# *In vitro* Assessment of Arsenic Immune Toxicity using Human Cord Blood and Murine Bone Marrow Cells

## Dissertation

**zur Erlangung des akademischen Grades**
**des Doktors der Naturwissenschaften**

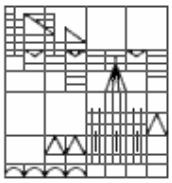
**Eingereicht am Fachbereich Biologie**
**der Universität Konstanz**

**vorgelegt von**

**Daniele Ferrario**

**Tag der mündlichen Prüfung: 21 September, 2009**
**Referent: Prof. Dr. Dr. T. Hartung**
**Referent: Prof. Dr. M. Leist**
**Referent: Prof. Dr. C. Urani**





Universität Konstanz

# Preface

This Ph.D. thesis is submitted for evaluation at the Department of Biology at the University of Konstanz, Germany. The work was carried out between May 2005 and October 2008 at the European Centre for Validation of Alternative Methods (ECVAM), Institute for Health and Consumer protection (IHCP) at the European Commission's Joint Research Centre (JRC) in Ispra, Italy.

My university supervisor for this thesis was Prof. Dr. Dr. Thomas Hartung (Department of Biology, University of Konstanz, Germany), my supervisor at ECVAM, Ispra, Italy, was Dr. Laura Gribaldo, and my former university supervisor was Prof. Dr. Marie Vahter (Department of Environmental Medicine, Karolinska Institute, Stockholm, Sweden).

The Ph.D. thesis focuses on the evaluation of the possible mechanisms of toxicity of arsenic and its metabolites on the progenitor cells of the immune system, comparing the results from both human and murine cells. Moreover, this work evaluates the possible gender differences in the toxic effect of arsenicals, and investigates the likely molecular mechanisms of such differences. *In vitro* and *ex vivo* methods were used to perform these investigations. This thesis consists of a review of the past and on-going available research studies in the field of arsenic and its metabolites immunotoxicity. Moreover, this thesis discusses the possible application of human cord blood cell models for *in vitro* assessment of developmental immunotoxicity including key-results from my own experimental work represented by the following manuscripts:

- **Toxicity of Inorganic Arsenic and its Metabolites on Hematopoietic Progenitors "In Vitro": Comparison between Species and Sexes.** Ferrario et al., *Toxicology* 2008, 249 (2-3): 102-108.
- **Combined in-utero and juvenile exposure of mice to arsenate and atrazine in drinking water modulates genes expression and clonogenicity of myeloid progenitors.** Ferrario et al., *Toxicology Letters* 2008, 180 (1): 59-66.
- **Arsenic induces telomerase expression and maintains telomere length in human cord blood cells.** Ferrario et al., *Toxicology* 2009, 260 (1-3): 132-141.

# Acknowledgements

I would like to express my deepest gratitude and sincerest respect to Prof. Dr. Thomas Hartung (University of Konstanz, Germany – Johns Hopkins Bloomberg School of Public Health, Baltimore, US). He made this study possible not only by giving me invaluable scientific advice and stimulating ideas, but also for taking over the supervision of this PhD thesis.

I want to address special thanks to my daily supervisor Dr. Laura Gribaldo (ECVAM, Ispra, Italy), who already supervised my Diploma thesis, for her enthusiasm, friendship, valuable scientific input, and strong trust and support in my work.

I am most grateful to Prof. Dr. Marie Vahter (Karolinska Institute, Stockholm, Sweden) for her supervision of my activities, and constructive criticism.

Many thanks to all the present and former members of the Haematotoxicity Key-area group at ECVAM for creating an excellent working spirit and a great teamwork. I especially would like to thank Dr. Maria Carfì for many interesting discussions, great lab-company and for introducing me into the Western Blotting technique, Gerard Bowe for his friendship, his excellent technical support with Flow-Cytometry and for his support in submitting my articles and this thesis, Dr. Cristina Croera for her help with the clonogenic assays, and for her ironic attitude to life. Greatest thanks to all my colleagues, which became friends, namely Cristian Pellizzer, Efrat Forti, Patricia Pazos, Erwin van Vliet, Helena Hogberg, Tina Stumann, Riccardo del Torchio, who made it possible to enjoy coming everyday to work. Thanks to Marina Hasiwa for her help with the german translation.

I am forever grateful to my beloved parents, who have always believed in me, and have always encouraged me throughout life. I really miss you.

Life blessed me with the opportunity to have met Dr. Valentina Campi, in which loving memory this thesis is dedicated. This work is just a small tribute to an exceptional colleague and friend. Her determination, sense of joy, and unforgettable smile I will always bring with me.

On the private side I wish to dedicate this thesis to the most important person of my life, my wife Roberta. She have walked by my side both through good and bad times. I doubt completing this work would have been possible without her persistent encouragement and unconditional love.

# List of Publications

**Manuscripts which are part of this thesis:**

**Ferrario, D.,** Gribaldo, L., Hartung, T. (2009). Arsenic Exposure and Immunotoxicity: A Review of the Influence of Age and Gender. Submitted to *Env. Research*.

**Ferrario, D.,** Collotta, A., Carfì, M., Bowe, G., Vahter, M., Hartung, T., Gribaldo, L. 2009. Arsenic induces telomerase expression and maintains telomere length in human cord blood cells. *Toxicology* 260, 132-41

**Ferrario, D.**, Croera, C., Brustio, R., Collotta, A., Bowe, G., Vahter, M., Gribaldo, L. (2008). Toxicity of inorganic arsenic and its metabolites on haematopoietic progenitors "in vitro": comparison between species and sexes. *Toxicology*, 249, 102-8.

Cimino-Reale, G., **Ferrario, D.**, Casati, B., Brustio, R., Diodovich, C., Collotta, A., Vahter, M., Gribaldo, L. (2008). Combined in utero and juvenile exposure of mice to arsenate and atrazine in drinking water modulates gene expression and clonogenicity of myeloid progenitors. *Toxicol Lett*.,180, 59-66.

**Manuscripts relevant for this thesis:**

Croera, C., **Ferrario, D**., Gribaldo, L. (2008). *In vitro* toxicity of naphthalene, 1-naphthol, 2-naphthol and 1,4-naphthoquinone on human CFU-GM from female and male cord blood donors. *Toxicol In Vitro.* 22, 1555-1561

Carfi', M., Croera, C., **Ferrario, D.,** Campi, V., Bowe, G., Pieters, R., Gribaldo, L. (2008). TBTC induces adipocyte differentiation in human bone marrow long term culture. *Toxicology*. 249, 11-8.

**Oral presentations relevant for this thesis:**

Sensitivity of human cord blood cells exposed to tetrachloroethylene: cellular and molecular endpoints. **Ferrario, D**., Diodovich, C., Gribaldo, L. 46$^{th}$ ETC International Meeting on "In Vitro Cytotoxicity Mechanisms", Verona, Italy, March 26-29, 2006.

Toxicity of Inorganic Arsenic on Human Cord Blood Cells *In Vitro*: Comparison between Sexes. **Ferrario, D**., Croera, C., Gribaldo, L. Gene Environmental Interactions, Postgraduate Course, Stockholm 13-17 June, 2006.

**Poster presentations relevant for this thesis:**

Toxicity of Inorganic Arsenic and its Metabolites on Hematopoietic Progenitors *In Vitro*: Comparison between Species and Sexes. **Ferrario, D**., Croera, C., Vahter, M., Gribaldo, L., FISV 2007, 9$^{th}$ Annual Congress, Riva del Garda, 26-29 September, 2007.

*In Vitro* exposure to arsenic of human cord blood and murine bone marrow cells. Comparison between genders and species. **Ferrario, D**., Croera, C., Malerba, I., Gribaldo, L. 43rd Congress of the European Societies of Toxicology, Dubrovnik, Croatia, September 20-24, 2006.

Arsenite Induces Telomerase and Telomere Modulation in Human Cord Blood Cells "*In Vitro*". **Ferrario, D.,** Carfi, M., Vahter, M., Bowe, G., Gribaldo, L. 45th Congress of the European Societies of Toxicology, Rhodes, Greece, 5-8 October, 2008.

**Other presentations:**

Role of Estrogen Receptors in Haematopoietic Stem Cells Development. Campi, V., Ferrario, D., and Gribaldo, L. 44th Congress of the European Societies of Toxicology, Amsterdam, The Netherlands , 7-10 October 2007.

# Abbreviations

| | |
|---|---|
| **[$\alpha^{33}$P]-dATP** | Deoxyadenosine 5"-triphosphate, [alpha-33P] |
| **1301** | Human, Leukemia, Acute Lymphoblastic T Cells |
| **18S rRNA** | 18S ribosomal RNA |
| **ANOVA** | Analysis of Variance |
| **AS3MT** | Arsenic Methyltranferase |
| **BSA** | Bovine Serum Albumine |
| **BSO** | Buthionine sulfoximine |
| **cDNA** | Complementary Deoxyribonucleic Acid |
| **CFU-GM** | Colony Forming Unit Granulocyte-Macrophage |
| **DCF** | 2',7'-di-chlorofluorescein |
| **$DMA^V$** | Dimethylarsinic acid |
| **DMPO** | 5,5-dimethyl-1-pyrroline-N-oxide |
| **DTT** | Dithiothreitol |
| **ER** | Estrogen Receptor |
| **FACS** | Fluorescence Activated Cell Sorter |
| **FBS** | Foetal Bovine Serum |
| **FCS** | Foetal Calf Serum |
| **FITC** | Fluorescein Isothiocyanate |
| **GAPDH** | Glyceraldehyde-3-phosphate dehydrogenase |
| **GM-CSF** | Granulocyte-Macrophage Colony Stimulating Factor |
| **GSH** | Glutathione |
| **GSTO1** | Glutathione S-transferase Omega 1 |
| **hTERT** | Human Telomerase Reverse Transcriptase |
| **$iAS^{III}$** | Inorganic Trivalent Arsenic |
| **$IC_{50}$** | 50% Inhibitory Concentration |
| **IL-3** | Interleukin 3 |
| **IL-6** | Interleukin 6 |
| **IMDM** | Iscove's Modified Dulbecco's Medium |
| **$MMA^{III}$** | Monomethylarsonous acid |
| **$MMA^V$** | Monomethylarsonic acid |
| **mRNA** | Messenger Ribonucleic acid |
| **MTT** | 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide |

| | |
|---|---|
| **PBS** | Phosphate buffered saline |
| **PI** | Propidium Iodide |
| **PNA** | Peptide nucleic acid |
| **PHSC** | Pluripotent haematopoietic stem cells |
| **ROS** | Reactive Oxygen Species |
| **RT PCR** | Reverse Transcriptase PCR |
| **SAM** | Significance Analysis of Microarrays |
| **SCF** | Stem Cells Factor |
| **SD** | Standard Deviation |
| **SDS** | Sodium Dodecyl Sulphate |
| **SEM** | Standard Error of the Mean |
| **UCB** | Umbilical Cord Blood Cells |

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# 1. Introduction

## 1.1. Arsenic Exposure and Immunotoxicity: A Review of the Influence of Age and Gender

Daniele Ferrario, Laura Gribaldo, and Thomas Hartung

European Centre for the Validation of Alternative Methods (ECVAM), T.P 580, IHCP, JRC,

European Commission, via Fermi 2749, 21027 Ispra (VA), Italy

Submitted to Environmental Research

### 1.1.1. Abstract

Increasing evidence suggests that inorganic arsenic, a major environmental pollutant, exerts immunosuppressive effects both in human and animal cells. However, the mechanisms remain unclear and little is known about variation in susceptibility depending on age and gender. The authors performed a review of the experimental and epidemiologic evidence on the association of arsenic exposure and immune diseases. The majority of the studies we reviewed reported that arsenic is a potent immunosuppressive compound. On the other hand, recently few studies have also reported an increase in allergy and autoimmune diseases, suggesting that arsenic may also act as immune system stimulator. However, the results provided limited information due either to the high concentrations of arsenic used in *in vitro* studies or to the extrapolation of animal data to predict human risks. Moreover, although there is emerging evidence that health effects of arsenic are manifested differently between male and female, we found very few studies that have focused on gender differences to the toxic outcomes of arsenic. For this reason, the relationship between gender and arsenic-induced toxicity presented in this review is partially inconclusive. In addition, in the epidemiological studies we reviewed insufficient attention has been directed towards the possibly immunotoxic effects of in utero arsenic exposure during pregnancy. In fact, almost all the studies on the health effects of arsenic were performed on the adult population, usually males, whereas very few studies exist on the potential toxic developmental effects. For this reason, experimental studies using concentrations relevant to human arsenic exposure to evaluate the immune dysfunction effects on developing immune system both in male and female should be a research priority.

***Keywords:*** Arsenic, Immune System, Immunotoxicity, Gender, Developmental Toxicity.

### 1.1.2. Introduction

Inorganic arsenic is a naturally occurring element widely distributed in the earth's crust (NRC, 1999, 2001; WHO, 2001). In the list drafted by the Agency for Toxic Substances and Disease Registry (ATSDR, 2007) of the most hazardous compounds in the environment, arsenic ranks first, and it is also the most frequently found at elevated concentrations in ground water (WHO, 2001; IARC, 2004). According to World Health Organization (WHO), the safe level of arsenic in water is below 10 µg/l, and the approved maximum contaminant level (MLC) is 50 µg/l (Basu et al., 2001; Brown et al., 2002). However, all over the world millions of people are exposed to arsenic above that value (Das et al., 1995). Usually, elevated concentrations of arsenic in groundwater have been found in Bangladesh, Taiwan, and Argentina (Vahter et al., 1995a; Aposhian et al., 2000; Meza et al., 2004). Arsenic compounds can be classified into three major groups: (1) inorganic arsenic compounds; (2) organic arsenic compounds; and (3) arsine gas. Arsenic exists in different valency states. The trivalent arsenic (AsIII) and the pentavalent arsenic (AsV) are widely present in natural waters (Feng et al., 2001) while arsenobetaine (AB) and arsenocholine (AC) are the most common forms in fish and crustaceans (NRC, 1999). Arsenic species can be methylated as monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), and eventually trimethylarsine oxide (TMAO) (Cullen 1979; Gadd et al., 1993) by humans and other mammals. The trivalent arsenicals are usually considered more toxic then the pentavalent ones (Styblo et al., 2000 2002; Vega et al., 2001; Schwerdtle et al., 2003a, 2003b). The major current uses of arsenic are in pesticides, herbicides and wood preservatives. Arsenic is also used as a decolorizing agent in the manufacture of glass (Peters et al., 1996), and as an additive in the production of several alloys to increase heat resistance. Gallium arsenide and indium arsenide have become important semiconductor materials, used in integrated circuits in the electronics industry and space research (Tanaka et al., 2004; IARC, 2006). Although many studies have evaluated the immunological effects of environmental toxic substances such as lead, cadmium and mercury, only a few studies on arsenic have been reported. In this review, we will highlight the toxic effects of arsenic on the immune system that might lead to compromised immune response. Moreover, where possible, we also focus on age and gender differences in health effect of arsenic. We hope it might serve to demonstrate the need for more accurate immunotoxic studies to assess the gender differences in exposure and toxicity of chemicals and environmental pollutants.

### 1.1.3. Arsenic Kinetics and Toxicity

Both human and animal data suggest, that following ingestion, more than 90% of trivalent and pentavalent arsenic is absorbed by the gastrointestinal tract (Bettley and O'Shea, 1975; Vahter and Norin, 1980; Marafante et al., 1981). Both the metabolites, MMA and DMA, are absorbed easily across the gastrointestinal tract (75-85%) (Buchet et al., 1981). After absorption, arsenic is transported by the blood and bound to the sulfhydryl groups (SH) of thiol-containing protein such as glutathione (GSH), and then transported to other parts of the body. In rats, arsenic is accumulated in the red blood cells, where it is bound to hemoglobin (Hisinaga et al., 1982). In humans, within 24 hours, arsenic is found mainly in the liver, kidneys, lungs, spleen, and skin (Bertolero et al., 1981). As(III) tends to accumulate in tissues, but As(V) and organic arsenic are rapidly and almost completely eliminated via the kidneys (Bertolero et al., 1987). Skin, bone, and muscle represent the major storage organs. Other experiments also indicate that the distribution of arsenic is dependent on the valence state of arsenic. Kadowaki (1960) reported an increase in arsenic levels in human fetus as pregnancy progressed. It has been demonstrated that arsenic passes through the placenta in hamsters after intravenous injections of sodium arsenate (Ferm et al., 1977). Moreover, a few years ago Concha and co-workers (Concha et al., 1998) also demonstrated that in a population of Andean women exposed to 200ppb of arsenic in drinking water, almost the same concentrations of arsenic were present either in cord blood or in maternal blood of the exposed population. For this reason, it was postulated that arsenic is able to cross the placental barrier and this early life exposure to arsenic may posing a risk to the normal development of the foetus.

Arsenic is known to be a carcinogen in humans (IARC, 1987; NRC, 1999), in fact it is well documented to cause cancer of the skin, lungs, urinary bladder, kidney and liver (IARC, 2004). Moreover, prolonged arsenic exposure through drinking water is associated with increased non-cancer diseases (cardiovascular diseases, hypertension, pigmentation changes, neurological disorders, and diabetes mellitus) (WHO, 2001). It has been reported that arsenic exposure can cause systemic immunodepression in several animal studies, as well as in humans (Patterson et al., 2004; Soto-Pena et al., 2006). Although there is a substantial amount of information reported on the immunosuppressive effects of arsenic in animal studies, there is a lack of information in humans, and uncertainty remains about the use of non-human data for predicting human risk. Moreover, the health effects of arsenic are usually documented in adults, whereas exposure to arsenic in polluted countries starts at the very beginning of life, and continue for many years, or even throughout life. Little is known about variation in susceptibility depending on age and

gender, thus modifications to adult testing has been suggested in response to the concern that adult exposure may not adequately predict early life exposure (Dietert and Piepenbrink, 2006). Concern over developmental immunotoxicity has increased in the last few years on the assumption that the developing immune system may be more sensitive than the adult one, above all in the response to some immunotoxic chemicals (Luebke et al., 2006). Several articles published on the immunosuppressive effects of arsenic in the last few years have made attempts to close the gap between animal data and human risk. However, investigations on the effects of arsenic at environmentally relevant concentrations on the human immune effector cells would be greatly welcomed in increasing the understanding of the mechanism behind the activity of arsenic on the immune system. Immune suppression with increased immunotoxic outcomes is not the only risk associated with modulation of the immune system. Immune stimulation resulting in enhanced risk of allergic and autoimmune diseases is also a concern. For example a tendency of the increased incidence of some allergies and asthma was observed in an epidemiological study among people exposed to arsenic (Soto-Pena et al., 2006).

Health effects of certain toxic metals are known to be manifested differently between males and female, due to differences in kinetics, arsenic and DNA methylation, susceptibility or mode of action (Loffredo et al., 2003; Vahter et al., 2007;). Nevertheless, gender differences were seldom evaluated in experimental studies. Gender sensitivity in response to arsenic toxicity has been described for human exposure, experimental animals and *in vitro* studies (Vega et al., 2004; Waalkes et al., 2006; Lindberg et al., 2007; Ferrario et al., 2008), probably due to influence of estrogens and sex steroid in the methylation of arsenic. Some studies indicate that men are more affected than women by arsenic-exposure related skin cancers, sometimes occurring at surprisingly low dose exposure and with evidence that the risks were also greater for those who might be malnourished (Tseng et al., 1977; Guha Mazumder et al., 1998; Chen et al., 2003). Rahman et al. (2006) showed that males exposed to arsenic for their lifetime, had twice the risk of obtaining skin lesions compared to females. The mechanism behind these results however is not clear. They postulate hormone interactions with arsenic as already mentioned above, which affects all cell types of importance for skin physiology (e.g. epidermal keratinocytes, dermal fibroblasts, melanocytes) (Thornton 2005). In addition, differences between the sexes in the metabolism of arsenic might have influenced the likelihood of developing skin lesions. Lindberg et al. (2008) showed that the well documented higher risk for men to develop arsenic-related skin lesions compared to women is mainly explained by the less efficient methylation capacity of arsenic, as defined by a higher fraction of MMA and lower fraction of DMA in the urine, among men. Generally, women especially at pregnancy, have better methylation capacity than their men

counterparts, probably due to the effect of estrogens (Waalkes et al., 2008; Agusa et al., 2009; Tseng et al., 2009). Watanabe et al. (2001) also reported males as more affected by skin diseases than females. However the mechanism by which arsenic manifested different toxicity between genders was not clarified. The authors speculated that two other confounding factors, sunlight exposure and smoking, may account for the observed sex-related difference in the dose–response relationship, with males having more severe skin manifestations.

Other studies reported that for arsenic-related kidney, lung and bladder cancers, as well as for diabetes, women might be at a higher risk then men (Wu et al., 1989; Steinmaus et al., 2005; Chiu et al., 2004, 2006; Yang et al., 2005). Moreover, in mice exposed in utero to arsenic (42-85 mg/L), marked sex related differences have been demonstrated (Waalkes et al., 2003). Female mice showed ovarian and lung tumors, while males showed higher incidence of liver and adrenal tumors. Although in the last few years more studies have been performed on gender-differences in response to arsenic, only increased susceptibility to arsenic-induced cancer has been evaluated, whereas studies on the toxicity of arsenic on the immune system are still lacking.

### 1.1.4. Hematopoietic And Immune System

All the cellular element of the blood, including the red blood cells and the white cells of the immune system, derive from the same progenitor, the hematopoietic stem cells in the bone marrow. As these stem cells can give rise to all types of blood cells, they are often known as pluripotent hematopoietic stem cells. Hematopoiesis is the process by which pluripotent hematopoietic stem cells (PHSCs) differentiate into many highly specialized circulating blood cells (Ogawa et al., 1993; Morrison et al., 1997). The long term PHSCs are capable of self-renewal as well as limited differentiation toward the lymphoid stem cells or myeloid multipotent stem cells. The myeloid progenitor is the precursor of granulocytes and macrophages of the immune system. The lymphoid progenitors give rise to two major types of lymphocytes known as B or T cells. The hematopoietic tissue has the capacity to respond quickly to an increased demand for mature cells as a response to an external stimulus (for example during infection or inflammation). Pluripotent stem cells and mature cells circulate in the blood stream where they are usually more exposed to xenobiotics than any other internal cell type. For this reason the hematopoietic and immune systems represent particularly sensitive xenobiotic targets. Xenobiotics exposure can lead to cytotoxic effects on cell function and commitment either directly or in concert with immune mechanisms. Xenobiotics may also interfere with complex regulation pathways that regulate differentiation and proliferation (Pessina et al., 2005). For this reason in the last few years increased concern over hematotoxicity and immunotoxicity has been

raised at least for some well characterized immunotoxic compounds (Luebke et al., 2006). Moreover, it is now widely accepted that the developing immune system represents a particularly sensitive xenobiotic target (Holladay and Smialowicz 2000; Dietert et al., 2000, 2002, 2005; Luster et al., 2005; Luebke et al., 2006). Several factors may account for this increased susceptibility such as functional immaturity of the immune system and the capacity of the xenobiotic to affect the developing immune system at lower doses than in adults (Heo et al., 1996; Snyder et al., 2000; Chen et al., 2004). In humans the effects of immunotoxicant exposure during development may be expressed immediately or later in life, increasing the severity of allergic and immune-related diseases (Holladay and Smith, 1994; Luster et al., 2003). Currently laboratory animals are widely used to predict hematotoxicity (Boorman et al., 1982). The prediction of hematotoxicity is based on clinical hematological parameters, such as peripheral blood cell counts and bone marrow cytology that are monitored in animal studies and then used to predict human hematotoxic effects. However, in recent years the use of alternative methods to animal models has increased, and *in vitro* techniques are now accepted as good options for pharmaco-toxicology (Gad et al., 1990).

Different agents like viruses, drugs or chemicals can interfere with the activity or the viability of the immune system cells. Based on the available clinical experience, immunotoxic effects are often divided into four categories: immunosuppression, immunostimulation, hypersensitivity, and autoimmunity. Each category is associated with relatively specific and clinically distinct adverse events (Descotes, 2004a).

