (16). Other estrogenic chemicals such as phthalates have also been reported to affect isoform usage for the *CAR* and *PXR* xenobiotic receptor genes in COS-1 human hepatocytes in vitro (17).

The aims of this study were to determine whether alternatively–expressed isoforms of the *ESRRA* gene exist in primary human tissues and if so, whether they respond differently to BPA in vitro and in vivo. We show here for the first time that the *ESRRA* gene produces both long and short transcripts, which show distinct expression patterns in human primary tissues. Furthermore, these isoforms respond differently to BPA at both high (50nM) and low (5nM) concentrations in vitro. Finally, in participants who had undertaken a week-long BPA avoidance diet, change in *ESRRA* short isoform expression was associated with change in urinary BPA levels, but only in participants who had demonstrated a reduction in urinary BPA. We discuss how the differential responses of *ESRRA* isoforms to BPA exposure may provide an explanation for the specific effects of BPA in different tissue types.

Materials and Methods

Cell lines and tissues

Jurkat E6.1 cells (HPA culture collections ECACC, Salisbury, UK) at passage 10 were used in our analysis for assessment of the effect of BPA on *ESRRA* gene expression. The E6.1 clone was derived from a blood sample from a patient with a T-cell leukemia and retains some immune function, in that it is able to secrete IL-2. For the generation of the *ESRRA* isoform tissue expression profile, a multi-tissue human expression panel including immune, metabolic, cardiovascular, endocrine, excretory, digestive and neural tissues was used (Becton, Dickinson & Co., Franklin Lakes, NJ, USA: BioChain Institute, Newark, CA, USA: Ambion®, Austin, TX, USA).

Human samples

Human peripheral blood samples were taken using the PAXgene system (Qiagen, UK) as part of a 'Citizen Science' engaged research project with schools in the South West of England, where students were both participants and researchers (18). Urine samples were taken into BPA-free urine collection tubes (Vacutest Kima, Italy) for BPA and creatinine quantification. Participating students were all aged 17-19 years and donated blood and urine samples for analysis of *ESRRA* isoform expression and BPA exposure on 2 occasions separated by 7 days. During this time period, student researchers undertook a "real world" diet designed to help them reduce their consumption of BPA by avoidance of processed foods, foods packaged in BPA containing plastics and canned foods and drinks. Information on sex, BMI, tobacco and alcohol usage and exposure to synthetic estrogens was also collected. Full details of our researcher participant cohort, food diaries and the BPA reduction diet are all described in (18).

Ethical permission was granted for this study by the University of Exeter Medical School Ethics Committee (reference number 15/07/074) and samples were taken with written informed consent.

Treatment of cell lines

Jurkat cells were established as suspension cultures in phenol red-free RPMI 1640 medium (Sigma Aldrich, St Louis, USA), due to the estrogenic activity of phenol red. Cells were seeded into 6 well plates at 2 x 10^6 cells per well for analysis and treated with either 1mM ethinyl estradiol (EE), 5nM BPA, 50nM BPA or solvent carrier (ethanol) alone (Sigma Aldrich, St Louis, USA). Time course experiments were undertaken with samples assessed at 0, 6, 24, 48 and 72 hours, in line with the dynamic of effect on gene expression that we have noted in our previous work (9, 10). Concentrations and time courses used are in line with other studies of this nature, with 5nM representing a physiological concentration since BPA has been found in human fluids in the range of 0.2 – 20nM (19). Experiments were each carried out in 8 biological replicates.

Measurement of BPA in urine samples

Detailed descriptions of analytical approach are given in more detail in (18). Briefly, samples were frozen on dry ice for transport to a commercial laboratory where analysis of total BPA was assessed by gas chromatography-tandem mass spectrometry. Experimental methods were validated in terms of linearity, detection limit and accuracy and specificity of quantification based on the Standard NF T 90-201 for determination of xenobiotics in water samples. QC was carried out after each batch analysis; a QC check of known standards injected every 6 samples at two levels of concentration (0.5 ng/ml and 5 ng/ml) was used each time and quantified along with the unknown samples. Water only samples were also included as negative controls to

identify contamination arising from collection plastics. Urinary creatinine was also measured by the Biochemistry service at the Royal Devon and Exeter Hospital using the Jaffe method on the Roche P800 platform (Roche, Mannheim, Germany), to allow correction for urine dilution. Results were expressed as a BPA:creatinine ratio.

