INVESTIGATION OF GENOTOXIC CHANGES IN MAMMARY EPITHELIAL CELLS AND BIOACTIVITY OF DIOXINS AT REPORTER GENE LEVEL IN BREAST MILK

by

Sami AĞUŞ

Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Master of Science in
Biotechnology

Yeditepe University
2015

INVESTIGATION OF GENOTOXIC CHANGES IN MAMMARY EPITHELIAL CELLS AND BIOACTIVITY OF DIOXINS AT REPORTER GENE LEVEL IN BREAST MILK

APPROVED BY:

Prof. Bayram YILMAZ .....
(Thesis Supervisor)

Prof. Gamze TORUN KÖSE .....

Assist. Prof. Burcu ŞEKER .....

DATE OF APPROVIAL:…/…/2015

“sevgili

eşime...”

ACKNOWLEDGEMENTS

I would like to thank Prof. Dr. Bayram YILMAZ. He suggested this project to me, answered all my needs, supported and treated me as no other advisor can do.

I am thankful to Assist. Prof. Burcu ŞEKER , Assist. Prof. Mehtap KACAR, Siğnem EYUBOĞLU, Sinem ETHEMOĞLU, Ahmet APAYDIN, Özge ATASAYAN and all people in Medicine Faculty of Yeditepe University.

Also I want to thank Yeditepe Univerity and TÜBİTAK for their technical and financial support to this study.

Finally, I greatly thank to my wife who showed great understanding during my work at laboratory and also home.

ABSTRACT

INVESTIGATION OF GENOTOXIC CHANGES IN MAMMARY EPITHELIAL CELLS AND BIOACTIVITY OF DIOXINS AT REPORTER GENE LEVEL IN BREAST MILK

Organochlorinated chemicals including dioxins and dioxin-like compounds threat environment and human health. They can easily contaminate living fields and bioaccumulate in adipose tissue in living organisms because of their chemical structures. Because these chemicals have toxic effect on several systems in the organism we considered that bioactivity of these substances should detect at reporter gene level using body fluid. Dioxins and dioxin-like compound can accumalated in breast milk which contain fat. So, human breast milk is one of the critical samples for both mother and baby in order to observe for dioxins.

In this study, 50 milk samples were collected from lactating mothers living in İstanbul. Mothers were asked to fill a questionnaire form including personal, medical and nutritional information. Exfoliated epithelial cells were isolated from collected breast milk. Comet assay was used to determine DNA damage in epithelial cells. DR-CALUX assay was used to determine dioxin level in the breast milk. Dioxin level of 27 of samples was above the detection limit. It was observed that DNA damage were high at samples which include high dioxins.

These results provide evidence that dioxins can cause genotoxicity in mammary epithelial cells. Furthermore, it was shown that CALUX assay can be used for human biomonitoring and also the human breast milk is very suitable biological sample for this purpose.

ÖZET

ANNE SÜTÜNDE DİOKSİN BİOAKTİVİTESİNİN REPORTER GEN SEVİYESİNDE İNCELENMESİ ve MEME EPİTEL HÜCRELERİNDE GENOTOKSİK DEĞİŞİKLİKLERİN ARAŞTIRILMASI

Dioksin ve dioksin benzeri organklorlu kimyasallar çevreyi ve insan sağlığını tehdit etmektedir. Bu maddeler kolaylıkla canlıların yaşadığı alanları kirletip kimyasal yapılarından dolayı canlı organizmaların yağ dokularında birikebilir. Canlı organizmasındaki bir çok system üzerinde toksik etkisi olduğundan dolayı bu maddelerin bioaktivitelerinin reporter gen düzeyinde belirlenmesi gerektiğini düşündük. Yağ içerdiği için dioksin ve dioksin benzeri maddeler anne sütünde birikebiliryorlar. Bu yüzden insane sütü hem anne için hem de bebek için dioksinlerin etkilerinin gözlenmesi yönünde çok kritik bir öneme sahiptir.

Bu çalışmada, İstanbul'da yaşayan 50 emziren anneden süt örnekleri topladık. Annelere doldurmaları için kişisel, hastalık geçmişi ve beslenme alışkanlıklarını içeren anket verdik. Toplanılan sütlerden döküntü epitel hücresi izole ettik. Bu epitel hücrelerde DNA hasarını tespit etmek için Comet assay yöntemini kullandık. Dioksin seviyelerini tespit etmek içinse DR-CALUX assay yöntemini kullandık. Toplanılan örneklerin 27'sinde dioksin seviyeleri tespit edilebilen seviyenin üzerindeydi. DNA hasarının dioxin seviyesi yüksek olan örneklerde yüksek olduğu gözlendi.

Bu sonuçlar dioksinlerin meme epitel hücresinde genotoksik etkisi olduğunu ispatlamaktadır. Ayrıca CALUX assay yönteminin insan biogörüntülemesi için kullanılabileceği ve anne sütünün de bu amaç için çok uygun bir biyolojik örnek olduğu gösterilmiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SYMBOLS/ABBREVIATIONS

AhR	Aryl hydrocarbon Receptor
Arnt	AhR nuclear translocator protein
CALUX	Chemically Activated LUciferase gene eXpression
DRE	Dioxin responsive elements
dl-PCBs	Dioxin-like Polychlorinated biphenyls
HAHs	Halogenated aromatic hydrocarbons
PAHs	Polycyclic aromatic hydrocarbons
PBS	Phosphate Buffer Saline
PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dibenzo-p-dioxins
PCDFs	Polychlorinated dibenzofurans
POPs	Persistent Organic Pollutants
WHO	World Helath Organization

1. INTRODUCTION

Xenobiotics, foreign substances in a living organism, include halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs). These man-made chemicals are toxicant substances that are distributed in all the world. Most of xenobiotics follow same molecular path way in a living cell. They firstly activate aryl hydrocarbon receptor (AhR).

Dioxins and dioxin-like compounds, the most infamous member of xenobiotics are widespread pollutants on plant and animal habitat. Level of these pollutants in environment increased by developing of industry in the world. This organochlorinated chemicals contaminated lots of food area and water resources in previous century. Dioxins and dioxin-like compounds have been detected in human in past 5 decades. Exposure of human to dioxins and dioxin-like compounds can show highly toxic effects in several system in the organism. Immunotoxicity, neurotoxicity, hepatotoxicity, endocrine disrupting and mutagenic effects are just some of reported toxicity effects of dioxins. According to World Helath Organization (WHO) developmental and neurodevelopmental effects are most sensitive health end-point of these pollutants.[1-4]

Dioxins and dioxin-like chemicals can travel from their main source to fields from where humans and animals feed and can bioaccumulate in living organisms due to their structural properties. That is why dioxin and dioxin-like contamination threats public health. In order to protect from dioxins and be aware of at which level public is expose to dioxins biomonitoring methods have to use in addition to chemical analysis.[5-8]

In this study, reflection of contamination of dioxins and dioxin-like compounds were demonstrated in breast milk samples collected from nursing women living in İstanbul. CALUX assay was used to detect bioactivity of dioxins. Also genotoxicty was investigated by single cell gel electrophoresis.

2. THEORETICAL BACKGROUND

Persistent Organic Pollutants (POPs) is a general name of a group of pollutants that include polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDDs and PCDFs), polychlorinated biphenyls (PCBs) dioxin-like PCBs (dl-PCBs) and polycyclic aromatic hydrocarbons (PAHs). PCDDs and PCDFs are called as "dioxins". Since dioxins and PCBs are fat-soluble substances they can easily accumulate in fat tissue of living organisms in nature. Because these chemicals are environmentally and biologically very stable they can stay in nature for long time.[9-11]

Because of bioaccumulation of dioxins and PCBs in fat tissue, these substances can join in food chain via fish and other animal food. In many studies, it has been shown that dioxins, PCBs and other POPs have neurotoxic, hepatotoxic, endocrine disrupter and mutagenic effects on living organisms. [11-13]

2.1 DIOXINS and DIOXIN-LIKE COMPOUNDS

The term "dioxins and dioxin-like substances" commonly refers to PCDDs, PCDFs and PCBs (WHO-2010). Dioxins and PCBs were included in the dirty dozen according to the Stockholm convention (2001). These organochlorinated chemicals are generally byproducts and waste products of industries of plastics, paint or pesticides. [5,13]

2.1.1 Mechanism of Toxicity

Different studies showed that AhR is expressed in numerous cell types and tissues such as human and mouse lung, kidney, liver, spleen and placenta, and also different tumor cell lines.[14] Human AhR is expressed with 848 amino acids. Its molecular mass is 96 kDa.

Ligands of AhR can be examined in two groups as man-made and natural compounds that are synthesized by microorganism, plants and animals. HAHs, PAHs, indole derivatives, alkaloids, polyphenols are known examples for both groups.

AhR is stable in cytoplasm with a couple protein complex in the absence of any ligands. This protein complex which includes two HSP90 molecules avoid AhR to translocate to the nucleus.

When a ligand, dioxins or others, bind to AhR it goes conformational changes. Although not fully understood phosphorylation/dephosphorylation balance for some serine residues on N-terminal nuclear localization signal domain of AhR causes translocation of the AhR into nucleus. In the nucleus, activated AhR binds to AhR nuclear translocater protein (Arnt). AhR/Arnt heterodimer then binds on specific region on DNA called dioxin responsive elements (DRE). This binding stimulates the expression of AhR-responsive genes.