Immunosuppression is the consequence of an inhibition of the host's immune response. Two major types of clinical adverse effects have been identified in relation to immunosuppression: the impaired resistance against microbial pathogens and the development of malignancies, both of which are associated with microbial infections. The possible occurrence of infectious diseases has not been extensively studied in humans exposed to occupational or environmental chemicals that are immunosuppressive in animals.

Immunostimulation usually is manifested as flu-like reactions, increased incidence of autoimmune diseases, increased incidence of hypersensitivity reactions to varied allergens (Vial and Descotes, 1995; Vial et al., 2002).

Hypersensitivity diseases usually reflect normal immune mechanisms directed to innocuous antigens. Hypersensitivity reactions are the most frequently reported immunotoxic effects of drugs and other chemicals in human beings.

Autoimmune disease occurs when a specific adaptive immune response is mounted against self antigens. Immunity causes chronic inflammatory injury to tissues that might also be lethal.

Although autoimmune diseases are relatively common in the general population, only a few epidemiological studies have identified drugs or chemicals as a possible cause of autoimmune disease.

### 1.1.5. Chronic Effects of Arsenic In Humans and Immune System Toxicity

When humans are chronically exposed to arsenic through their drinking water, they exhibit increased rates of several cancers, such as bladder, liver and kidney cancers and cancers of other internal organs (Kitchin et al., 2001; Abernathy et al., 2003; IARC, 2004). Tseng et al. (1977) demonstrated a clear dose-response relationship between Blackfoot disease and skin cancer in people exposed to arsenic in drinking water in Taiwan. Invasive in situ cell carcinomas (Bowen's disease, BD) are known to be associated with chronic arsenic exposure (ATDSR, 1990). There is evidence from epidemiological studies that early-life exposure to arsenic increases the health risks later in life. In fact, Smith and co-workers (2006), demonstrated that in utero exposure to arsenic has pulmonary effects, increasing the mortality in young adults from lung disease. Hematopoietic depression, and liver damage were also observed (Webb et al., 1966). Long-term exposure to inorganic arsenic, through drinking water, medication, or in occupational situations, has resulted in disturbances of the hematopoietic system (Kyle and Pease, 1965; Westhoff et al., 1975; Feussner et al., 1979): the blood picture in these situations often resembles that in acute intoxications. Bone marrow examination shows disturbed erythropoiesis, and occasionally megaloblastic changes. Severe granulocytopenia may also be present, with possible effects on resistance to bacterial infections. Harrison and McCoy (2001) suggested that apoptosis might be an important mechanism of arsenic-induce immunosuppression, whereas Frenkel et al. (2002) reported that arsenic impairs the immune system. Biswas et al. (2008) demonstrated that arsenic in exposed individuals can cause immunosuppression through a significant decrease in the T-cell proliferation, due to a reduced level of secreted cytokines by the T cells (TNF-alpha, IFN-gamma, IL2, IL10, IL5, and IL4). In a recent study (Liao et al., 2009 ) it has been demonstrated that people affected with BD showed both cutaneous and systemic immune dysfunctions probably due to the decreased expression of CD4 + cells, that are important factors for the recognition of antigens on the surface of a virus infected cell. In a pilot study Raqib et al. (2009) evaluated the impact of in utero arsenic exposure on child immunity in Bangladesh. The observations suggested that in utero exposure to arsenic caused acute respiratory effects in male children, and impaired child thymic development, possibly due to immunosuppression.

In clinical investigations, it has been demonstrated that arsenic may also impact adversely on the immune system, which may later predispose to abnormal inflammatory-like immunotoxicity in

humans (Hall et al., 2002; Soto-Pena et al., 2006). In fact, it has been reported in several epidemiological study that arsenic exposure increased the incidence of autoimmune-mediated diseases, such as diabetes mellitus (Tseng et al., 2004), cardiovascular disease mediated by arsenic-induced vascular inflammation by increased expression of Tumor Necrosis Factor-α (TNFα) and Interleukin-8 (IL-8) (Wu et al., 2003). Various severe inflammatory clinical observations such as hypertrophy of the liver and/or the spleen, were found at rates of over 70% in chronic arsenic poisoning patients (Guha-Mazumder et al., 1995). Inhibition of lymphocyte proliferation has been reported in adults exposed to arsenic-contaminated drinking water (412µg/l) (Gonsebatt et al., 1994) and in children (Soto-Pena et al., 2006). Study of gene expression in samples taken from humans exposed to arsenic in arseniasis-endemic areas in Taiwan indicate an over expression of inflammatory molecules such as cytokines or growth factors (Wu et al., 2003). Genomic DNA methylation is also a proposed mechanism of arsenic toxicity, as observed by a study of Pilsner et al. (2009), which indicates that hypomethylation of leukocyte DNA is associated with increased risk for inflammatory skin lesions. Arsenic has also been shown to cause anemia (Parish et al., 1979; ATSDR, 2000) caused by the disruption of normal regulatory mechanism exerted by macrophages and T-cells (Sathe et al., 1990; Gascon et al., 1993). Moreover, a study in patients suffering from Black Foot Disease (Lin and Yang, 1988) as a result of chronic arsenic consumption (Tseng et al., 1977, 1989; Chen et al., 1988b) revealed that arsenic was able to decrease the concentration of zinc and selenium. The deficiency of these two micronutrients has also been associated with alterations of the immune system (McMurray et al., 1990; Vega et al., 1999; Frenkel et al., 2002). A study in Mexico (Rosales-Castillo et al., 2004) assessed a relationship between chronic arsenic exposure, human papilloma virus (HPV) and nonmelanoma skin cancer (NMSC), and concluded that arsenic might cause suppression in the cell-mediated immune functions, enhancing the susceptibility to viral infection.

### 1.1.6. Immune System Toxicity In Animal Studies

Arsenic has been shown to be embryotoxic and teratogenic in animals, acting through foetal growth retardation and neurotoxicity (Wang et al., 2006). Results of animal experiments show effects on the haematopoietic system similar to those observed in man. A decrease in haematocrit and in haemoglobin has been observed in female rats exposed to arsenite in their feed (250 mg As/kg diet) for 2 years (Byron et al., 1967) and in rats given sodium arsenate in their feed (50 mg As/kg diet) for 10 weeks (Mahaffey & Fowler, 1977). The same effects were observed in cats given arsenite or arsenate in the diet in doses of 1.5 mg As/kg body weight (Massmann and

Opitz, 1954). Immunotoxic effects of arsenic exposure have been shown in animal models as well. Blakely et al. (1980) reported that arsenite at the concentration of 0.5-10 ppm in drinking water produced immunosuppressive effects in mice. Inhalation of arsenic trioxide by mice caused injury to alveolar macrophages (Aranyi et al., 1985). B6C3F1 female mice exposed to a single dose of gallium arsenide (GaAs 200mg/kg) suppressed the IgM and IgG antibody-forming cells response, inhibited T-cell proliferation, macrophage activity (Sikorski et al., 1989; Burns and Munson, 1993; Patterson et al., 2004), and altered macrophage function decreasing adhesion, migration, and phagocytic properties (Lewis et al., 1998; Bishayi et al., 2003). CD57BL6N male mice exposed to sodium arsenite at environmentally relevant concentrations for 30 days (from 1 to 0.01 mg/kg/day) showed that arsenic interfered with the activation of T-cell, affecting the pathway of T-cell receptor activation (Soto-Pena et al., 2008). States et al. (2009) in a Microarray studies of liver mRNA in mice exposed in utero suggested that a permanent state of stress is induced by arsenic exposure. Furthermore, the livers of the arsenic-exposed mice have activated pathways involved in immune responses suggesting a pro-inflammatory condition. Other studies demonstrated that arsenic is immunosuppressive and that it enhances susceptibility to infections, inhibiting the activity of macrophages (Lantz et al., 1994; Dai et al., 1999). Moreover, acute pulmonary inflammation was observed after a single exposure to GaAs (100mg/kg) in rats (Webb et al., 1986). Aberrant expression of inflammatory molecules, such as granulocyte-macrophage colony-stimulating factor, or tumor necrosis factor-α, after exposure to arsenic was noted both in *in vivo* and *in vitro* study (Germolec et al., 1997; Chen et al., 2001; Yih et al., 2002). On the contrary, in a previous study we demonstrated that mice exposed to arsenic at the concentration of 1mg/l did not modulate the number of bone marrow granulocyte-macrophage progenitor cells (Cimino Reale et al., 2008). Nevertheless, a gender dimorphism in response to arsenic toxicity has been observed in the mRNA expression of cell adhesion, cell cycle, and intra-cellular modulator genes, with females being more sensitive to this over-expression.

### 1.1.7. *In Vitro* Experimental Studies

Although there is a substantial amount of studies of arsenic immunotoxicity in animals, *in vitro* models based on human cells are still lacking. Yoshida et al. (1987) reported immunological effects of arsenic compounds on mouse spleen cells *in vitro*. In fact, arsenic exerted cytotoxicity against precursors of suppressor T-cells. Gonsebatt et al. (1992) investigated the effect of arsenic on human lymphocyte stimulation and proliferation using concentrations of arsenic similar to those found in blood. When human lymphocytes collected from healthy donors (two men, two

women) were exposed to arsenite and arsenate (10–7, 10–8 and 10–9 M) a dose-related inhibition of proliferation was observed. The results show that, at the concentrations tested, arsenite and arsenate impaired lymphocyte stimulation and proliferation and confirm that chronic exposure to arsenic can affect the proliferation of whole-blood lymphocytes. Meng et al. (1994) reported that low concentrations (from 1μM to 10 μM) of inorganic arsenicals, arsenite and arsenate increased the DNA synthesis of human peripheral blood lymphocyte. Recently, some authors have demonstrated that arsenic can act differently producing either inhibition or induction of proliferative responses in human cells depending upon the concentration tested. In fact, micromolar concentrations of inorganic arsenic *in vitro* inhibit machrophagic differentiation of human blood-derived monocytes (Sakurai et al., 2005, 2006), and inhibit the proliferative response of lymphocytes (Gonsebatt et al., 1994; Vega et al., 1999; Meng and Meng, 2000; Galicia et al., 2003; Vega et al., 2004) and machrophages (Sakurai et al., 2005). Lemarie et al. (2006) demonstrated that arsenic at 1μM had a double effect on immune system both blocking differentiation of human monocytes into macrophages and impairing endocytosis and phagocytosis of macrophages. Arsenic was shown to decrease the adhesion molecules and differentiation of monocytes and macrophages (Lemarie et al., 2006). Bourdonnay et al. 2009 demonstrated that micro-molar environmentally relevant concentrations of arsenic exerted immunosuppressive effects by impairing the expression of macrophage-specific genes, that are essential for the correct differentiation program of human macrophages. Vernhet et al. 2009 showed that low concentrations of arsenic (0.1-5 microM) inhibited *in vitro* proliferation of CD34+ stem cells and their differentiation into various hematological cell lineages. They concluded that arsenic exposure can induce suppression of human hematopoiesis by decreasing survival of CD34+ progenitor cells. Very low arsenic concentrations (nM range) can induce lymphocytes proliferation (Vega et al., 1999; Meng et al., 2000). Exposure of human lymphocytes *in vitro* to arsenic has also been shown to inhibit secretion of Interleukin 2 (IL-2), fundamental for immune development (Vega et al., 1999; Galicia et al., 2003), as well as modulation of the basal proliferative response of lymphocytes (Cooper et al., 2007; Wetzler et al., 2006). Arsenic inhibited murine enzymatic activity of lysosomal protease cathepsin L, an enzyme that plays an important role in antigen processing for stimulating T cell response to inflammation (Harrison et al., 2001). Arsenic suppressed the immune response in mice spleen cells *in vitro* by causing exposed cells to undergo apoptosis (Yoshida et al., 1986). In our previous study *in vitro* we observed that arsenic at the concentration of 1μM decreased the production and proliferation of CFU-GM colonies both in male and female human cord blood cells and in murine bone marrow cells (Ferrario et al., 2008). These results indicate that the

pathway of arsenic toxicity is conserved in both species and in both genders. In the same experiment, we observed that at the concentration of 0.0001 μM arsenic was able to increase the proliferation of CFU-GM colonies only in female cells, suggesting that low concentrations stimulation is probably gender related. Other gender differences in response to arsenic toxicity have also been observed both in exposed population, and in experimental animals (Vega et al., 2004; Waalkes et al. 2006; Lindberg et al., 2007). We also observed that arsenic at 1μM was able to decrease the telomerase mRNA and protein expression and telomere length enhancing the apoptotic pathway in a ROS dependant manner in human cord blood cells (Ferrario et al., 2009). The results observed were the same for both male and female. On the contrary, the concentration of 0.0001 μM increased the expression of telomerase, with maintained telomere length only in female progenitors. These results confirmed that hematopoietic and immune cells are sensitive targets for arsenic toxicity; moreover, health effects gender differences of arsenic do exist, and they are probably related to the concentration of arsenic used, as well as to the possible interaction between arsenic and sex hormones and estrogen receptors.

### 1.1.8. Conclusions

The concentrations of harmful chemicals in the environment are generally below the levels that produce direct toxic effects. However, the sensitivity of the haematopoietic and immune system to even very low concentrations of chemicals, make it an appealing system to study the effects of chemical toxicity (Selgrade et al., 2007). In principle, the exposure to an immunotoxic compound may result either in an enhancement of the immune response that may lead to allergy or autoimmunity, or into immunosuppression that may increase cancer susceptibility, and risk of infections (IPCS, 1996). The evidence from the present review is consistent with a role of inorganic arsenic exposure and immunotoxicity. However, the mechanisms of immune suppression are not yet clear, even if findings suggest that methylation, apoptosis and generation of oxygen species could be major mechanisms of arsenic-induced immunosuppression. On the other hand, recently some studies have reported an increase in allergy and autoimmune diseases after exposure to arsenic (Tseng et al., 2004; Soto-Pena et al., 2006), suggesting that arsenic may also act as a pro-allergenic compound. For this reason, as the pattern of arsenic toxicity is complex, much more needs to be done in order to better understand the role of arsenic as both an immunosuppressive and as an immuno-stimulating compound. In the last few years the increase of other diseases attributed to an alteration in the immune system were observed in arsenic-exposed populations, such as cardiovascular disease (Engel et al., 1994; Simeonova et al., 2004) caused by over expression of Tumor Necrosis Factor-α and Interleukin-8, and diabetes mellitus

in childhood and adolescence caused by autoimmune destruction of pancreatic β-cells (American Diabetes Association, 2004). Despite the relatively small number of subjects, Soto-Pena and co-workers (2006) were able to detect a tendency in the increase of the incidence of immune-related conditions (asthma, allergies, and parasitic infections) among individuals with arsenic values higher than 50 μg/l in their urine.

For this reason, it is likely that chronic exposure to arsenic may increase the incidence of those autoimmune diseases as a result of impairing the normal function of the immune system that may have been predisposed to viral or bacterial infections.

The hypothesis is put forward that several of those diseases resulting from alteration of the immunological surveillance have not yet been directly attributed to arsenic toxicity. Thus, an exploration into this area is appropriate. Recently, an alternative mechanism of action of arsenic's role in tumorigenesis has been suggested; this mechanism suggests that arsenic may induce damage to immune cells, which impairs their ability to respond to transformed cells, as well as to chronic and opportunistic pathogens (Andres et al., 2005; Wiger et al., 2005). Although none of these effects have been clearly demonstrated, it has been reported that arsenic exposure increases the incidence of autoimmune-mediated diseases, such as diabetes mellitus (Tseng et al., 2004), as well as other immunosuppressive diseases, such as the presence of skin cancer similar to that induced in immunosuppressed populations as a result of organ transplantation or HIV infection.

Nevertheless, these observations have been questioned in recent reviews. The authors reported limitations in the epidemiologic literature on arsenic exposure of both diabetes and cardiovascular outcomes, and revealed that the association of arsenic exposure with diabetes and cardiovascular diseases were inconclusive because of limitations in the epidemiological literature that added uncertainty (Navas-Acien et al., 2005, 2006). For these reasons the authors stressed that more accurate studies using relevant arsenic concentrations to assess the possible association between arsenic and autoimmune diseases should be a research priority.

Although there is increasing evidence that health effects of arsenic are manifested differently between male and female (Guha Mazumder et al., 1998; Watanabe et al., 2001; Chen et al., 2003; Waalkes et al., 2003; Shen et al., 2006; Rahman et al., 2006; Vahter et al., 2007), very few studies have focused on gender differences to the toxic response of arsenic. Thus, possible mechanisms related to sex hormone interaction, were not detected. The evidence of the relationship between gender and arsenic-induced toxicity presented in this review is partially inconclusive. On the one hand the evidence suggests that gender differences in response to arsenic induced-toxicity do exist and on the other hand few gender-related studies exist. For most

of the epidemiological studies on arsenic, the health risk assessment have been based on data from occupationally exposed men, and the results used as being representative of the general population, including children (Vahter et al., 2007). These studies were also limited to show differences in the population, without addressing the mechanisms behind these differences. Most studies suggested that women have a better methylation capacity than men (Chung et al., 2008; Huang et al., 2008; Lindberg et al., 2008a). This gender difference in arsenic methylation capacity could probably be partially explained by the effect of estrogens, and has been proposed as a possible mechanism of gender differences in arsenic toxicity outcomes. Inorganic arsenic is metabolized in the body and the end products methylarsonic acid (MMA) and dimethylarsinic acid (DMA) are readily excreted in urine. Intermediate reduced forms of the methylated metabolites, MMA(III) and DMA(III) are highly toxic, and may be responsible for arsenic toxicity (Schwerdtle et al., 2003). The methylation of arsenic is well known to be influenced by gender and age (Vahter et al., 2002; Loffredo et al., 2003; Waaalkes et al., 2008). Recently, it has been demonstrated that women during childbearing years are more efficient at As methylation than men (Lindberg et al. 2007; Agusa et al., 2009), particularly during pregnancy. This is likely due to the de novo synthesis of choline by the phosphatidylethanolamine methyltransferase (PEMT) pathway (Vahter 2007), which can probably be up-regulated by estrogens. However, some confounding effects in epidemiological studies still exist (age, occupational co-exposure, diet, women usually do not smoke and tend to drink less water than men), or other not yet identified factors cannot be completely excluded for gender differences in the methylation of arsenic. For this reason we propose that gender-related animal studies might decrease these factors, and might better reflect the toxic effects of arsenic alone. However, considering the differences between the species these results should be then properly extrapolated to humans. Clarification of the basis of gender-related differences in response to arsenic toxicity should be a research priority to better understand the mechanism of arsenic toxicity and to take countermeasures for prevention and treatments.

In spite of the large number of people being exposed to arsenic and the numerous studies on the health effects of arsenic on the adult population, generally, the epidemiological studies reviewed gave insufficient attention toward the risk of arsenic-induced immune dysfunction in utero. This information is essential, since exposure in polluted regions usually starts very early in life (or even during gestation) and continues throughout life. Even if studies are few, significant effects on the developing immune system have been described (Price et al., 1976; Tendron et al., 2002). Evidence indicates that fetal chemical exposure in utero could affect the development of human diseases during adulthood (Luster et al., 2008), since transplacental xenobiotics can negatively

interact with fetal immune stem cells maturation (Holladay et al., 1999). Although the mechanism by which arsenic induces adverse developmental health effects has not clearly elucidated, several pathways of toxicity have been suggested, such as inhibition of DNA repair, alterations in DNA methylation and other epigenetic mechanisms (Liu and Waalkes, 2008). Methyl groups from S-adenosylmethionine (SAM) are essential to both arsenic and DNA methylation. DNA methylation status is a well-defined controlling factor in gene expression. Moreover, alterations in DNA methylation is an epigenetic mechanism related with carcinogenesis in various systems (Waalkes et al., 2004; Pilsner et al., 2007). DNA methylation is essential for normal development and function of the immune system (Strickland and Richardson, 2008). A failure to maintain epigenetic homeostasis in the immune response leads to aberrant gene expression, contributing to immune dysfunction and in some cases to the development of immune diseases.

Although there is a substantial amount of proof that arsenic can negatively interfere with immune system development, in the last few years integration of parameters that address the immune system in developmental toxicology studies has been poorly investigated, and immune organs are still not routinely included as potential sensitive organs in most developmental toxicity protocols (Luebke et al., 2006). Therefore, we propose that use of *in vitro* methods employing human umbilical cord blood cells taken from both sexes might better reflect the possible mechanism behind arsenic immunosuppression and immunotoxicity to very early life exposure.

Taken together, there is emerging evidence of immune alteration caused by relevant exposure to arsenic from *in vitro*, *in vivo* and human studies. Preliminary data suggest gender differences especially for low arsenic exposures. Despite several proposed mechanisms it is not clear how arsenic exerts these effects.

# 2. Aims of the Study

*In vitro* clonogenic assays have been developed and widely used, for many years, to investigate the proliferation and the differentiation of pluripotent haematopoietic stem cells (PHSC), and of the different progenitors of blood cell lineages, such as granulocytes-macrophages (CFU-GM), and erythrocytes (BFU-E/CFU-E). The use of these techniques has increased rapidly, and appears to be very useful in the investigation of pathogenic mechanisms of drug induced blood disorders, and also for screening compounds during preclinical safety studies. Moreover, the clonogenic assays give the possibility to establish humanised *in vitro* tests, which may reduce problems of interspecies differences in safety evaluations and may better predict human chemical and drug hazards. The use of laboratory animals may also be reduced by the use of *in vitro* clonogenic tools.

The work presented in this Ph.D thesis aims to evaluate the possible mechanisms behind the toxicity of arsenic and its metabolites on hematopoietic and immune progenitor cells, such as human cord blood or murine bone marrow cells. Moreover, a comparison between the toxicity of arsenic on male and female donors was also evaluated.

The specific aims of the experimental work were carried out in three parts:

1. **To investigate the toxicity of inorganic arsenic and its metabolites on haematopoietic progenitors *in vitro* in two sexes and two species, human and mouse**.

    - To investigate the possible adverse effects of arsenic and dimethylarsinic acid ($DMA^V$), monomethylarsonic acid ($MMA^V$) and monomethylarsonous acid ($MMA^{III}$) on the progenitors of the blood forming system (CFU-GM).
    - To compare the results obtained on the toxicity of progenitors between genders and species.
    - To provide proof that the validated *in vitro* CFU-GM assays may be of help when applied to the field of toxicological evaluation of environmental pollutants.

2. **To investigate the effects of combined *in-utero* and juvenile exposure of mice to arsenate and atrazine in drinking water on the clonogenicity of myeloid progenitors.**
    - To explore the potential trans-placental effects of arsenic on offspring developmental immune progenitor cells.

- To investigate possible differences in health effects both on cellular and molecular endpoints between the two sexes.
- To investigate the effects of co-exposure on immune cells development.

3. **To further explore the possible arsenic toxicity mechanism on cell viability, telomerase expression, telomere length, and reactive oxygen species production in cord blood cells.**
    - To investigate the possible mechanisms of arsenic immune toxicity and immune stimulation on cord blood cells.
    - To assess whether the previously observed biphasic, concentration-dependent mechanism of arsenic toxicity, could be detected in early phases at the molecular level.
    - To compare molecular endpoints of arsenic activity between genders.

# 3. Manuscripts

# 3A. Toxicity of Inorganic Arsenic and its Metabolites on Haematopoietic Progenitors "*in vitro*": comparison between Species and Sexes.

