3. CONTENTS

3.1. Introduction

The prevalence of diabetes is sharply increasing, in Europe for example, in 2016, 60 million people had diabetes and in some countries, 10 to 15% of the total population is affected by this metabolic disease[1]. Traditionally, the increasing incidence of diabetes (in particular Type 2 diabetes-T2D) was mainly attributed to lifestyle factors, including diet and obesity[2]. However, emerging evidence suggests that environmental contaminants, particularly endocrine disrupting chemicals (EDCs) may also play an important role[2]. In fact, according to the Endocrine Society[3] there is enough evidence to suggest that some EDCs act as diabetogens. Up to date, there is already an important body of research concerning the associations between EDCs exposure and diabetes, particularly T2D[4], although some studies on type 1 also disclosed a positive association with the exposure with EDCs[5]. Despite the growing number of evidences associating diabetes with EDCs exposure few studies have addressed gestational diabetes (GD). The incidence of GD has also increased in the past decades, and currently one in seven births is affected by GD[6]. This is a significant public health concern, as GD is associated with dramatic adverse consequences, including preeclampsia, birth complications, cesarean delivery, as well as long-term risk of type 2 diabetes, obesity, and cardiovascular disease for both the mother and child[7]. Thus, it is critical to identify alternative root causes of GD[8]. Given the compelling evidences already available between exposure to phenolic compounds with T2D[4] and the extensive exposure of pregnant women to these chemicals, we aim to study the levels of these chemicals in pregnant women with and without GD.

3.2. Procedure

3.2.1. Sampling

This study was approved by the Ethics Committee of Centro Hospitalar do Baixo Vouga (in which Aveiro Hospital is included) and by the Portuguese National Data Protection Agency. Due to the late approval of the project, recruitment started latter than expected and as a consequence it was not possible to collect the samples from the 50 pregnant women with GD, as initially programed. Hence, samples from 27 pregnant women with GD and 10 samples from pregnant women without GD were collected. From these women, morning spot urine samples were collected during the first consultation of the 1st trimester. An aliquot of each sample was transported to CMES in cool conditions and preserved at -20^{o}C until analysis.

3.2.2. Chemical analysis – urine samples

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 (2010)[9] 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 sample after being hydrolised with β-glucuronidase/aryl-sulfatase for 16h at 37^0C. 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 dried for 15 min. The eluted target compounds were evaporated to dryness under nitrogen flux and re-dissolved with acetonitile 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 4^0C and before injection into the LC-MS/MS, Milli-Q water was added to a final volume of 1mL.

The ISs mixture contained 100 ng/mL of Triclosan-$^{13}C_6$, 20 ng/mL of Triclocarban-$13C_6$, 20 ng/mL of Methyl paraben-$^{13}C_6$ and 20 ng/mL of Butyl paraben-$^{13}C_6$).

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).

3.4. Results

Of the 19 compounds analyzed, seven were not detected in any sample being always bellow their respective detection limit (Triclocarban, Benzophenone-2, Bisphenols AF, B, C and Z and TBBPA). Overall, it was possible to detect at least three EDCs in each sample, with an average of 7.9 compounds per sample. The most frequent compounds were Benzophenone-1 and Ethylparaben, detected in 100% of the samples, followed by Bisphenol-F and Methylparaben, detected in 96.2 and 92.3% of the samples (Figure 1).

