3. CONTENTS

3.1. Introduction

Similarly to what happened last year, the current coronavirus pandemic precluded the collection of the target samples, as the hospital services were mostly entirely dedicated to treat Covid-19 patients and non-urgent consultations were postponed and/or were reduced to a minimum. For this reason, the collection of urine samples from early menopause women could not be performed. As a contingency plan, previous urine samples already collected from volunteers from Aveiro district were analyzed and data computed in order to understand any possible changes in the use of consumer products with endocrine disruptors. Particular attention was drawn to Bisphenol A (BPA) and substitutes. BPA is generally used in manufacture of polycarbonate (PC) plastics, epoxy resins and other polymeric materials, as well as paper products (thermal receipts, e.g.) and other consumer products including dental sealants. Due to their widespread use, humans are continuously exposed and given the endocrine disrupting and immunotoxic potential of this compound and the public awareness of its toxicity, BPA regulations have been implemented over the last decade and as a result, other bisphenols have been increasingly used[1]. In January 2011, the European Commission prohibited the use of BPA in the manufacture of polycarbonate infant feeding bottles. In February 2018, the EU introduced stricter limits on BPA in food contact materials. Currently (as of February 2022), in the European Union (EU), BPA is still permitted for use in food contact materials under Regulation 10/2011/EU, relating to plastic materials and articles intending to come into contact with foodstuff, except some countries like France that have already completely banned the use of BPA in food packing, containers and utensils. In January 2020, EU-wide ban has entered into force on the use of BPA in thermal paper (in a concentration equal to or greater than 0.02% by weight). Recently (21st December 2021), the Court of Justice in the EU confirmed that BPA must be listed as a “substance of very high concern” for its hormone disrupting properties in humans[2]. Given the molting evidences on BPA toxicity, the European Food Safety Agency (EFSA) proposed in December 2021 new safety standards for BPA, radically decreasing the recommended exposure limit by a factor of 100,000, to a recommended value of 0.04 ng per kg of body weight per day, this proposal was until 22th February under public consultation. This new standard is so low that if approved, it will probably ban the use of BPA in any products that come into contact with food. Therefore a drastic decrease in BPA levels are expected in the future.

Hence, the aim of this report is to describe the levels of phenolic compounds, namely triclosan, triclocarban, parabens, benzophenones and bisphenols in urine samples from volunteers living is Aveiro district, to describe exposure levels and to understand any potential shift in the exposure to EDCs, particularly bisphenols.

3.2. Procedure

3.2.1. Sampling

This study was approved by the Ethics Committee of Centro Hospitalar do Baixo Vouga and by the Portuguese National Data Protection Agency. Samples from volunteers inhabiting Aveiro district were collected between 2017 and 2019. Three different groups were selected: men (n=24), women (n=20) and pregnant women (n=28). Urine samples were collected directly into sterile containers and several aliquots of 1.5ml were prepared and immediately frozen. Two aliquots of each sample were transported to CMES in cool conditions and preserved at -20^{o}C until analysis.

3.2.2. Chemical analysis

At CMES, levels of triclosan, triclocarban, parabens (methyl, ethyl, propyl, butyl), benzophenones 2-OH-4-MeO-BP (BP-3); 2,4-diOH-BP (BP-1); 2,2'-diOH-4-MeO-BP (BP-8); 2,2',4,4'-tetraOH-BP (BP-2); 4-OH-BP) and bisphenols (BPA, BPB, BPC, BPF, BPS, BPZ, BPAF, TBBPA) were quantified in urine samples. The target compounds were analyzed following the protocol described by Kunisue, et al. [3] after some modifications and optimization by the team members (see LaMer 2017/18 report). Briefly, 50 µL of Internal Standards (ISs) Mixture was added to each urine sample after being hydrolised with β-glucuronidase/aryl-sulfatase for 16h at 37^{0}C. Afterwards, cold methanol, ultrapure water and ammonia solution (5% NH_4OH) were added. The samples were then loaded into a pre-conditioned OASIS MAX cartridge (MTBE, Methanol and ultrapure water). Prior to elution with formic acid:MTBE:methanol = 0.2: 3: 7 (v/v/v), the cartridges were washed with 5% NH_4OH, 5% NH_4OH in methanol, Milli-Q-water: methanol = 0.2: 6: 4 (v/v/v) and afterwards completely dried for 15 min. The eluted target compounds were evaporated to dryness under nitrogen flux and re-dissolved with acetonitrile and 50 µL of mixture of naproxen-$^{13}C,d_3$ and ketoprofen-$^{13}C,d_3$ (20 ng/mL each) was added. The samples were preserved in amber LC glass vials at 4^{0}C and before injection into the LC-MS/MS, Milli-Q water was added to a final volume of 1mL. The internal standard (ISs) mixture contains 100 ng/mL of Triclosan-$^{13}C_6$, 20 ng/mL of