Daniele Ferrario, Cristina Croera, Roberta Brustio, Angelo Collotta, Gerard Bowe, Marie Vahter and Laura Gribaldo

European Centre for the Validation of Alternative Methods (ECVAM), T.P 580, IHCP, JRC,

European Commission, via Fermi 2749, 21027 Ispra (VA), Italy

Toxicology 2008, 249 (2-3): 102-108

## 3A.1 Abstract

Inorganic arsenic (iAs) and its metabolites are transferred to the foetus through the placental barrier and this exposure can compromise the normal development of the unborn. For this reason, we assessed the toxicity of sodium arsenite ($iAs^{III}$) and its metabolites Dimethylarsinic Acid ($DMA^{V}$), Monomethylarsonic Acid ($MMA^{V}$) and Monomethylarsonous Acid ($MMA^{III}$) on human haematopoietic cord blood cells and murine bone marrow progenitors *in vitro*, looking at the effects induced at different concentrations in the two genders. The expression of two enzymes responsible for arsenic biotransformation Arsenic Methyltranferase (AS3MT) and Glutathione S-transferase omega 1 (GSTO1) was evaluated in human cord blood cells. Cord blood and bone marrow cells were exposed *in vitro* to $iAs^{III}$ at a wide range of concentrations: from 0.0001 μM to 10 μM. The methylated arsenic metabolites were tested only on human cord blood cells at concentrations ranging from 0.00064 μM to 50 μM. The results showed that $iAs^{III}$ was toxic on male and female colony forming units to about the same extent both in human and in mouse. Surprisingly, very low concentrations of $iAs^{III}$ increased the proliferation rate of both human and murine female cells, while male cells showed no significant modulation. $MMA^{V}$ and $DMA^{V}$ did not exert detectable toxicity on the cord blood cells, while $MMA^{III}$ had a marked toxic effect both in male and female human progenitors. AS3MT mRNA expression was not

induced in human cord blood cells after $iAs^{III}$ exposure. GSTO1 expression decreased after $MMA^{III}$ treatment. This study provides evidence that exposure to $iAs^{III}$ and $MMA^{III}$ at μM concentrations is associated with immunosuppression *in vitro*.

***Key Words:*** CFU-GM, Arsenic, Monomethylarsonic acid, Monomethylarsonous acid, Dimethylarsinic acid, AS3MT, GSTO1.

**3A.2 Introduction**

Millions of people world-wide are chronically exposed to arsenic, often due to naturally occurring arsenic in groundwater (WHO 2001; Watanabe et al., 2001; Bhattacharyya et al., 2003). Arsenic occurs in drinking-water primarily as inorganic arsenic (iAs). Levels of arsenic in affected areas may range from tens to hundreds or even thousands of μg/L, whereas in unaffected areas levels are typically below a few μg/L (IARC 2004).

The World Health Organization (WHO) guideline value for arsenic in drinking-water is 10 μg /L (WHO 2004), nevertheless that concentration is associated with an appreciable risk of cancer (NRC 2001). Chronic exposure to inorganic arsenic may give rise to cancer of the skin, lung, bladder, kidney (IARC 2004; Chen et al. 2003; Rey et al. 2004) as well as increased risk to develop lymphoma and myelogenous leukaemia (Chen, et al., 2003; Hall, et al., 2002 Kjeldsberg and Ward 1972; ATSDR,1990; Luh, et al.,1973) and immunosuppression (Sakurai et al., 2006; Hall et al., 2002), since human macrophages, lymphocytes and monocytes seem to constitute a sensitive target of iAs exposure, (Lemarie et al., 2006; Soto-Pena et al., 2006; Sakurai et al., 2006). Arsenic is able to cross the placental barrier. Concha et al., 1998 have previously demonstrated that the concentration of arsenic in cord blood can be as high as in the blood of the exposed women. Thus, the exposure to arsenic may start very early in life, which poses a risk for impaired foetal development (DeSesso et al., 1998). Indeed, consumption of well water with arsenic at the concentration < 10μg /L during pregnancy increased foetal loss and infant death, mainly due to infectious diseases, possibly indicating an effect of prenatal arsenic exposure on the immune function (Kapaj et al., 2006; Rahman et al., 2007).

Following ingestion, iAs undergoes biotranformation to mono and dimethylated metabolites ($MMA^{III-V}$, $DMA^{III-V)}$, which are excreted in urine, mostly as dimethylarsinic acis ($DMA^{V}$) (Vahter et al., 2002). The dimethylated metabolites were found to be the main forms of arsenic in blood of newborns whose mothers consumed water contaminated with arsenic (Devesa et al., 2006; Concha et al., 1998). Biomethylathion of inAs has been thought to decrease arsenic toxicity (Sax and Lewis, 1989), however this interpretation has been later questioned (Yamanaka

et al., 1997). Nowadays, trivalent arsenic forms, rather than the pentavalent ones, are associated with increased toxicity of iAs (Styblo et al., 2000; Thomas et al., 2007). Moreover trivalent arsenicals are more reactive than pentavalent, and bind with high affinity to thiol groups present in protein and glutathione (GSH) (Vahter and Marafante 1983; Suzuki et al., 2004).

The haematopoietic system is mainly committed to give rise to all blood cells type, including myeloid and lymphoid cells. With its rapid cell renewal, haematopoietic tissue is one of the most sensitive targets to environmental toxicants (Gribaldo et al., 1999). Since recent review reports have stressed that early-life exposure to xenobiotics poses a great risk for the immune system (Dietert et al., 2002, 2006; Holladay 1999; Holladay and Smialowicz 2000; Luebke et al 2006), we investigated the potential immunotoxic effects of $iAs^{III}$ and its methylated metabolites on human granulocyte-macrophage progenitors derived either from human cord blood cells or murine bone marrow cells. As there is increasing evidence for gender differences in the metabolism and toxicity of arsenic (Vahter et al., 2006; Lindberg, Kumar et al. 2007; Waalkes et al., 2007), we also evaluated the sensitivity to this metal in female and male donors from different species.

The biotrasformation of iAs in humans involves a series of reduction and methylation reactions. Two conceptual models for iAs methylation have been proposed (Cullen et al., 1984; Hayakawa et al., 2005). However, in both methods the methylation of iAs is enzymatically catalyzed by Arsenic (+3 oxidation state) methyltransferase (AS3MT). In fact there is strong evidence that AS3MT catalyzes the transfer of a methyl group using *S*-adenosyl-L-methionine (Ado Met) as the methyl donor to trivalent arsenicals producing methylated and dimethylated arsenicals (Marafante and Vahter 1984; Lin et al., 2002 Thomas et al., 2007). AS3MT is the only methyltransferase identified in humans (Lin et al., 2002).

The reduction reactions seem to occur already in the blood cells, using thiols as electron donors (Marafante et al., 1985; Vahter and Envall 1983). So far only one reductase has been identified in humans called Glutathione S-transferase omega 1 (GSTO1) (Zakharian et al., 2001).

For this reason the presence AS3MT and GSTO1 were both investigated to assess whether cord blood cells are capable of arsenic biomethylation and reduction "*in vitro"*.

## 3A.3. Materials and Methods

### *3A.3.1. Chemicals*

Trivalent inorganic arsenic (sodium (meta) arsenite [NaAso2], MW 129.91; abbreviated as $iAs^{III}$) and dimethylarsinic acid ($DMA^{V}$- $(CH_3)_2AsO(OH)$, MW 138) were obtained from Sigma-

Aldrich (Sigma, USA), and monomethylarsonic acid ($MMA^V$- $(CH_3)AsO(OH)_2$, MW 139.97) from Tri Chemical (Japan). Monomethylarsonous acid ($MMA^{III}$- $(CH_3)As(OH)_2$ MW 126) was kindly supplied by Dr. M. Styblo. The purity of $MMA^{III}$ was > 95%.

The chemicals were dissolved in bi-distilled water to a final concentration of $10^{-2}$M, and these stock solutions were stored at -20°C until required.

### *3A.3.2. Source of human progenitor cells*

Human umbilical cord blood cells (UCB) were used as source of progenitor cells supplied frozen by Biopredic International (France), according to a protocol approved by the Institutional Review Board (IRB). The cryotubes were stored in liquid nitrogen. Three different donors for each gender were used for three independent experiments, each performed in triplicate. Immediately before use, the cells were quickly thawed at 37° C in a water-bath, swirling gently for 1-2 minutes. After wiping the outside of the vial with 70% ethyl alcohol on an absorbent paper, the cell suspension was transferred, drop by drop, to 10 mL of IMDM medium + Glutammax (Gibco, Italy) containing 10% FBS (Gibco, Italy). It was then centrifuged at 300 g at room temperature for 10 minutes. The supernatant was removed and the cells gently resuspended in IMDM with 30% Foetal Calf Serum (FCS) and counted using Trypan blue to assess the cell viability that was usually 95% or greater. Cell suspension was adjusted to achieve the viable cell density required: 5 x $10^5$ cells/mL.

### *3A.3.3. Isolation of murine bone marrow cells*

Three-week old CD-1 SPF/VAF mice, weighing 14-16g, were purchased from Charles River Italia (Charles River laboratories, Calco-Mi, Italy). The mice were housed in cages with stainless steel grid floors and lids, at a temperature of 22-24$^o$C and with a relative humidity of 45-55%, and a 12-h light/dark cycle. Mice were fed with rodent chow and mineral still water was provided *ad libitum.* These studies were carried out under established guideline for the care and use of animals for experimental and other scientific purposes, approved by the Council Directive 86/609/EEC, 24$^{th}$ November 1986.

Progenitor cells were flushed from femurs of three different mice for each gender and used as source of murine progenitors cells. For each different donor an experiment was performed in triplicate. This procedure was performed under rigorous sterile conditions on untreated mice. Following animal sacrifice by cervical dislocation, intact femora were isolated by cutting muscle ligaments, cleaned and placed in 100 mm Petri dishes containing ice-cold 10 ml IMDM supplemented with antibiotics (Penicillin 100U/ml – Streptomycin 100 µg /ml, Sigma-Aldrich

S.r.l., Milano, Italy). The ends of each femur were cut just below the head and bone marrow was flushed with 3 ml of IMDM without antibiotics. A single cell suspension was produced by gently and repeatedly drawing the marrow cells through a syringe fitted with a 23-gauge needle. Bone marrow cells were then filtered through a 100 µM cell strainer and washed by centrifugation at 400 x g for 10 min at 20°C.

The pellet was resuspended in medium (3 ml IMDM + 30 % FCS) and 10 µl of cells were diluted with 90 µl Trypan blue and counted in a haemocytometer. Viability was usually 95% or greater. The original cell suspensions were diluted to achieve the correct number of cells per ml for the assay to be performed as described below.

### *3A.3.4. Human CFU-GM assay*

Human Cord blood cells were seeded in MethoCult-H4534 medium (StemCell Technologies, Vancouver, BC, Canada). This medium is specific for human cells and contains methylcellulose (1%), Foetal Bovine Serum (FBS 30%), Bovine Serum Albumine (BSA 1%), 2-mercaptoethanol ($10^{-4}$ M), glutamine (2mM), Interleukin 3 (IL-3 10 ng/ml), Granulocytes-Macrophages-Colony Stimulating Factor (GM-CSF 10 ng/ml) and Stem Cell Factor (SCF 50 ng/ml).

Briefly, control tubes (linearity controls) and dose-response curve tubes with different arsenic dilution were prepared. To each tube containing 4.4 mL of methylcellulose culture medium, were added 78 µl of IMDM and 300 µl of cells (0.74-1.1 x $10^6$ cells/ml). 22 µl of water were added to the control tubes, and 22 µl of arsenic dilution were added for dose-response curve. Each tube was used to prepare three culture dishes. All the toxicants dilutions were prepared at X200 the final dilution, in order to obtain the final fold dilution. Then 1 ml of methylcellulose-cells suspension was seeded in 35 mm Petri dishes. Cultures were incubated at 37°C in 5% $CO_2$ for 14 days.

### *3A.3.5. Murine CFU- GM assay*

Murine progenitors, collected as described above, were washed, diluted in 30% FBS-IMDM and then seeded in MethoCult-M3534 medium (StemCell Technologies, Vancouver, BC, Canada) for the GM-CFU assay. These media are specific for murine cells and contain methylcellulose (1%), FBS (15%), BSA (1%), bovine pancreatic insulin (10 µg/ml), human transferrin iron-saturated (200 µg /ml), 2-mercaptoethanol ($10^{-4}$ M), and glutamine (2mM). The procedure was similar to that followed for human assays. To each tube containing 4,4 ml of methylcellulose culture medium, were added 78 µl of IMDM and 300 µl of cells (0.74-1.1 x $10^6$ cells/ml). 22 µl of water were added to the control tubes, and 22 µl of arsenic dilution were added for dose-response

curve. Each tube was used to prepare three culture dishes. All the toxicants dilutions were prepared at X200 the final dilution, in order to obtain the final fold dilution. Finally, 1 ml methylcellulose cell suspension was seeded in 35 mm dishes and the cultures were incubated at 37°C in 5% $CO_2$ for 7 days.

### *3A.3.6. Colony scoring*

Human CFU-GM colonies were scored using an inverted microscope after 14 days of incubation, whereas murine CFU-GM colonies were scored after 7 days of incubation.

A CFU-GM colony was defined and scored as an aggregate containing at least 50 or more cells (Pessina et al., 2001).

### *3A.3.7. RNA isolation and real time PCR*

Human cord blood cells coming from three different donors for each gender were thawed at 37° C in a water-bath and re-suspended IMDM culture medium supplemented with 10% FBS. After 24 hours of culture, cells were treated for 6 and 24 hours with $iAs^{III}$, $MMA^{III}$, $MMA^{V}$, and $DMA^{V}$ at the concentrations of 1 and 0.0001 μM. Cells were then harvested and lysed using the lysing RLT buffer (Qiagen, USA) + 1% b-mercaptoethanol and used for RNA extraction using a Qiagen Micro Kit, following the manufacture's protocol.

500 ng of total RNA was reverse transcripted using a mixture (1:1) of random hexamer and oligo dT primers (Promega) and Moloney murine leukaemia virus reverse transcriptase (M-MLV, Promega, Italy). The quality and quantity of RNA was checked using the Agilent 2100 bioanalyzer (Agilent, Palo Alto, California). The mRNA levels of AS3MT and GSTO1 were analysed by RT-PCR (TaqMan probes). The relative gene expression profile was normalised against the three most stable housekeeping genes (calculated with the geNorm system), TATA, B2M, ActB, as suggested by Vandesompele et al., 2002. All primers and probes were obtained from Applied Biosystems (California, USA), "Assay on demand" gene expression products. Primers for these genes were designed and labeled at the 5'-end with a reporter dye (FAM) and a quencher dye (TAMRA) at the 3'-end. Three separate experiments using cells from different donor were performed in triplicate in 96-well plates using TAQMAN Universal Master Mix.

Real-time PCR amplification was performed using a Gene Amp 7000 Sequence Detection System according to the manufacturer's protocol. PCR conditions were 50°C for 2 min, 95°C for 10min, then 40 cycles at 95°C for 15 seconds, and 60°C for 1 min. Fluorescence data were processed and analysed with ABI PRISM Sequence Detection software (version 1.6 software, Applied Biosystems). The quantification of the PCR assay was based on relative quantification

(ΔΔCT method). The CT is the "threshold cycle" when the system begins to detect the increase in the fluorescent signal associated with an exponential growth of PCR product during the log-linear phase.

The average CT values from each experiment were calculated, and the results were graphed with the corresponding standard deviation indicated with error bars in the figures (FIG 6 and 7). Briefly, the CT values indicate the fractional cycle number for which the amount of amplified target reaches a fixed threshold. This amount is a constant depending on the primer set. The difference (CT) between the CT of the target gene (CT t) and the reference gene (CT r) depends on the RNA relative copy number between the target and the reference gene. Standard curves were generated by using 10-fold serial dilution of pooled cDNA with five measuring points in order to verify the efficiency of the PCR. The linear correlation coefficient (R2) was between 0.994 and 0.999, and the PCR efficiency between 88.7 and 107. 3%.

### *3A.3.8. Data analysis*

The numbers of colonies (CFU-GM) in triplicate cultures from at least three separate donors were considered (Prism, Graph Pad, USA). Cell proliferation was expressed as a percentage of growth, with 100% corresponding to the number of CFU-GM colonies in the control dishes. Inhibitory concentrations of CFU-GM colonies ($IC_{10}$, $IC_{50}$ and $IC_{90}$) were calculated from the regression line using the statistical programme Prism (Graph Pad, USA) and data were expressed as mean ± standard error of the mean (SEM) (Table 1 and 2). The concentrations, which inhibit 50% of CFU-GM colonies' growth (IC50), were also interpolated according to the Reed and Muench formula (Reed and Muench, 1938) of at least three experiments carried out in triplicate.

A two way Analysis of Variance was performed to evaluate the statistical significance of the data and values of *$p<0.05$ were considered statistically significant, whereas **$p<0.01$ were considered high significant.

## 3A.4. RESULTS

### *3A.4.1. Human and murine CFU-GM*

A methylcellulose colony-forming unit-granulocyte/macrophage (CFU-GM) assay was used to evaluate the toxicity of $iAs^{III}$ on myeloid progenitors of male and female human cord blood cells and murine bone marrow cells. Our data showed a significant toxicity of $iAs^{III}$ both in human and in murine progenitor cells ($p< 0.05$), without significant differences between sexes. The IC10, IC50 and IC90, values are reported in Tables 1A and 2A. The IC10, IC50 and IC90 refer

to the number of CFU-GM colonies, each containing at least 50 or more cells, present in the plates. $iAs^{III}$ was toxic to about the same extent in both species and without differences between sexes (IC50 of 1.34 $\pm$ 0.43 μM and 1.22 $\pm$ 0.13 μM for male mice and male human respectively, and 0,95 $\pm$ 0.26 μM μM and 1,34 $\pm$ 0.43 μM for female mice and female human respectively). After exposure to low concentrations of $iAs^{III}$ (0.0006 μM for female human and 0.0001 μM for female murine), there was a significant increase in the number of colonies in both human and murine female cells, whereas no changes were observed in the male cells exposed to the same concentrations (Figures 1A and 2A).

| | Human | | | Murine | |
|---|---|---|---|---|---|
| | **Male** | **Female** | | **Male** | **Female** |
| **iASIII** | | | **iASIII** | | |
| **IC10** | 0.17 ± 0.03μM | 0.32 ± 0.01μM | **IC10** | 0.21 ± 0.06μM | 0.11 ± 0.05μM |
| **IC50** | 1.22 ± 0.13μM | 1.45 ± 0.16μM | **IC50** | 1.34 ± 0.43μM | 0.95 ± 0.26μM |
| **IC90** | 5.11 ± 0.84μM | 6.39 ± 0.40μM | **IC90** | 6.01 ± 0.27μM | 6.18 ± 0.54μM |

**Table 1A**. CFU-GM assay IC10, IC50 and IC90 values of $iAs^{III}$ exposure in three different male and female human cord blood cells donors and three different male and female murine bone marrow cells donors ± SE

| **MMAIII** | | **MMAV** | | **DMAV** | |
|---|---|---|---|---|---|
| **IC10** | 0.06 ± 0.01μM | **IC10** | > 50μM | **IC10** | > 50μM |
| **IC50** | 0.21 ± 0.03μM | **IC50** | > 50μM | **IC50** | > 50μM |
| **IC90** | 0.59 ± 0.03μM | **IC90** | > 50μM | **IC90** | > 50μM |

**Table 2A.** CFU-GM assay IC10, IC50 and IC90 values of $MMA^{III}$, $MMA^{V}$, and $DMA^{V}$ exposure in three different male and female human cord blood cells donors ± SE.

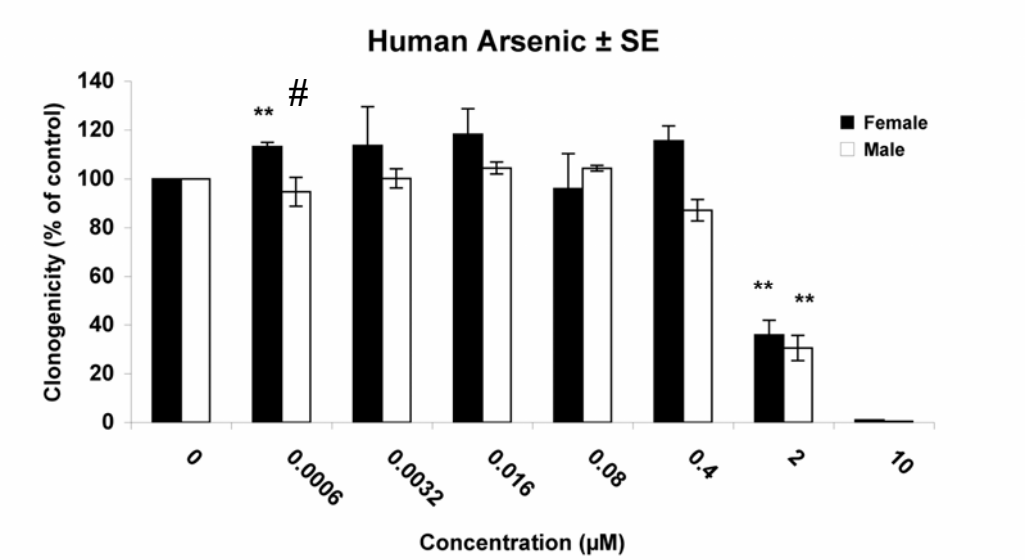


**Figure 1A.** Dose dependent curve of colony formation by myeloid progenitors (CFU-GM) resulting from in vitro $iAS^{III}$ exposure of human cord blood cells taken from three different male and female donors. Each column expresses the mean + SE of three independent experiment performed in triplicate. High statistical significance between samples and controls is expressed as ** $p < 0.01$. Significance between female and male is expressed as $^{\#}$ $p<0.05$.

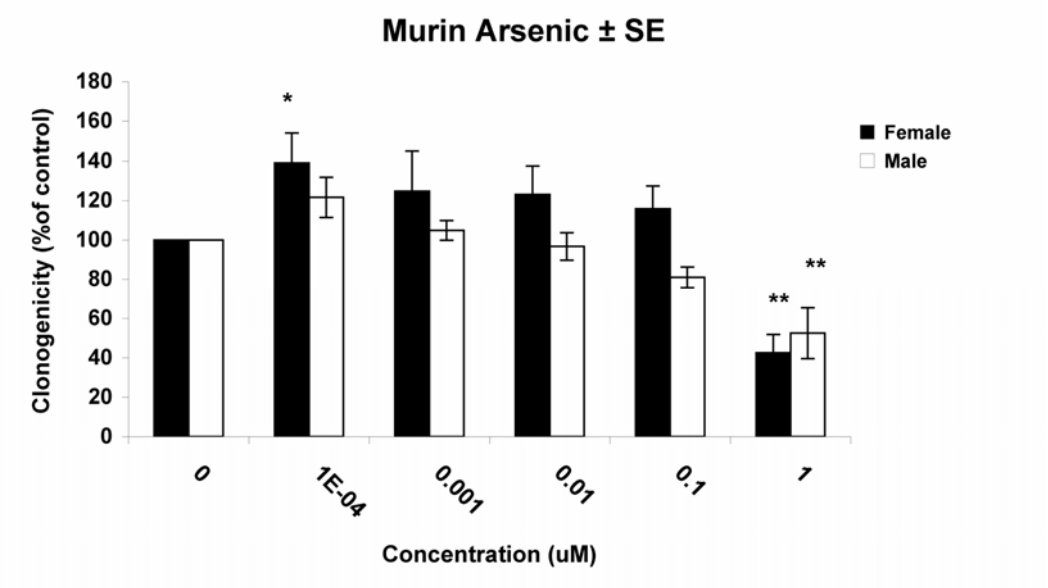


**Figure 2A.** Dose dependent curve of colony formation by myeloid progenitors (CFU-GM) resulting from in vitro $iAS^{III}$ exposure of murine bone marrow cells taken from three different male and female donors. Each column expresses the mean + SE of three independent experiment performed in triplicate. Statistical significance between samples and controls is expressed as *$p< 0.05$. High statistical significance between samples and controls is expressed as **$p< 0.01$.

The toxicity of the arsenic metabolites $DMA^V$, $MMA^V$, and $MMA^{III}$ was also evaluated in male and female human cord blood progenitors and described as concentration-response curves (Figures 3A, 4A, and 5A). The toxicity of the pentavalent methylated arsenic metabolites in cord blood cells was very low, and it was not possible to determine the IC50 values for $MMA^V$ and $DMA^V$ (> 50 μM in both male and female cells) (Table 2A). Similarly, there was no increase in the number of colonies neither in male, nor in female cells at any of the concentrations of $MMA^V$ tested (Figure 3A), whereas $DMA^V$ caused a slight, but not significant, increase in female CFU-GM colonies at all concentrations tested. No increase was observed in male progenitors (Figure 4A). In contrast, $MMA^{III}$ caused a marked and significant ($p < 0.05$) decrease in the number of CFU-GM in both male and female human progenitor cells (figure 5). The IC50 values were 0.21 $\pm$ 0.03 μM and 0.13 $\pm$ 0.02 μM, respectively (Table 2A). No cells proliferation was observed in either male or female progenitors after $MMA^{III}$ exposure.