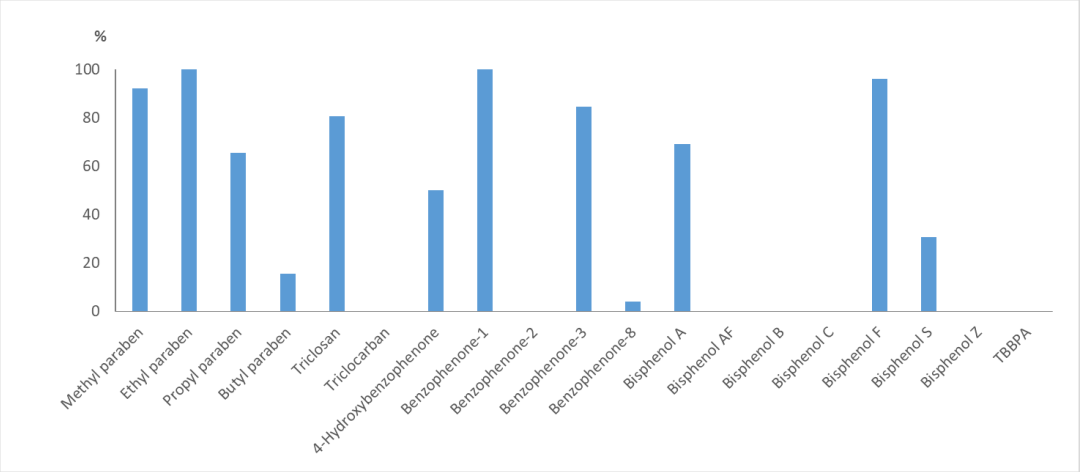

Figure 1. Percentage of detection of each EDC in all the urine samples analyzed (n=26).

Generally, the highest levels were associated with benzophenone-3 (average= 92.5 ng/mL), methylparaben (40.2 ng/mL) and triclosan (27.8 ng/mL). Of the parabens, and as expected, methylparaben was the one with the highest concentrations (Figure 2).

As for the antimicrobials only triclosan was detected with values ranging from <MDL (0.15 ng/mL) up to 440 ng/mL. In what concerns the benzophenon UV filters, benzophenone-1 was detected in all the samples with values ranging between 0.02 and 135 ng/mL and benzophenon-3 was detected in 85% of the samples with one sample registering values as high as 2400 ng/mL.

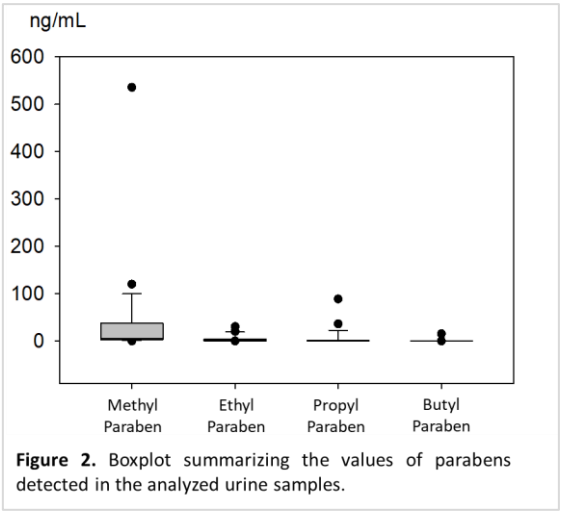

Figure 2. Boxplot summarizing the values of parabens detected in the analyzed urine samples.

Of the bisphenols, interestingly, BPA was not the most prevalent compound nor the one that registered the highest concentrations. In fact, it was detected in 69.2% of the samples with an average value of 3.03 ng/mL whereas its substitute BPF was detected in 96.2% of the samples with an average value of 4.95 ng/mL.

Amongst the analyzed EDCs a significant correlation was obtained between levels of bisphenols and UV filters (Spearman rank order correlation: r=0.445, p=0.0226). For the remainder groups of EDCs no correlations were found ($p>0.05$).

In what concerns the possible associations with clinical parameters no significant correlations could be established between any group of EDCs and glucose levels, gestational age and mothers'

age, with p values higher than 0.3 in most categories analyzed. The only exception was the correlation obtained between gestational age and the levels of parabens (Spearman rank order correlation: *r*=0.351, *p*=0.0784). However, due to the limited number of samples analyzed so far these results are merely indicative and need to be further explored with more samples.

3.5. Future perspectives

We are continuing the recruitment so that a larger data set can be analyzed in the future and consequently a research paper can be prepared and submitted by 2020. We are preparing a paper with the study design of the DEGAS project that will acknowledge the funds by LaMer Project.