Triclocarban-13C$_6$, 20 ng/mL of Methyl paraben-$^{13}C_6$, 20 ng/mL of Butyl paraben-$^{13}C_6$, 20 ng/mL of BP-3-d$_5$, 100 ng/mL of Bisphenol A-d$_{16}$, 100 ng/mL of Bisphenol F-$^{13}C_6$, 20 ng/mL of Bisphenol S-$^{13}C_{12}$, 20 ng/mL of Bisphenol AF-d$_4$ and 20 ng/mL of Tetrabromobisphenol A-$^{13}C_{12}$. The activity of β-glucuronidase/aryl-sulfatase was 290 units per mL of urine. The solution was prepared twice a week by adding 4.7 ml of 1.0 mol/L ammonium acetate; 5.3 mL of 1.0 mol/L acetic acid and 50 µL of β-glucuronidase/aryl-sulfatase solution (116,000 units/mL). The crude mixture of β-glucuronidase/sulfatase from Helix pomatia (Type HP-2, aqueous solution, 116,000 units/mL glucuronidase and 1020 units/mL sulfatase) was purchased from Sigma-Aldrich (St. Louis, MO, USA); (G7017). The method detection limit was 0.147 ng/mL for Triclosan, 0.073 ng/mL for Triclocarban, 1.101 ng/mL for Methyl paraben, 0.036 ng/mL for Ethyl paraben, 0.64 ng/mL for Propyl paraben, 0.015 ng/mL for Butyl paraben, 0.015 ng/mL for BP-1, 0.025 ng/mL for BP-2, 0.471 ng/mL for BP-3, 0.046 ng/mL for BP-8, 0.223 ng/mL for 4-OH-BP, 2.2 ng/mL for Bisphenol A, 0.076 ng/mL for Bisphenol AF, 0.325ng/mL for Bisphenol B, 2.175 ng/mL for Bisphenol C, 0.910 ng/mL for Bisphenol F, 0.199 ng/mL for Bisphenol S, 0.438 ng/mL for Bisphenol S, and 0.386 ng/mL for TTBBPA.