The best known target gene of AhR/Arnt signaling is *CYP1A1*. *CYP1A1* gene regulates expression of enzyme complex of cytochrome P450. [14-17]

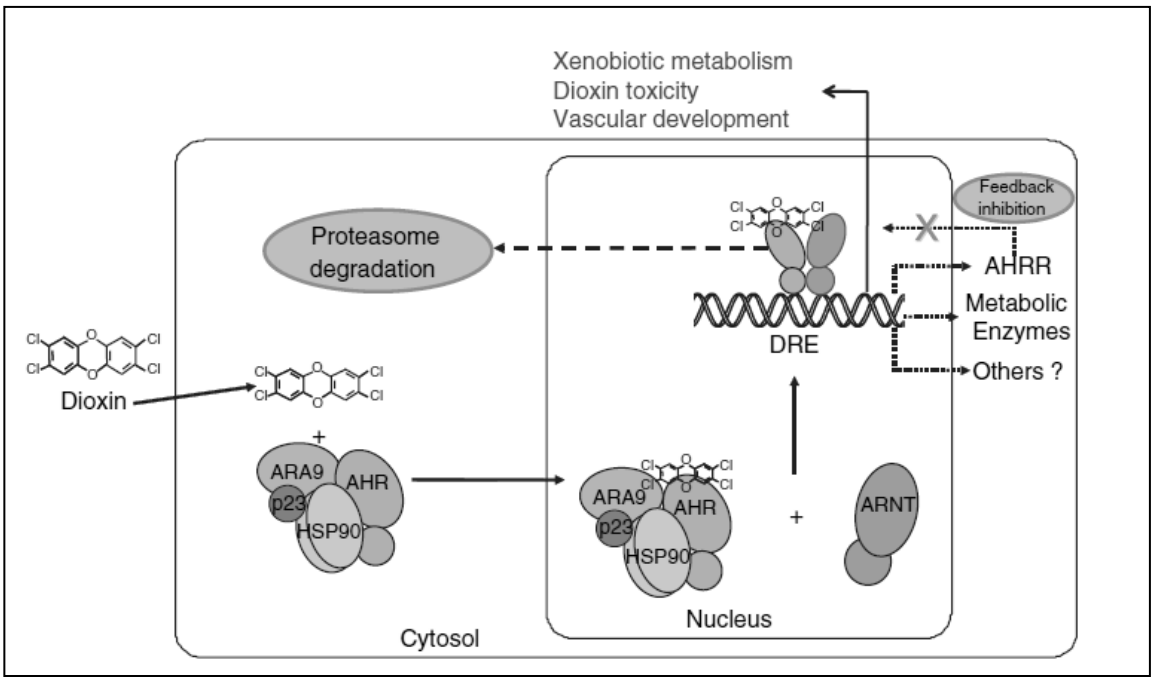

Figure 2.1. Molecular pathway of dioxins and dioxin-like compounds toxicity [14]

2.2 CHEMICAL STRUCTURE

2.2.1 Dioxins

There are more than 200 different dioxins (PCDDs and PCDFs) in nature. PCDDs and PCDFs have two benzene rings bounded oxygen atoms. Rings are bounded with two oxygen atoms in PCDD and with one oxygen and one carbon atom in PCDFs. 7 of PCDDs and 10 of PCDFs have chlorine substation at position 2,3,7 and 8. From among these substances, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is the most toxic known compound which is used generally as a standard in assays in order to detect dioxins. [18-20]

Dioxins and dioxin-lika compounds are colourless in pure solid or crystal form. Since they are not in use they can be transported as residue bounded to related particles. Dioxins and dioxin-like compounds are hydrophobic and strongly lipophilic. Thanks to their chlorine content they are soluble in organic contents. This feature help them to travel in food chain easily.[21]

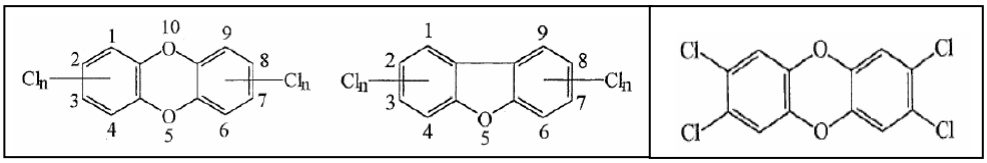

Figure 2.2. Chemical structure of PCDDs, PCDFs and 2,3,7,8-TCDD respectively [19]

2.2.2 Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls have very similar to dioxins. PCBs are also lipophylic and have toxicity mechanism like dioxins. 12 of 209 PCBs congeners are called as dioxin-like compounds since they have same stereo-configuration with dioxins.[5,6,19]

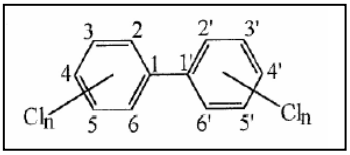

Figure 2.3. Chemical structure of PCBs [19]

2.3 ENVIRONMENTAL CONTAMINATION and FOOD CHAIN

Dioxins are emitted to the nature as natural product of combustion or by products of different industrial processes. Dioxins and PCBs are not used for any production in industry. Although PCBs releasing is disallow by Stockholm Convention PCBs are still detected in nature because of before usage in various equipment.[18,20-22]

PCDDs and PCDFs are released after manufacture of pesticides and herbicides and melting of many metals in mine industry. Also natural disaster such as forest fires and volcanic explosion can cause dioxins emission to nature. Due to feature of low electrical conductivity PCBs have been used in many equipment including industry and domestic usage. All organic chemicals including chlorine give dioxins to nature when they are combusted.[23,24]

Because of their hydrophobic feature dioxins and dioxin-like compounds attach to any organic material such as microorganisms, animals or plants in aquatic environment. There are lots of contaminant area especially water resources in the world. In Turkey there are some studies which show that some water resources such as rivers, some sea areas at where people are fishing and some agricultural areas are contaminated with dioxins and dioxin-like compounds. Usage of pesticides under any expert control mostly cause this contamination.[25-28]

When a habitat including sea and rivers is contaminated with dioxins and dioxin-like compounds they can be easily involved in food chain because of bioaccumulation in fat-tissue. There is lots of studies show that animals especially fishes which feed from contaminated fields have dioxins and dioxin-like compounds in their fat tissue. Dioxins and dioxin-like compounds were also detected in animal milk and eggs. The level of dioxins in some areas are higher than limits of WHO. [5,29-31]

When people consume contaminated animals and animal products such as milk and eggs these toxic substances take place in human fat tissue. Dioxins and PCBs have been detected also in human milk. It is also known from previous studies dioxins and dioxin-like compounds can transfer to fetus through placenta and to babies with breast milk. [32-37]

Lots of studies showed that living organisms can be exposed PCBs with inhalation. PCBs were detected in atmosphere in different areas of the world including Marmamara Area.[38]

2.4 DETERMINING TOXICITY and GENOTOXICTY of DIOXINS (BIOMONITORING)

Gas Chromatography with combination of High Resolution Mass Spectrometry (GC-HRMS) was used to determine the concentration of dioxins and PCBs as a traditionally method. Because this method is expensive and labor-dependency a new bioassay was developed to process many samples at same time and with low-cost using molecular pathway of dioxins inside the cell. The CALUX (Chemically Activated LUciferase gene eXpression) assay allow to use of genetically engineered cells to generate light when they expose to dioxins and dioxin-like compounds. Since the CALUX assay allow to detect bioactivity of dioxins and dioxin-like compounds extracted from body fluid it can be used as a human biomonitoring method. [39,40]

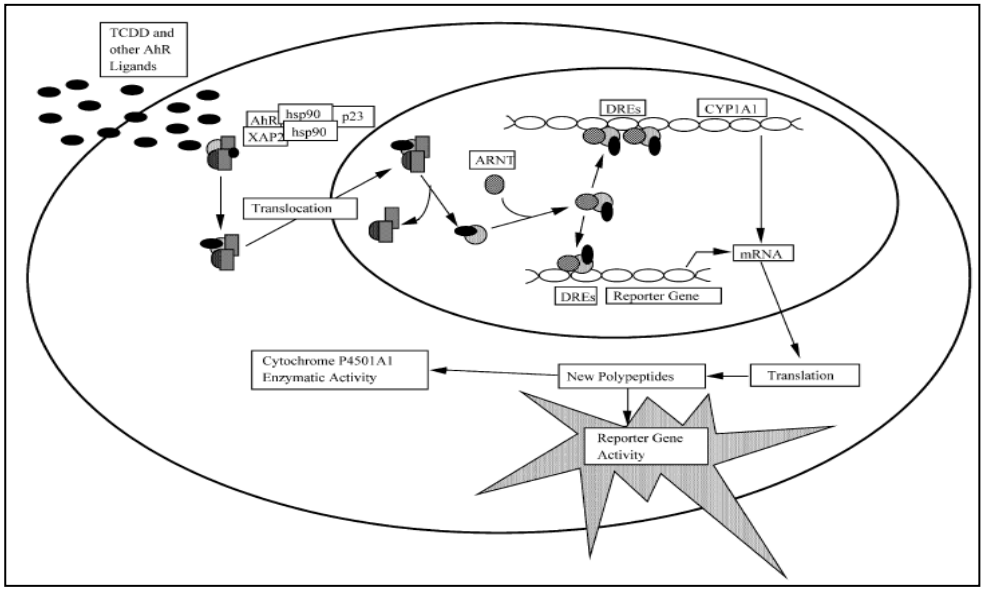

Figure 2.4 Luciferase pathway in the CALUX assay [42]

The Comet assay is a simple and sensitive method to measure DNA breaks in a small amount of cells. This method is generally used for human biomonitoring especially in occupational exposure studies. The Comet assay is the most sensitive and applicable method to understand dioxins and dioxin-like compounds has genotoxic effect on mammary epithelial cells. [41,42]

3. MATERIALS and METHODS

3.1. MILK SAMPLES

50 healthy lactating mothers attended to the study from different places of İstanbul. The mothers were asked to aspirate their breast milk by hand into a sterilized jar given by us. They were also asked to fill a questionnaire form including several information such as demographical, drug using, nutritional habitat and medical history. Also BMI values of the women were calculated from the given information (Figure 3.1). Milk samples were transported to the laboratory under cold condition immediately. Collected milk volume ranged from 2,5 ml to 31 ml.

