Plastics Additives and Human Health: A Case Study of Bisphenol A (BPA)

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ABSTRACT

Plastics are useful and versatile materials that bring many societal benefits, but concern has been raised about the potential of additive substances, including chemicals classified to be of concern to human health, to migrate from packaging and enter the human body. Human biomonitoring of global populations has identified exposure to a range of plastic additives, detectable in some cases in the majority of people. Whilst the concentrations involved are frequently within regulatory guidelines for tolerable daily exposure limits, the potential nonetheless exists for chronic, low dose and mixture effects. In this chapter, plastics additives in common use are identified and some of the factors that influence their migration out of plastics are discussed. Using the endocrine disrupting chemical bisphenol A (BPA) as a case study, the routes of exposure and potential for interventions to reduce exposure are discussed. Mechanisms of toxicity, including the possibility for effects mediated by changes in gene expression or epigenetic changes are illustrated using the estrogen related receptor α (ESRRA) as an example.

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†Electronic supplementary information (ESI) available: The names of the study participants. The authors confirm that all participants agreed to have their names published in the chapter in accordance with their role as researcher participants. See DOI: 10.1039/9781788013314-00131.

Issues in Environmental Science and Technology No. 47
Plastics and the Environment
Edited by R.E. Hester and R.M. Harrison

Published by the Royal Society of Chemistry, www.rsc.org

1 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 synthetic fibres, foams, adhesives and coatings with broad applications in construction, and the manufacture of clothing and other goods. The growing popularity of plastics has however come at a cost; not only is there continuous exposure of the population to plastics used in the manufacture of food contact materials and consumer items, but there is also widespread leakage of plastic waste into the environment, for example from 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 25.8 million tonnes per year in Europe alone,[1] 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.[2] Much of that concern lies in the potential for the continuous interactions with plastic items that most people experience in their daily life and could lead to uptake of plastics additives across the skin or airways, or through ingestion of contaminated food and drink.[3] Contamination of food may come from various sources, either from direct contact with packaging or processing materials during food manufacture or could be the consequence of leaching of additives and degradation of plastic litter into the environment and the food chain.[4]

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.[5] 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.[6] 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.

This chapter presents a brief overview of the most commonly encountered types of plastics and the chemical additives and monomers present in them that may pose a risk to human health. It is beyond the scope of this chapter to consider the health risks of multiple materials and their various permutations and additives and instead, a case study is presented of the health

risks associated with exposure to the plastics additive and constituent monomer, bisphenol A (BPA).

1.1 Plastics and Their Additives

The word plastic is used to describe polymers, mostly those made out of hydrogen and carbon rich substances.[7] Generally, most modern plastics are made out of monomers derived as by-products from the petroleum industry (US EPA http://www.epa.gov/waste/conserve/materials/plastics.htm). Monomers of different kinds are blended with various additives that improve the performance, stability or durability of the plastic and/or the products it is made into. The most commonly used additives include plasticisers, pigments and dyes, anti-static and anti-inflammatory agents, light and heat stabilisers and lubricants. Anti-block and anti-slip agents are used in plastics films to prevent them from sticking together whilst fillers such as kaolin, clay or calcium carbonate are used to add strength or to alter texture.[8] Additives are not bound to the polymer matrix (except for some flame retardant compounds) and they may leach out of the plastic polymer, particularly if they are of low molecular weight, into the surrounding water, air, food substance or body tissue.[9,10] Inventory lists of substances found in plastics include many thousands of different chemicals that are added as intentional starting products, but the final contents may be transformed further during manufacture to form by-products and degradation products that are referred to in regulatory terms as non-intentionally added substances.[11] Certain polymers contain significantly higher concentrations of additives than others, for example polypropylene, which is used in the manufacture of plastic packaging and drinks bottles and is vulnerable to oxidation and contains UV stabilisers and antioxidants,[12] whilst PVC (which is used to make diverse items including clothing, credit cards and pipes for water and gas), contains more additives, including plasticisers and heat stabilisers, than most other polymers.[13] Table 1 provides some examples of plastic additives in common use.

1.2 Migration of Chemical Substances Out of Plastics

Plastics may pose a hazard due to the release of unreacted monomers and dendrimers retained within the polymer matrix and monomers released during breakdown of the plastic polymer chains themselves. Examples of the former include the release of residual styrene monomers from polystyrene food packaging into food at concentrations sufficient to raise concern[14] or the release of vinyl chloride monomers from PVC.[15] Biodegradation is not a major route for the breakdown of most polymers in everyday use and breakdown of the polymer chain is most likely to be caused by abiotic factors such as mechanical or chemical abrasion, heat and UV light. Breaking of the bonds in the polymer backbone is followed by chain scission and depolymerisation, and stripping and release of side chains. The rates at which

Table 1 Examples of plastics additives in common use. w/w = weight to weight; PVC = polyvinylchloride. Derived from Hansen *et al.*[21] and Hahladakis *et al.*[8]