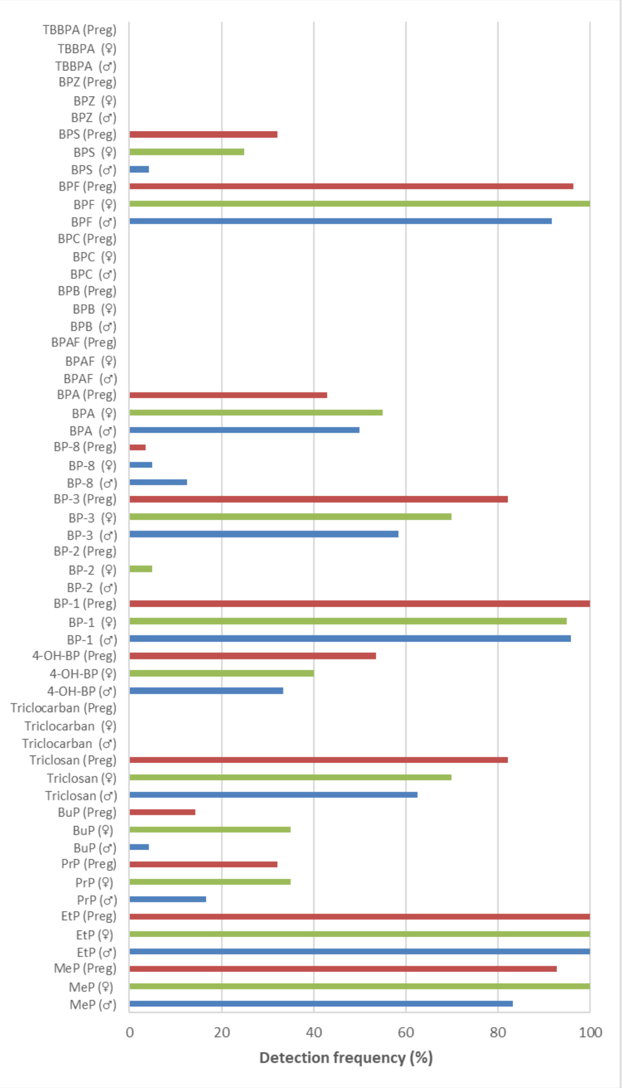

Figure 1 - Detection frequency (%) of each EDC in the urine samples from men (n=24, blue bars), women (n=20, green bars) and pregnant women (n=28, red bars).

3.3. Results

Of the 19 compounds analyzed, only triclocarban, namely BPAF, BPB, BPC, BPZ and TBBPA were not detected in all groups analyzed. BP-2 was not detected in men and pregnant women, and all the remaining EDCs were detected in at least one individual of each group (*Figure 1*). Ethyl paraben was the only EDC detected in all the all samples analyzed for all groups. The EDCs with the highest detection frequencies in men were Ethyl paraben (100%), Benzophenone-1 (96%), Bisphenol F (92%) and Methyl paraben (83%). In women, the EDCs with the highest detection frequencies were Ethyl paraben (100%), BPF (100%), Methyl paraben (100%) and Benzophenone-1 (95%). In pregnant women, the EDCs with the highest detection frequencies were Ethyl paraben (100%), Benzophenone-1 (100%), BPF (96%), Methyl paraben (93%), Triclosan (82%), and Benzophenone-3 (82%).

The profiles for each phenolic compounds detected in urine and dust samples are described in Figures 2-4. Overall, in urine samples the highest average concentration detected was for Methyl paraben in women and pregnant women (Figure 2), followed by benzophenone-3 in pregnant women (Figure 3).

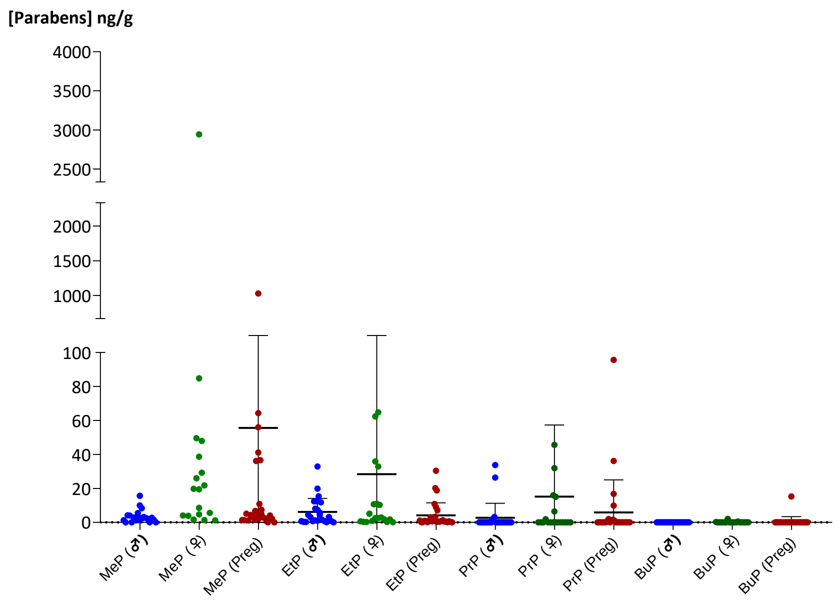

Figure 2 - Scatter plot summarizing the values of parabens in the analyzed urine samples in men (n=24, blue bars), women (n=20, green bars) and pregnant women (n=28, red bars).

Regarding bisphenols (Figure 4), generally men displayed the lowest levels. Interestingly, in pregnant women the levels of BPA were lower than the levels of BPF and BPS, two widely used alternatives for BPA. This tendency is probably associated with the concern of women to avoid BPA products containing BPA.