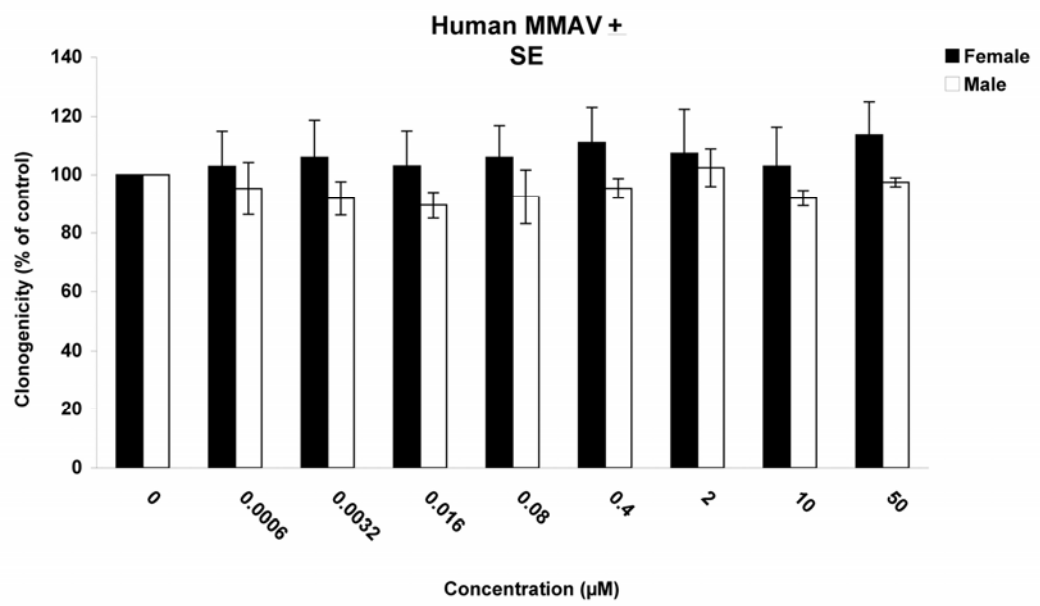


**Figure 3A.** Dose dependent curve of colony formation by myeloid progenitors (CFU-GM) resulting from in vitro $MMA^V$ exposure of human cord blood cells taken from three different male and female donors. Each column expresses the mean + SE of three independent experiment performed in triplicate.

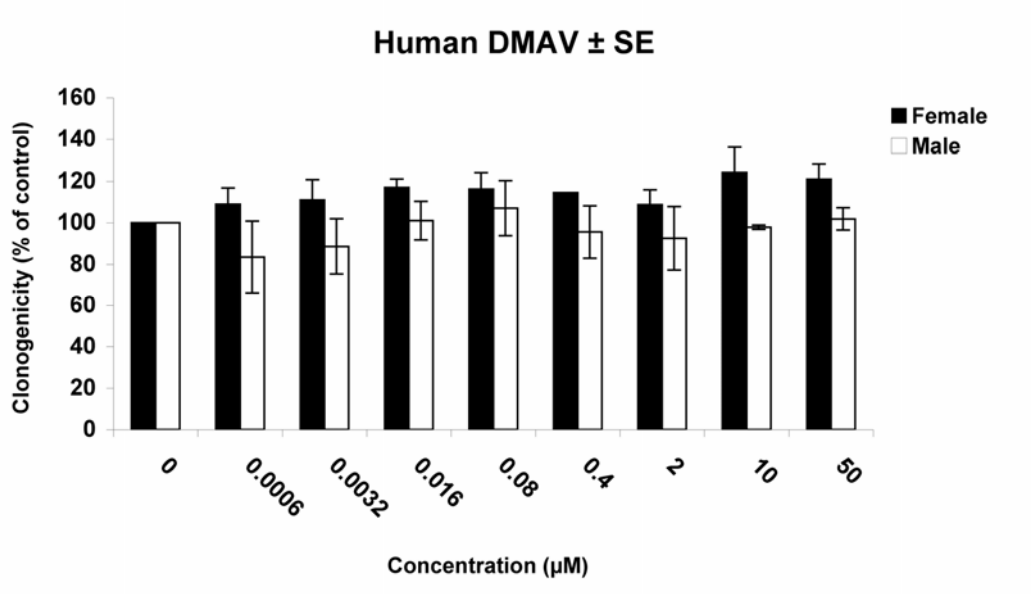


**Figure 4A.** Dose dependent curve of colony formation by myeloid progenitors (CFU-GM) resulting from in vitro $DMA^V$ exposure of human cord blood cells taken from three different male and female donors. Each column expresses the mean + SE of three independent experiment performed in triplicate.

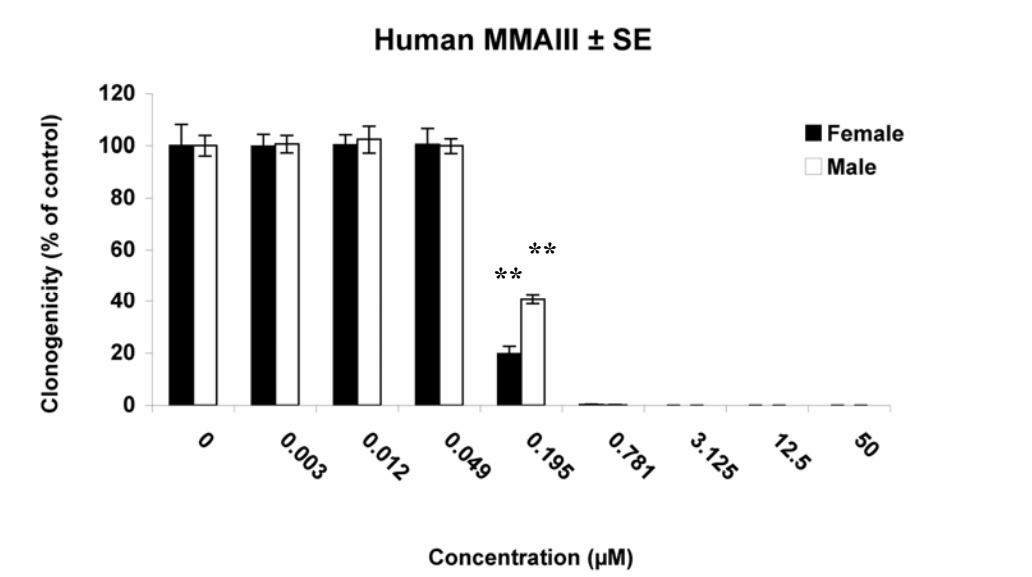


**Figure 5A.** Dose dependent curve of colony formation by myeloid progenitors (CFU-GM) resulting from in vitro $MMA^{III}$ exposure of human cord blood cells taken from three different male and female donors. Each column expresses the mean + SE of three independent experiment performed in triplicate. Statistical significance between samples and controls is expressed as ** $p < 0.01$.

### *3A.4.2. Real Time PCR*

Quantitative PCR was used to evaluate the presence of AS3MT and GSTO1 in the human cord blood cells. We used the HepG2 cell line (Human Hepato-carcinoma cell line) as a positive control for AS3MT gene. PCR was performed in triplicate for each run, and two separate experiments were carried out.

The results showed that in human cord blood cells AS3MT mRNA was present in very low numbers of copies, almost undetectable (Table 3A). Following $iAS^{III}$ exposure after either 6 or 24 hours at a concentration of 0.0001 μM and 1 μM, the AS3MT mRNA expression was not induced either in male or in female human cord blood cells (Table 3A).

GSTO1 mRNA was expressed at basal levels in the control sample and strongly down-modulated by $MMA^{III}$ treatment, both after 6 and 24 hours of exposure at the concentrations of 0.0001 and 1 μM (Figures 6A and 7A). Treatment with $iAs^{III}$, $MMA^{V}$ or $DMA^{V}$ at both concentrations did not induce significant modulation after both 6 and 24 hours.

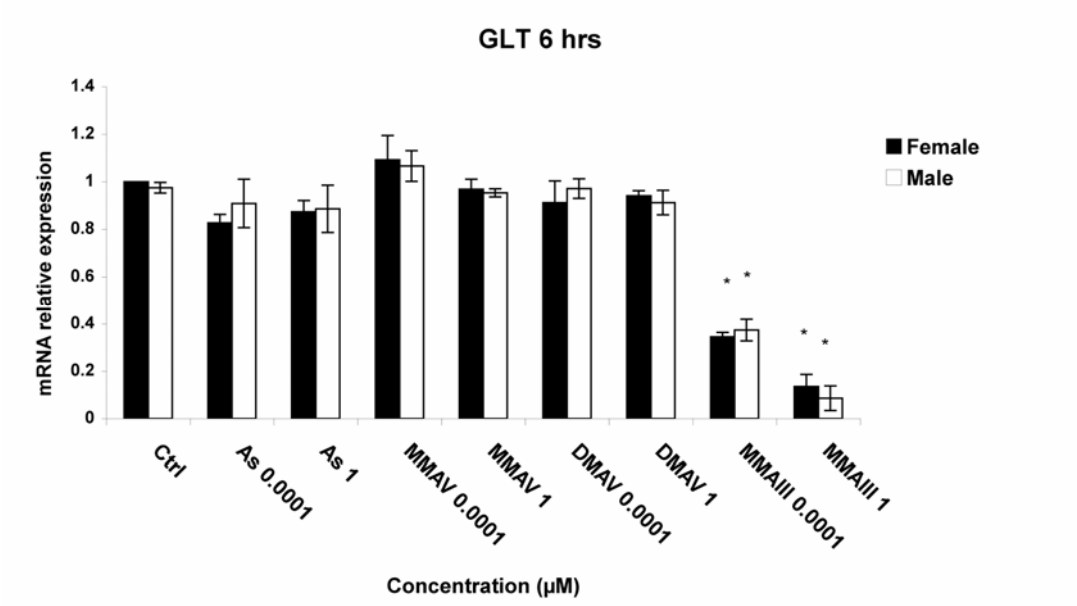


**Figure 6A.** Real Time PCR analysis (Taqman) of Glutathione-S-Tranferase Omega 1 (GLTO1) mRNA levels in cord blood cells after 6 hours in untreated (Ctrl), 0.0001 and 1 μM $iAs^{III}$, 0.0001 and 1 μM $MMA^{V}$, 0.0001 and 1 μM $MMA^{III}$. Statistical significance between samples and controls is expressed as * $p < 0.05$.

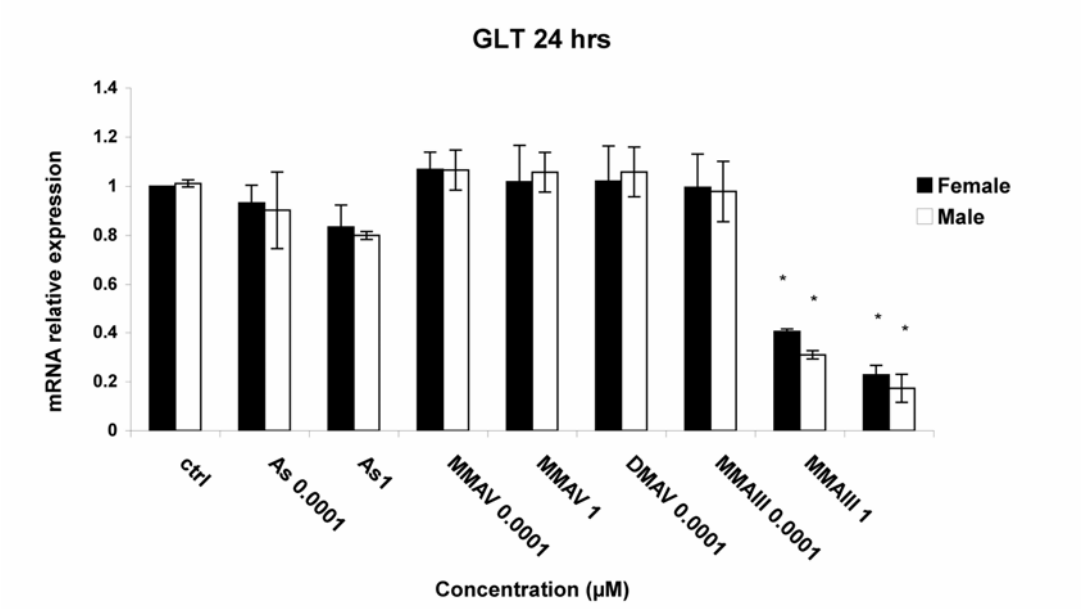


**Figure 7A.** Real Time PCR analysis (Taqman) of Glutathione-S-Tranferase Omega 1 (GLTO1) mRNA levels in cord blood cells after 24 hours in untreated (Ctrl), 0.0001 and 1 μM $iAs^{III}$, 0.0001 and 1 μM $MMA^{V}$, 0.0001 and 1 μM $MMA^{III}$. Statistical significance between samples and controls is expressed as * $p < 0.05$.

| | HepG2 cells | Female CB cells | Male CB cells |
|---|---|---|---|
| **Ctrl** | 1 | 0.0029 ± 0.0031 | 0.0032 ± 0.0031 |
| **iAsIII 0.0001μM** | 1.35 ± 0.09 | 0.0028 ± 00.28 | 0.003 ± 0.0028 |
| **iAsIII 1μM** | 1.59 ± 0.13 | 0.005 ± 0.0054 | 0.004 ± 0.004 |

**Table 3A.** Real Time PCR analysis (Taqman) of Arsenic-3-Methyl-Trasferase (AS3MT) mRNA levels after 24 hours in untreated (Ctrl), and 1 μM $iAs^{III}$ in human cord blood cells and HepG2 cell line.

### 3A.5. Discussion

Sakurai et al., 2006, already demonstrated the hypothesis that inorganic arsenic toxicity can target granulocytes and macrophages. They observed that arsenic was able to inhibit the CSF-induced "*in vitro*" maturation of monocytes into macrophages at sub μM levels of exposure, inducing cytolethality. In our work, we demonstrated a high toxicity of $iAs^{III}$ "*in vitro*" both on human cord blood and on murine bone marrow colony forming unit granulocytes-macrophages, without significant differences between the two species. The inhibition observed in CFU-GM assay indicates an immunosuppressive effect of $iAs^{III}$ on myeloid progenitors at the concentration around 1 μM. This range of μM concentrations is very close to the peripheral blood concentration of $iAs^{III}$ found in exposed population who suffer of inflammatory-like immune responses (Pi et al., 2000, Wu et al., 2003). At the same concentrations, the toxicity and DNA damage caused by $iAs^{III}$ were already observed in cultured human cells (Schwerdtle et al., 2003).

Although, previously published works on cellular sensitivity in response to inorganic arsenic used relatively high concentrations, the chronic health effects experienced by millions of people to iAs are the results of prolonged sub-toxic, low concentrations exposures (Boffetta et al., 1993).

We demonstrated that after exposure to very low μM concentrations of $iAs^{III}$ , a significant increase of granulocytes-macrophages colonies was observed in female progenitors, whereas male were completely unaffected by the same concentrations.

Since recent findings showed that the foetus can be exposed to the full range of arsenic species, generated during arsenic metabolism (Hall et al., 2007), we also tested the effects of the metabolites on human cord blood progenitors. Pentavalent DMA and MMA metabolites did not exert toxicity at the μM concentrations tested in either male or female human progenitors, whereas trivalent MMA was about five times more toxic than trivalent arsenic on human cord blood cells. No increase in the number of granulocytes-macrophages colonies was observed at all concentrations tested both for pentavalent and trivalent metabolites in both sexes.

To our knowledge this is the first attempt to assess the myelotoxic effects of arsenic and its metabolites on human cord blood cells cultures.

AS3MT mRNA was present in very low number of copies, almost undetectable in cord blood cells and not modulated after arsenic treatment. Furthermore, the expression of GSTO1 mRNA was strongly modulated after exposure to trivalent MMA, whereas no modulation was observed for either $iAs^{III}$ or pentavalent metabolites.

Gender differences on health effects of chronic exposure to inorganic arsenic via drinking water have been demonstrated (Vahter et al. 2007), and are probably due to different capacity of methylation between men and women (Lindberg et al., 2007).

In this study we did not find any differences between either the sexes or the two species in the toxic effects exerted by $iAs^{III}$ on the granulocytes-macrophages; it is likely that our "*in vitro*" system is not capable of arsenic biomethylation.

Somewhat surprising was the capacity of $iAs^{III}$ at very low concentrations to increase the granulocytes-macrophages colonies' number only with female progenitors, observed in both species, leaving male unaffected at the same concentrations tested. In fact, a significant increase has been observed at sub μM concentrations in female human cord blood cells and female murine bone marrow cells; this result suggests that this kind of arsenic stimulation is conserved across species. Our findings support data of other authors, where the toxicity of arsenic, always assumed as a linear dose response curve, is likely to be non-linear at very low concentrations (Schoen, et al., 2004). The changes in CFU-GM response at very low concentrations are different from those observed at higher, more toxic concentrations, following a biphasic curve, the so called U or J shape (Calabrese et al., 2001), indicative of a hormetic response. Evidence of this "hormetic" response after iAs treatment has been already observed on cell proliferation (Soucy et al., 2003) and base excision repair (Snow et al., 2003). Furthermore, it has been observed that exposure to low concentrations of inorganic arsenic increased the viability of cells in culture, whereas higher concentrations gave a cytotoxic effect (Calabrese and Baldwin 2003b). This modulation at low concentrations seems to be the result of different mechanisms of response to oxidative stress, apoptosis, increased DNA excision repair activity, as well as increased telomerase activity (Droge at al., 2002; Snow et al., 2003; Zhang et al., 2003). Nevertheless, the increase in cell proliferation may allow mutant cells to survive, preventing cellular senescence, apoptosis and eventually death, leading female to be more susceptible to cancer risk than male. However, since our study do not address a mechanistic explanation of this increased proliferation of female progenitors, more studies are needed to confirm whether and how $iAs^{III}$ toxicity acts differently in male and female at very low concentrations. We hypothesize that $iAs^{III}$ at low concentrations in female donor cells is able to stimulate the expression of pro-inflammatory and growth-promoting cytokines, above all GM-CSF (granulocyte/macrophage colony-stimulating factor), as already shown by other authors in different *"in vitro"* model (Vega et al., 2001, Germolec et al., 1996, 1998). Studies showed that some metabolites, above all $MMA^{III}$, are much more toxic than inorganic arsenic compounds (Kligerman and Tennant 2006; Petrick et al. 2000; Schwerdtle et al. 2003; Styblo et al. 2000;

Vega et al. 2001). Our results are in accordance with these findings; in fact trivalent MMA exerted the most toxic effects among all arsenicals, decreasing the number of granulocytes-macrophages colonies of human progenitors at lower concentrations with respect to $iAs^{III}$. We also confirm that pentavalent DMA and MMA, did not exerted either toxicity or increase the proliferative rate of male and female human granulocytes-macrophages progenitors up to the maximum concentration tested.

The marked toxicity caused by $MMA^{III}$, is probably due to a different mechanism of action with respect to $iAs^{III}$, such as increased oxidative stress, and damage to the DNA structure (Nesnow et al., 2002). Moreover its higher reactivity with respect to $iAs^{III}$ and other pentavalent metabolites allows $MMA^{III}$ to bind with more affinity to the tissues (Lindberg et al., 2007). Furthermore, $MMA^{III}$ is likely to be more membrane permeable to cord blood cells, and the higher uptake of the trivalent methylated arsenicals with respect to $iAs^{III}$ may be responsible for the reported greater cytotoxic effects of this compound (Dopp et al., 2004; 2005; Schwerdtle et al., 2003; Yamanaka et al., 2004).

In addition, $MMA^{III}$, has been shown to inhibit enzymes, such as glutathione reductase, and glutathione peroxidase altering the capacity of arsenic biotransformation (Styblo et al., 1997; Lin et al., 2001; Schuliga et al., 2002). In this study we confirmed that $MMA^{III}$ was able to decrease the expression of GSTO1.

GSTO1 is able to reduce both $MMA^{V}$ to $MMA^{III}$ and arsenate $As^{V}$ to arsenite $As^{III}$ (Zakharyan et al. 2001; Schmuck et al., 2005). In this reaction glutathione (GSH) is required for the catalytic activity of GSTO1 (Zakharyan et al., 2005). Data obtained with RT-PCR, indicate that $MMA^{III}$ was able to inhibit GSTO1 mRNA expression in cord blood cells, whereas either pentavalent metabolites or trivalent arsenic were not. The inhibition of GSTO1 observed in hematopoietic progenitor might be one of the possible explanations of the increased rate of toxicity of $MMA^{III}$ with respect to $iAs^{III}$: that down-modulation may cause an imbalance between the conjugation of $MMA^{III}$ and GSH with consequent increase in $MMA^{III}$ cells accumulation if not methylated, leading to increased cytotoxicity. On the other hand $MMA^{III}$ could inhibit GSTO1 through a mechanism of negative feedback, to avoid the accumulation of trivalent MMA into the cells. Nevertheless the decreased expression of GSTO1 after $MMA^{III}$ exposure deserves a deeper investigation in order to understand if the modulation of this gene might represent increased risk factors for arsenic-dependent immunotoxicity.

The expression of AS3MT mRNA in cord blood cells was detected at extremely low levels after PCR amplification, as was expected. Being AS3MT one out of the enzymes responsible for inorganic arsenic methylathion, it is likely that cord blood cells are not capable of arsenic

methylation "*in situ",* and for this reason a high exposure and accumulation of the more toxic $MMA^{III}$ in cord blood might increase the toxicity caused by arsenic on myeloid progenitors differentiation. In fact as already observed an higher proportion of MMA indicates a lower methylating capacity and probably also a higher concentration of the toxic $MMA^{III}$ in tissue leading to higher retention of arsenic in the body causing higher prevalence of cancer chromosomal aberration (Chen et al., 2005b; Steinmaus et al 2006a; Yu et al., 2000).

In conclusion, this study provides evidences that exposure to $iAs^{III}$ and $MMA^{III}$ at μM concentrations is associated with immunosuppression "*in vitro*". $iAs^{III}$ at very low concentrations increased the proliferative rate of female donor progenitors, supporting the fact that arsenic's different mode of action in the two genders at low concentrations exists. However, further investigations are needed to better understand the likely interactions of arsenic with oestrogen receptors and sex steroids.

Moreover, we hypothesis that additional concern about transplacental transfer of inorganic arsenic and its trivalent metabolites during the gestational period might exist, and in light of these findings, newborns exposed to high concentrations of $MMA^{III}$ through the cord blood, are likely to be at greater risk of arsenic-induced immune disease.

# 3B. Combined in-utero and juvenile exposure of mice to arsenate and atrazine in drinking water modulates gene expression and clonogenicity of myeloid progenitors

Graziella Cimino-Reale*, Daniele Ferrario*, Barbara Casati, Roberta Brustio, Cristina Diodovich, Angelo Collotta, Marie Vahter, and Laura Gribaldo.