Additive type	Example substance	Used in which plastics?
Plasticisers	Short, medium and long chain chlorinate paraffins. Phthalates: Bis (2-ethylhexyl)phthalate (DEHP), dibutylphthalate (DBP), dipehnylphthalate (DPP). Adipates: diheptyl adipate (DHA), heptyl adipate (HAD), heptyl octyl adipate (HOA).	Mostly used in PVC and cellulose based polymers where they can make up to 75% w/w of the final product.
Flame retardants	Brominated flame retardants; polybrominated diphenylethers (PBDEs), decabromodiphenylethane. Phosphorous flame retardants; tris (2-chloroethyl)phosphate (TCEP), tris (2-chlorisopropyl)phosphate (TCPP).	Brominated compounds can reach 25% w/w of the final polymer.
Stabilisers, ultraviolet stabilisers, antioxidants	Bisphenol A (BPA) Cadmium and lead compounds Nonylphenols, octylphenols Butylated hydroxytoluene	Up to 3% w/w; phenolics generally added at lower amounts.
Slip agents	Fatty acid amides Fatty acid esters Zinc stearate	Added at up to 3% w/w depending on the polymer type.
Biocides	Organotins Arsenic compounds Triclosan	Added primarily to soft PVC and polyurethane foams.
Inorganic pigments	Cadmium, chromium and lead compounds Zinc oxide Iron oxide Titanium dioxide Lead carbonate Aluminium and copper powders	Non-fluorescing substances show lower migration rates.
Organic pigments	Cobalt(II) diacetate	Insoluble, low migration tendencies.
Fillers	Calcium carbonate Zinc oxide Barium sulphate Glass microspheres Nanomaterials Clays	Can make up to 50% w/w.

these reactions proceed vary depending on the type of polymer, its porosity and size, oxygen, temperature and light conditions, with polyester and polycarbonate more prone to depolymerisation reactions than, for example, polypropylenes and polyethylene.[3,16] Migration rates can be measured directly, for example by using simulated foodstuffs or solvents to determine leaching rates into food[17] or simulated using partition models that incorporate desorption rates with physicochemical characteristics of the polymers and the diffusing molecules.[18] Health risks are possible if migration of monomers and oligomers and other low molecular weight additives occur from plastic packaging into food and into the bodies of humans or animals in sufficient quantities to cause harm, or from plastic products into water, food, air, saliva or sweat, all of which have been identified to occur under laboratory settings.[19]

Migration rates of chemical additives into food have been comprehensively reviewed.[8,20] These reviews identify the main factors governing migration rates, including diffusion of the chemical through the polymer, desorption from the polymer surface, sorption at the polymer:receiving matrix interface (*e.g.* food substance, body fluid, tissue or water) and absorption into the receiving matrix. Mass diffusion processes follow Fick's law, and generally, migration rates are higher for smaller compounds and *vice versa*, with compounds such as vinyl chloride and butadiene exhibiting relatively rapid migration rates.[21] Migration rates are strongly influenced by the nature of the polymer framework, including its thickness and crystallinity and the nature of the surface.[21] This can be exploited in the design of low migration derivatives, for example the migration rates of additives including the antimicrobial triclosan were found to be up to six fold lower when nanoclay fillers were incorporated into the polymer during manufacture. The nanoclay molecules became interspersed within the polymer layers and reduced migration by creating a so-called tortuosity effect.[22]

1.3 *Hazard* Versus *Risk*

A comprehensive hazard ranking performed to cover 55 of the most widely encountered plastics in everyday use[13] utilised data on the hazardous potential of the constituent monomers, additives and degradation products to rank each polymer. This hazard ranking identified the polymers to be of higher hazard as including PVC, polyurethane, epoxy resins and polystyrenes, driven largely by the classification of their constituent monomers as being carcinogenic or mutagenic, and also the high percentage of additives in the case of PVC. An important gap in this approach was the lack of hazard ranking for chemicals classified as endocrine disrupting chemicals and hence common plastic associated additives including phthalates and BPA were excluded from the analysis. Endocrine disrupting chemicals are compounds that are taken up into the body through food, drink, from the air or across the skin, generally unintentionally, and that interfere with the normal functioning of hormones in the body. Hormones are responsible for

homeostatic, reproductive and developmental processes and exposure to endocrine disruptors has been implicated, through experiments on laboratory animal, clinical observations and epidemiological analyses with numerous endocrine health-related effects. These include; male and female reproductive abnormalities, incidence of hormone-sensitive cancers including of the breast and prostate, neuroendocrinological abnormalities and behavioural conditions including autism, obesity-related conditions including diabetes, and cardiovascular dysfunction (this topic is comprehensively reviewed in Gore *et al.*[23]).