RNA extraction

For the cell work, total RNA was extracted from approximately one million cells per sample, using the MirVana kit (Ambion™/ThermoFisher, MA, USA) according to the manufacturer's instructions. RNA purity and concentration was assessed by Nanodrop spectrophotometry (NanoDrop/ThermoFisher, MA, USA). RNA was extracted from researcher participant blood samples using the PaxGene system (Qiagen, UK) according to manufacturer's instructions.

Reverse transcription

Complementary DNA (cDNA) was produced by reverse transcription from 100ng of total RNA for both cells and blood samples, using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen™/ThermoFisher, MA, USA) in 20µl reactions, according to the manufacturer's instructions.

Determination of total ESRRA expression

For qRTPCR, 0.5µl cDNA was added to a 5µl reaction including 2.5µl TaqMan® Universal Master Mix II, no UNG (ThermoFisher, MA, USA) and 0.25µl of probe and primer mix (corresponding to 900nM each primer and 250nM probe) as supplied by the manufacturers. Total *ESRRA* expression was measured using an assay described previously (9, 10). Isoform specific assessment of the expression of long and short *ESRRA* isoforms was carried out using real-time PCR assays custom designed specific to unique regions of the isoforms in question

(see figure 1). One assay was designed to amplify both of the long isoforms (transcripts NM_001282451.1 and NM_004451) which are expressed from the 5' promoter and are almost identical, differing in only a single amino acid, and the other was designed to the short isoform expressed from the 3' promoter (transcript NM_001282450) and has a different first exon. The custom assays were validated for efficiency, sensitivity and accuracy by standard curve analysis with 7 serial 1:2 dilutions of Jurkat cDNA template. Primer sequences and control gene assay identifiers are available on request. Assays to the endogenous control genes β-2-microglobulin (*B2M*), Beta-glucorinidase (*GUSB*), Hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), Isocitrate dehydrogenase [NAD] subunit beta (*IDH3B*) and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and cyclophilin (*PP1A*) were obtained as off-the-shelf assays from Thermo Fisher (MA, USA); assay identifiers are available on request. Reactions were run in triplicate on 384-well plates using the 7900HT Fast Real-Time PCR System (Thermo Fisher, MA, USA). Amplification conditions were a single cycle of 95°C for 10 minutes followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Gene expression levels were calculated using the Comparative Ct approach relative to housekeeping genes chosen empirically from the list on the basis of stability (*B2M*, *IDH3B* for the cell work and *B2M*, *HRPT*, *IDH3B* for blood work). Relative levels of long and short isoforms in each tissue calculated by the comparative Ct approach and were expressed relative to the geometric mean of long isoforms across the tissue panel (in vitro work) or the geometric mean of long isoform levels at visit 1 (in vivo work).

Figure 1*: Gene structure, regulatory motifs and location of promoter regions of the ESRRA gene.*

The position of the 5' terminal exons of the three putative isoforms of the ESRRA gene are indicated by black boxes. The position of the large ESRRA CpG island is indicated by mid-grey hatched boxes. The direction of transcription is marked by a grey arrow. The positions of the isoform-specific PCR primers are given by black arrows. Active regulatory regions as indicated by H3K27Ac histone acetylation marks is given by light grey areas, with potential alternative promoter regions ESRRA(1) and ESRRA(2) indicated by dark grey boxes.

Statistics

Responses of total *ESRRA* expression to 1mM EE, 5nM BPA or 50nM BPA and characterisation of *ESRRA* isoform usage in response to 5nM and 50nM BPA treatment were all carried out by pairwise two-tailed Students t-tests. Associations between change in BPA (ΔBPA) and change in usage of long or short *ESRRA* isoforms was carried out by linear regression, adjusted for sex, tobacco usage, alcohol usage, BMI and exposure to synthetic estrogens. Statistics were performed using SPSS v.22 (IBM, USA).

Results

ESRRA isoforms demonstrate distinct tissue profiles.

To assess the authenticity of different *ESRRA* isoforms in human tissues, we quantified their expression by qRTPCR using isoform-specific probes. Isoform-specific assays proved accurate and sensitive over a linear range of 7 serial dilutions, with efficiencies of 86% and 83%, and r^2 between technical replicates of 0.96 and 0.97 respectively. We determined that the cDNA sequences represented in the transcriptome databases do indeed represent genuine *ESRRA* isoforms, which encode identical proteins, but differ in their 5' regulatory regions (figure 1). Both long and short isoforms of the *ESRRA* gene were present in all tissues tested, but in differing proportions (figure 2). The long isoforms were predominant in endocrine/metabolic

tissues (with the exception of liver), digestive, muscular, neuronal and excretory tissues. Levels of the short isoform were more abundant in immune and reproductive tissues and were predominant in liver, thymus, whole blood, uterus, testes, ovary and placenta (figure 2).