3.6. Achievements

Oral Communications by Invitation

- Sousa ACA, Ikenaka Y, Ichise T, Pastorinho MR, Barros R, Taborda-Barata L, Barroso CM, Coelho SD, Miranda S, Marques A, Nakayama SMM, Tanoue R, Takahashi S, Kunisue T, Ishizuka M, Tanabe S (2018) Levels of endocrine disruptors in Portugal: results from the joint collaboration between Portugal and Japan. 2nd Chemical Hazard Symposium, Ehime University, 8 December 2018, Matsuyama, Japan
- Sousa ACA (2018) Environmental contaminants and the obesity epidemic: insights on how to reduce exposure. CBEM 2018-33º Congresso Brasileiro de Endocrinologia e Metabologia, 7-11 August 2018, Belo Horizonte, Brazil (Keynote lecture)
- Sousa ACA (2018) Environmental contaminants and endocrine disruption: The story of obesogens. 20th European Congress of Endocrinology. Symposium 4: "Environmental Effects on Endocrine Function", S4.2. 20 May 2018, Barcelona, Spain
- Sousa ACA (2018) Environmental pollution - a biotechnological perspective. IV *Encontro Nacional de Estudantes de Biotecnologia*, University of Beira Interior, Portugal

Oral Communications

- Sousa ACA, Miranda S, Marques A, Valente C, Tanoue R, Kunisue T, Tanabe S, Patinha C, Ferreira da Silva E, Silva T, Henriques I, Amaro R, Pereira CC, Teixeira JP, Freire MG, Tabord-Barat L, Pastorinho MR (2018) Multidisciplinary approach to understand the role of environmental contaminants in respiratory diseases in Estarreja Region - multiRESPIRA. Séminaire 2018 Labex DRIIHM, 8 – 10 October, La bastide des Joncas La

couronne (Martigues), France

Posters in Conferences/Workshops

- Sousa ACA, Miranda S, Silva T, Marques A, Valente C, Amaro R, Pereira CC, Teixeira JP, Henriques I, Tanoue R, Kunisue T, Tanabe S, Taborda-Barata L, Noack Y, Freire MG, Pastorinho MR (2018) Levels of selected pharmaceuticals and personal care products (PPCPs) in human and environmental samples collected under the framework of MultiRespira Project. Séminaire 2018 Labex DRIIHM, 8 – 10 October, La bastide des Joncas La couronne (Martigues), France
- Sousa ACA, Miranda S, Marques A, Valente C, Amaro R, Pereira CC, Teixeira JP, Silva T, Henriques I, Kunisue T, Tanabe S, Taborda-Barata L, Pastorinho MR (2018) RESPIRA Project: Understanding the role of environmental contaminants in respiratory diseases. 1st Workshop on Human Biomonitoring in Portugal, 11th May 2018, Lisbon, Portugal. p. 47

Peer-reviewed abstracts in international conferences

- Sousa ACA (2018) Environmental contaminants and endocrine disruption: the story of obesogens. Endocrine Abstracts 56, S4.3; http://dx.doi.org/10.1530/endoabs.56.S4.3

Book chapters:

- Sousa ACA, Pastorinho MR (submitted) The use of cats and dogs as indicators of PPCPs (Pharmaceuticals and Personal Care Products) exposure. In: Pets as sentinels, forecasters and promoters of human health. Sousa ACA & Pastorinho MR (Eds) Springer Nature (scheduled to be published March 2019)

References:

(1) WHO; Word Health Organization: 2016.

(2) Chevalier, N.; Fénichel, P. Diabetes Metab. 2015, 41, 107.

(3) Gore, A. C.; Chappell, V. A.; Fenton, S. E.; Flaws, J. A.; Nadal, A.; Prins, G. S.; Toppari, J.; Zoeller, R. T. Endocr. Rev. 2015, 36, E1.

(4) Song, Y.; Chou, E. L.; Baecker, A.; You, N.-C. Y.; Song, Y.; Sun, Q.; Liu, S. Journal of Diabetes 2016, 8, 516.

(5) Thayer, K. A.; Heindel, J. J.; Bucher, J. R.; Gallo, M. A. Environ Health Perspect 2012, 120.

(6) International Diabetes Federation; Federation, I. D., Ed.; International Diabetes Federation: 2018.

(7) Kessous, R.; Shoham-Vardi, I.; Pariente, G.; Sherf, M.; Sheiner, E. Heart 2013, 99, 1118.

(8) Ehrlich, S.; Lambers, D.; Baccarelli, A.; Khoury, J.; Macaluso, M.; Ho, S.-M. Amer J Perinatol 2016, 33, 1313.

(9) Kunisue, T.; Wu, Q.; Tanabe, S.; Aldous, K.M.; Kannan, K. Anal Methods 2010, 2, 707−713.