In addition, whilst hazard ranking can identify potential concern based on what is present in a polymer, the presence of a compound alone does not pose a risk if there is no potential for exposure, and hence consideration must also be given to the myriad factors that can influence the potential release of these compounds and their bioavailability to humans and animals. Regulatory requirements to protect populations from unintended exposure include the European Food Standard Agency Specific Migration Limits for additives within plastics used for food packaging of 10 mg dm^{-2} of the contact material, with a lower limit of 0.01 mg kg^{-1} food material for a substance of concern.[24] These migration rates could equate to an individual being exposed to individual chemicals from food packaging of up to 0.25 mg kg^{-1} body weight per day.[25]

Examples of compounds of potential concern that have been studied in relation to their potential migration from plastic products and into humans, animals and the environment include compounds classified as endocrine disrupting chemicals; phthalates,[26] brominated flame retardants,[27] BPA, 4-nonylphenol;[28] heavy metals (lead, cadmium, tin),[29] benzene and other volatile organic compounds.[30] In many cases, the migration of each substance has been found to be within the regulatory limits. Guidelines, however, do not always consider the low level exposures at which endocrine disrupting chemicals may be active, nor the possibility for mixture effects.[31,32]

1.4 Human Biomonitoring

Central to assessing any risks to human health is to know exactly what chemicals and plastics are actually getting into people. In addition to exposure through food and drink, most people are exposed to complex and variable mixtures of chemicals and other substances throughout their normal daily activities, such as handling and using consumer products and through interactions with the wider environment; inhaling chemicals through the 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[33] 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 recent 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 chapter, NHANES includes the measurement in population representative samples of numerous chemicals associated with the use or manufacture of plastics, polymers and resins including BPA, 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 and use of plastic 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 susceptibilities. 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.[34,35]

2 A Case Study of BPA

2.1 BPA: an Endocrine Disrupting Chemical

One such chemical is BPA, a synthetic compound with estrogenic properties that is widely used as a monomer in the synthesis of polycarbonate, and as an additive in other plastics including PVC. It is one of the world's highest production volume chemicals with a current yearly global production in excess of 8 million metric tonnes (https://www.prnewswire.com/news-releases/global-bisphenol-a-market-overview-2016-2022). It is predominantly found in food packaging (polycarbonate plastics, epoxy can linings), thermal paper and dental sealants.[36] It is labile within plastics, particularly when in contact with lipid-rich foods or during heating[37] and can readily leach into

the contents of the packaging, as reviewed by Chen *et al.*.[38] In the Western world, there is near ubiquitous exposure, with greater than 95% of people showing measureable levels of BPA metabolites in their urine.[39,40] Concern has been raised about the public health consequences of widespread exposure because BPA is classified as an endocrine disrupting chemical which has been linked to reproductive, developmental and other health disorders in cell and animal models. Although not a definitive proof of causality, exposure to BPA has been associated with adverse human health outcomes, including type 2 diabetes, cardiovascular disease,[40,41] obesity[42] and abnormalities of sex hormone levels in cross-sectional studies[43] and prospective studies; as reviewed in Ranciere *et al.*[44] The safety of BPA to the general public has received continuous scrutiny, with the European Food Standards Agency (EFSA) noting that sufficient uncertainty remains that it is not possible to exclude effects on the reproductive, immune, nervous, metabolic and cardiovascular systems and on cancer development.[45] The classification of BPA by the European Chemical Agency is as a chemical of very high concern due to its endocrine disrupting properties,[46] whilst the US Food Safety Alliance for Packaging included BPA in an industry-led list of substances/groups of substance and solvents recommended by food producers that should not be used in packaging where alternatives exist.[47]

The biological activity of BPA is attributed to its estrogenic properties. Estrogens are steroidal sex hormones that control sexual and reproductive functions, especially in women, and are produced in the gonads, mainly the ovaries, but also by fat cells and the adrenal gland. In addition to their role in female sexual functions, estrogens function in the regulation of bone growth, cardiovascular function and the maintenance of tissues and organs in both sexes. In common with other steroid hormones, estrogens exert their effects through binding to ligand-inducible nuclear transcription factors termed estrogen receptors. *In vitro* cell and *in vivo* laboratory studies in animals have shown that BPA can interact not only with estrogen receptors, but also with other steroid hormone receptors, exhibiting estrogenic, androgenic and anti-androgenic activities, and can inhibit the expression of aromatase, an enzyme crucial in the synthesis of estrogen and other steroidal enzymes.[48] Other receptor mediated effects include those mediated by thyroid hormone disruption,[49] alterations to pancreatic beta cell function and obesity promoting effects.[50] The estrogen related receptor α (ESRRA) gene has also been identified as a molecular target of BPA both *in vivo* and *in vitro*.[51,52]

2.2 *Routes of Exposure and Potential Interventions*

Given the human health concerns expressed over exposure to BPA it is unsurprising that the major routes of entry into the body and the potential for reducing individual exposure have received attention. The main source of exposure to BPA is believed to be through food and drink contaminated with BPA during production and storage. BPA can enter food products after

leaching from polycarbonate containers or from the epoxy resin linings of canned goods after manufacture, or by hydrolysis of the polymer itself.[53] The migration rate of BPA increases with temperature,[54] and with time and duration of use, for example, with repeated use of polycarbonate drinks bottles.[55] Exposure to BPA can also occur through ingestion of dust and absorption through the skin.[56] BPA is metabolised quickly in the gut wall and in the liver, forming the major metabolite BPA-glucurinide and after circulating in the blood stream it is removed *via* the kidneys with a short half life in the body of around 6 hours.[57] Hence, any intervention to reduce exposure has the potential to reduce circulating levels of BPA rapidly. Concentrations of unconjugated BPA in human blood and tissues are in the range of 0.1–10 $\mu g\,L^{-1}$ [58] and it is also present in amniotic fluid and human milk.[59]