Figure 2. *Expression patterns of ESRRA isoforms in human tissues*

Expression of ESRRA isoforms is calculated relative to a panel of endogenous control genes and normalised to the geometric mean of levels of the long isoforms across the panel as a whole (see methods). Error bars refer to the range of measurement as calculated from the standard deviation of the triplicate measurements. Levels of the long isoforms (NM_001282451 and NM_004451), captured by a single probe are given in dark grey, whilst levels of the short isoform (NM_001282450) are given in light grey. Tissue characterisation is given on the X axis, and is labelled as follows: A. Metabolic/endocrine tissues B. Immune tissues, C. Digestive tissues D. Excretory tissues. E. Respiratory tissues F. Brain tissues G. cardiovascular/muscle tissues and H. reproductive tissues.

Total ESRRA expression in human Jurkat T cells is responsive to 17α-ethinyl estradiol and BPA.

We next examined the effect of BPA on alternative expression of *ESRRA* isoforms in vitro. Firstly, we confirmed the estrogen responsiveness of the Jurkat cell cultures to 1mM 17α-ethinyl estradiol (EE). Up-regulation of total gene expression of the *ESRRA* gene in response to 1mM EE (figure 3A). We then assessed changes in total *ESRRA* expression following exposure to 5nM and 50nM BPA (figures 3B and 3C). Although no significant differences in *ESRRA* expression were noted at 5nM BPA, this could result from the isoforms responding

reciprocally to BPA exposure. Changes in *ESRRA* expression were seen following treatment with 50nM BPA which were similar to those seen in our previous work (10).

Figure 3 *– Expression changes in ESRRA gene expression levels in response to 17α-Ethinyl Estradiol (EE), 5nM BPA and 50nM BPA.*

*The expression responses of the ESRRA gene in response to 17-α ethinyl estradiol (**A**), 5nM BPA (**B**) or 50nM BPA (**C**) are given. Data are presented as stem and whisker plots representing the median value and interquartile range at each time point. Expression data are given on the Y axis and represent total ESRRA expression relative to the geometric mean of a panel of endogenous control genes that included B2M, GAPDH, GUSB, HPRT, IDH3B and PP1A. Data are normalised to the levels of ESRRA expression seen at baseline. Levels of statistical significance are given by stars, and * = p = <0.05, ** = p = <0.005, *** = p = <0.0005.*

Treatment with 5nM and 50nM BPA leads to a change in ESRRA isoform usage

We noted a reciprocal change in the expression of transcripts encoding long and short *ESRRA* isoforms in response to both 5nM and 50nM BPA. At 5nM BPA, the *ESRRA* isoform profile switches towards the long isoforms and away from the expression of the short isoform as soon as 6 hours after treatment, and returns to baseline levels at the 48 hour time point (figures 4A and 4B). These results explain the lack of changes in total *ESRRA* expression seen at 5nM BPA, since the differential effects of long and short isoforms are reciprocal. At 50nM BPA, we saw similar, but more marked changes, with the up-regulation of long *ESRRA* isoforms being elevated at 6 hours and remaining high thereafter (figure 4C). The expression of the short

isoform demonstrates a biphasic expression at 50nM BPA, with levels being initially lower at 6 hours, but then elevated after more chronic exposure at 48 and 72 hours (figure 4D).

Figure 4 *– Changes in ESRRA isoform usage at 5nM and 50nM BPA.*

This figure illustrates the changes in the relative expression of alternatively-expressed ESRRA isoforms in response to 5 and 50nM BPA Data are presented as stem and whisker plots representing the median value and interquartile range at each time point. Expression changes to the level of long ESRRA isoforms (NM_001282451 and NM_004451) in response to 5nM and 50nM BPA are given in ***(A)*** *and* ***(C)****. Changes in short ESRRA isoform (NM_001282450) expression in response to 5nM and 50nM BPA are given in* ***(B)*** *and* ***(D)****. Expression levels of each isoform at each time point are calculated relative to the endogenous control genes (HPRT, B2M and IDH3B) and are normalised to levels of the long isoforms at baseline. Levels of statistical significance are given by stars, and * = p = <0.05, ** = p = <0.005, *** = p = <0.0005.*