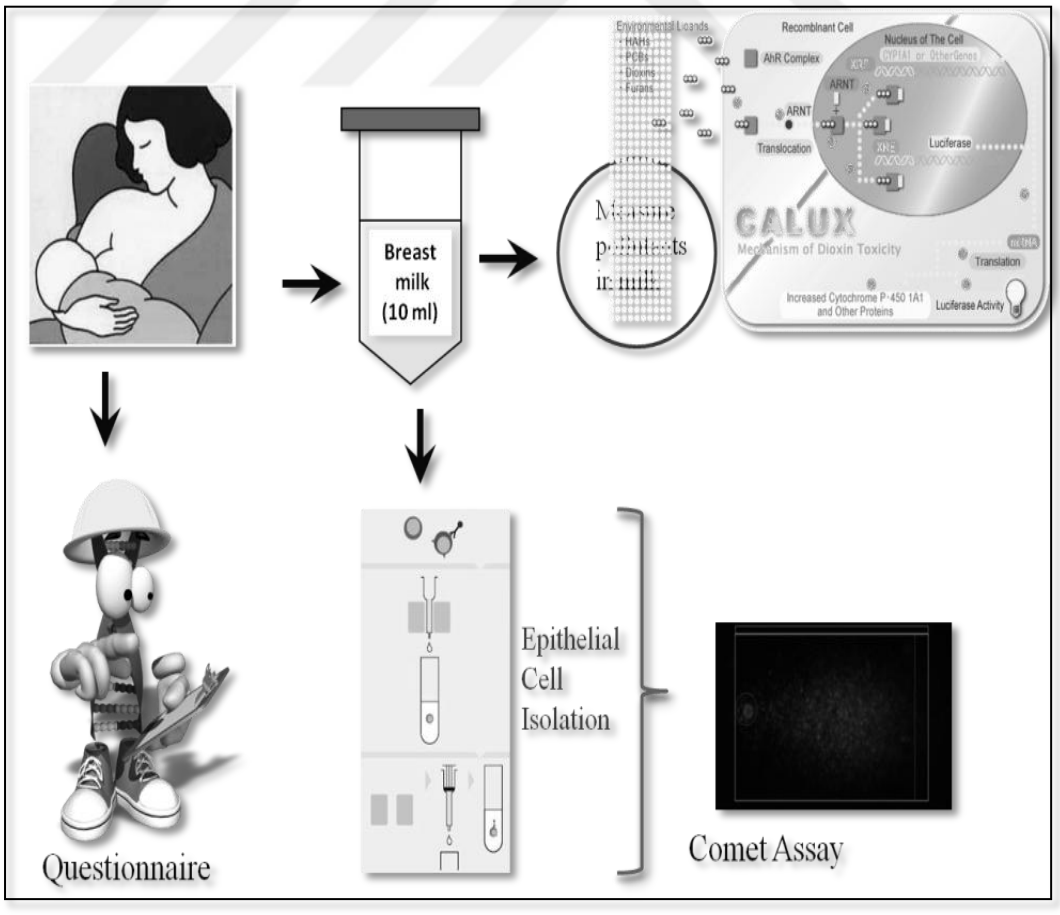

Figure 3.1 Schematically representation of the study

3.2. ISOLATION of MAMMARY EPITHELIAL CELLS

3.2.1. Sample Preparing

Collected milk samples were dived two. Half of the samples were stored for dioxin analysis at -20 ^{o}C. Remaining samples were diluted 1:1 with Phosphate Buffer Saline (PBS) (Invitrogen, life technologies, USA) in a falcon tube. The diluted samples were centrifuged at 2000 RPM for 10 minutes. The supernatant part including fat and milk serum was removed and stored in a amber jar. The pellet including total exfoliated cells were washed 2 ml of PBS and centrifuged at 1500 RPM for 5 minutes. After centrifugation the supernatant was removed and the pellet was diluted with 2 ml of PBS and transferred into 2 ml eppondorf tubes.

3.2.2. Cell Counting

0.4% Tryphan blue (Invitrogen, life technologies, USA) was used to determine cell viability. 10 µl of samples were diluted with 10 µl of 0.4% tryphan blue and total cells in the samples were counted by using hemocytometer. About 1 X 10^5 of cells were taken after counting to use in comet assay genotoxic analysis.

3.2.3. Cell Isolation

Mammary epithelial cells were isolated using magnetic beads and columns (Miltenyl Biotec, Germany). The samples in the eppondorf tubes were centrifuged at 1500 RPM for 5 minutes. After centrifugation the supernatant was removed. The pellet was washed by 60 µl of PBS including 0.5% Bovine Serum Albumin (BSA) and 2mM EDTA. 20 µl of reaction reagent (Miltenyl Biotec, Germany) and 20 µl of human epithelial magnetic anti-body (Miltenyl Biotec, Germany) were added into the eppondorf tubes. The samples were incubated at +4 ^{o}C for 30 min. Magnetic columns were placed at magnetic stands (Miltenyl Biotec, Germany) and washed 500 µl of PBS-0.5% BSA during incubation. After incubation the samples were centrifuged at 1500 RMP for 5 min. Supernatant was removed and the pellet was dissolved with 500 µl of PBS-0.5%BSA. The eppondorf tubes were

marked with (-) and placed under the magnetic columns in order to collect epithelial depleted cells. The samples were transferred into the magnetic columns and cell separation started. 500 µl of PBS-0.5%BSA was added into the columns during separation. When 1,5 ml of samples was collected in the eppondorf tubes, the magnetic column was removed from magnetic stands. 1 ml of PBS-0.5%BSA was added into the column and column syringe was pushed immediately. The samples were collected into a new eppondorf tubes marked with (+). These tubes included enriched epithelial cells.

Figure 3.2 Schematically representation of the cell isolation

3.3. COMMET ASSAY

3.3.1. Slide Preparation and Lysis

The exfoliated cells were mixed with 0.65% Low Melting Agarose (LMA) (Invitrogen, life technologies, USA) at 37 ^{o}C. The cell suspension was layered onto slides precoated with 0.65% High Melting Agarose (HMA) (Invitrogen, life technologies, USA). Two slides were prepared for each sample. The slides were covered with a largecover slip immediately and incubated at 4 ^{o}C for 10 minutes to allow the agarose solidify. After incubation, cover slips were removed and the slides were immersed in freshly prepared lysis solution (2.5M NaCl, 100mM EDTA, 10mM Tris, pH 10, with 1% Triton X-100 and 10%DMSO). The slides were incubated in lysis solution at +4 ^{o}C for 1 hour.

3.3.2. Electrophoresis

After lysis, the slides were placed in a horizontal gel tank containing fresh alkaline electrophoresis buffer solution (1mM EDTA, 300mM NaOH (pH > 13). The slides were incubated in the electrophoresis buffer for 20 min. in order to allow DNA unwinding. The electrophoresis was run at 25V (0,83/cm) and 300 mA for 25 min. after incubation. After the electrophoresis the slides were incubated three times with neutralizing buffer at 4 ^{o}C for 5 min. for each. The slides were dried and stained with 20 µg/ml of ethidium bromide.

3.3.3. Slide Scoring

At least fifty cells per slide and two slides per sample scored to determine DNA damage. The slides were examined under a fluorescent microscope (Zeiss, Germany) equipped with suitable filters at 200 X magnification. The cells were scored by using a software (Comet Assay IV, Perspective Instruments, UK). Tail intensity and tail moment values were used represent DNA Damage.

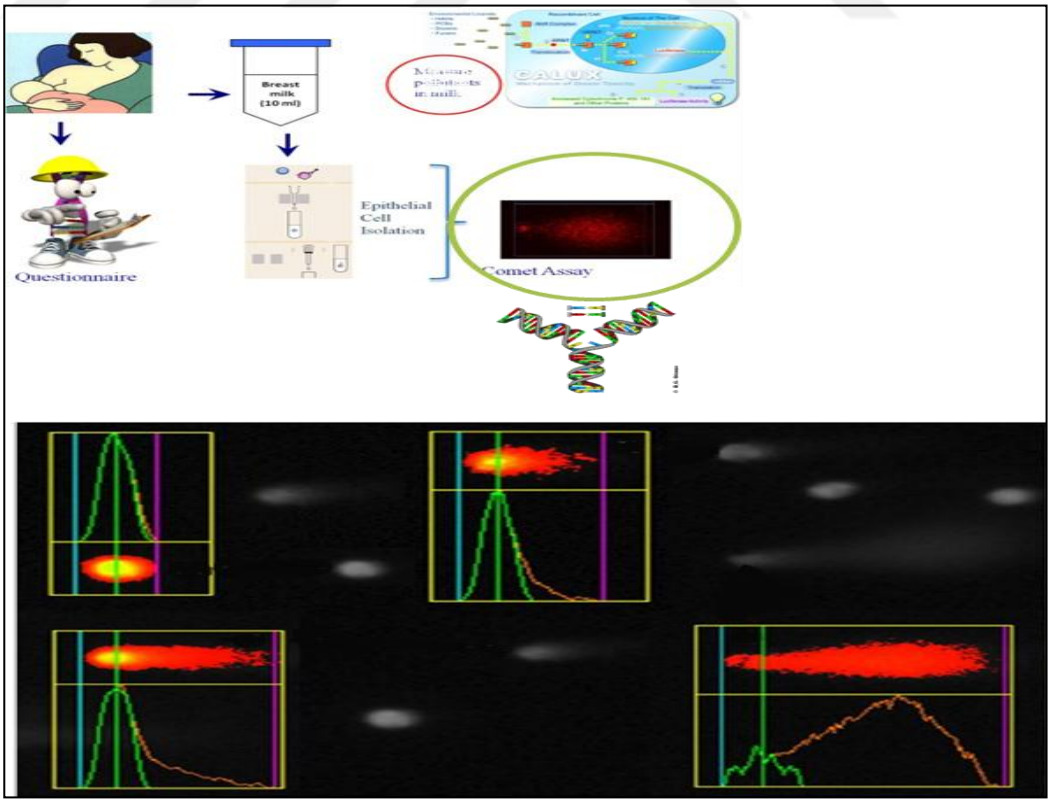

Figure 3.3 Schematically representation of the comet assay

3.4. DIOXINS and PCB ANALYSIS

Half of each sample were processed at BioDeteciton System (BDS) Company in Amsterdam for DR-CALUX. The method used in BDS was shake extraction with organic solvent (hexane); the extracts were cleaned on an acidic slica column. The cleaned extracts were dissolved in DMSO. The DR-CALUX activity was determined (after 24h exposure). The evaluation was done on the maximum level for PCDD/F, from which a cut off value has been established to determine if a sample is compliant or suspected. After the evaluation, an estimation is given of the sample in the form of a BEQ outcome.