The concentrations of BPA in common food types has been reported in the range of 0.46–700 $ng\,g^{-1}$, with higher concentrations reported for canned foods.[60] Dietary interventions studies have involved a study of 22 volunteers who were provided with full dietary replacement of fresh, unpackaged foods over 3 days.[61] The study subjects achieved an average reduction of 66% in urinary BPA excretion over the course of the study. A study in which households followed written instructions on how to reduce exposure were unable to achieve such a reduction, and there was no significant change in their exposure status.[62] More recently, a citizen science approach was used in which teenagers in the UK enrolled onto an intervention trial and designed and followed their own reduced-BPA diet over 7 days, following official guidelines designed to help individuals to reduce their own exposure.[63] A total of 94 teenagers provided diet diaries and urine and blood samples during the study and creatinine adjusted urinary BPA concentrations were determined, whilst information about the food and drink they consumed was used to devise a risk score for each participant. The presence of BPA in the urine was confirmed for 86% of the teenagers prior to starting the dietary trial. There was no statistically significant change in urinary BPA before and after the trial, although there was a positive association between individuals who showed a drop in their urinary BPA concentration after the trial and their initial BPA level. Feedback from the study participants was that they would be unlikely to keep to the intervention in their diet long term, because it was too difficult to identify food that was likely to be free of BPA, reflected in the lack of association between the risk score devised from their food diaries and concentrations of urinary BPA. This study illustrates that for some plastic associated chemicals, it is extremely difficult to avoid continuous exposure during normal daily life.

2.3 *Genetic and Epigenetic Mechanisms of Effect*

Exposure to BPA has been widely reported to be associated with gene expression changes in animal models and in human cells.[64–66] A study of the genes and proteins shown to be affected by exposure to BPA in the

Comparative Toxicogenomics Database identified 1232 reported interactions, including genes associated with inflammation and with reproductive and sexual functions.[67] In addition to changes in gene expression, environmental chemicals such as BPA have been reported to induce epigenetic changes, which are heritable changes in gene expression that are independent of changes in gene sequence. Epigenetic changes include changes to the amount of methylation of DNA, modifications to histones and expression of non-coding RNAs (including microRNAs).[68] In the context of environmental chemical exposure, most research has involved studying DNA methylation patterns. These epigenetic modifications can affect the gene expression profiles and healthy function of most organs and tissues and can persist from early exposures, for example *in utero* and persist throughout life, even persisting through to the next generation. BPA is considered to be epigenetically toxic, based on the results from numerous animal and cell studies.[68,69] This is illustrated in a study of mice in which maternal exposure to BPA resulted in a change in the colour distribution of offspring, which was associated with a decrease in the CpG methylation pattern of a transposable sequence upstream of the Agouti gene. The Agouti gene participates in the control of coat colour selection, hence the change in colour distribution of the offspring.[70] In a study of the epigenetic effects of mixtures, a mixture of plastic derived compounds including BPA and phthalates was shown to promote epigenetic transgenerational inheritance of adult onset disease and associated DNA methylation permutations in a rodent model. There was an increased incidence of pubertal abnormalities and obesity related indicators in F1 and F3 generation animals following the exposure of gestating F0 parents.[71]

2.4 *ESRRA and BPA*

The ESRRA gene has previously been identified as a molecular target of BPA *in vivo* and *in vitro*.[51,52] This gene has a key role in cardiac function, immune response and energy sensing.[72–74] 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 not only overall gene expression, but also 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.[75] 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.[76]

BPA has also been shown to alter the splicing patterns of other target genes such as vascular endothelial growth factor, *VEGF*, in the reproductive tissues of both Fisher and Sprague Dawley rats.[77] Other estrogenic chemicals such as phthalates have also been reported to affect isoform usage for xenobiotic receptor genes *CAR* and *PXR* in COS-1 human hepatocytes *in vitro*.[78]

2.5 *Expression of ESRRA* In Vitro *Following Exposure to BPA*

We were interested to know 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*. To explore this, we quantified their expression in human tissues using quantitative real time PCR, using isoform-specific probes. Using cDNA sequences from transcriptome databases, the ESRRA isoforms were shown to encode identical proteins that differ in their 5′ regulatory regions (Figure 1). Both long and short isoforms of the ESRRA gene were present in all of the tissues tested but were expressed 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 the liver, thymus, whole blood, uterus, testes, ovary and placenta (Figure 2).