In vivo assessment of BPA levels

Full details of the effect of the dietary intervention on BPA levels has been previously published (18). Briefly, BPA levels were quantified in samples taken before and after participation in the intervention trial from 94 individuals, with a limit of detection of 0.1ng/ml. Samples scoring positive for BPA but quantifying at or around the limits of detection (LOD) were scored as LOD/√2 (=0.07ng/ml) according to the method of Hornung and Reed (20). No BPA contamination from the urine collection containers themselves was noted in any of the controls. Following the dietary intervention, 50/94 participants showed lower urinary BPA levels at visit 2 (18). In these 50 participants, mean urinary BPA at visit 1 was 2.41ng/ml (95%

382 CI 1.9 to 2.9 ng/ml), whereas mean urinary BPA at visit 2 was 1.02 ng/ml (95% CI 0.74 to
383 1.30ng/ml). The mean drop in BPA between visit 1 and visit 2 in the 50 participants was 1.41
384 ng/ml. Cohort characteristics for BPA measurement for these 50 participants are given in table
385 1.

386 **Table 1**. Characteristics of student researchers in the BPA study cohort. The characteristic of
387 the student participant cohort are given here. Numbers in parentheses refer to the standard
388 deviation of measurement.

Characteristic	Measurement
Mean BPA at visit 1	2.41 (1.80) ng/ml
Mean BPA at visit 2	1.02 (1.00) ng/ml
Mean change in BPA	-1.41 (1.53) ng/ml
Mean *ESRRA* long isoform expression at visit 1	1.13 (0.47)
Mean *ESRRA* long isoform expression at visit 2	1.09 (0.57)
Mean change in *ESRRA* long isoform expression	-0.04 (0.53)
Mean *ESRRA* short isoform expression at visit 1	1.20 (0.63)
Mean *ESRRA* short isoform expression at visit 2	1.07 (0.63)
Mean change in *ESRRA* short isoform expression	-0.11 (0.59)
Mean BMI	21.5 (3.17)
% synthetic estrogen exposure	16%
% male	45%
% tobacco usage	8%
% alcohol usage	36%

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Change in urinary BPA level is positively correlated with change in levels of the short ESRRA isoform in vivo.

There was no cross-sectional correlation between expression of long or short isoforms of *ESRRA* before or after the intervention trial. The degree of change in urinary BPA concentration before and after intervention was however, positively correlated with change in the expression of the short *ESRRA* isoform NM_001282450, but not the long isoforms NM_001282451.1 and NM_004451 (beta coefficients 0.42 and 0.49; p = 0.06 and 0.02 for the long and short isoforms respectively; figure 5). The samples showing the largest decrease in urinary BPA excretion between visits demonstrated the largest change in expression of the short isoform of *ESRRA*. No correlation between change in isoform levels and change in urinary BPA concentration was noted for individuals reporting an increase in urinary BPA at visit 2.

Figure 5 *– Correlation between change in urinary BPA and change in the expression of the short isoform of the ESRRA gene.*

This scatter plot illustrates the relationship between change in urinary BPA and change in the expression of the short isoform of the ESRRA gene before and after participation in the dietary intervention trial. Urinary BPA is expressed as a BPA:creatinine ratio in ng/ml whilst expression units are arbitrary measures representing the amount of the short isoform of ESRRA expressed relative to the endogenous control genes and normalised to the level of the reference isoforms.

Discussion

Exposure to BPA has been widely reported to be associated with gene expression changes in animal models and in human cells (21-23). We report here that the *ESRRA* gene is expressed as three alternatively-expressed isoforms in man, which exhibit tissue-specific expression. Furthermore, exposure to physiologically-relevant concentrations of BPA (5nM) can cause a switch in isoform usage towards the long isoforms of *ESRRA* transcripts in human T-cells. Furthermore, in human blood mRNA samples from individuals who demonstrated a change in urinary BPA levels following an intervention diet, changes in the expression of the *ESRRA* gene were noted that are consistent with the switch in isoform usage that we noted in vitro.

The long isoforms of *ESRRA* are expressed in tissues such as heart, pancreas and adipose tissue whilst the short isoform of the *ESRRA* gene is predominant in many reproductive tissues such as ovary and testes involved in sex hormone signalling. Exposure to BPA has been associated with adverse human health outcomes, including type 2 diabetes, cardiovascular disease, obesity and abnormalities of sex hormone levels and reproductive function in cross-sectional studies (4-7) and prospective studies; reviewed in (8).