3.5. STATISTICS

For statistical data comparisons, SPSS software (SPSS Inc., Chicago, IL, USA) was used. Correlations were calculated by one-way ANOVA and independent simple t-test. All values were given as mean ±SD. P values <0,05 are considered statistically significant.

4. RESULTS

4.1. DEMOGRAPHIC DATA

4.1.1. Personal Information

Age of the mother in this study ranged from 19 to 42. Mean age (±SD) of the donors is 28,26 ±4,88. BMI values range from 19,8 to 44,7. The mean of the BMI of the mothers is 26,3 ±4,82. 8 of the mothers in the study smoke and 18 of the mothers are exposed smoke in their home.

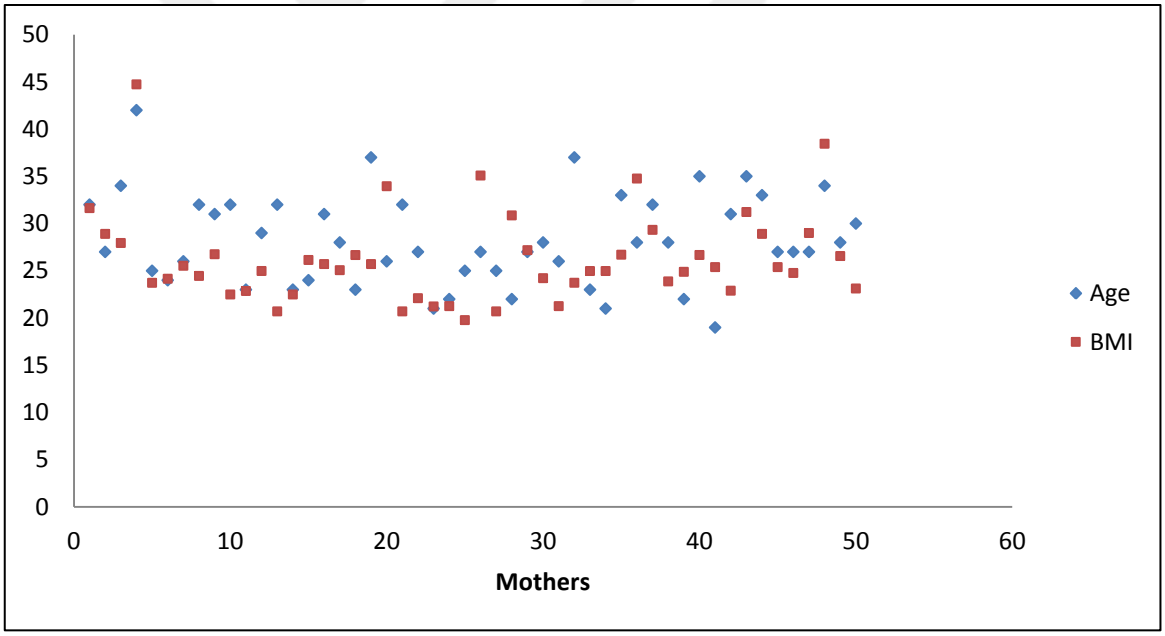

Figure 4.1.1. Age and BMI values of mothers in the study.

4.1.2. Nutritional Information

26 of the mothers eat fish monthly, 14 of them eat weekly, 6 of them eat weekly, 2 of them eat every day and 2 of them do not eat fish.

39 of the mothers consume milk and milk products every day and 11 of them consume weekly milk and milk products.

32 of the donors eat red meat weekly, 16 of them eat meat monthly and 2 of them eat meat every day.

43 of the mothers eat white meat (chicken etc.) weekly, 11 of them eat white meat yearly, 5 of them eat white meat monthly.

35 of the donors eat egg every day, 14 of them weekly and 1 subject never eats egg.

25 of the mothers consume barbecue yearly, 17 of them consume monthly, 6 of them consume weekly and 2 subjects never consume barbecue.

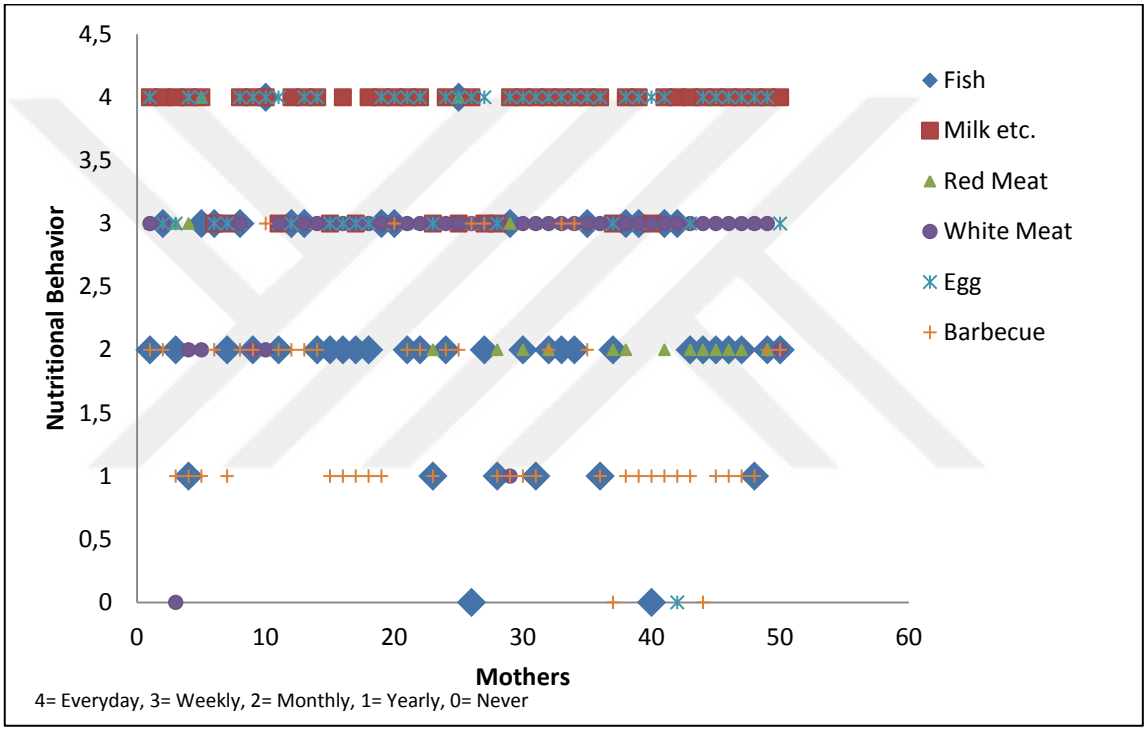

Figure 4.1.2 Nutritional behavior of mothers in the study.

20 of the subjects drink coffee or tea rarely, 12 of them drink 3-4 cups a day, 9 of them drink 1-2 cups of day and 9 subjects drink coffee or tea more than 4 cups a day.

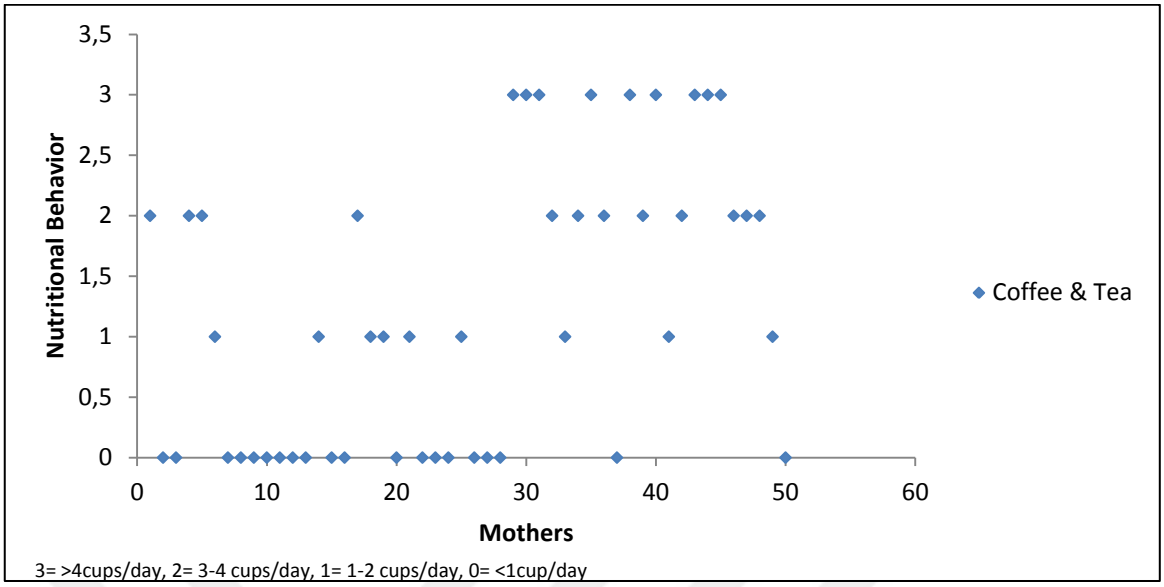

Figure 4.1.3 Coffee and tea consumption of mothers in the study.