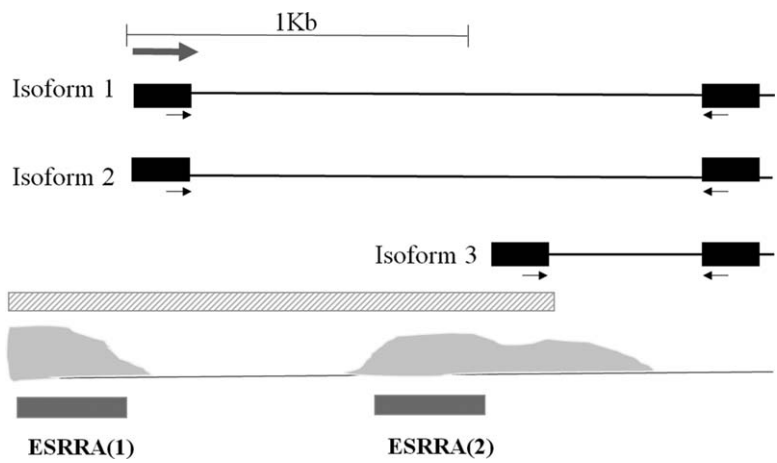


Figure 1 Gene structure, regulatory motifs and location of promoter regions of the ESRRA gene. The position of the 5′ terminal exons of the three putative isoforms of the ESRRA gene are indicated by black boxes. The position of the large ESRRA CpG island is indicated by mid-grey hatched boxes. The direction 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 are given by light grey areas, with potential alternative promoter regions ESRRA(1) and ESRRA(2) indicated by dark grey boxes.

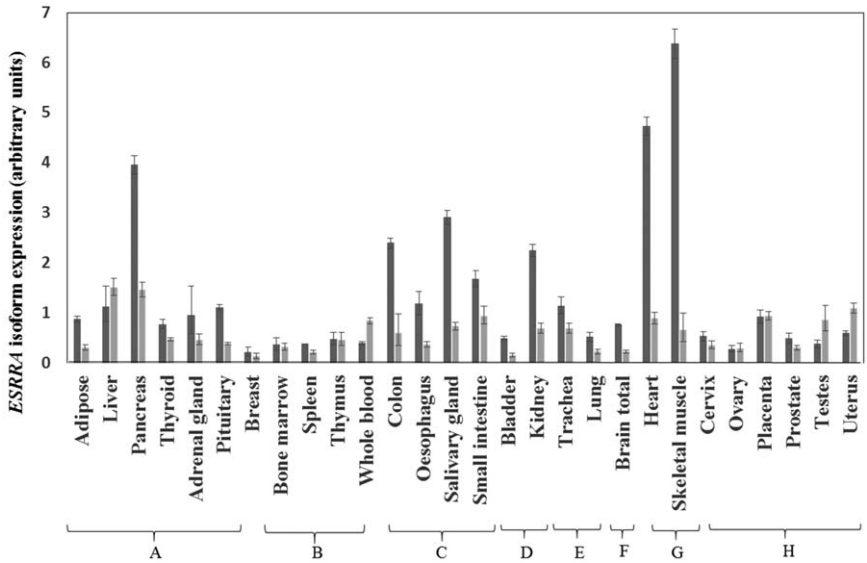


Figure 2 Expression patterns of ESRRA isoforms in human tissues. Expression is calculated relative to a panel of endogenous control genes and normalised to the geometric mean of long isoform expression across all tissues. Error bars are calculated from the standard deviation of triplicate measurements. 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, 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.

We next examined the effect of BPA on alternative expression of ESRRA isoforms *in vitro*, using Jurkat cells, an immortalised line of human T lymphocytes, comparing any changes in expression to 17α-ethinyl estradiol as a positive estrogenic control (Figure 3a). Concentrations of BPA were chosen to represent low and high doses relative to the exposure levels of the general adult population. Changes in ESRRA expression were seen following treatment with 50 nM BPA as in previous studies,[52] but not at 5 nM (Figure 3b and c). Although no significant differences in ESRRA expression were noted at 5nM BPA, it was of interest to note that the long and short isoforms responded in a reciprocal manner, showing biphasic expression at both 5 nM and 50 nM BPA (Figure 4a–c).

2.6 *Expression of ESRRA* In Vivo *Following Dietary Intervention to Reduce BPA Exposure*