European Centre for the Validation of Alternative Methods (ECVAM), T.P 580, IHCP, JRC,

European Commission, via Fermi 2749, 21027 Ispra (VA), Italy

*These authors contributed equally to this work

Toxicology Letters 2008, 180 (1): 59-66

## 3B.1. Abstract

The effects of arsenate and atrazine on myeloid progenitors (CFU-GM) cells derived from bone marrow were studied in male and female mice after combined in-utero and juvenile exposure. Female adult mice were treated with arsenate in drinking water during gestation. Then, separate groups of males and females' offspring were exposed for 4 months to atrazine, to additional arsenate or to co-exposure of atrazine and arsenate together in drinking water. In male mice, arsenate and the combined exposure did not modulate the percentage of CFU-GM progenitors, whereas atrazine significantly decreases the clonogenicity of myeloid cells. In females, the percentage of CFU-GM significantly decreased after atrazine exposure, did not change with arsenate treatment, but dramatically increased after the combined exposure.The expression of estrogen receptors alpha and beta in bone marrow cells was investigated, and an up-regulation of receptor beta was observed in both genders. A gene expression profile was generated using nylon membranes spotted with 1185 cancer related genes. Results from microarrays indicate that atrazine alone did not stimulate the expression of any of the genes analyzed in both male and

female. Arsenic induced gene expression modulation only in female. Major significant changes on the gene expression resulted following the co-exposure to arsenic and atrazine in both male and female.

***Key Words*:** Arsenate; Atrazine; CFU-GM; Estrogen receptors.

## 3B.2. Introduction

Human health protection most frequently employs exposure limits based on criteria for single agents (WHO, 2002). However, populations from urbanised and industrialised sites are often exposed to mixtures of chemical contaminants and heavy metals (ATDSR, 2004). The interactions occurring during these multiple exposure might result in additive, synergistic or antagonistic effects with regard to the toxic outcome. Nevertheless, information on the toxicity of the mixture is often lacking.

Atrazine (Atr) is a widely used chloro-S-triazine herbicide and its toxic effects have been extensively studied both in experimental animals and in humans (Gressel, 1984; Wetzel et al., 1994; Rodriguez et al., 2005). Atrazine has been reported to disrupt the estrous cycle in various laboratory rat strains (Cooper et al., 1996). In other animal studies, lifetime exposure to Atrazine caused premature reproductive senescence in addition to early onset and increased the incidence of mammary tumours in females Sprague-Dawley rats (Stevens et al., 1994). The developmental immunotoxicity of Atrazine was recently evaluated (Rooney et al., 2003). Atrazine exposure appears to be detrimental to the immune system of juvenile mice by decreasing cellularity and affecting lymphocyte distribution, with certain effects persisting long after exposure has been terminated (Filipov et al., 2005). Atrazine was also shown to be hematotoxic on murine progenitors (Mencoboni et al., 1992).

Inorganic Arsenic (iAs) is a worldwide water contaminant, and population chronically exposed to toxic levels of inorganic arsenic have been associated with a large number of health effects, such as skin lesions, neurotoxicity, cancer of the skin, lung, bladder, kidney, lymphoma as well as myelogenous leukaemia (IARC, 2004; WHO, 2001; NRC, 2001; Chen et al., 2003; Bates et al., 2004). iAs is also able to exert immunotoxic and immunodisruptive effects on human monocytes and macrophages (Sakurai et al., 2006).

Based on evidence that iAs is able to cross the placental barrier (Concha et al., 1998), gestation in mammalian can be a period of hypersensitivity to chemical carcinogenesis (Anderson et al., 2000). Moreover significant “in utero” arsenic exposure occurs in human population, suggesting that transplacental toxic risks defined in rodents may predict similar effect in humans.

A series of chronic carcinogenesis experiments in mice have been recently performed, that involved maternal oral exposure to iAs during gestation. In these studies it has been observed that a remarkable carcinogenic response in the offspring occurred after they had become adults and long after arsenic exposure had ceased (Waalkes et al., 2003; Waalkes et al., 2004; Liu et al., 2007).

Moreover there is increasing evidence that health effects of toxic metals are manifested differently between sexes (Vahter et al., 2007). For this reason, it has been postulated that estrogens might play a role. Recent studies have suggested that iAs can interfere with the action of estrogen receptors (ERs) acting as an endocrine disruptor, although the mechanism is poorly understood (Chen et al., 2002, Chow et al., 2004; Liu et al., 2007). Arsenic has been shown to modulate the expression of estrogen receptor α (ERα) both in human and in mice (Davey et al., 2007; Waalkes et al., 2004). It has also been demonstrated that inactivation of the estrogen receptor beta (ERβ) gene in mice leads to a chronic myeloid leukemia-like syndrome (Medina et al., 2001), suggesting a novel role for ERβ in regulating the differentiation of pluripotent hematopoietic progenitor cells.

Haematopoietic tissue is a complex system mainly addressed to the production of mature blood cells, where a limited number of stem cells give rise to progenitors of different lineages (Gribaldo, 2002). The capacity of the hematopoietic tissue to respond quickly to an increased demand for mature cells, as well as the complexity of the system, makes it a major target for xenobiotic toxicity (Gribaldo et al., 1996), e.g. arsenic (Woods and Fowler, 1977). Xenobiotic exposure can lead to cytotoxic effects on cell function or to cytolysis, either directly or in concert with immune mechanisms (Pessina et al., 2005).

In the present study, the clonogenicity of myeloid progenitors (CFU-GM), and the modulation of gene expression of 1185 cancer-related genes (DNA microarrays) in mice bone marrow, were used to investigate in young male and female mice the combined effects of continuous exposure to arsenate and atrazine in drinking water. The modulation of estrogen receptor alpha and beta was verified after exposure to atrazine, arsenate, and co-exposure. The overall goal of the study was to investigate the interactions between the biological effects of these two classes of contaminants, commonly present in industrial, urban, and natural environments on myeloid commitment. The hypothesis proposed was that co-exposure to these two agents can modulate the biological effects of exposure to the single substances and, ultimately alters their toxicity in a gender specific manner.

## 3B.3. Materials and Methods

### *3B.3.1 Animals and treatments*

Male and female CD-1mice (Charles River laboratories, Calco-Mi, Italy) were used. The mice were housed in cages with stainless steel grid floors and tops, at a temperature of 22-24$^{o}$C and with a relative humidity of 45-55%, and a 12-h light/dark cycle. Mice were fed with rodent chow and still mineral water was provided *ad libitum.*

These studies were carried out under established guideline for the care and use of animals for experimental and other scientific purposes, approved by the Council Directive 86/609/EEC, 24th November 1986.

Female adult mice were treated with sodium arsenate [(NaHAsO4) M.W 311, 98] directly in drinking water (1 mg /l) for 10 days before mating and during gestation. Separate groups of arsenate exposed male and female offspring were exposed for 4 months to 1mg /l of atrazine [(Atr) M.W 216], to 1 mg /l of additional arsenate (As) or to atrazine and arsenate together in drinking water (As+Atr). The doses were selected on the basis of the level of exposure of population in highly contaminated areas. Four animals were used for each treatment. Control mice (n=4) without any treatment were also analysed (Ctrl).

At the end of the treatments, the mice were sacrificed and the femora were rapidly removed, cleaned of tissue, and the ends removed. Bone marrow cells were collected from one femur for the CFU-GM assay as described below. The second femur was similarly flushed with 1 ml of RNA-Later (Ambion Inc., Austin, Texas, U.S.A.) to stabilize the RNA for later isolation.

The collected cells were kept at 4°C for 24 hours, and then stored at –20°C until analysis.

### *3B.3.2. RNA extraction and analysis*

Total RNA was extracted from bone marrow cells according to the RNeasy protocol (Qiagen Co., U.S.A.). The quality of the RNA was checked using the Agilent 2100 Bionalyzer (Agilent, Switzerland). An amount of 1μg of total RNA was converted into [33P]-labelled cDNA using Super Script III Reverse Transcriptase (Invitrogen, U.S.A.), in the presence of Mouse Cancer 1.2 CDS primer mix (Clontech, U.S.A.) and [□33P]-dATP (Amersham, U.K.). The cDNA obtained was purified by gel filtration using NucAwayTM spin columns (Ambion, U.S.A.) and the radioactivity linked to the high-molecular weight fraction was measured by □-counter (PerkinElmer Life and Analytical Sciences Inc., U.S.A.). The [33P]-cDNA samples were then heated at 95°C for 5 minutes and aliquots of 2.5x106 cpm added to the pre-hybridised cDNA microarray nylon membranes (Mouse Cancer 1.2 Array, BD AtlasTM, Clontech, U.S.A.)

according to the procedures described in the Array Advantage AA 1857 Ambion protocol. The hybridisation step was prolonged for about 16 hours at 50°C in a controlled temperature oven/shaker (GE Healthcare, UK) holding up to 6 glass tubes in which the membranes were inserted and rotated at 8 rev/minute. At the end of the hybridisation process, each membrane was washed twice with 50 ml aliquots of a low stringency wash buffer (2x SSC, 1% SDS) and twice with 50 ml aliquots of a high stringency wash buffer (0.5x SSC, 1% SDS), sealed into Saran wrap plastic film, and exposed for 21-24 hours on a phosphor-image screen (PerkinElmer, U.S.A.).

### *3B.3.3. Expression array analysis*

The images produced were recorded in a Cyclone instrument (PerkinElmer, U.S.A.) and analysed by Atlas Image software (BD AtlasTM). The spot intensities, corrected for the surrounding background intensity, were expressed as the number of pixels per spot, normalised as the percentage of the total pixels detected on the membrane. The percentages were transformed into angles (angle=asin (%value/100)1/2 x 57.29578). The angles were then used in Significance Analysis of Microarrays (SAM) analyses in order to find out the differences in the gene expression in treated, with respect to control samples (www-stat.stanford.edu/~tibs/SAM (Tusher et al., 2001). SAM is a supervised learning statistical software used to identify genes with significant changes in gene expression. A transcript was considered differentially expressed if the p-value was ≤0.05 (Student's t-test, n=4).

### *3B.3.4. Cell cultures*

Murine bone marrow cells were collected from mice CD-1, according to the procedure described below. Mice were treated with arsenate and atrazine, then sacrificed by cervical dislocation (without anaesthesia) and washed thoroughly with 70% ethyl alcohol. Femora were isolated intact by cutting the muscle ligaments and put in a 35-mm Petri dish containing 10 ml IMDM supplemented with antibiotics (100U/ml of penicillin and 100µg/ml of streptomycin). Femora were then cleaned from the articular knee cartilage and both ends cut just below the head. The marrow cells were recovered from the femora by flushing with IMDM (Gibco, Milan, Italy) containing 10% Foetal Bovine Serum (FBS) without antibiotics by inserting the needle of a syringe into one end. Marrow cells were collected in a 15-ml round–bottomed tube, dispersed with the syringe by repeated flushing and transferred into a 50-ml sterile tube by filtering them through a 100µm disposable cell strainer. Cells were then centrifuged at 800xg for 10 minutes, supernatant discarded and cells resuspended in IMDM supplemented with 30% of FBS. After a

cell viability evaluation, cells were diluted to 6.0 x 105 viable cells/ml, to achieve a final concentration of 4.0 x 104 cells/ml per plate.

### *3B.3.5. Murine CFU-GM assay*

Murine progenitors, collected from treated animals were washed, diluted in 30% FBS-IMDM and then seeded in "MethoCult-GF-M3534" (StemCell Technologies, Vancouver, BC, Canada) for the CFU-GM assay. This medium is specific for murine cells and contains methylcellulose (1%), FBS (15%), Bovine Serum Albumine (1%), bovine pancreatic insulin (10 µg/ml), human transferrin iron-saturated (200 µg g/ml), 2-mercaptoethanol (10-4 M), glutamine (2mM) and contains interleukin-3 (IL-3 10 ng/ml), interleukin-6 (IL-6 10 ng/ml) and Stem Cells Factor (SCF 50 ng/ml) to stimulate CFU-GM growth. Finally, 1ml methylcellulose-cells suspension was seeded in 35mm dishes and the cultures were incubated at 37°C in 5% CO2 for 7 days. All experiment was performed in triplicate.

### *3B.3.6. Colonies scoring and data analysis*

Murine CFU-GM colonies were scored after 7 days of incubation, using an inverted microscope. A CFU-GM colony was defined as an aggregate containing 50 or more cells (Pessina et al., 2001). Morphologically, four classes of CFU-GM colonies can be observed: compact, diffuse and spread multicentric, multifocal colonies. A compact colony presents a central dense nucleus and a peripheral halo. Diffuse and spread colonies are without an apparent nucleus.

A multicentric colony appears with two or more dense nuclei nearby, with a common peripheral halo growing at the same depth in the plate. Multifocal colonies are aggregates of several colonies or clusters with or without a peripheral halo.

Data were expressed as mean ± S.E. values of at least three experiments carried out in triplicate. Statistical analysis was performed by two way ANOVA followed by post-ANOVA tests (Fisher PLSD and Scheffe F-test). Values of $p<0.05$ were considered statistically significant.

### *3B.3.7. Real-time PCR analysis (TaqMan®)*

An amount of 500 ng of total RNA was reverse transcribed using random hexamer primers High-Capacity cDNA Archive Kit (Applied Biosystems) and Moloney murine leukaemia virus reverse transcriptase (M-MLV, Promega, Italy). The mRNA levels of estrogen receptors alpha and beta were analysed by RT-PCR in four adult male and four adult female mice. The relative gene expression profile was normalized against the most stable housekeeping gene 18s (calculated with the geNorm system), as suggested by (Vandesompele et al., 2002). All primers and probes

were obtained from Applied Biosystems (California, USA), "Assay on demand" gene expression products. Primers for these genes were designed and labeled at the 5'-end with a reporter dye (FAM) and a quencher dye (TAMRA) at the 3'-end. Two separate experiments were performed in triplicate in 96-well plates using TAQMAN Universal Master Mix (Applied Biosystems).

Real-time PCR amplification was performed on a Gene Amp 7900 Sequence Detection System according to the manufacturer's protocol. PCR conditions were 50°C for 2 min, 95°C for 10min, then 40 cycles at 95°C for 15 s, and 60°C for 1 min. Fluorescence data were processed and analysed with ABI PRISM Sequence Detection software (version 1.6 software, Applied Biosystems).

The quantification of the PCR assay was based on $\Delta\Delta C_T$ values. The average CT values from each experiment were calculated, and the results were graphed with the corresponding standard deviation indicated with error bars in the figures (Figures 3 and 4). Briefly, the CT values indicate the fractional cycle number for which the amount of amplified target reaches a fixed threshold. This amount is a constant depending on the primer set. The difference (CT) between the CT of the target gene (CT t) and the reference gene (CT r) depends on the RNA relative copy number between the target and the reference gene. Standard curves were generated by using 10-fold serial dilution of pooled cDNA with five measuring points in order to verify the efficiency of the PCR.

## 3B.4. Results

### *3B.4.1. Pathological changes and murine CFU-GM assay*

No gross pathological changes were observed at necropsy in both treated and control mice. The validated methylcellulose colony-forming unit-granulocyte/macrophage (CFU-GM) assay (Pessina et al., 2003) was used in this study to evaluate the toxicity of atrazine, arsenate and atrazine-arsenate mixture on myeloid progenitors in mice. These results were obtained from eight groups of mice: male and female control (non treated mice), arsenate treated mice, atrazine treated mice, arsenate and atrazine treated mice.

As shown in figure 1B, in male mice the percentage of CFU-GM significantly decreased after exposure to atrazine (colonies' mean 81 + 13). The exposure to arsenate alone did not modulate the percentage of CFU-GM (colonies' mean 105 + 6), whereas the co-exposure to arsenate+atrazine reduced in a non- significant manner the number of CFU-GM (colonies' mean 92 + 12). In female mice, the percentage of CFU-GM decreased significantly after atrazine exposure (colonies' mean 77 + 7) , slightly increased with arsenate treatment (colonies' mean

109 + 9) , but dramatically increased after the combined administration (colonies' mean 138 + 12), suggesting a strong interaction between the biological effects of the two compounds.

The mean of absolute CFU-GM colonies number taken as 100% were 123 ± 5.3 for female murine and 117 ± 8 for male murine respectively.

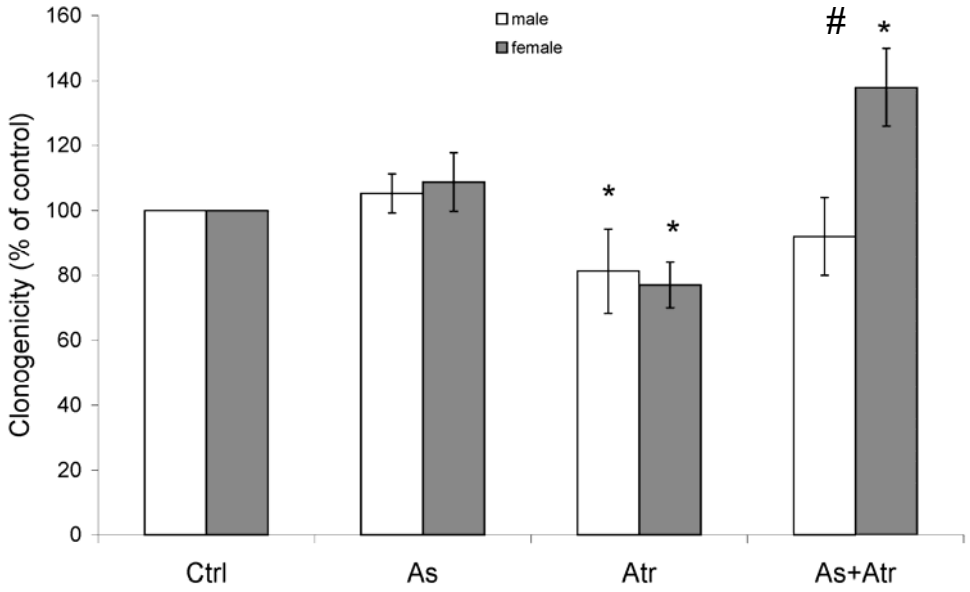


**Figure 1B** - CFU-GM colony formation resulting from in vivo exposure of mice (male and female) to arsenic, atrazine and arsenic + atrazine (at the concentration of 1mg/l in drinking water). Data are expressed as percentage on control colonies and represent the mean ± SE of five experiments done in triplicate. Statistical significance between samples and controls is expressed as * $p < 0.05$. Significance between female and male is expressed as $^{\#}$ $p<0.05$.

### *3B.4.2. Microarray analysis*

Atlas™ mouse 1.2 cDNA nylon filters (1185 genes) were used to evaluate global changes in gene expression induced by single and combined exposures to atrazine and arsenate, in male and female mice following 4 months of oral exposure. RNA was isolated by mice bone marrow cells and subjected to microarray analysis.

As shown in Figure 2B, in male mice the exposure to arsenate (As) or to atrazine (Atr) did not result in significant changes on the gene expression in bone marrow cells. On the contrary, the co-exposure to arsenate and atrazine (As+Atr) resulted in an up-modulation of 20 differentially expressed genes selected on the criteria that there was a significant difference ($p<0.05$, Student's *t* test, n=4) between the expression of the gene in untreated and in treated bone marrow cells.

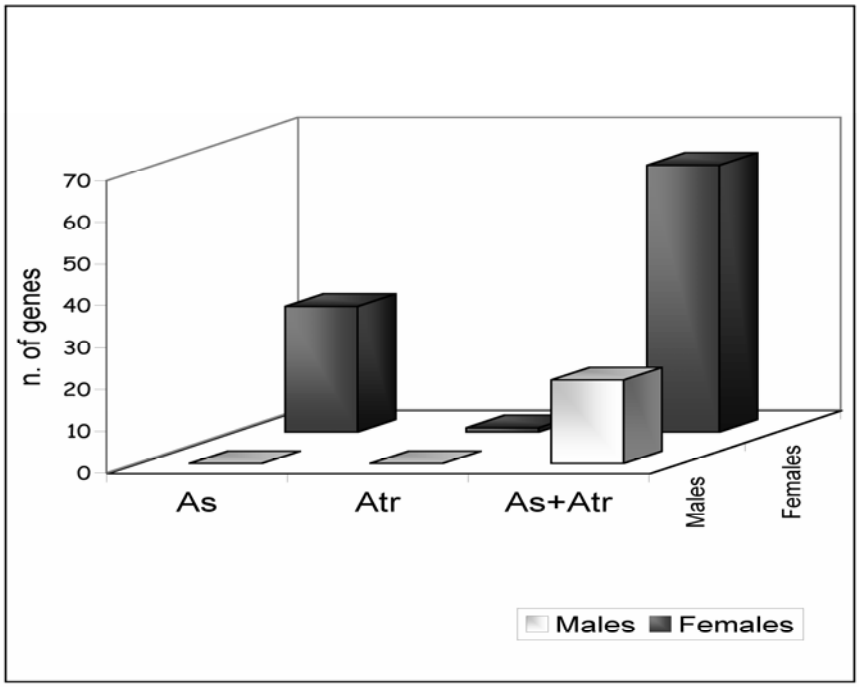


**Figure 2B -** Number of modulated genes by Arsenic and/or Atrazine treatments in male and female mice.

The selected 20 genes were classified in Gene Families that can be clearly visualised in Table 1B.

Five of these up-regulated genes code for the biosynthesis of chemokines and cytokines (Fst, Fgf10, Ccl7, Smad2 and Tmsb4x**)**, three for the adhesion cell-cell receptors (Ephb2, EphA1 and Fath) and 4 genes code for receptor (A2a, Cd28, Csf3r and IL1r1). Three genes (Dffa, Cnih2 and Hint1) code for intra cellular modulator; two genes (Serpinf2 and Ubb) code for protein turnover, two (Prg1 and Adcyap1) for signaling/extra cellular communications and the last one gene (Btg1) codes for cell cycle transcription. These genes were subjected to Panther (Protein Analysis Through Evolutionary Relationship) Classification System v.5.0 (https://panther.applied biosystems.com/) in order to classify the modulated genes according to Biological Processes (Table 2B). The major gene population (45%, p value= 4.0E-07) was categorised as cell surface receptor. The signal transduction process involved nine of these modulated genes (p value= 5.7E-05). Six of the genes (p value= 6.1E-04) were associated with

developmental processes, and four (p value= 9.7E-03) with immunity and defence. It is to note that a gene can be involved in more than one biological process.

Significance analysis of microarray (SAM) identified 30 up-modulated genes in female mice exposed to arsenate (As). The functional roles exerted by the genes modulated after arsenate treatment were in cell adhesion (9 genes) and in the biosynthesis of chemokines and cytokines (7 genes). The other up-modulated genes code for intracellular modulator (6 genes), cell cycle (2 genes). The families of DNA binding, extracellular transporters, oncogene and tumor suppressor, protein turnover and signaling present each 1 gene (Table 1B).

The only gene up-modulated in Atrazine treated female mice is a serine proteinase inhibitor (Serpine 1) that belongs to the protein turnover family.

The co-exposure to arsenate and atrazine (As+Atr) resulted in an up-modulation of expression of 64 genes. The classification in the Gene Family indicates that the more representative genes code for adhesion molecules (26.6%), for intracell modulator (12.5%), for receptor (10.9%), and for protein turnover (9.4%). As with the male mice, no down-modulated genes were detected in any treatments.