4.1.3. Medical Record

4 subjects have a relative who suffered from breast cancer and 1 subject has a relative who suffered from ovary cancer.

4.1.4. Lactation Information

2 of the subjects got pregnant six times, 2 of them got pregnant five times, 7 of them got pregnant four times, 5 of them got pregnant three times, 15 of them got pregnant two times and 19 of subjects have their first children in their first pregnancy.

4 of the mothers nursed their 4^{th} children, 9 of them nursed their 3^{rd} children, 11 of them nursed their 2^{nd} children and 26 of the mothers nursed their 1^{st} child while they donate their breast milk for this study.

41 of the mothers in this study was post-partum and 9 of the mothers nursed their babies for more than 1 month.

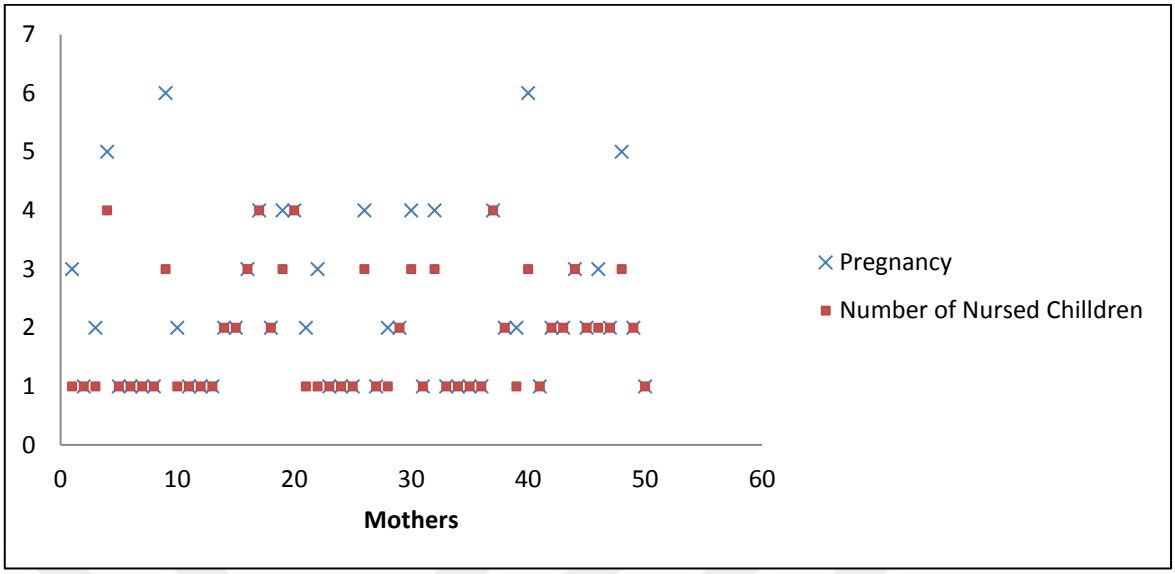

Figure 4.1.4 Lactation information of mothers in the study.

4.2. GENOTOXIC ANALYSIS

Genotoxicty was presented as Tail Intensity (TI) and Tail Moment (TM). TI ranges from 3,04 to 37,80. The mean value of TI is 14,60 ±9,66. TM ranges from 0,34 to 8,48. The mean value of TM is 2,81±2,17.

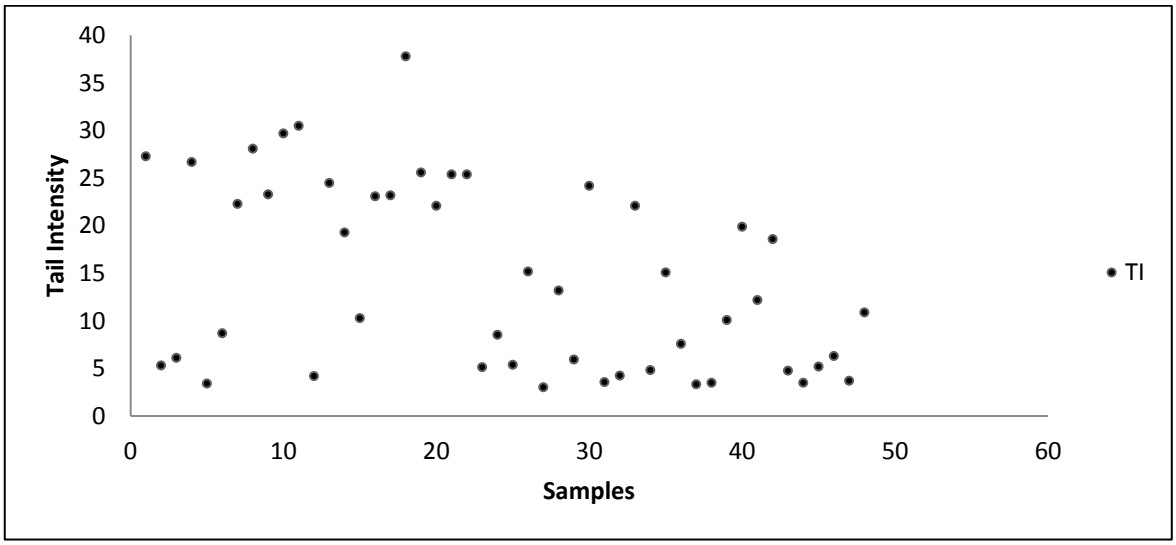

Figure 4.2.1 Tail intensity values of total exfoliated cells from milk samples.

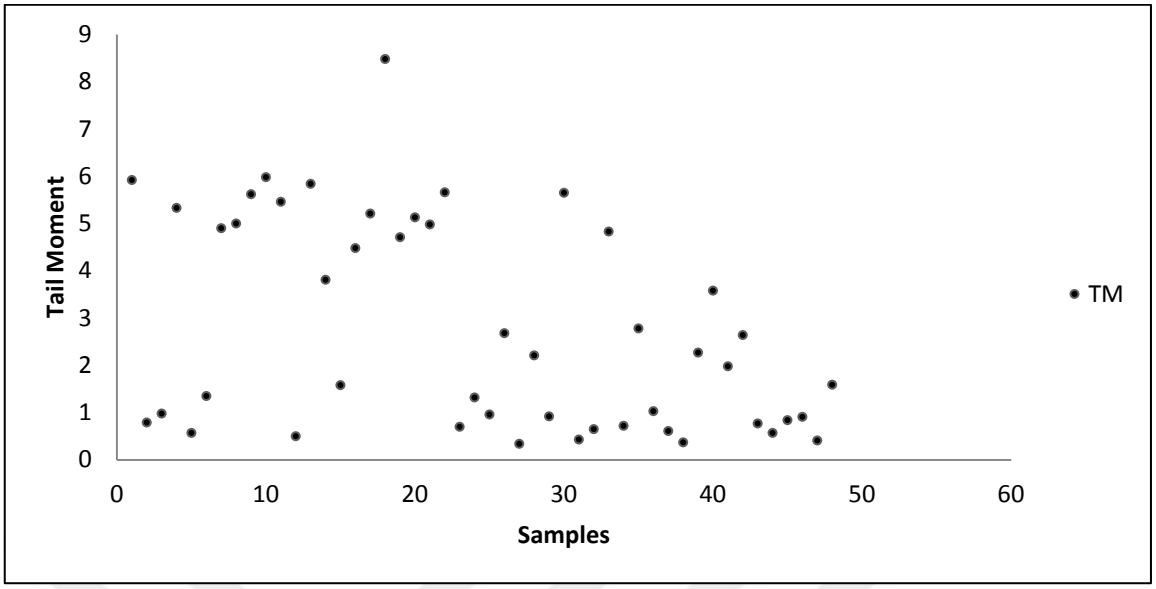

Figure 4.2.2 Tail moment values of total exfoliated cells from milk samples.

4.3. DIOXIN and PCB ANALYSIS

Dioxin and PCBs levels of the milks in the study were expressed as *pg 2,3,7,8-TCDD BEQ/g fat*. Dioxin level of 11 samples is under limit of detection (LOD) and dioxin level of 12 samples is between LOD and Limit of Quantification (LOQ) that can be measured. Dioxin level of remained 27 samples ranges from 3,16 *pg 2,3,7,8-TCDD BEQ/g fat* to 123,32 *pg 2,3,7,8-TCDD BEQ/g fat*. The levels of fat content in the milk samples were also detected during this procedure to analyze dioxins and PCBs levels because of their lipophilic feature. Percentage of fat content in the milk samples ranges from 0,21 to 13,77.

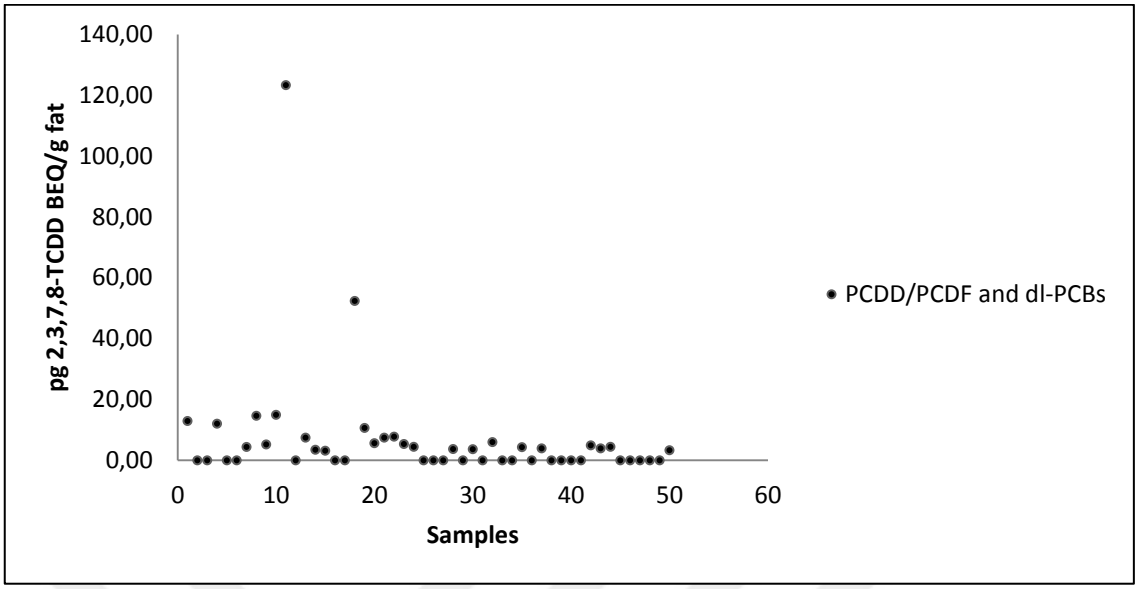

Figure 4.3.1 Dioxin and PCBs level of milk samples.