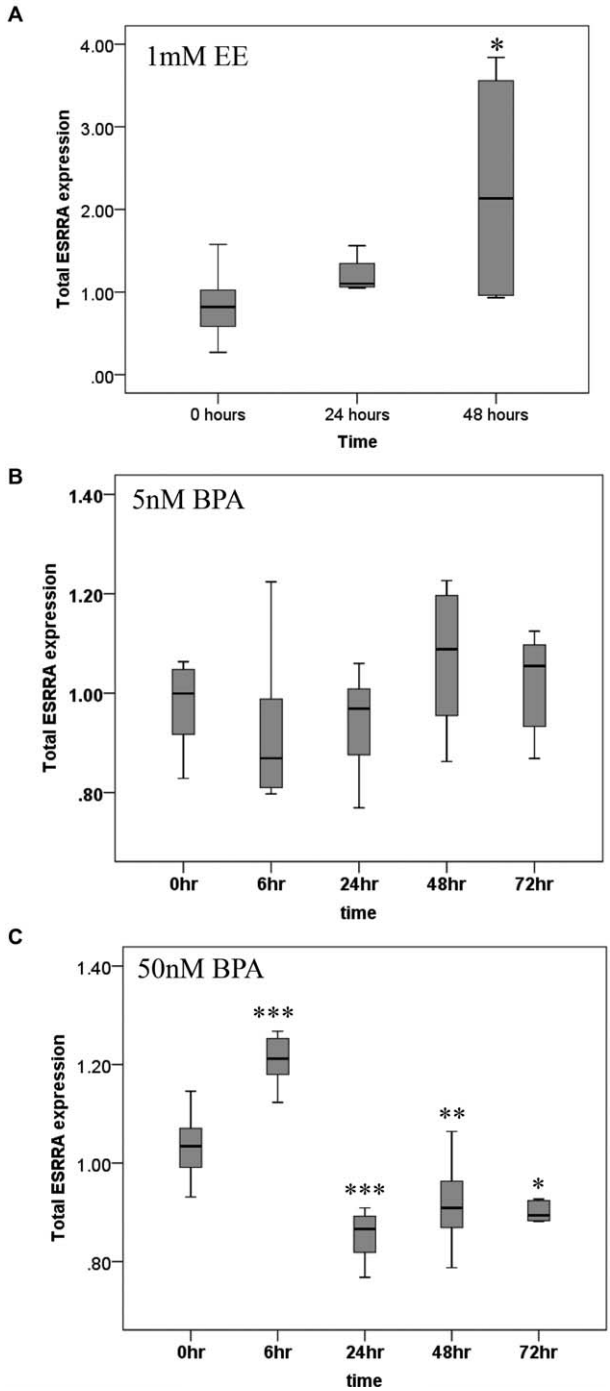
Given the interesting observations that ESRRA appears responsive to BPA *in vitro*, we were motivated to explore the possibility that similar effects

might manifest *in vivo*. When considering exposure to a potentially endocrine disrupting chemical, it is not ethical to propose an intervention trial to *increase* exposure. The design of such a study is further complicated by the lack of individuals not exposed to BPA, given the high percentage of the population with detectable BPA in their urine. Instead, the epigenetic effects of *reduced* exposure were investigated in student volunteers after completing a 7 day trial on a 'low BPA' diet (as described further in Section 2.2).

Full details of the effect of the dietary intervention on BPA levels have been previously published.[63] Briefly, BPA levels were quantified in samples taken before (visit 1) and after (visit 2) participation in the intervention trial from 94 individuals, with a limit of detection for urinary BPA of 0.1 $ng\,ml^{-1}$. Samples scoring positive for BPA but quantifying at or around the limits of detection (LOD) were scored as $LOD/\sqrt{2}$ ($=0.07\ ng\,ml^{-1}$). Following the dietary intervention, 50 out of 94 participants showed lower urinary BPA levels at visit 2[62]. In these 50 participants, the mean urinary BPA at visit 1 was 2.41 $ng\,ml^{-1}$ (95% CI, 1.9–2.9 $ng\,ml^{-1}$), whereas mean urinary BPA at visit 2 was 1.02 $ng\,ml^{-1}$ (95% CI, 0.74–1.30 $ng\,ml^{-1}$). The mean drop in BPA between visit 1 and visit 2 in the 50 participants was 1.41 $ng\,ml^{-1}$. Details for these 50 participants are given in Table 2.

Peripheral blood samples taken from volunteers at both visits were used to extract mRNA and to measure the expressions of each of the ESRRA isoforms. 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 a change in the expression of the short ESRRA isoform, but not the long isoforms (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 the change in isoform levels and the change in urinary BPA concentration was noted for individuals reporting an increase in urinary BPA at visit 2.

These changes in the expression of the ESRRA gene are consistent with the switch in isoform usage that were noted *in vitro*. By measuring change in the expression of ESRRA isoforms in relation to change in BPA levels within the same subjects, we were able to use them as their own controls, allowing examination of potential relationships without the confounding influence of other genetic or environmental factors that could influence ESRRA isoform expression. This preliminary analysis suggests that individuals showing a reduction in BPA exposure during a dietary intervention had correspondingly lower ESRRA short isoform expression. There was 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.



2.7 *Are There Physiological Implications for Changes in the Expression of ESSRA Isoforms?*

As shown from these results, the long isoforms of ESRRA are expressed in tissues such as the heart, pancreas and adipose tissue, whilst the short isoform of the ESRRA gene is predominant in many reproductive tissues such as the ovary and testes which are involved in sex hormone signalling. The ESRRA gene product, ERRα, has pivotal roles in cellular metabolism and energy sensing, particularly in tissues with high energy demand.[79] This is particularly evident in tissues such as the heart, for which whole body ERRα knockout mice showed a reduced ability to respond to increased bioenergetic demand, impaired functional adaptation to cardiac stress and neonatal cardiac defects.[80,81] ERRα also has roles in immune function through 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.[82] Given these findings, it is interesting to note the range of adverse health outcomes with which exposure to BPA has been associated, including type 2 diabetes, cardiovascular disease, obesity and abnormalities of sex hormone levels, immune and reproductive function.[40–44]