We noted no cross-sectional associations between expression levels of either long or short isoforms of *ESRRA* and urinary BPA concentrations in intervention trial volunteers. The analysis was underpowered to detect such associations given our small numbers, differences in genetic background and the presence of circulating estrogens in the female members of our cohort. However, measuring change in expression in relation to change in BPA levels allows examination of potential relationships without the confounding influence of other genetic or environmental factors that may influence *ESRRA* isoform expression. This analysis suggests that individuals showing a reduction in BPA exposure during a dietary intervention had correspondingly lower *ESRRA* short isoform expression. We identified no relationship between

change in urinary BPA levels and change in isoform expression in individuals who did not demonstrate a drop in BPA during the intervention trial, perhaps indicating a threshold effect.

The *ESRRA* gene product, ERRα, has pivotal roles in cellular metabolism and energy sensing, particularly in tissues with high energy demand (24). This is particularly evident in tissues such as the heart, where whole body ERRα knockout mice has shown defects in response to bioenergetics and functional adaptation to cardiac stress and neonatal cardiac defects (25, 26). ERRα also has roles in effector T cell activation and differentiation; Inhibition of ERRα results in blocks to T effector cell growth and differentiation following immunisation and in experimental models of autoimmunity (27). Given these findings, it is interesting to note the range of adverse health outcomes with which exposure to BPA has been associated.

The physiological consequences of *ESRRA* isoform changes are difficult to predict, given that all three isoforms code for the same protein; the first exon is non-coding and the translation initiation codon is present in exon 2. The isoforms have distinct 5' regulatory regions and may therefore show different temporal or spatial expression, as suggested by our tissue specificity data (figure 2). Alternative promoter usage has previously been shown to have profound effects on the stability or translation potential of mRNA species, even when the encoded protein products are identical (28, 29). ERRα acts as a transcriptional activator of downstream genes involved in energy management, by virtue of its interaction with the peroxisome proliferator-activated receptor γ coactivator 1a (PGC-Iα), which acts as a ligand independent coactivator (30). The long isoforms of *ESRRA* contain several regulatory elements not found in the short isoforms. Firstly, the *ESRRA* gene responds to estrogen (and BPA) through a conserved hormone response element consisting of a 34bp sequence present in its proximal promoter region. Studies show that this sequence is a target for ERRγ transactivation that is enhanced by

the binding of PGC-1α (31). Examination of the sequence around the putative second promoter reveals that this motif is not present, which may explain why the short isoform demonstrates reduced expression in response to BPA at early time points whilst the long isoforms are upregulated. There is also evidence that this regulatory element is able to bring about an ERRα/PGC-1α dependent autoregulation of *ESRRA* by itself. Interestingly, this motif is polymorphic, with evidence that the number of repeats influences the degree of *ESRRA* activation (32). The lack of this regulatory region in the distal promoter is likely to result in a different activation dynamic of short isoforms of the *ESRRA* gene, which could have profound consequences for tissues where *ESRRA* expression is predominantly of this type.

The strengths of our study are the tandem analysis of gene expression both in vitro and in vivo. The limitations of our study are our use of T cells as our model system rather than other cell types. T cells are however a valid and important BPA target tissue as evidenced by the effects of BPA on autoimmunity and immune response (33, 34) and the role of ERRα in determination of T effector cell proliferation and differentiation (27). It would be of particular interest given our findings to expand future studies to include other metabolically active cell types, given the observed tissue specificity of *ESRRA* isoforms. The consequences of these changes and their potential effects on human health must be interpreted in the context of existing data on the effects of chronic BPA exposure in populations on health outcomes, and also the in vivo roles of the genes in question.

Conclusions

We conclude from our studies that BPA may influence expression of some of its target genes by modulating the usage of alternatively-expressed isoforms in human T cells in vitro and potentially, in mRNA from peripheral blood in vivo. When considered in the context of existing

data on the known role of the *ESRRA* gene in cardiac metabolism (25, 26), and the known associations between BPA exposure and cardiovascular outcomes in longitudinal studies (35), the production of *ESRRA* isoforms with different potential for transactivation or autoregulation in response to BPA may help to explain some of the phenotypes associated with exposure to this chemical.

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603 **Supporting information**

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605 Supporting information file 1. Members of the BPA Schools study consortium

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