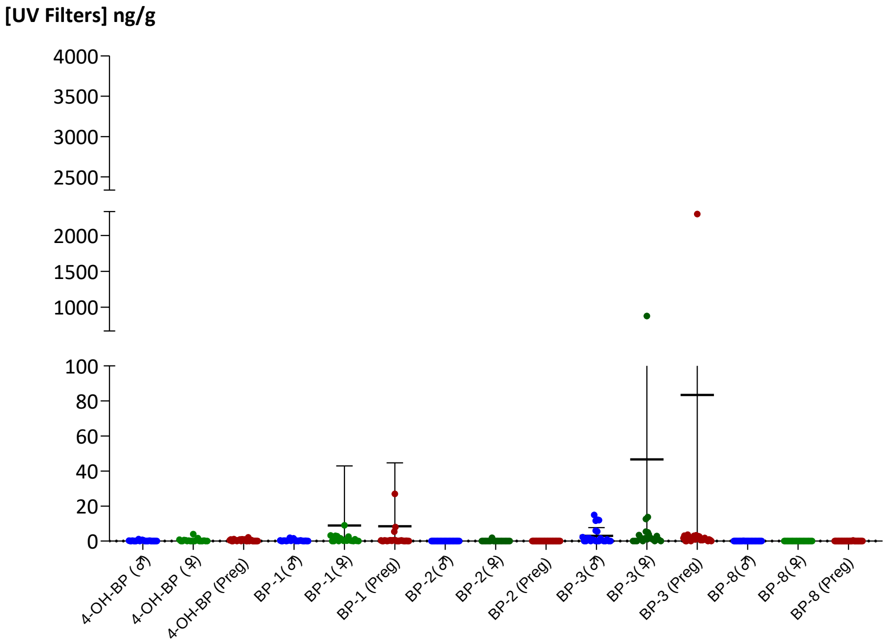

Figure 3 - Scatter plot summarizing the values of UV-filters in the analyzed urine samples in men (n=24, blue bars), women (n=20, green bars) and pregnant women (n=28, red bars).

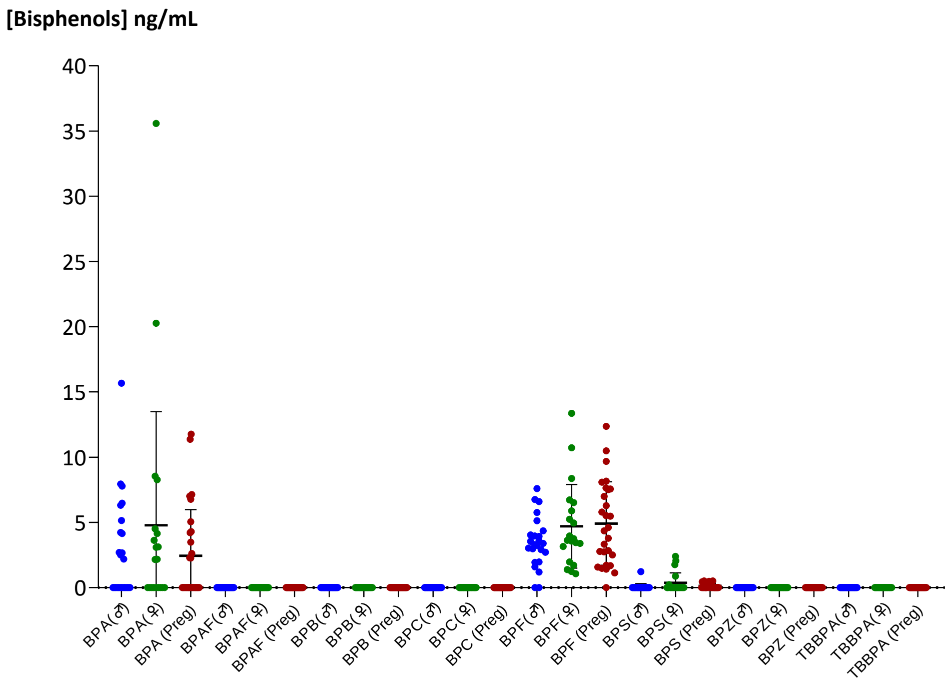

Figure 4 - Scatter plot summarizing the values of bisphenols in the analyzed urine samples in men (n=24, blue bars), women (n=20, green bars) and pregnant women (n=28, red bars).