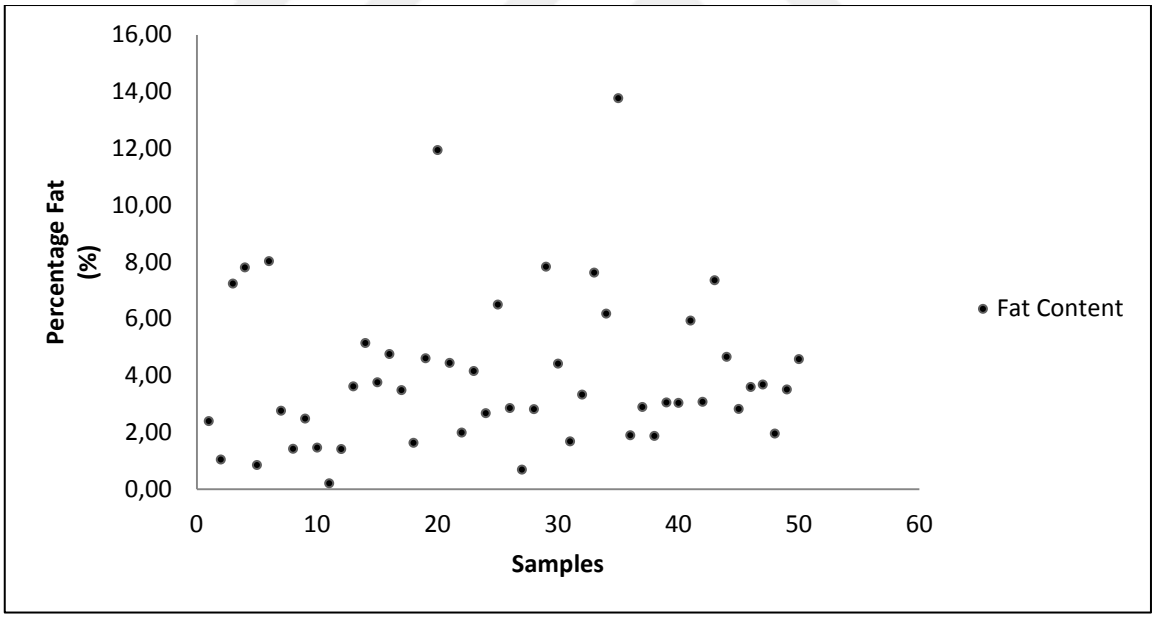

Figure 4.3.2 Fat content of milk samples.

It has been shown that there is a high significant correlation between DR-CALUX and comet assay results ($p<0,001$). High DNA damage was seen in the samples that contain high dioxins.

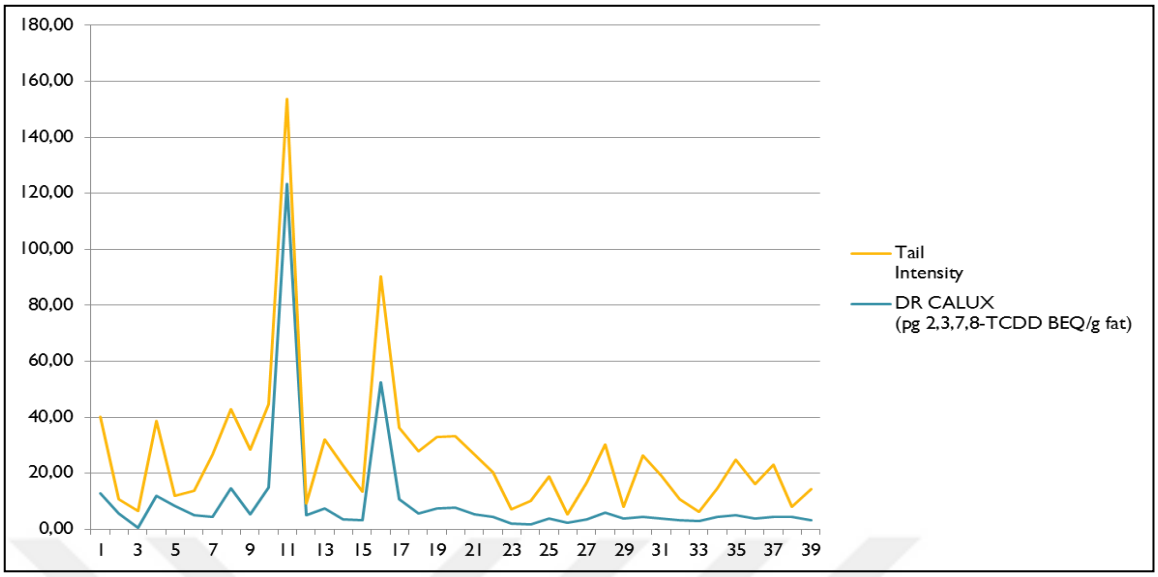

Figure 4.3.3 Compare of DR-CALUX and Comet Assay results

When BMI and DR-CALUX results were compared, a significant correlation was seen ($p<0,001$). High dioxin level was seen in the samples collected from mothers with high BMI.

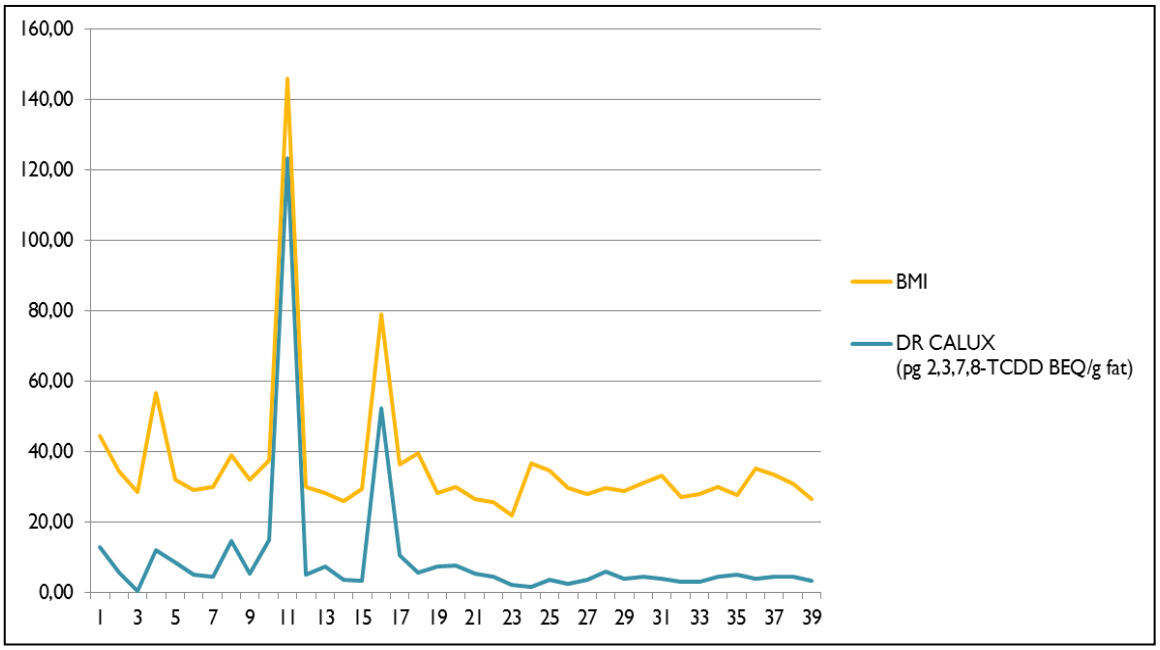

Figure 4.3.4 Compare of DR-CALUX results and BMI values

5. DISCUSSION

Dioxins and dioxin-like compounds tend to accumulate in fat tissue of human body because of lipophicity feature. When a cell is exposed to dioxins or dioxin-like compounds AhR is activated and molecular pathway is resulted in expression of cytochrome P4501A1 (CYP1A1). It is known that they have very serious biological and toxic effects on physiological systems. WHO highlights of their effects on neurodevelopment, neurotoxic and genotoxic effects.

The Joint Food and Agriculture Organization of the United Nations (FAO)/WHO Expert Committee on Food Additives (JECFA) established a provisional tolerable intake of 70 pg/kg body weight per month for PCDDs, PCDFs and coplanar PCBs. The release of dioxins and PCBs to nature is being tried to restricted by authorities. When the emission of dioxins and dioxin-like compounds is controlled it is also critical to monitorize the effects of dioxins and dioxin-like compounds in the population. Suitable biomonitoring methods are needed to be used in order to see reflection of toxicity of dioxins.

DR-CALUX assay was used for examination of bioactivity of dioxins and dioxin-like compounds. Also comet assay was chosen as a biomonitoring method for genotoxic effect of dioxins.

Significant increase in DNA damage was seen in the samples that contain high level of dioxin and dioxin-like compounds. Also same significant result was seen in comparison of BMI and DR-CALUX results.

Although there was not any significant correlation between nutritional and smoking behavior of donors and their DR-CALUX results, the highest values of dioxin level was seen in mothers consume fish and red meat relatively more than others.

6. CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

In this study, breast milk was collected from 50 healthy post-martum mothers. They were asked to fill a questionnaire form including their personal and medical information and nutritional behaviors. The Comet assay method was used to investigate DNA damage on exfoliated mammary epithelial cells which were isolated from milk samples. DR-CALUX assay was used to examine bioactivity of dioxins and dioxin-like compounds. DNA damage significantly was high in the samples with high level of dioxins and dioxin-like compounds. DR-CALUX results were significantly high with high BMI values.

These results provide that dioxins and dioxin-like compounds can cause DNA damage in mammary epithelial cells. Also it was shown that high weight can cause accumulation of dioxins and dioxin-like compounds in adipose tissue. DR-CALUX and Comet assays are reliable methods for human biomonitoring in the exposure studies.

6.2 RECOMMENDATIONS

As a future work, milk samples can be collected from same mothers and it can observe that if any genotoxic change occurs in different nursing time. Also large number of samples would give more significant results especially for nutritional habitat.

Same study can be done with samples from obese people since dioxins and dioxin-like compounds accumulate in adipose tissue.

REFERENCES

1. D. O. Carpenter, K. F. Arcaro, B. Bush, W. D. Niemi, S. Pang, D. D. Vakharia. Human health and chemical mixtures: an overview. *Environmental Health Perspectives*, 106; 1263-1270, 1998.

2. D. O. Carpenter. Health effects of persistent organic pollutants: the challenge for the Pacific Basin and for the world. *Reviews on Environmental Health*, 26; 61-9, 2011.

3. D. O. Carpenter. Polychlorinated biphenyls (PCBs): routes of exposure and effects on human health. *Reviews on Environmental Health*, 21; 1-23, 2006.

4. A. Colles, G. Koppen, V. Hanot, V. Nelen, M. C. Dewolf, E. Noël, R. Malisch, A. Kotz, K. Kypke, P. Biot, C. Vinkx, G. Schoeters. Fourth WHO-coordinated survey of human milk for persistent organic pollutants (POPs): Belgian results. *Chemosphere*, 73; 907-14, 2008.

5. WHO Dioxin Report. Exposure to dioxins and dioxin-like substances: a major public health concern, www.who.int/ipcs/features/dioxins.pdf [retrieved 05 January 2015]

6. WHO Polychlorinated dibenzodioxins, polychlorinated dibenzofurans, and coplanar polychlorinated biphenyls. In: Safety evaluation of certain food additives and contaminants. Geneva, World Health Organization "WHO Food Additives Series, No. 48" http://www.inchem.org/documents/jecfa/jecmono/v48je20.htm [retrived 05 JAnuary 2015]

7. K. Breivik, R. Alcock, Y. F. Li, R. E. Bailey, H. Fiedler, J. M. Pacyna. Primary sources of selected POPs: regional and global scale emission inventories. *Environmental Pollutions*, 128; 3-16, 2004.

8. J. C. Larsen. Risk assessments of polychlorinated dibenzo- p-dioxins, polychlorinated dibenzofurans, and dioxin-likepolychlorinated biphenyls in food. *Molecular Nutrition and Food Researchs*, 50; 885-96, 2006.

9. F. K. Arcaro, and J. Gierthy. Assessing modulation of estrogenic activity of environmental and pharmaceutical compounds using MCF-7 focus assay. *Methods in Molecular Biology*, 176; 341-351, 2001.

10. M. Aube, C. Larochelle, P. Ayotte. Differential effects of a complex organochlorine mixture on the proliferation of breast cancer cell lines. *Environmental Research.*, 111; 337-47, 2011.

11. C. Dietrich, B. Kaina. The aryl hydrocarbon receptor (AhR) in the regulation of cell-cell contact and tumor growth. *Carcinogenesis*, 31; 1319-28, 2010.

12. R. Golden, R. Kimbrough. Weight of evidence evaluation of potential human cancer risks from exposure to polychlorinated biphenyls: an update based on studies published since 2003. *Critical Reviews in Toxicology*, 39; 299-331, 2009.

13. S. Knerr, D. Schrenk. Carcinogenicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in experimental models. *Molecular Nutrition and Food Research*, 50; 897-907, 2006.

14. J. Abel, T. Haarmann-Stemmann. An introduction to the molecular basics of aryl hydrocarbon receptor biology. *Biological Chemistry*, 391; 1235-48, 2010.

15. T. Kung, K. A. Murphy, L. A. White. The aryl hydrocarbon receptor (AhR) pathway as a regulatory pathway for cell adhesion and matrix metabolism. *Biochemical Pharmacol*ogy 77; 536–546, 2009.

16. M. S. Denison and S.R. Nagy. Activation of the Aryl Hydrocarbon Receptor by Structure Diverse Exogenous and Endogenous Chemicals. *Annual Review of Pharmacology and Toxicology*, 43; 309-334, 2003.

17. A. J. Papoutsis, J. L. Borg, O. I. Selmin, D. F. Romagnolo. BRCA-1 promoter hypermethylation and silencing induced by the aromatic hydrocarbon receptor-ligand TCDD are prevented by resveratrol in MCF-7 cells. *The Journal of Nutritional Biochemistry*, 23; 1324-32, 2012.

18. J. Vijgen, P.C. Abhilash, Y. F. Li, R. Lal, M. Forter, J. Torres, N. Singh, M. Yunus, C. Tian, A. Schäffer, R. Weber. Hexachlorocyclohexane (HCH) as new Stockholm Convention POPs--a global perspective on the management of Lindane and its waste isomers. *Environmental Science and Pollution Research International*, 18; 152-62, 2011.

19. R. Weber, A. Watson, M. Forter, F. Oliaei. Review Article: Persistent organic pollutants and landfills - a review of past experiences and future challenges. *Waste Management and Research*, 29; 107-21, 2011.

20. K. Norström, G. Czub, M. S. McLachlan, D. Hu, P. S. Thorne, K. C. Hornbuckle. External exposure and bioaccumulation of PCBs in humans living in a contaminated urban environment. *Environment International*, 36; 855-61, 2010.

21. H. Y. Yu, Y. Guo, E. Y. Zeng. Dietary intake of persistent organic pollutants and potential health risks via consumption of global aquatic products. *Environmental Toxicology and Chemistry*, 29; 2135-42, 2010.

22. M. Warner, P. Mocarelli, S. Samuels, L. Needham, P. Brambilla, B. Eskenazi. Dioxin exposure and cancer risk in the Seveso Women's Health Study. *Environmental Health Perspectives*,119;1700-5, 2011.

23. M. Günindi, Y. Tasdemir. Atmospheric polychlorinated biphenyl (pcb) inputs to a coastal city near the Marmara Sea. *Marine Pollution Bulletin*, 60; 2242-50, 2010.

24. J. F. Hsu, H. R. Guo, H. W. Wang, C. K. Liao, P. C. Liao. An occupational exposure assessment of polychlorinated dibenzo-p-dioxin and dibenzofurans in firefighters. *Chemosphere*, 83; 1353-9, 2011.

25. O. Humblet, P. L. Williams, S. A. Korrick, O. Sergeyev, C. Emond, L. S. Birnbaum, J. S. Burns, L. Altshul, D. G. Patterson, W. E. Turner, M. M. Lee, B. Revich, R. Hauser. Predictors of serum dioxin, furan, and PCB concentrations among women from Chapaevsk, Russia. *Environmental Science and Technology*, 44; 5633-40, 2010.

26. S. Jenkins, C. Rowell, J. Wang, C. A. Lamartiniere. Prenatal TCDD exposure predisposes for mammary cancer in rats. *Reproductive Toxicology,* 23(3); 391-6, 2007.

27. H. Lılıenthal, A. Fastabend, J. Hany, H. Kaya, A. Harer, L. Dunemann, G. Winneke. Reduced levels of 1,25-Dihydroxyvitamin D3 in rat dams and offspring after exposure to a reconstituted PCB mixture. *Toxicological Sciences*, 57; 292 – 301, 2000.

28. U. Manuwald, M. G. Velasco, J. Berger, A. Manz, X. Baur. Mortality study of chemical workers exposed to dioxins: follow-up 23 years after chemical plant closure. *Occupational and Environmental Medicine,* 69; 636-42, 2012.

29. O. S. Okay, B. Karacik, S. Başak, B. Henkelmann, S. Bernhöft, K. W. Schramm. PCB and PCDD/F in sediments and mussels of the Istanbul strait (Turkey). *Chemosphere,* 76; 159-66, 2009.

30. L. Wang, S. Weng, S. Wen, T. Shi, G. Sun, Y. Zeng, C. Qi, W. Chen. Polychlorinated dibenzo-p-dioxins and dibenzofurans and their association with cancer mortality among workers in one automobile foundry factory. *The Science of the Total Environment,* 443; 104-11, 2013.

31. E. M. Petro, A. Covaci, J. L. Leroy, A. C. Dirtu, W. De Coen, P. E. Bols. Occurrence of endocrine disrupting compounds in tissues and body fluids of Belgian dairy cows and its implications for the use of the cow as a model to study endocrine disruption. *The Science of the Total Environment*, 408; 5423-8, 2010.

32. G. Bianco, R. Zianni, G. Anzillotta, A. Palma, V. Vitacco, L. Scrano, T. R. Cataldi, Dibenzo-p-dioxins and dibenzofurans in human breast milk collected in the area of Taranto (Southern Italy): first case study. *Analytical and Bioanalytical Chemistry*, 405; 2405-10, 2013.

33. I. Çok, C. Yelken, E. Durmaz, M. Uner, B. Sever, F. Satır. Polychlorinated Biphenyl and Organochlorine Pesticide Levels in Human Breast Milk from the Mediterranean city

Antalya, Turkey. *Bulletin of Environmental Contamination and Toxicology*, 86; 423-7, 2011.