Also in females, the significative modulated genes were classified according to biological processes using Panther Classification System (Table 3B).

| Family Name | Males | | | Females | | |
|---|---|---|---|---|---|---|
| | As | Atr | As+Atr | As | Atr | As+Atr |
| Adhesion | 0 | 0 | 3 | 9 | 0 | 17 |
| Apoptosis | 0 | 0 | 0 | 0 | 0 | 5 |
| Cell cycle | 0 | 0 | 1 | 2 | 0 | 3 |
| DNA binding/chromatin | 0 | 0 | 0 | 1 | 0 | 1 |
| Extracell transporters/carriers | 0 | 0 | 0 | 1 | 0 | 0 |
| G.F.,Cytok.,Chemokines | 0 | 0 | 5 | 7 | 0 | 3 |
| Intracell modulator | 0 | 0 | 3 | 6 | 0 | 8 |
| Matrix | 0 | 0 | 0 | 0 | 0 | 2 |
| Metabolism (xenobiotic) | 0 | 0 | 0 | 0 | 0 | 2 |
| Oncogenes and tumor suppressors | 0 | 0 | 0 | 1 | 0 | 5 |
| Post-translational modification | 0 | 0 | 0 | 0 | 0 | 1 |
| Protein turnover | 0 | 0 | 2 | 1 | 1 | 6 |
| Receptors | 0 | 0 | 4 | 0 | 0 | 7 |
| Signaling | 0 | 0 | 2 | 1 | 0 | 1 |
| Transcription | 0 | 0 | 0 | 0 | 0 | 3 |
| Unclassified | 0 | 0 | 0 | 1 | 0 | 0 |

**Table 1B**. Gene family classification of modulated genes in male and female treated mice.

| **Biological Processes** | As+Atr | (p-value) |
|---|---|---|
| Cell surface receptor mediated signal transduction | 9 | 4.00E-07 |
| Cytokine and chemokine mediated signaling | 2 | 8.03E-03 |
| Developmental processes | 6 | 6.07E-04 |
| Ectoderm development | 3 | 5.48E-03 |
| Immunity and defense | 4 | 9.74E-03 |
| Ligand-mediated signaling | 3 | 1.17E-03 |
| Mesoderm development | 3 | 3.28E-03 |
| Neurogenesis | 3 | 3.63E-03 |
| Receptor protein tyrosine kinase signaling | 3 | 2.09E-04 |
| Signal transduction | 9 | 5.71E-05 |
| T-cell mediated immunity | 2 | 8.60E-03 |

**Table 2B.** Biological Processes identified by Panther in Male As+Atr treated mice

| **Biological Processes** | As | As (p-value) | Atr | Atr (p-value) | As+Atr | As+Atr (p-value) |
|---|---|---|---|---|---|---|
| Cell adhesion | 3 | 1.30E-02 | 0 | 9.79E-01 | 8 | 2.25E-05 |
| Cell communication | 8 | 3.21E-06 | 0 | 9.59E-01 | 15 | 6.50E-09 |
| Cell proliferation and differentiation | 6 | 7.65E-05 | 0 | 9.69E-01 | 9 | 6.50E-05 |
| Cell surface receptor mediated signal transduction | 7 | 1.27E-03 | 0 | 9.27E-01 | 16 | 2.13E-06 |
| Developmental processes | 11 | 3.74E-07 | 0 | 9.28E-01 | 23 | 4.49E-12 |
| Intracellular signaling cascade | 5 | 5.90E-04 | 0 | 9.71E-01 | 10 | 5.98E-06 |
| Mesoderm development | 5 | 9.83E-05 | 0 | 9.81E-01 | 8 | 1.75E-05 |
| Neurogenesis | 5 | 1.09E-04 | 0 | 9.80E-01 | 9 | 2.30E-06 |
| Protein metabolism and modification | 6 | 3.05E-02 | 1 | 1.05E-01 | 13 | 4.32E-03 |
| Receptor protein tyrosine kinase signaling | 3 | 7.86E-04 | 0 | 9.93E-01 | 5 | 7.61E-05 |
| Signal transduction | 14 | 2.95E-07 | 0 | 8.68E-01 | 28 | 8.94E-11 |

**Table 3B**. Biological Processes identified by Panther in Female treated mice.

### *3B.4.3. Real-time PCR (Taqman®)*

We investigated the modulation in gene expression of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) in bone marrow cells of four male and female mice exposed *in vivo* to arsenate, atrazine and arsenate and atrazine together in drinking water, using a quantitative RT-PCR.

ERβ was present at relatively low number of copies (mean value Ct 31.77) in respect to 18s housekeeping gene (mean Ct value 14.90), whereas ERα was expressed at relatively high number of copies (mean Ct value 23.22).

The ERα gene expression was not modified significantly after chemical treatments in both males and females

On the contrary the ERβ gene expression was up-modulated in both genders. In males the exposure to arsenate, atrazine and the co-exposure modulated the expression of receptor beta in a significant way as shown in figure 3B. In females, due to very high variability among the individuals, only the co-exposure to arsenate and atrazine modulated the expression of the receptor beta in a significant manner, as shown in figure 4B.

In groups treated with arsenate or atrazine alone, a high variability among individuals, both in controls and in treated mice, has been observed.

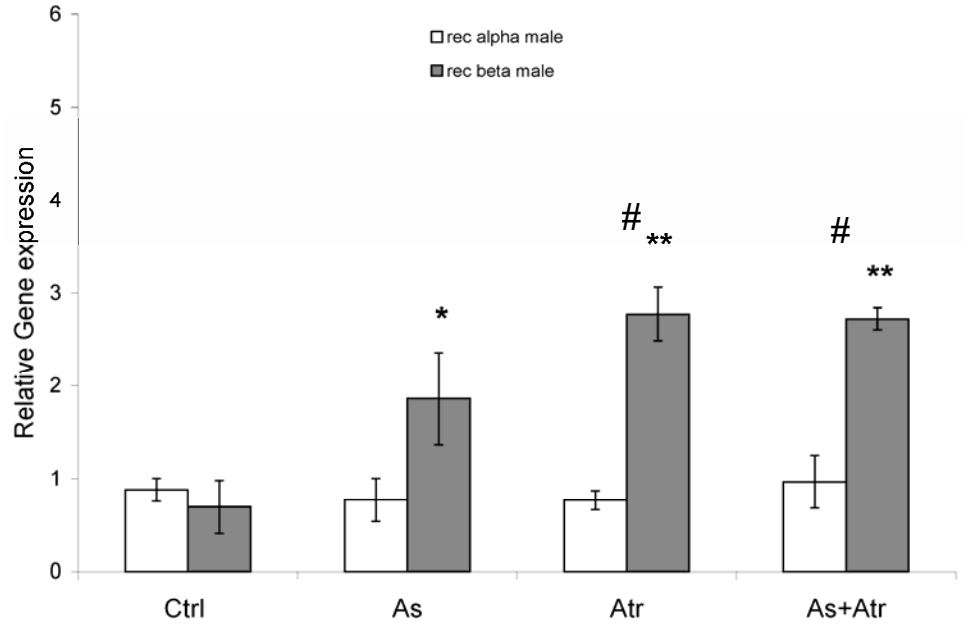


**Figure 3B** - Relative mRNA expression of estrogens receptor alpha (ERα) and beta (ERβ) in male murine bone marrow cells, before and after treatment with arsenic and atrazine. Statistical significance between samples and controls is expressed as * $p < 0.05$. High statistical significance between samples and controls is expressed as ** $p < 0.01$. Significance between female and male is expressed as $^{\#}$ $p<0.05$.

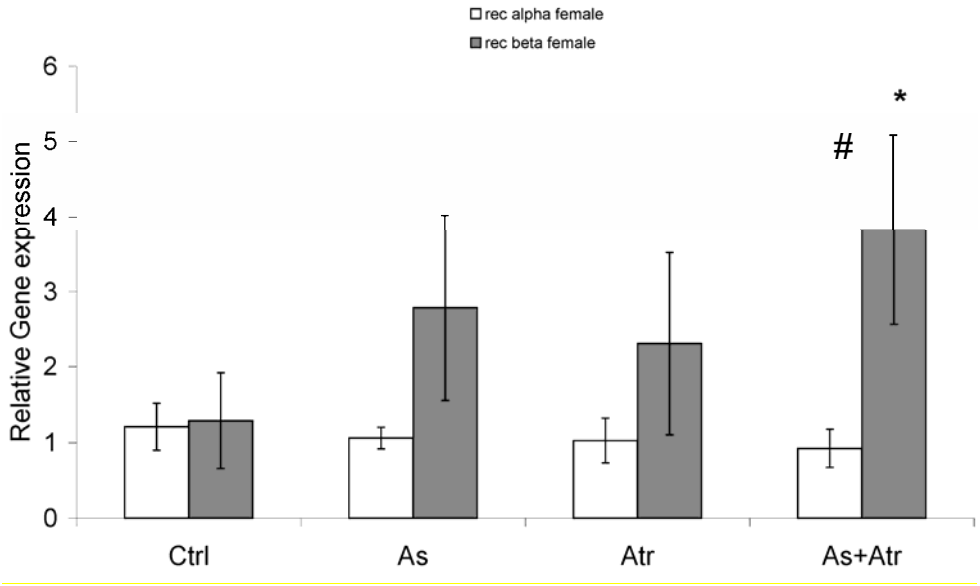


**Figure 4B** - Relative mRNA expression of estrogens receptor alpha (ERα) and beta (ERβ) in female murine bone marrow cells, before and after treatment with arsenic and atrazine. Statistical significance between samples and controls is expressed as * $p < 0.05$. Significance between female and male is expressed as $^{\#}$ $p<0.05$.

**3B.5. Discussion**

Arsenic and atrazine are well known contaminants, both able to interact with the immune system (Filipov et al., 2005, Sakurai et al., 2006). The present study investigated wheater in utero and juvenile exposure to atrazine, arsenic and co-exposure to both of them results in modulation of the capacity of granulocytes-macrophages progenitors to give rise to colonies, as an early marker of myelotoxicity. The aim of this investigation was to demonstrate that metals and pollutants might induce a deregulation in the bone marrow tissue differentiation, through different pathways implicated in inflammation responses, cellular proliferation, and epigenetic mechanisms in a gender-dependent manner.

Usually, the simplest exposure paradigm is the single substance and single dose at an enviromentally relevant concentration once during pregnacy. However, the real-life exposure is often for extended period, during gestation and after birth. Moreover, since interactivity may increase the potential toxicity of a single compound, it should be considered in assessing the risk associated with mixture of chronic toxicants.

The results obtained with the clonogenic assay indicate that in-utero and juvenile exposure to atrazine induced a significant inhibition of CFU-GM colonies both in male and female mice. Arsenate did not modulate the clonogenic capabilities of progenitors in both the sexes, whereas the co-exposure increased the progenitors' proliferation only in female.

The potential immunotoxicity of atrazine has been extensively studied using adult exposure models (NTP, 1994; Pruett et al., 2003), however there is a substantial literature reporting that developing immune system is more sensitive to the toxic insult of xenobiotics (Blaylock et al., 1992, Holladay, 1999 and Theus et al., 1992). In this study, the detrimental effect of atrazine on myeloid progenitors confirms data observed by others authors (Rowe et al., 2007, Filipov et al., 2005, Mencoboni et al., 1992), showing that atrazine can damage the immune system of juvenile mice by decreasing cellularity and affecting lymphocyte distribution. No differences were observed between the two sexes following atrazine exposure alone.

Speculations about the possible mechanism of how prenatal and juvenile exposure to atrazine can causes the toxicity to the offspring's immune system have been raised. In the first hypothesis atrazine could interact directly with the immune cell progenitors of the foetus. The second one is that these changes could take place via epigenetic mechanisms (Rowe et al.,2007). In our study no gene modulation was observed in treated males, whereas only one gene out of 1185, Serpin-1 was up-modulated in females. This gene is a serine protease inhibitor that belongs to the protein turnover family. Its role is mainly related to the mediation of innate immune responses, and when it is given as purified protein, it markedly inhibits vascular monocyte invasion (Viswanathan et al., 2006, Dai et al., 2003). The down-modulation exerted in the granulocytes-

macrophages progenitors after atrazine exposure, raises the possibility that atrazine might induce a direct effect on the development of the immune progenitor cells rather than through epigenetic modulation. Howeverer, it is likely that atrazine can cause immunotoxicity inducing also developmental endocrine disfuntion as already reported by other authors (Cooper et al., 2000).

Nevertheless, because our investigation does not address an exhaustive explanation of the immunotoxic effect exerted by atrazine, further studies are needed to better understand the toxicological pathways behind atrazine toxicity during the gestational and juvenile exposure.

The exposure to arsenate induced neither inhibition nor increase in the number of CFU-GM colonies. These results suggest that arsenic is not able to cause myelotoxicity at the concentrations used in bone marrow progenitors either in male or in female, whereas the microarrays analysis evidenced 30 out of 1185 genes modulated after treatment in females', leaving males completely unaffected. The functional role exerted by the genes modulated after treatment in females, is related to cell adhesion, and biosynthesis of chemokines and cytokines. The cell adhesion molecules mediate the migration of cells to sites of inflammation and the effector functions of cells within the lesions. The increased expression of either cell adhesion and cytokines related genes suggest that arsenic is able to create inflammation at the dose tested. However, this modulation should be further investigated looking at the effects on the protein expression. The increased expression of inflammatory mediators in a gender dependant manner, suggesting female mice as the most sensitive gender with respect to male to arsenic toxicity.

More interestingly, the clonogenicity of myeloid progenitors was significantly stimulated following the co-exposure to arsenate and atrazine only in female mice, supporting a gender-specific co-operative role of these two chemicals in promoting cell proliferation as already observed in different species (Shen et al., 2006; Rahman et al., 2006). Only a slight decrease was observed in male progenitors. A significant difference between genders has been observed in microarrays analysis as well, where only few genes (Epha1, Fath, Cnih2 and Csf3r) were commonly modulated. Eph receptor tyrosine kinases (Epha1), plays a pivotal role in regulating cell migration and recently it has been shown also in lymphoid cells, raising the possibility that Eph receptors may similarly regulate lymphocyte migration (Sharfe et al., 2002). Granulocyte colony-stimulating factor (G-CSF) is the principal growth factor regulating granulopoiesis (Jacob et al., 1998). As observed by some authors (Richards et al., 2003) the positive or negative regulation of G-CSF levels may provide a mechanism for directing primitive haematopoietic progenitors into the common myeloid lineage in response to environmental stresses. The gene coding for the receptor of G-CSF (Csf3r) was found up-modulated after co exposure both in males and females, even though only females showed an increase in the progenitors "growth".

Furthermore, in female mice the co-exposure with arsenate and atrazine show an up-modulation of a series of other genes involved in apoptosis, cell cycle, oncogenes and tumor suppressor genes.

Bcl-2, Cyclin B1, and programmed cell death 1 (Pdcd2) were up-regulated. The complex of Cdk and cyclin B is called maturation promoting factor (MPF) that stimulates the mitotic and meiotic cell cycles. Pdcd1 has been postulated to have essential roles in the regulation of autoimmunity and introduces tolerance for lymphocytes (Okazaki and Honjo, 2006); moreover it has been demonstrated that this gene is able to exert inhibitory functions on T cells, causing the progression of self-reactive immune disease (Kroner et al., 2005).

A higher level of expression of Pdcd1 has been also observed in patients with autoimmune disease such as Sjogren's syndrome (SS) (Kobayashi et al., 2005). Retinoblastoma-like 1-2, T-cell lymphoma invasion and metastasis 1 were modulated as well. These oncogenes are involved in the pathway of cell proliferation, and it is likely that the higher rate of gene up-modulation observed after co-exposure of arsenic and atrazine in female might explain the increase of CFU-GM proliferation. Those results suggest that as for arsenic exposure alone, also for the co-exposure female is the most sensitive sex to xenobiotic exposure. In female mice the co-exposure to arsenic and atrazine can create a synergistic effect, pushing the myeloid progenitors towards increased proliferation rather than decrease the number of myeloid cells as observed for atrazine single exposure. Although the precise mechanism of these observation remains unclear, it is likely that gender dimorphism in proliferation could result either through direct action on gene expression or mediated via action of gender hormones or receptors (Davey et al., 2007).

Lea and co-workers (Lea et al.,1999) underlined that some cytokines transcripts that are involved in the pathway of the granulocytes-macriphages (GM) proliferation are differentially expressed in the presence of gender hormones "*in vivo*"; the expression of these genes was increased in estrogens deficient rats and above all the production of GM increased after ovariectomy. The action of gender hormones could be directly manifested trough their receptors present on bone marrow cells and may exert differential effects on bone marrow hematopoiesis (Van Merris et al., 2004; Capellino et al., 2006, Lim et al., 1999). For this reason, we investigated if the expression of Erα and ERβ in the bone marrow of treated mice were differently expressed between the sexes.

Our work indicates that arsenate, atrazine and the co-exposure are able to modulate the expression of estrogen receptors genes in bone marrow cells of mice chronically exposed to these toxicants, as observed by other studies (Waalkes et al., 2004). Atrazine, arsenate and co-exposure modulated mainly the expression of ERβ gene, leaving the Erα unaffected, suggesting

that primarily ERβ regulates proliferation and differentiation in the hemopoietic system (Islander et al., 2003).

In males, ERβ was significantly up-modulated after exposure to arsenate, atrazine, and co-exposure. In females we still observed an increase in gene expression, even though the interpretation of results was less clear, due to the high variability among the individuals, and only the co-exposure caused a significant up-modulation of ERβ. Our data confirm previous observations (Shim et al., 2006), suggesting a new unknown role for ERβ in regulating the differentiation of pluripotent hematopoietic progenitor cells. However, it should be noted that the degree of ERβ expression was much lower than that of ERα, as already observed (Lim et al., 1999) and for this reason we cannot conclude that the modulation observed in ERβ alone can play a key role in regulation of immune response in our system.

Previous investigations were undertaken to study if gender dimorphism in the growth of a T cell tumor could be associated with a gender-dependent differential myelopoiesis of bone marrow cells (Gupta and Singh, 2007). Bone marrow cells of male and female showed a differential expression of the cell cycle and apoptosis regulatory protein p53 and macrophage-colony stimulating factor (M-CSF) genes. Bone marrow cells of male tumor-bearing hosts showed a predominant differentiation in the macrophage lineage whereas those of female tumor-bearing mice were in the granulocyte lineage. Bone marrow-derived macrophages (BMDM) from male and female tumor-bearing mice also showed the existence of gender dimorphism with respect to their differentiation and activation. The ERβ mRNA expression was up-modulated either after arsenate or atrazine single exposure as well as after the co-exposure in males, whereas in females only the co-exposure up-regulated ERβ. These results suggest a gender-dependent relationship between colonies proliferation and differentitation and ERβ mRNA expression. However, little is known about ERβ function, and we cannot conclude that ERβ can be responsible for the increased proliferation of female GM colonies. Further studies on the mechanisms of activation of ERβ in the modulation of the immune system are necessary. On the other hand, it is authors' opinion that the up-modulation of ERβ in concert with the higher rate of gene modualtion observed only in female mice after the co-exposure might be resposonsible for the increased proliferation observed in myeloid colonies.

In conclusion, we found a gender dimorfism in the synergistic effect of simultaneous exposure to atrazine and arsenic in comparison with indipendent exposure to them. Moreover, in female mice, ERβ mRNA was up-modulated only after co-exposure, suggesting a gender-dependent role of ERβ in the pathway of proliferation and differentiation of pluripotent haematopoietic progenitor cells.

### *3.B.6. Acknowledgments*

We thank Gerard Bowe for critical reading of the manuscript.

# 3C. Arsenic induces telomerase expression and maintains telomere length in human cord blood cells.

Daniele Ferrario, Angelo Collotta, Maria Carfi', Gerard Bowe, Marie Vahter, Thomas Hartung, and Laura Gribaldo

European Centre for the Validation of Alternative Methods (ECVAM), T.P 580, IHCP, JRC,

European Commission, via Fermi 2749, 21027 Ispra (VA), Italy

Toxicology 2009, 260(1-3): 132-141

## 3C.1 Abstract

Inorganic arsenic (iAs) is a human carcinogen, well known as a clastogenic compound. To evaluate the molecular mechanism of arsenite ($iAs^{III}$) toxicity, we investigated the effects on cell growth and apoptosis, telomere length, telomerase expression, as well as the formation of reactive oxygen species (ROS) in male and female human cord blood cells *in vitro*. Incubation with $iAs^{III}$ at the concentration of 0.0001 μM increased telomerase mRNA and protein expression maintained both telomere length and cellular growth, and induced mRNA over-expression of the two oncogenes *ras* and *myc*. Our results suggest that female cord blood cells are more sensitive than male ones to $iAs^{III}$ induced telomerase stimulation at low concentrations, possibly related to the increased expression of *ras* and *myc* oncogenes. On the contrary, at the concentration of 1 μM, $iAs^{III}$ decreased telomerase expression and telomere length, and induced apoptosis, necrosis and production of reactive oxygen species. Buthionine sulfoximine (BSO), an inhibitor of glutathione (GSH) synthesis, markedly increased the percentage of apoptotic cells, suggesting that GSH is fundamental for detoxification of $iAs^{III}$ in cord blood cells. The reactive oxygen species (ROS) scavenger, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), protected cord blood cells from $iAs^{III}$ toxicity, and prevented telomere shortening and telomerase down-modulation. It can be concluded that telomerase expression and telomere length are associated with $iAs^{III}$ induced cell death, via production of reactive oxygen species, as well as with $iAs^{III}$ induced effects on cell differentiation processes and rate of cell growth.

***Keywords***: *In vitro*, Arsenic, hTERT, ROS, BSO, DMPO

## 3C.2. Introduction

Arsenic is a ubiquitous contaminant, and it is considered one of the top environmental health threats based on the population's potential exposure from contaminated drinking water and the high number of diseases arsenic has been associated with (NRC 2001, Abernarty et al., 2003). Exposure to inorganic arsenic (iAs) is linked with increased risks of malignant skin lesions, skin and internal cancers, as well as cardiovascular diseases (WHO 2001, IARC 2004; Yoshida et al., 2004). Moreover, arsenic has been shown to cause increases in the incidence of different cancers in experimental animals after transplacental exposure (Waalkes et al., 2004, 2006). Arsenic is not considered a direct mutagen; nevertheless, it may increase DNA damage and mutation indirectly by altering DNA repair machinery (Andrew et al., 2003, 2006). Several studies have demonstrated that arsenic is an immunotoxic compound (Burns and Munson, 1993; Hall et al., 2002; Soto Pena et al., 2006; Patterson et al., 2004, Sakurai et al., 2004). Arsenic was also shown to cross the placental barrier (Concha et al., 1998; Golub et al., 1998), extending the risk of toxicity to the foetus (Jin et al., 2006; Waalkes et al., 2007). However, the mechanisms of arsenic toxicity in early life exposure are largely unknown. Both *in vivo* and *in vitro* studies have shown that arsenic is able to induce chromosome instability, aberration, and telomere attrition (Barrett et al., 1989), and formation of reactive oxygen species (ROS) (Chen et al., 1998; Liu et al., 2003).

Human telomeres consist of long repetitive TTAGGG subunits, which are associated with a variety of telomere-binding proteins (Blackburn, 2000). Telomeres play a critical role in maintaining chromosome stability by protecting the chromosome ends from degradation and fusions (Bouffler et al., 2001; Shay et al., 2001). Eukaryotic cells are not able to copy the 3' end of the chromosome during cellular replication. For this reason, a gradual loss of telomeric sequence happens at each replication, leading to chromosomes shortening with progressing cell division. A special ribonucleoprotein reverse transcriptase, called *telomerase* (hTERT) is activated to maintain the telomere length. Telomerase synthesises telomere repeats TTAGGG onto chromosome ends to overcome the loss of sequence during normal replication. Even though, in most somatic cells telomerase activity is lacking, haematopoietic cell precursors exhibit a detectable telomerase activity since the expression of this protein supports the constant replacement of blood cells (Engelhardt et al., 1997); hence, it has been proposed that the expression of telomerase promotes continued proliferation of haematopoietic cells bypassing cellular senescence (Chiou et al., 1996). On the other hand, telomere dysfunction in haematopoietic cells is implicated in the pathogenesis of blood disorders, such as anaemia and

acute leukaemia (Leteurtre et al., 1999; Engelhardt et al., 2000; Campbell et., 2006). Furthermore, murine models suggested that telomere shortening could contribute to the replicative exhaustion of haematopoietic stem cells observed after serial transplantation of haematopoietic stem cells (Allsopp et al., 2003; Chen et al., 2004).

Our previous *in vitro* studies, performed on colony forming unit granulocytes macrophages (CFU-GM), showed that $iAs^{III}$ is able to exert ubiquitous effects depending on the concentration and the sex of the donor of the cord blood cells (Ferrario et al., 2008). We observed a decrease in the proliferation of haematopoietic progenitor cells in both sexes at concentrations of about 1μM, but an increase in proliferation at 0.0001 μM only in female progenitor cells.

For this reason, the aim of the present study was to further elucidate whether the previously observed effect in progenitor cells could be related to modulation of telomerase expression and telomere length, and the association with cell viability, apoptosis, reactive oxygen species formation, and the expression of two oncogenes, known to interfere with proliferation pathways (*ras* and *myc*). We used both male and female donor cells, to increase our understanding of gender difference in the mode of action of $iAs^{III}$.