3.5. Future perspectives

Due to the Covid pandemic the computation of clinical data (that was performed by a Medical doctor) was delayed and for this reason the multivariate statistical analysis that was scheduled to be finish in 2021 are still ongoing. The research papers projected in the previous report to be published in 2021 will only be published in 2022. Nevertheless, we have published a review paper, an encyclopedia entry and submitted another review paper (see achievements section). Furthermore, the manuscript entitled "The RESPIRA Project: Unraveling the environmental determinants of high burden respiratory diseases in Estarreja Region" is going to be submitted in the beginning of March (see Achievements section) and another paper entitled "Risk perception and attitudes of pregnant women regarding exposure to endocrine disrupting chemicals: a narrative review" is currently under preparation. All these manuscripts will acknowledge LaMer funding.

We expect to continue sampling and to obtain a representative number of samples so that in collaboration with CMES we can conclude the study on the effects of exposure to phenolic endocrine disruptors and human reproductive health. Besides female reproductive health, a new study will be implemented focusing on male reproductive health.

Furthermore, based on the results here described, and given the new regulation on BPA that will likely enter into force in 2022 or 2023, we also aim to perform a new study to evaluate the temporal trends in bisphenols levels in the same individuals.

3.6. Achievements

Oral Communications by Invitation

- **Sousa ACA**, Pastorinho MR, Coelho SD, Isobe T, Tanoue R, Kunisue T, Tanabe S (2021) Hormones messed up - a tale of endocrine disruptors in the XXI century (**Invited lecture**). XXI SPB - National Congress of Biochemistry: Tuning Biochemistry with Life Sciences and Society. 14-16 November 2021, Évora, Portugal, p. 36

Publications

- **Sousa ACA**🖂, Souto-Miranda S, Valente C, Tanoue R, Kunisue T, Tanabe S, Taborda-Barata L, Pastorinho MR and the RESPIRA team (under preparation) The RESPIRA Project: Unraveling the environmental determinants of high burden respiratory diseases in Estarreja Region. To be submitted by March 10th
- Rato L, **Sousa ACA**🖂 (2021) The impact of endocrine-disrupting chemicals in male fertility: focus on the action of obesogens. Journal of Xenobiotics 11, 163–196; **Feature Paper** https://doi.org/10.3390/jox11040012

- **Sousa ACA** and Rato L (2022) The Impact of Endocrine-Disrupting Chemicals in Male Fertility. Scholarly Community Encyclopedia. https://encyclopedia.pub/20789

Editorial activities

- Special Issue: "Monitoring of Organic Contaminants", Processes, Guest Associate Editor
- Special issue: "Obesogens in the XXI century: emerging health challenges", Frontiers in Endocrinology, Guest Associate Editor
- Special Issue: "Environmental Exposures and Epidemiological Studies on Maternal and Child Health", International Journal of Environmental Research and Public Health, Guest Associate Editor

Outreach activities

- **Sousa ACA** (2021) Imposex and Obesity: the story of an environmental contaminant. Lecture for the general public under the framework of the 6th Coffee with Science program. Centro de Ciência Viva de Lagos. 2 July 2021. Online, available at: https://www.facebook.com/watch/?extid=WA-UNK-UNK-UNK-IOS_GK0T-GK1C&v=262701795803074 (LaMer ackowlegment min 53:40)
- Sousa ACA (2021) Environmental Contaminants and endocrine disruptors in the global obesity epidemic. *Ciclo de Palestras do CEBE*, 22th May 2021, online

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2. General Court of the European Union. Confirmation of the inclusion of Bisphenol A as a substance of very high concern on account of its properties as a substance toxic for reproduction. In: General Court of the European Union, ed. T-185/17. Luxembourg, 2021.
3. Kunisue T, Wu Q, Tanabe S, et al. Analysis of five benzophenone-type UV filters in human urine by liquid chromatography-tandem mass spectrometry. *Analytical Methods* 2010;2(6):707-13. doi: 10.1039/B9AY00324J