34. I. Çok, M. K. Donmez, M. Uner, E. Demirkaya, B. Henkelmann, H. Shen, J. Kotalik, K. W. Schramm. Polychlorinated dibenzo-p-dioxins, dibenzofurans and polychlorinated biphenyls levels in human breast milk from different regions of Turkey. *Chemosphere*, 76; 1563-71, 2009.

35. O. Erdoğrul, A. Covaci, N. Kurtul, P. Schepens. Levels of organohalogenated persistent pollutants in human milk from Kahramanmaraş region. Turkey, *Environment International*, 30; 659-66, 2004.

36. P. Furst. Dioxins, polychlorinated biphenyls and other organohalogen compounds in human milk. Levels, correlations, trends and exposure through breastfeeding. *Moleculat Nutrition and Food Research*, 50; 922-33, 2006.

37. B. Yılmaz, S. Sandal, H. Ayvaci, N. Tug, A. Vitrinel. Genotoxicity profiles in exfoliated human mammary cells recovered from lactating mothers in Istanbul; relationship with demographic and dietary factors. *Mutation Research*, 749; 17-22, 2012.

38. F. Telli-Karakoc, L. Tolu, B. Henkelmann, C. Klinn, O. Okay, K. W. Schramm. Polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) distributions in the Bay of Marmara sea: Izmit Bay. *Environmental Pollution*, 119; 383-397, 2002.

39. J. P. Giesy, K. Hilscherova, P. D. Jones, K. Kannan, M. Machala. Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. *Marine Pollution Bulletin*, 45; 3–16, 2002.

40. G. He, T. Tsutsumi, B. Zhao, D. S. Baston, J. Zhao, S. Heath-Pagliuso, M. S. Denison. Third-Generation Ah Receptor-Responsive Luciferase Reporter Plasmids: Amplification of Dioxin-Responsive Elements Dramatically Increases CALUX Bioassay Sensitivity and Responsiveness. *Toxicological Sciences*, 123; 511-522, 2011.

41. F. L. Martın, K. J. Cole, D. Harvey, G. Weaver, J. Williams, B C. Millar, D. H. Phillips, P. L. Grover. DNA damage in human breast milk cells and its induction by 'early' and 'late' milk extracts. *Carcinogenesis*, 21; 799-804, 2000.

42. F. L. Martın, K. J. Cole, G. Weaver, J. A. Williams, B. C. Millar, P. L. Grover, D. H. Phillips. Genotoxicity of human milk extracts and detection of DNA damage in exfoliated cells recovered from breast milk. *Biochemical and Biophysical Research Communications*, 7; 319-26, 1999.

APPENDIX A: Questionnaire Form

ANNE SÜTÜ – EPİGENETİK ÇALIŞMASI ANKET FORMU

A. Üreme sağlığı

A1. İlk adeti (menstüasyon) kaç yaşında gördünüz?

9 10 11 12 13 14 15 16 >17

A2. Toplam kaç kez gebe kaldınız?

1 2 3 4 5 6 7 >8

A3. İlk doğum yaptığınızda kaç yaşındaydınız? _____

A4. Son doğum yaptığınızda kaç yaşındaydınız? _____

A5. Kaç çocuk emzirdiniz?

1 2 3 4 5 6 7 >8

A6. Her bir çocuğu toplam kaç ay emzirdiniz?

Birinci çocuk _____ ay

İkinci çocuk _____ ay

Üçüncü çocuk _____ ay

Dördüncü çocuk _____ ay

Beşinci çocuk _____ ay

Altıncı çocuk _____ ay

A7a. Doğum kontrol hapı kullandınız mı?

Evet Hayır

A7b. Hormon içeren rahim içi araç (IUD) kullandınız mı?

Evet Hayır

A8. Yukarıdaki sorulara cevabınız evet ise, ne kadar süreyle kullandınız? ……

B. Genel Sağlık

B1. Herhangi bir reçeteyle verilen ilaç kullanıyor musunuz?

Hayır

Evet ise adı: ……..

B2. Herhangi bir ağrı kesici, vitamin veya bitkisel destekleyici ilaç aldınız mı?

Hayır

Evet ise adı: ……..

B3. Son bir ay içinde grip geçirdiniz mi?

Evet

Hayır

C. Kendiniz Hakkında

C1. Yaşınız _____

C2. Mesleğiniz _____

C3. Halihazırdaki kilonuz ……. ve boyunuz ……..

C4. Kaç yıldır İstanbul'da (veya çevresinde) yaşıyorsunuz? _____

C5. Yukarıdaki soruya cevabınız 5 yıldan az ise daha önce nerede yaşıyordunuz? _____

C6. Hâlihazırda sigara içiyor musunuz?
Evet Hayır

C7. Evinizde sigara kullanan var mı?
Evet Hayır

C8. Vejetaryen misiniz?
Evet Hayır

C9. Aşağıdaki besinleri hangi sıklıkla aldığınızı belirtiniz:

	Her gün	bir veya iki defa			Hiç
		Haftada	Ayda	Yılda	
Balık ve deniz ürünleri					
Süt ve yoğurt					
Kırmızı Et					
Tavuk, hindi veya ördek					
Yumurta					
Mangal					

C10. Kahve ve benzeri kafein içeren içecekleri hangi sıklıkla tüketiyorsunuz?

Nadiren (Günde bir fincandan daha az)	Günde 1 – 2 fincan	Günde 3 - 4 fincan	Günde 4 fincandan daha fazla

C11. Çocuk sahibi olmadan önceki dönemde hangi sıklıkla egzersiz (yürüyüş veya takım sporları dahil) yapardınız?

Sedanter	Ayda bir	Haftada bir	Her gün

D. Aileniz Hakkında

D1a. Ailenizde meme kanseri öyküsü olan kimse var mı?
Hayır
Evet

D1b. Cevabınız evet ise size yakınlık derecesi nedir? …….

D1c. Cevabınız evet ise kaç yaşında meme kanseri gelişmiştir? ……...

D2a. Ailenizde ovaryum kanseri öyküsü olan kimse var mı?
Hayır
Evet

D3b. Cevabınız evet ise size yakınlık derecesi nedir? …….

D2c. Cevabınız evet ise kaç yaşında ovaryum kanseri gelişmiştir? ……...

D3. Bu çalışma için süt örneğinin alındığı Tarih: _____

Saat: _____

D4. Bu süt örneğini vermeden en son kaç saat önce bebeğinizi emzirdiniz? _____

Bu çalışmaya katıldığınız ve zaman ayırdığınız için teşekkür ederiz.

APPENDIX B: Ethical Committee Approval

YEDİTEPE ÜNİVERSİTESİ HASTANESİ

YEDİTEPE ÜNİVERSİTESİ KLİNİK ARAŞTIRMALAR ETİK KURULU KARAR FORMU

	SONUÇ RAPORU	☐
	GÜVENLİLİK BİLDİRİMLERİ	☐
	DİĞER	☐

KARAR BİLGİLERİ	Karar No: 302	Tarih:05.03.2013
	Prof.Dr.Bayram Yılmaz ve Prof.Dr.Ayça Vitrinel sorumluluğunda yapılması tasarlanan ve yukarıda başvuru bilgileri verilen klinik araştırma başvuru dosyası ve ilgili belgeler araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş, gerçekleştirilmesinde etik bir sakınca bulunmadığına toplantıya katılan etik kurulu üyelerinin oy çokluğu ile karar verilmiştir.	

ETİK KURULU BİLGİLERİ

ÇALIŞMA ESASI	Klinik Araştırmalar Hakkında Yönetmelik, İyi Klinik Uygulamaları Kılavuzu, Yeditepe Üniversitesi Tıp Fakültesi, Klinik Araştırmalar Etik Kurulu Kuruluş ve Çalışma Esasları.

ETİK KURUL BAŞKANI UNVANI/ADI/SOYADI: Prof. Dr. R. Serdar ALPAN

ETİK KURULU ÜYELERİ

Unvanı/Adı/Soyadı	Uzmanlık Alanı	Kurumu	Cinsiyet		İlişki *		Katılım **		İmza
Prof. Dr. R. Serdar Alpan	Farmakoloji	YÜTF	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Prof. Dr. M. Reha Cengizlier	Pediatri	YÜTF	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Prof. Dr. Serdar Öztezcan	Biyokimya	YÜTF	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Doç. Dr. Baki Ekçi	Genel Cerrahi	YÜTF	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Prof. Dr. Ferda Özkan	Patoloji	YÜTF	E ☐	K ☒	E ☐	H ☐	E ☐	H ☐	MAZERETLİ
Prof.Dr.Nural Bekiroğlu	Biyoistatistik	MÜTF	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Doç. Dr. Esra Can Say	Diş Has. Ted.	YÜDF	E ☐	K ☒	E ☐	H ☐	E ☐	H ☐	
Doç. Dr. Meriç Köksal	Eczacılık	YÜEF	E ☐	K ☒	E ☐	H ☐	E ☐	H ☐	İZİNLİ
Prof. Dr. Ali Rıza Okur	Hukuk	YÜHF	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Prof. Dr. Başar Atalay	Beyin Cerrahi	YÜTF	E ☒	K ☐	E ☐	H ☐	E ☐	H ☐	MAZERETLİ
Yrd.Doç.Dr.Nesrin Sarıman	Göğüs Hastalıkları	MÜTF	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Yrd.Doç.Dr.Esin Öztürk Işık	Biyomedikal Mühendisi	YÜTF	E ☐	K ☒	E ☐	H ☐	E ☐	H ☐	MAZERETLİ
Bilge Firuzbay	Sivil Üye/Emekli		E ☐	K ☒	E ☐	H ☐	E ☐	H ☐	MAZERETLİ

* : Araştırma ile İlişki
** : Toplantıda Bulunma

Önemli Not: Çalışmanızın Klinik Araştırmalar Etik Kurulu tarafından onaylanan protokole göre yürütülmesi ve çalışma protokolündeki değişiklilerin kurulumuza bildirilmesi gerekmektedir.