The physiological consequences of ESRRA isoform changes are difficult to predict, given that all three isoforms code for the same protein. The isoforms have distinct 5′ regulatory regions; alternative promoter usage can have profound effects on the stability or translation potential of mRNA species, even when the encoded protein products are identical.[83,84] 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.[85] 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 34 bp 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.[86] 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

Figure 3 Change in ESRRA gene expression in response to 17-α ethinyl estradiol (EE), 5 nM BPA and 50 nM BPA. Expression of the ESRRA gene in response to 17-α EE (A), 5 nM BPA (B) or 50 nM BPA (C). 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 * = $p = < 0.05$, ** = $p = < 0.005$, *** = $p = < 0.0005$.

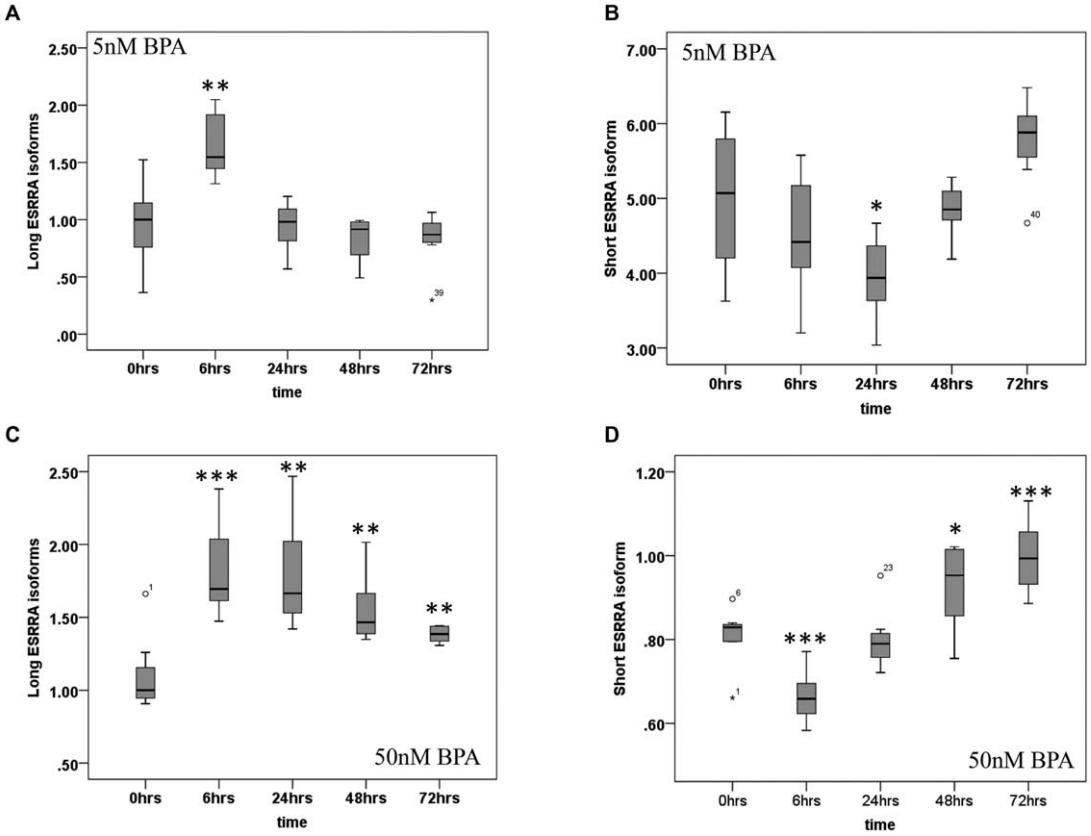


Figure 4 Change in ESRRA isoform usage following exposure to 5 nM and 50 nM BPA. Data are presented as stem and whisker plots representing the median value and interquartile range at each time point. Changes in long ESRRA isoforms (NM_001282451 and NM_004451) expression in response to 5 nM and 50 nM BPA are given in (A) and (C). Changes in short ESRRA isoform (NM_001282450) expression in response to 5 nM and 50 nM 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 $* = p = < 0.05$, $** = p = < 0.005$, $*** = p = < 0.0005$.

Table 2 Characteristics of student volunteers in the BPA dietary intervention trial. Numbers in parentheses refer to the standard deviation of measurement.