## 3C.3. Materials and Methods

### *3C.3.1. Source of human progenitor cells*

The mononucleated cell fraction isolated from male and female human umbilical cord blood (huUCB), was supplied frozen by Stem Cells Technologies (Vancouver, British Columbia, Canada) and used as a source of stem cells. For every single experiment on cell viability, PCR, Western Blot, telomere length detection, ROS, a vial coming from one single donors for male and female was used, and each experiment was performed in triplicate. Three independent experiments were performed. Immediately before use, the cells were quickly thawed at 37° C, swirling gently for 1-2 minutes. After wiping the outside of the vial with 70% ethanol, the cell suspension was transferred, drop by drop, to 10 ml of IMDM medium + Glutammax (Gibco Company; Grand Island, USA) containing 10% Foetal Bovine Serum (FBS) (Gibco). It was then centrifuged at 300 g for 10 minutes at room temperature. The supernatant was removed and the cells gently resuspended in IMDM 30% FBS. HuUCB cells were cultured in 5% $CO_2$ at 37˚C in IMDM medium. The medium was supplemented with 10% FBS and 1% human penicillin/streptomycin (P/S), human interleukin 6 (IL-6 50ng/ml), human interleukin 3 (IL-3 20ng/ml), human stem cells factor (SCF 20ng/ml), and human granulocyte-macrophage colony stimulating factor (GM-CSF 50ng/ml) (Sigma-Aldrich, S. Louis, Mo, USA).

### *3C.3.2. Cell treatment and viability*

Trivalent Inorganic Arsenic (Sodium (meta) arsenite [NaAso2], MW 129.91, abbreviated as $iAS^{III}$ (purity > 98 %) was supplied by Sigma-Aldrich (Sigma), and was solved in bidistilled water to a final concentration of $10^{-2}$M. This stock solution was freshly prepared for every test. Serial dilutions were prepared starting from the stock solution.

24 hours after cell seeding, the cells were counted by flow cytometry FACSaria (BD San Jose, Ca, USA) to assess cell viability with a solution of fluorescein isothiocyanate (AnnexinV-FITC) and propidium iodide (PI) (Sigma). When the cells viability was ≥ 80%, $iAs^{III}$ was added to the cultures to final concentrations of 0.0001 µM and 1 µM. The access to the primary human cells is rather limited and costly. Therefore, only a limited number of arsenic concentrations and exposure time could be tested. Preliminary experiments confirmed biological effects at the effective concentrations of 0.0001 and 1 µM on CFU-GM progenitors (Ferrario et al., 2008).. The concentration of 0.0001 µM was capable of increasing the clonogenic capacity of Granulocytes-Macrophages in female progenitors, whereas the concentration of about 1µM (1.33±0.43 µM for female and 1.22 ± 0.13 µM for male) was the concentration which inhibited 50% of colonies growth ($IC_{50}$ value) calculated for both genders after 14 days in culture. An even higher concentration, 10 µM (10 times higher than the $IC_{50}$), was used in real time PCR as a positive control to assess the telomerase mRNA expression down modulation.

### *3C.3.3. Apoptosis Measurement*

To determine the apoptotic effect of $iAs^{III}$ in human cord blood cells, we incubated HuUCB cells with a solution of Fluorescein Isothiocyanate labelled with Annexin V (AnnexinV-FITC) and Propidium Iodide (PI). Cells were treated with $iAs^{III}$ at concentrations of 0.0001 and 1 µM for 24 hours and 7days. The control samples and the treated cells were harvested, centrifuged, washed twice in PBS buffer and resuspended in Binding Buffer (1X, Sigma-Aldrich) diluted 1:10 to $10^6$cells/ml. HuUCB cells were then stained for 10 min with 5 µM FITC Annexin V- and 10 µM propidium iodide (Sigma-Aldrich).

The percentage of apoptotic cells was determined by FITC positive staining, and necrotic cells were detected by PI positive staining. For each sample, 10000 events were recorded using a FACSAria cell sorter and the percentage of live, apoptotic, and necrotic cells were calculated using Flowjo software (Flowjo Tree star Inc., Ashland, OR, USA).

Furthermore, in order to assess the role of glutathione (GSH) in $iAs^{III}$ detoxification, an inhibitor of GSH, bovine buthionine sulfoximine (BSO) (Sigma) was added to the control and treated cells at a concentration of 100 µM. Moreover to assess the role of oxigen radicals (ROS) generated by

$iAs^{III}$ on cells growth, apoptosis and necrosis, the ROS scavenger 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (Sigma) was added to the control culture and treated cells at a concentration of 250 μM. Cell viability, and apoptosis were assessed to determine the effects of BSO and DMPO on $iAs^{III}$ treated cells.

### *3C.3.4. Measurement of Telomerase Expression, Ras, Myc by Real Time PCR*

Telomerase expression in human cord blood cells was measured using "Quantitative Real-Time PCR" (TaqMan, Applied Biosystems, Foster City, CA, USA) - HuUCB cells were treated for 30' minutes, for 2, 15, and 24 hours and for 7 and 14 days with $iAs^{III}$, at concentrations of and 0.0001, 1, 10 μM. After 7 days in culture, cells were collected, centrifuged, and then the old medium was replaced with fresh medium. Arsenic was also added to the treated samples.

In order to evaluate if the modulation on telomerase expression might be mediated by perturbation in the proliferative pathways, two well known oncogenes *ras* and *myc* were also investigated. For this purpose, the cells were treated for 6 and 24 hours and for 14 days at concentrations of 0.0001 and 1 μM and then mRNA expression was evaluated. Cells were then lysed using the lysing RLT buffer (Qiagen, Milan, Italy) + 1% β-mercaptoethanol and used for RNA extraction using a Qiagen Micro Kit, following the manufacture's protocol.

500 ng of total RNA was reverse transcribed using a mix (1:1) of random hexamer and oligo dT primers (Promega, Madison, USA) and Moloney Murine Leukaemia virus reverse transcriptase (M-MLV, Promega). The quantity of RNA was measured by a photometer, and the quality of RNA was checked using the Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA, USA). The mRNA levels of *htert, ras*, and *myc* were analysed by real time-PCR (TaqMan primers & probes). The relative gene expression profile was normalised against the three most stable housekeeping genes (calculated with the geNorm software, Primer Design Ltd, Southampton UK), *tata, b2m, actb*, as suggested by Vandesompele et al., 2002. All primers and probes were obtained from Applied Biosystems ("Assay on demand" gene expression products). Primers for these genes were designed and labelled at the 5'-end with a reporter dye (FAM) and a quencher dye (TAMRA) at the 3'-end. All experiments were performed in triplicate in 96-well plates using TAQMAN Universal Master Mix (Applied Biosystems).

Real-time PCR amplification was performed on a Gene Amp 7000 Sequence Detection System according to the manufacturer's protocol. PCR conditions were 50°C for 2 min, 95°C for 10min, then 40 cycles at 95°C for 15 s, and 60°C for 1 min. Fluorescence data were processed and analysed with ABI PRISM Sequence Detection software (version 1.6 software, Applied Biosystems). Quantification of the PCR assay was based on relative quantification ($2^2$CT

method). CT is the "Cycle Threshold" when the system begins to detect the increase in the fluorescent signal associated with an exponential growth of PCR product during the log-linear phase. The average CT values from each experiment were calculated, and the results were graphed with the corresponding standard deviation indicated with error bars in the figures. Briefly, the CT values indicate the fractional cycle number for which the amount of amplified target reaches a fixed threshold. This threshold was set for each primer set. The difference (ΔCT ) between the CT of the target gene (CT t) and the reference gene (CT r) depends on the RNA relative copy number between the target and the reference gene. Standard curves were generated by using 10-fold serial dilution of pooled cDNA with five measuring points in order to verify the efficiency of the PCR. The linear correlation coefficient ($r^2$) was between 0.991 and 0.999, and the PCR efficiency between 89,7 and 107,3%.

***3C.3.5. Western Blotting Analysis***

HuUCB cells were seeded at 15 x $10^6$ cells in 75$cm^2$ cell culture flasks (BD) and grown under the same conditions as described above. Cells were treated with $iAs^{III}$ at the concentration of 0.0001 and 1 μM for 24 hours and 7 days. After treatment, cells were washed twice in PBS and collected as dried pellet. For huUCB cells lysing and protein extraction, the pellet was incubated in Ripa–like buffer (50mM Tris pH 7.4, 0.1% SDS, 250mM NaCl, 2mM dithiothreitol (DTT), 0.5% Nonidet P-40) (Bio-Rad protein assay kit, Bio-Rad, Milan, Italy) with protease inhibitors (1 μg/ml leupeptin, 1 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotitin, 1 μg/ml pepstatin A) (Sigma) for 30min on ice. After centrifugation (15 min at 13000rpm, at 4ºC) the supernatant was collected and protein quantified using the Lowry test (Bio-Rad). 50ug of protein for each sample were resolved by 10% NuPAGE Bis-Tris Gel (Invitrogen, LifeTechnologies, San Diego, USA) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Massachusset, USA). The membranes were then incubated in blocking buffer (5% non-fat milk in PBS and 0.1% Tween 20) for 1 hour at room temperature. The primary antibodies used were: anti hTERT 1ug/ml (Rockland Immunochemicals, Inc. Gilbertsville, PA, USA) and GAPDH 1μg/ml (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). GAPDH housekeeping expression was simultaneously estimated in each sample as the internal control by Western blotting technique. The primary antibody was diluted 1:500 following the manufacture's protocol. The membranes were incubated with the primary antibody overnight at 4 ºC. After wash in rinsing buffer (PBS and 0.1% Tween 20) for 40 minutes (changed every 10 minutes) at room temperature, membranes were incubated with the secondary antibody. The secondary antibody used was horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1/3000 (Chemicon, San

Diego, CA, USA). Membranes were incubated with Immobilon Western, chemiluminescent HRP substrate (Millipore, MA, US) for 5 minutes. Then images were captured using Gel Logic 2200 Imaging System and analysed using Kodak Molecular Imaging Software (Eastman Kodak Company, Rochester, USA).

### *3C.3.6. Telomere Length Measurement*

To determine the telomere length, the "Telomere PNA/Kit FITC probe (Dako, DK-2600 Glostrup, Denmark) was used. PNA probe is a synthetic DNA/RNA analogue capable of binding to DNA/RNA in a sequence-specific manner. In the PNA probe, the sugar phosphate backbone has been replaced by a neutral peptide/polyamide backbone. This PNA probe is superior to DNA probes in terms of sensitivity and specificity. Moreover the PNA probe is highly resistant to degradation by DNases, RNases and Proteinases. The fluorescence intensity of the cells is directly correlated to the length of telomeres.

Cord blood cells were harvested in IMDM + 10% FBS + 1% p/s. Preparations for FACS analysis were made by exposing the cells to $iAs^{III}$ at concentrations of 0.0001 and 1 μM for 24 hours, and 7 days. Cells were counted, and then for each sample 2 x $10^6$ test cells were added to 2 x $10^6$ control cells. As control cells, we used the human 1301 T-cell leukaemia line (American Type Culture Collection, ATCC, Manassas, VA, US), which is tetraploid, so easily distinguished from diploid HuUCB cells and has very long telomeres (> 30 kilobases).

HuUCB cells were then washed twice in PBS. Then 300μl of "Hybridisation solution" (containing 70% formamide) was added to control tubes, and 300μl of "Telomere PNA Probe/FITC in Hybridization Solution" (containing 70% formamide) to the test tubes. HuUCB cells were incubated in a heating block at 82˚C for 10 minutes and then the tubes were placed over night in the dark at room temperature for hybridization.

The day after, the cells were washed twice in washing solution (PBS 1X), stained with staining solution (containing Propidium Iodide and RNase diluted 10:1 in water). After 4-5 hours cells were analysed with a FACSaria cell sorter using a logarithmic scale for probe fluorescence and linear scale for DNA staining. Telomere fluorescence of at least 10.000 $G_o/G_1$ gated events was measured. The Relative Telomere Length (RLT) was calculated as the ratio between the telomere signal of each HuUCB cells sample and the control cells (human 1301) with the correlation for the DNA index of $G_{0/1}$ cells.

### *3C.3.7. Reactive Oxygen Species Analysis*

To assess the Reactive Oxygen Species (ROS) formed by $iAs^{III}$ exposure in human cord blood cells cultures, the ROS detection Reagent (Molecular Probes, Invitrogen) was used; Chemically reduced and acetylated forms of 2',7'-di-chlorofluorescein (DCF) are non-fluorescent until the acetate groups are removed by intracellular esterase and oxidation occurs within the cell. Oxidation of these probes can be detected by monitoring the increase in fluorescence with a fluorometer, using excitation source and filters for FITC.

Shortly before performing the experiments the ROS detection Reagent was reconstituted with 100% ethanol to make a concentrated stock solution. After 24 hours or 7 days of $iAs^{III}$ exposure at a concentration of 0.0001 or 1 µM, with or without BSO or DMPO, cells were collected and washed twice in PBS and resuspended in pre-warmed PBS containing the probe at the final concentration of 5 µM. The cells were then incubated for 30 minutes at 37 ºC. Thereafter, the medium was removed and the cells were returned to pre-warmed IMDM, mixed with 10% FBS to allow a short recovery time for the cellular esterases to hydrolyse the acetate groups and render the dye responsive to oxidation. Positive controls for oxidative stress were created by exposing the HuUCB cells to hydrogen peroxide ($H_2O_2$) at the concentration of 100 µM.

After incubation, the cells were seeded in 12-well plates (Corning, Lowell, MA, USA) at 5 x $10^5$ cells/well and analysed using a Fluoroskan Ascent (Thermo Scientific, Barrington IL, USA) microplate fluorometer, excitation wavelength of 485 nm and fluorescence emission wavelength of 535 nm. To assure that the added $iAs^{III}$ would not quench the dye, the absorbance spectrum of the compound was examined to make sure that the absorbance peak did not overlap with either the excitation or the emission peak of the oxidized dye. The blank value was subtracted from each sample and results were expressed as percentage of relative fluorescence units (% RFU).

### *3C.3.8. Data Analysis*

Statistical analysis was performed using the GraphPad Prism 5 software (Prism Software Solutions, Inc. MN, US). Data obtained by real time PCR, Apoptosis, Western blot, ROS, and telomere length analysis were expressed as means of three independent experiments performed in duplicate ± standard error of the mean (SEM). The statistical comparison were calculated with raw data.

All data were initially analysed to prove the Gaussian distribution with the D'Agostino and Pearson normality test. For each time points to evaluate the statistical significance of comparisons between different concentrations, and genders a two-way analysis of variance (ANOVA) followed by a post-hoc Bonferroni's multiple comparison test was used. Two-way

ANOVA works by comparing the differences among group means with the pooled standard deviations of the groups. Values of $p<0.05$ were considered statistically significant (indicated in tables and figures as *). Values of $p<0.01$ were considered highly significant (indicated in tables and figures as **). Significance between male and female at <0.05 is indicated in the graphs as #.

## 3C.4. Results

### 3C.4.1. *Arsenic-induced Apoptosis*

To assess the toxic effect on HuUCB cells viability, we investigated the capacity of $iAS^{III}$ to increase the production of apoptotic and necrotic cells. The $iAS^{III}$ induced apoptosis and necrosis of male and female human cord blood cells was evaluated by FITC-Annexin-V/PI, using a flow cytometry as described above, and the results are summarised in the tables 1C and 2C. After 24 hours of $iAS^{III}$ exposure at both the concentration of 0.0001 and 1 μM no modulation of cell viability was observed compared to control cells of respective sexes.. After 7 days of exposure to $iAS^{III}$ at 0.0001 μM, we did not see toxicity in either sex. An increase in the number of apoptotic and necrotic cells was observed in both male and female samples, exposed to 1 μM (Table 2C).

When 100 μM of BSO, an inhibitor of GSH synthesis, was added to the culture treated with $iAS^{III}$ at the concentration of 0.0001 μM, we did not observe an increase in the production of apoptotic cells in both genders after 24 hours of exposure. After 7 days of culture, an increase in the apoptotic cells was observed in female but not in male. On the contrary, when BSO was added to the culture treated with $iAS^{III}$ at the concentration of 1 μM we observed a significant increase of apoptotic cells of both male and female after both 24 hours and 7 days of cultures. When DMPO a ROS scavenger was added to the cultures at the concentration of 250 μM, it tended to protect against $iAS^{III}$ induced toxicity, increasing the cell viability of samples treated with 1 μM. These data suggest a highly toxic effect of 1μM $iAS^{III}$ on cell viability after 7 days, increasing the apoptotic and necrotic cells in both genders. Moreover, GSH is effective in the detoxification pathways of HuUCB cells, in fact when BSO was added at the cultures, an increase in the toxic outcome of $iAS^{III}$ was observed even at the concentration of 0.0001 μM in females.

| | Female 24h | | | Male 24h | | |
|---|---|---|---|---|---|---|
| **Sample** | **Live** | **Apoptotic** | **Necrotic** | **Live** | **Apoptotic** | **Necrotic** |
| | **%** | **%** | **%** | **%** | **%** | **%** |
| **Control** | **84,9** | **8,1** | **6,4** | **80,6** | **9,1** | **7,7** |
| ± *sd* | *2,8* | *2,4* | *0,2* | *2,1* | *0,9* | *1,0* |
| **Control BSO** | **80,9** | **10,6** | **7,9** | **81,8** | **8,3** | **7,9** |
| ± *sd* | *1,3* | *2,0* | *0,2* | *1,6* | *0,5* | *0,4* |
| **Control DMPO** | **80,1** | **12,5** | **7,1** | **81,1** | **8,6** | **8,6** |
| ± *sd* | *1,5* | *1,8* | *1,1* | *1,3* | *1,1* | *0,5* |
| **iAs 0.0001µM** | **84,7** | **8,2** | **6,5** | **80,6** | **9,2** | **9,0** |
| ± *sd* | *3,2* | *2,2* | *1,6* | *4,5* | *2,2* | *2,3* |
| **iAs 0.0001µM BSO** | **77,3** | **11,4** | **7,1** | **79,2** | **10,6** | **7,5** |
| ± *sd* | *1,1* | *1,8* | *0,6* | *1,6* | *0,6* | *1,2* |
| **iAs 0.0001µM DMPO** | **81,3** | **10,2** | **8,3** | **80,3** | **10,3** | **8,5** |
| ± *sd* | *3,9* | *2,1* | *1,6* | *1,8* | *0,6* | *0,9* |
| **iAs 1µM** | **78,4** | **11,6** | **9,0** | **78,2** | **11,0** | **10,2** |
| ± *sd* | *3,7* | *3,2* | *1,6* | *2,5* | *0,4* | *2,7* |
| **iAs 1µM BSO** | **70.6 *** | **15.6 *** | **12.6 *** | **75,3** | **14.2 *** | **9,0** |
| ± *sd* | *4,5* | *3,1* | *2,8* | *2,3* | *0,9* | *1,0* |
| **iAs 1µM DMPO** | **79,1** | **10,7** | **9,6** | **80,4** | **10,4** | **8,1** |
| ± *sd* | *2,8* | *1,4* | *2,1* | *1,0* | *0,5* | *0,8* |

**Table 1C. Flow cytometry analysis of cord blood cells exposed to $iAs^{III}$ for 24 hours**.
The table shows the cell viability, apoptosis, and necrosis of female and male cord blood cells. Three independent experiments were performed and the results are given as mean ± standard deviation (sd). Statistical significance between samples and controls is expressed as $^{*}p < 0.05$.

| | Female 7 days | | | Male 7 days | | |
|---|---|---|---|---|---|---|
| **Sample** | **Live** | **Apoptotic** | **Necrotic** | **Live** | **Apoptotic** | **Necrotic** |
| | **%** | **%** | **%** | **%** | **%** | **%** |
| **Control** | **90,1** | **4,8** | **4,0** | **88,1** | **6,0** | **5,2** |
| ± *sd* | *2,4* | *1,0* | *1,1* | *5,3* | *2,6* | *2,7* |
| **Control BSO** | **89,6** | **5,1** | **5,0** | **87,5** | **6,0** | **6,2** |
| ± *sd* | *2,1* | *1,1* | *1,5* | *6,2* | *2,7* | *3,3* |
| **Control DMPO** | **86,7** | **7,4** | **5,3** | **83,9** | **8,8** | **5,9** |
| ± *sd* | *1,9* | *1,4* | *0,5* | *2,0* | *0,5* | *0,3* |
| **iAs 0.0001µM** | **90,5** | **4,9** | **4,2** | **88,5** | **6,0** | **5,4** |
| ± *sd* | *3,3* | *1,7* | *1,4* | *4,2* | *1,6* | *2,6* |
| **iAs 0.0001µM BSO** | **81,1*** | **11,1*** | **5,3** | **81,8** | **7,3** | **6,0** |
| ± *sd* | *3,4* | *1,8* | *1,5* | *3,2* | *0,8* | *1,6* |
| **iAs 0.0001µM DMPO** | **87,0** | **6,7** | **5,8** | **85,4** | **6,2** | **5,5** |
| ± *sd* | *1,7* | *0,9* | *0,9* | *1,7* | *1,2* | *0,7* |
| **iAs 1µM** | **56.3 *** | **22.5 *** | **16.7 *** | **56.3 *** | **21.1 *** | **18.9 *** |
| ± *sd* | *2,8* | *2,1* | *1,8* | *5,1* | *4,0* | *2,1* |
| **iAs 1µM BSO** | **50.4 *^** | **24.4 *^** | **20.9 *** | **51.9 *^** | **25.6 *^** | **20 *** |
| ± *sd* | *2,1* | *0,8* | *1,6* | *2,1* | *0,4* | *1,4* |
| **iAs 1µM DMPO** | **60.5 *^** | **19.6 *^** | **18 *** | **60 *^** | **20.8 *^** | **16.9 *** |
| ± *sd* | *4,6* | *2,1* | *1,7* | *2,4* | *2,1* | *2,1* |

**Table 2C. Flow cytometry analysis of cord blood cells exposed to $iAs^{III}$ for 7 days.**
The table shows the cell viability, apoptosis, and necrosis of female and male cord blood cells. Three independent experiments were performed and the results are given as mean ± standard deviation (sd). Statistical significance between samples and controls is expressed as $^{*}$ $p < 0.05$. Statistical significance between samples treated with $iAs^{III}$ 1 µM + BSO and $iAs^{III}$ 1 µM + DMPO is expressed as ^ $p < 0.05$.

### *3C.4.2. Effect of Arsenic on Telomerase, Ras and Myc mRNA Expression*

To investigate the genotoxic effect of $iAs^{III}$ we assessed whether $iAS^{III}$ could interact with the normal expression of telomerase and oncogenes at the concentrations used. The telomerase mRNA expression (*htert*) was assessed from 30 minutes up to 2 weeks of $iAs^{III}$ exposure. The *htert* expression in control cells increased over time, with the maximum expression after 7 days in culture. Then *htert* decreased to half the maximum values at 14 days (table 3C). As described in figure 1C-1A, after 30 min of $iAs^{III}$ exposure, a slight down-modulation was observed at both 1 and 10 μM, whereas the lower concentration of 0.0001 μM significantly increased *htert* in both sexes. After 2 hours $iAs^{III}$ exposure (figure 1C-1B) a significant down-modulation was found at the concentrations of 1 and 10 μM in both female and male progenitors. Notably, at the concentration of 0.0001 μM an up-modulation of *htert* was observed for both sexes, but significant only for female. After 15 and 24 hours exposure (figure 1C-1C and 1D) *htert* was down modulated at concentrations of 1 and 10 μM, whereas no significative modulation was found at the concentration of 0.0001 μM.

After 7 and 14 days of culture in the presence of cytokines, two different cell populations were identified: one of adherent "mesenchymal" cells, and the second one of "floating" cells. The adherent population showed very low *htert* with respect to non-adherent cells (figure 1C-1E and F). After 7 and 14 days of treatment with $iAs^{III}$ a dose-response decrease in *htert* in the suspended population was observed for the concentrations of 1 and 10 μM. An increase in *htert* was observed at the concentration of 0.0001 μM after 7 days for both sexes (figure 5C). Notably, after 14 days of $iAs^{III}$ exposure, the concentration of 0.0001 μM gave a significant increase in *htert* only in female progenitors (figure 6C).