Characteristic	Measurement
Mean BPA at visit 1	2.41 (1.80) ng ml^{-1}
Mean BPA at visit 2	1.02 (1.00) ng ml^{-1}
Mean change in BPA	−1.41 (1.53) ng ml^{-1}
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%

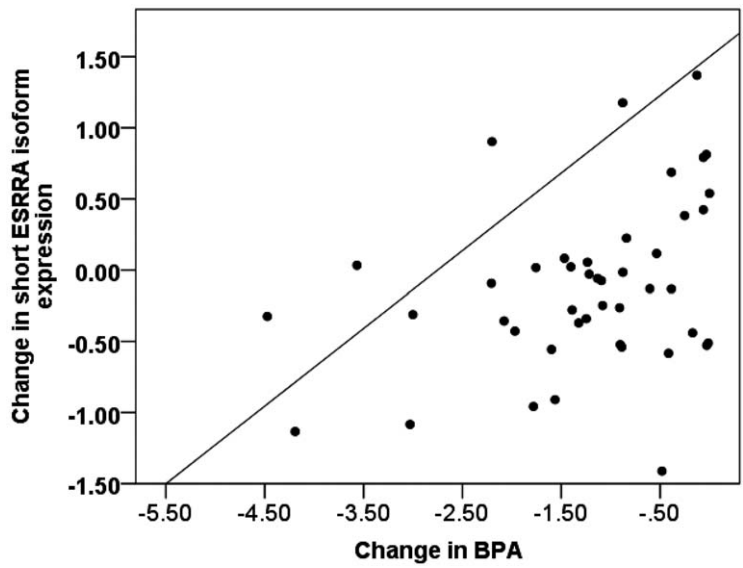


Figure 5 Correlation between change in urinary BPA and change in the expression of the short isoform of the ESRRA gene after participation in a dietary intervention trial. Urinary BPA is expressed as a BPA:creatinine ratio in ng ml^{-1} whilst expression units are arbitrary measures representing the amount of the short isoform of ESRRA expressed relative to the endogenous control genes and normalised to the level of the reference isoforms.

BPA 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.[87] 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.

3 Conclusions and Future Perspectives

The results presented in the BPA case study above are of interest in illustrating potential mechanisms by which exposure to endocrine disrupting chemicals such as BPA may affect biological endpoints, in this case by influencing expression of target genes through modulating the expression of genetic isoforms. The production of ESRRA isoforms with different potential for transactivation or autoregulation in response to BPA could potentially help to explain some of the phenotypes associated with chronic exposure. For example, as noted above, the ESRRA gene plays a role in the regulation of cardiac metabolism.[80,81] Exposure to BPA has been associated with an elevated risk of heart disease in cross-sectional epidemiological studies.[88,89] Data on urinary BPA concentrations for 1455 adults aged 18 to 74 years from the US National Health and Nutrition Examination Survey (NHANES) 2003–2004 was used to show an association between BPA exposure and cardiovascular diagnoses (odds ratio [OR]) per 1-SD increase in BPA concentration = 1.39; 95% confidence interval [CI], 1.18–1.63; $P = 0.001$, a finding which was closely replicated in an independent study population of 493 adults from NHANES 2005–2006.

These cross-sectional analyses were supported in a longitudinal study over 10 years of 1919 adults, including 758 individuals who developed incident coronary artery disease and 861 controls. Increased exposure to BPA was associated with an elevated incidence of cardiovascular disease independent of all the other risk factors that were measured, including education, occupational social class, body mass index category, systolic blood pressure, lipid concentrations, and exercise.[90] This is interesting because it suggests an independent mode of action, although what pathways are involved in this mechanism remain unknown. A mechanism that involves estrogenic or antiandrogenic effects has some plausibility given the role of sex hormones in healthy cardiac function, but a direct link between BPA, estrogen receptor binding and risk of cardiac disease has not been made. Exploring the potential involvement of epigenetic effects such as altered patterns of ESRRA isoform expression in the health effects of exposure to chemicals such as BPA remains a tantalising avenue for future studies.

BPA is just one example of the plastic associated chemicals that have received attention due to concerns about their effects on human health and potential to migrate from plastic items. As discussed in Section 1.2, the concentrations of substances including phthalates,[26] BPA and nonyphenol,[28] brominated compounds,[27] metals[29] and volatile organics[30] that are released are low compared with guidelines for migration limits or tolerable daily exposure limits, but it is notable that such guidelines are often

not designed to consider the low concentrations at which endocrine disrupting chemicals may exert effects, or to consider the effects of mixtures. New recommendations and green chemistry developments are however increasingly addressing these issues. To improve risk assessment for food contact materials recent recommendations have suggested evaluation of potential low-dose endocrine-mediated effects for all chemicals that come into contact with food substances. In particular, Muncke and colleagues[91] recommend that toxicological assessment be performed on finished materials used for food packaging, which would include the complete mixture of substances as used in the finished product.[91] When combined with voluntary actions, such as the guidelines in the publication by Seltenrich[47] for minimising or eliminating substances of concern from food packaging, and exciting new developments in materials science for cutting migration rates of additives from packaging,[92] considerable reductions in the unnecessary exposure of the human population could be achieved.

Acknowledgements

We acknowledge funding from a Wellcome Trust People Award to LWH and TG (grant number 105162/Z/14/Z), an award from the Northcott Devon Medical Trust to LWH and a NERC award to TG (grant numbers NE/L007010 and NE/N006178/1). We also acknowledge the work of the NIHR Exeter Clinical Research Facility in aiding the collection of the urine samples. The authors would like to acknowledge Florence Emond and Henry Lloyd Laney for technical assistance.

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