To evaluate if $iAS^{III}$ toxicity might involve a modulation of mRNA expression of *ras* and *myc* oncogens, cells were exposed to $iAs^{III}$ at 0.0001 or 1 μM for three different time periods: 6, and 24 hours and 14 days. The low concentration, 0.0001 μM, induced expression of *ras* mRNA at each time points in female progenitors (figure 2C-2A). In male, the *ras* mRNA expression was significantly modulated only at 24hours of $iAs^{III}$ exposure. Similarly, *myc* was significantly up modulated at the concentration of 0.0001 μM, but these changes were only observed after 14 days of exposure (figure 2C-2B). These results suggest that *htert* is differently expressed in cells belonging to different lineages, and this pathway of expression is conserved both in male and female. Moreover, at the concentration of 1 μM, $iAs^{III}$ is able to interact with the normal mRNA expression of *htert* without gender differences. On the contrary, we observed a gender differences with females being more sensitive in the up-modulation of oncogenes after low concentration $iAs^{III}$ exposure.

| Samples | Relative expression | SD ± | Relative expression | SD ± |
|---|---|---|---|---|
| | Female | | Male | |
| Ctrl 0 h | **1,00** | *0,11* | **0,85** | *0,26* |
| Ctrl 24 h | **1.95 *** | *0,10* | **1,14** | *0,20* |
| Ctrl 7 days | **10.22*** | *0,34* | **7.48*** | *0,65* |
| Ctrl 14 days | **4.70*** | *0,36* | **3.56*** | *0,67* |

**Table 3C. Real time PCR analysis of untreated cord blood cells.**
The table shows the relative mRNA basal expression of htert of female and male cells at time 0 hours, 24 hours, 7 days, and 14 days in culture. Three independent experiments were performed and the results are given as mean ± standard deviation (sd). Statistical significance between samples is expressed as $^{*}p < 0.05$.

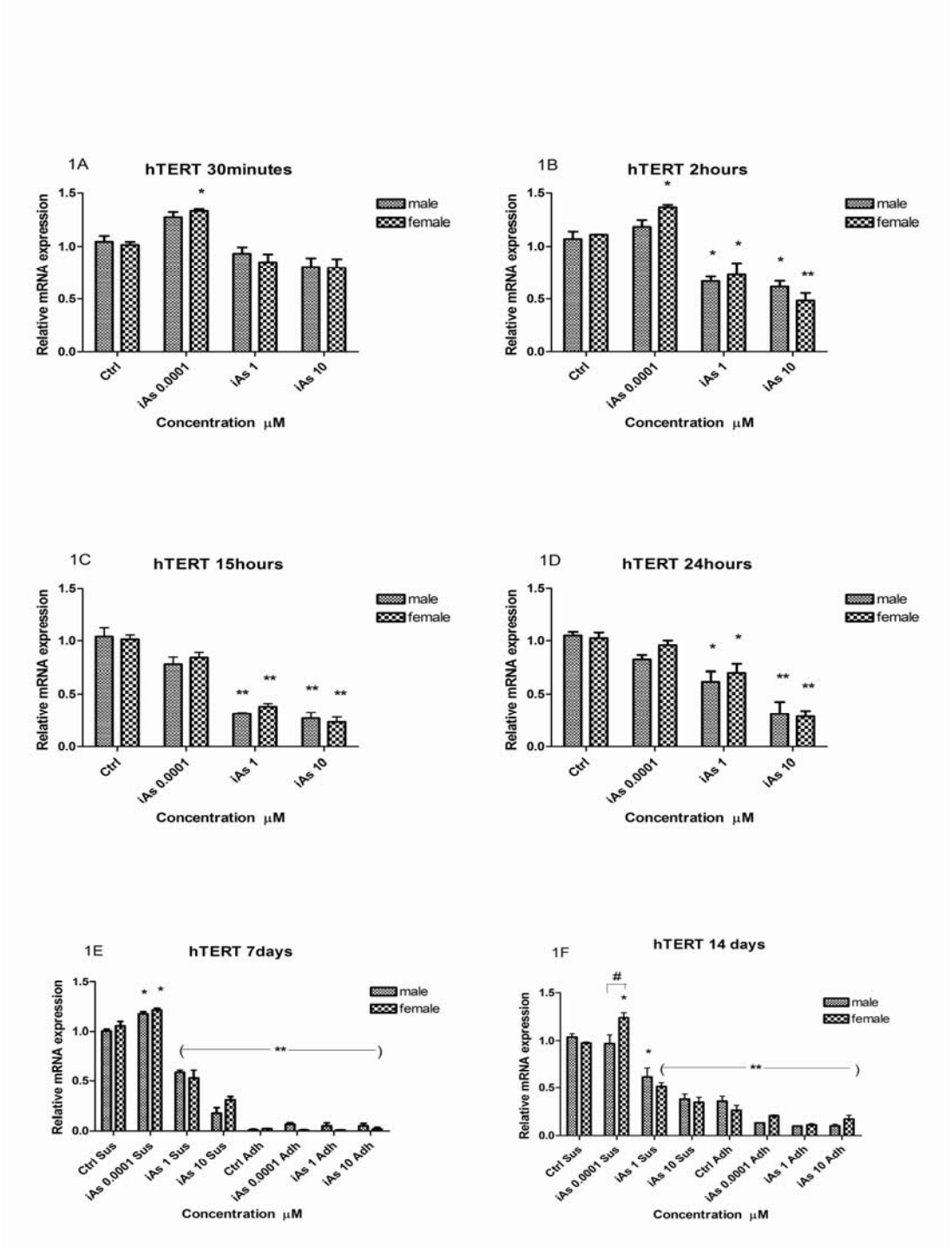


**Figure 1C. Real Time PCR analysis of cord blood cells exposed to iAsIII.**
Relative hTERT mRNA expression at time 30 minutes (A), 2 hours (B), 15 hours (C), 24 hours (D), 7 days (E) and 14 days (F) of iASIII exposure. Concentrations are expressed in μM. At 7 and 14 days of exposure "Sus" means "suspended population", Ad represents "adherent population". Statistical significance between samples and controls is expressed as * $p < 0.05$. High statistical significance between samples and controls is expressed as ** $p < 0.01$. Statistical significance between female and male is expressed as # $p<0.05$. In brackets is expressed the statistical significance of all the samples compared to controls.

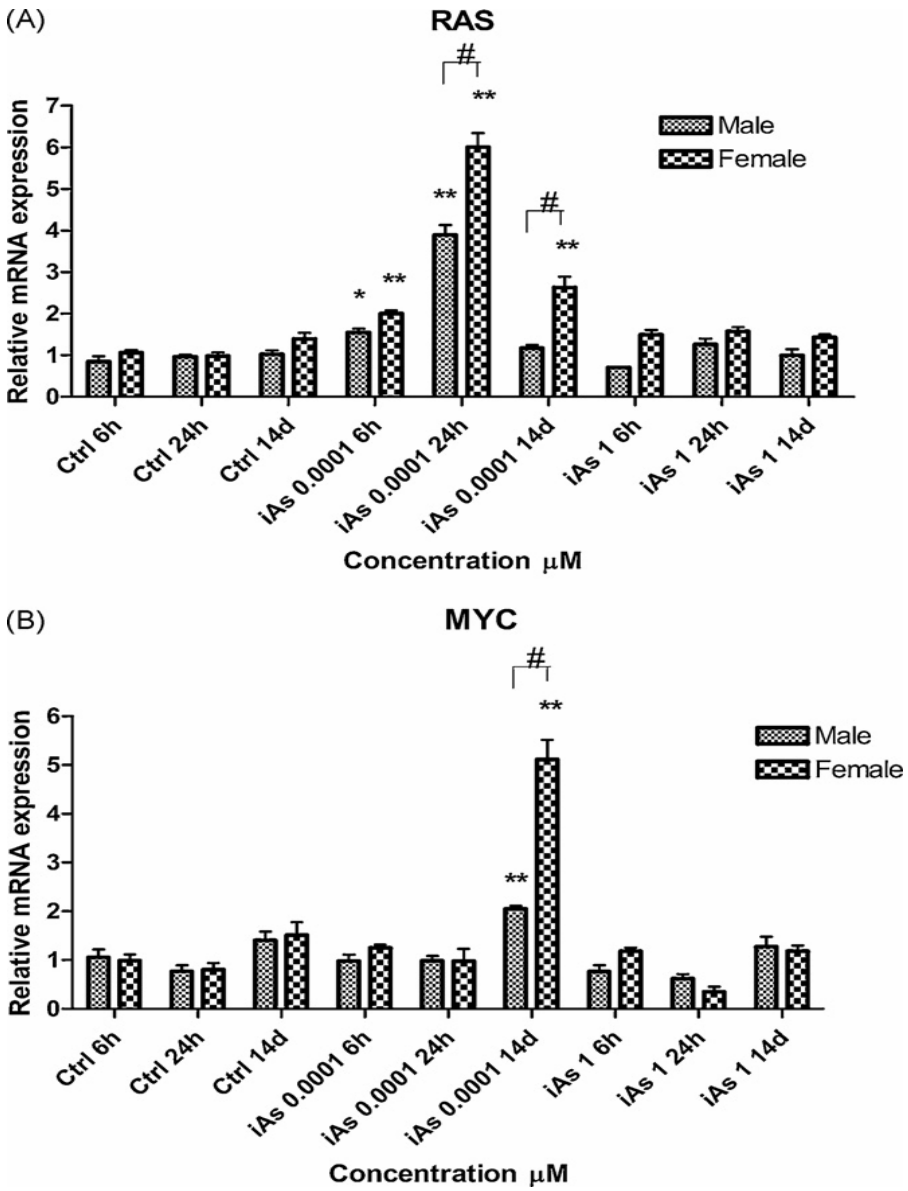


**Fig. 2C.** Real-time PCR analysis of cord blood cells exposed to iAsIII . (A) Relative ras mRNA expression at time 6 h, 24 h, 14 days of iASIII exposure. (B) RelativemycmRNA expression at time 6 h, 24 h, 14 days of iASIII exposure. Concentrations are expressed in μM. Statistical significance betweensamples and controls is expressed as *$p < 0.05$. High statistical significance between samples and controls is expressed as **$p < 0.01$. Statistical significance between female and male is expressed as #$p < 0.05$.

### *3C.4.3. Effect of Arsenic on telomerase Protein Expression (Western Blotting)*

Since $iAS^{III}$ was able to interact with the basal *htert* mRNA expression, we investigated whether $iAS^{III}$ could also modulate the basal expression of the hTERT protein. The expression of hTERT protein was measured in human cord blood cells after exposure to $iAs^{III}$ using the Western Blot assay. Protein analysis revealed that hTERT was expressed in both genders in control samples after 24 hours of incubation. Treatment with $iAS^{III}$ 0.0001 μM caused a slight decrease in the expression of telomerase in both male and female progenitors (figure 3C). After treatment with 1 μM $iAs^{III}$, telomerase expression had decreased to a major extent both in female and male progenitors.

After 7 days in culture, the protein was almost not expressed in control cells of the adherent population, whereas the protein was highly expressed in control samples in the "suspended" fraction of cell population of both male and female, suggesting that telomerase is expressed differently in specific cell lineages. Moreover, as shown in figure 5C, in female cells hTERT expression was stimulated by 0.0001 μM $iAs^{III}$ compared to the control cells, whereas in male cells no significant modulation was observed. After 7 days of exposure to $iAS^{III}$ at 1 μM, hTERT was down-modulated in the "suspended" cell populations of both sexes (figure 4C). No expression of hTERT was found in the adherent population after exposure to 1 μM (data not shown). These results suggest that $iAs^{III}$ exerts its toxicity on hTERT expression both at the transcriptional and translational level.

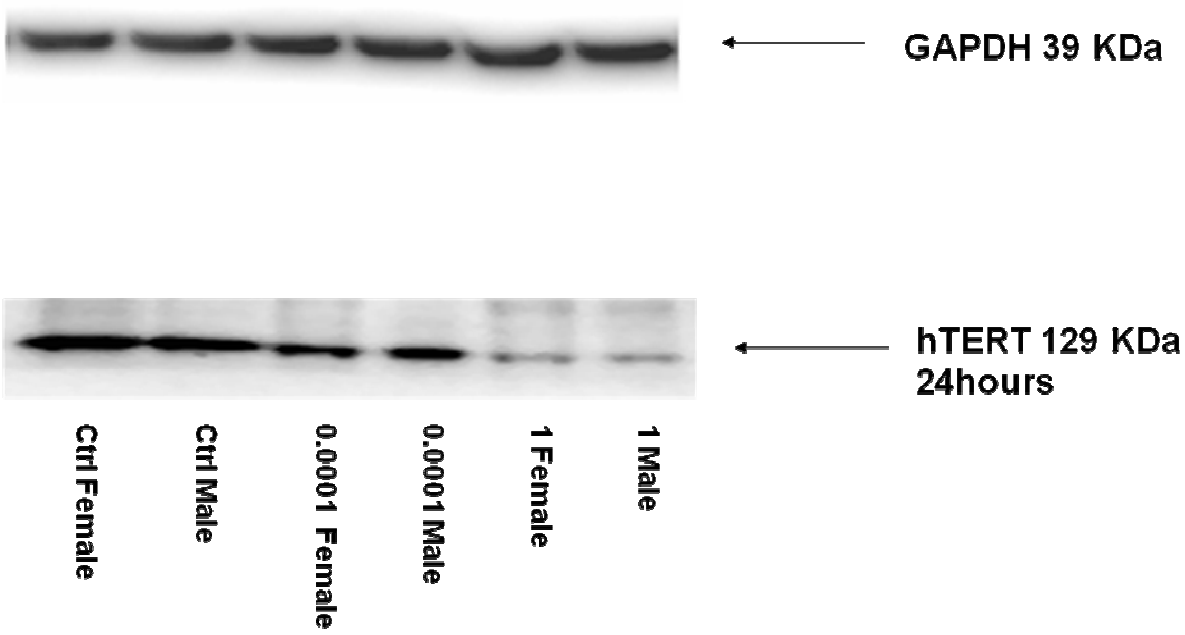


**Figure 3C. Western blot analysis of cord blood cells exposed to $iAs^{III}$ for 24 hours.**
The figure shows hTERT protein expression (MW 129 KDa) in female and male control cells and after exposure to $iAS^{III}$. hTERT expression was normalised against GAPDH housekeeping protein (MW 39 KDa). Concentrations are expressed in μM.

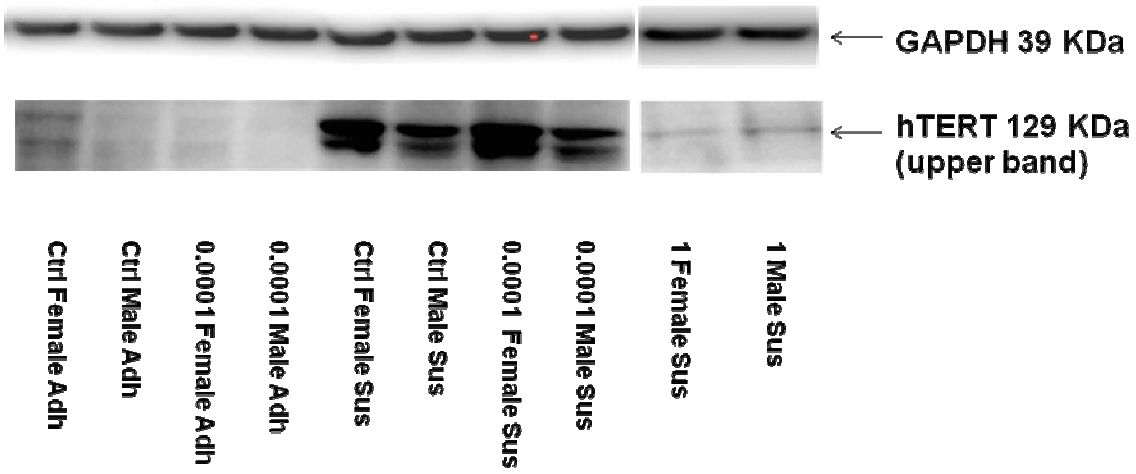


**Figure 4C. Western blot analysis of cord blood cells exposed to $iAs^{III}$ for 7 days.**
Western blot analysis of hTERT protein expression (MW 129 KDa) in female and male control cells and after exposure to $iAS^{III}$. hTERT expression was normalised against GAPDH housekeeping gene (MW 39 KDa). Ad = Adherent population, Sus = Suspended population. Concentrations are expressed in μM.

### *3C.4.4. Arsenic Effect on Telomere Length*

To assess a potential genotoxicity of $iAS^{III}$ in cord blood cells, we measured the effect of $iAs^{III}$ on telomeres length (TL) by flow cytometry using PNA probes as described above. The human leukaemia cell line 1301 was used as the control cell line, because it has very long telomeres and because it is tetraploid, thereby easily distinguished from diploid cord blood cells. Telomere length was then evaluated after 24 hours and 7 days of treatment with $iAs^{III}$ at 0.0001 and 1 μM. The results (summarised in figure 5C) show that after 24 hours of culture, the telomere length of untreated cord blood cells are 18.7 $\pm$ 1.1 % and 18.0 $\pm$ 1.9 % of that in 1301cells in male and female , respectively. After 24 hours of $iAs^{III}$ exposure at 0.0001 μM, a slight increase in the telomere length was observed in both sexes (21.8 $\pm$ 0.5% and 21.1 $\pm$ 1.5% for males and females, respectively). The same increase was observed in both genders (20.7 $\pm$ 1.3% and 20.3 $\pm$ 1.3% for males and females, respectively) after exposure to 1 μM.

After 7 days in culture, telomere length decreased in the control cells of both donors (17.6 $\pm$ 2.6% and 17.4 $\pm$ 1.0% for males and females, respectively). The concentration of 0.0001 μM tended to increase the telomere length with respect to the control in both genders but this increase was significant only in female progenitors (18.8 $\pm$ 1.5% and 23.9 $\pm$ 0.6% for males and

females, respectively). At the concentration of 1 μM, a strong decrease in the telomere length was observed in both genders (12.5 ± 1.4% and 9.5 ± 0.6% for males and females respectively). These results suggest that the decrease of hTERT expression observed both at mRNA and protein level after exposure to 1 μM of $iAS^{III}$ is also able to interact with the normal process of telomere synthesis, by decreasing the expression of the telomere in both genders.

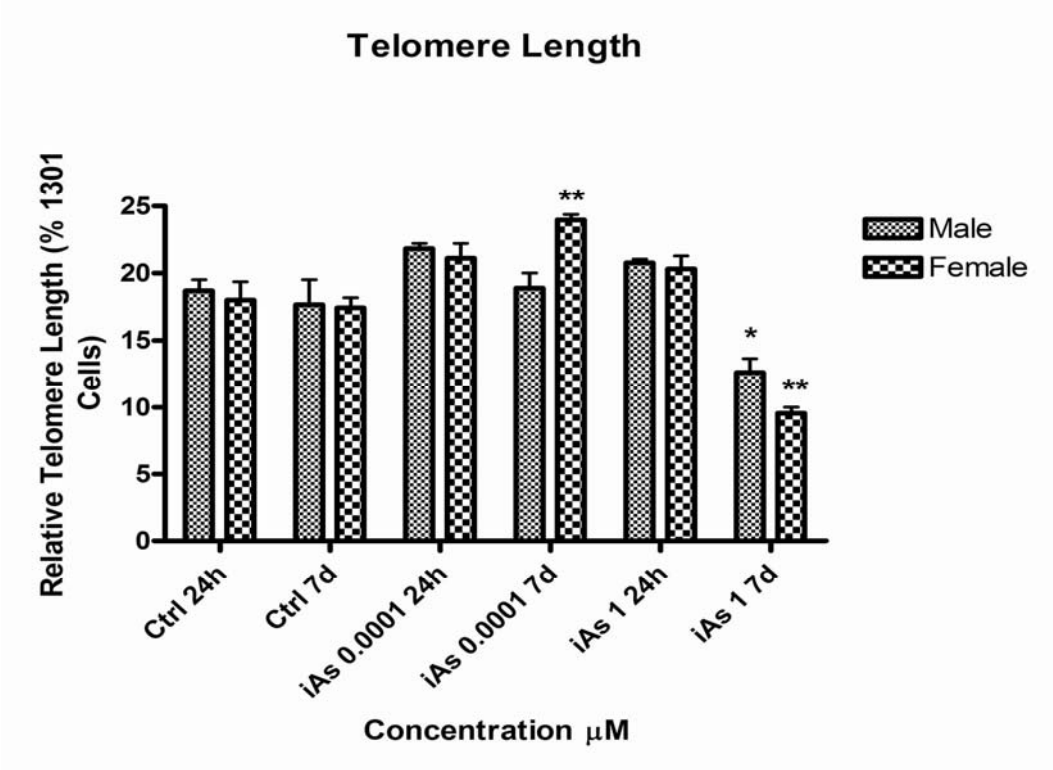


**Figure 5C. Flow Cytometry measurement of Telomere length after exposure to $iAS^{III}$ for 24 hours.**
Telomere length is expressed as percentage of telomere length in the control cells 1301 (set as 100% length). Concentrations are expressed in μM. Statistical significance between samples and controls is expressed as $^{*}p < 0.05$. High statistical significance between samples and controls is expressed as $^{**}p < 0.01$.

### *3C.4.5. Reactive Oxygen Species*

To investigate the potential mechanisms of $iAS^{III}$ toxicity on telomere length and telomerase expression, we put forward the hypothesis that $iAS^{III}$ might increase the production of oxygen radicals. The induction of reactive oxygen species (ROS) in male and female human cord blood cells was evaluated after 24 hours and 7 days of $iAS^{III}$ exposure at concentrations of 0.0001 and 1 μM. Moreover, as for cell viability analysis, we added either BSO or DMPO to cell cultures, in order to assess if these two compounds can modulate the formation of ROS in cord blood cells. The results are summarised in the figure 6C-6A and 6C-6B.

The unstained samples (controls) are expressed as relative fluorescence unit, corresponding to the value of the fluorescence in the control dishes. The RFU measured in unstained controls were 44.5 ± 2.8 and 58 ± 3.3 for male and female, respectively, at 24 hours, and 56.8 ± 4.9 and 63.8 ± 0.8 for male and female, respectively, at 7 days. After 24 hours of $iAS^{III}$ exposure for male cells we observed only a slight increase in the production of ROS, both for $iAS^{III}$ alone and $iAS^{III}$ + DMPO (both concentrations). On the contrary, when BSO was added to the culture, a strong increase in the production of ROS was observed at both concentrations (see figure 6C-6A). In females, we observed an increase in the production of ROS in all the samples tested, at both concentrations, but the production of ROS was more strongly modulated in the samples with BSO.

After 7 days of $iAS^{III}$ exposure at 1 μM, and in combination with BSO we observed a significant increase of ROS production both in male and female cells (figure 6C-6B). On the contrary, only in female samples treated with $iAS^{III}$ at the concentration of 0.0001 μM with added BSO, we observed an increase in the production of ROS. These results suggest that BSO increases the toxicity of $iAS^{III}$ by blocking the action of GSH, since BSO is a well known inhibitor of GSH synthesis. The samples with added DMPO in concert with $iAS^{III}$ at 0.0001 μM caused no significant variation with respect to the control. On the other hand, the concentration of 1 μM in addition with DMPO gave an increase in the production of ROS for both genders. However, in the samples treated with DMPO + $iAs^{III}$ 1μM the extent of toxicity was lower in comparison with samples treated with 1μM. The results show that female cells after 24 hours of exposure are more sensitive to the production of ROS after exposure to low concentration of $iAS^{III}$. The increased production of oxidative radicals after exposure to 1 μM of $iAS^{III}$ is likely to contribute to the toxic effect of $iAS^{III}$ on telomerase activity and telomere synthesis. Moreover, the results confirm that GSH is fundamental for $iAS^{III}$ detoxification, and when the concentration of GSH is reduced, the toxic outcome of $iAS^{III}$ is increased.

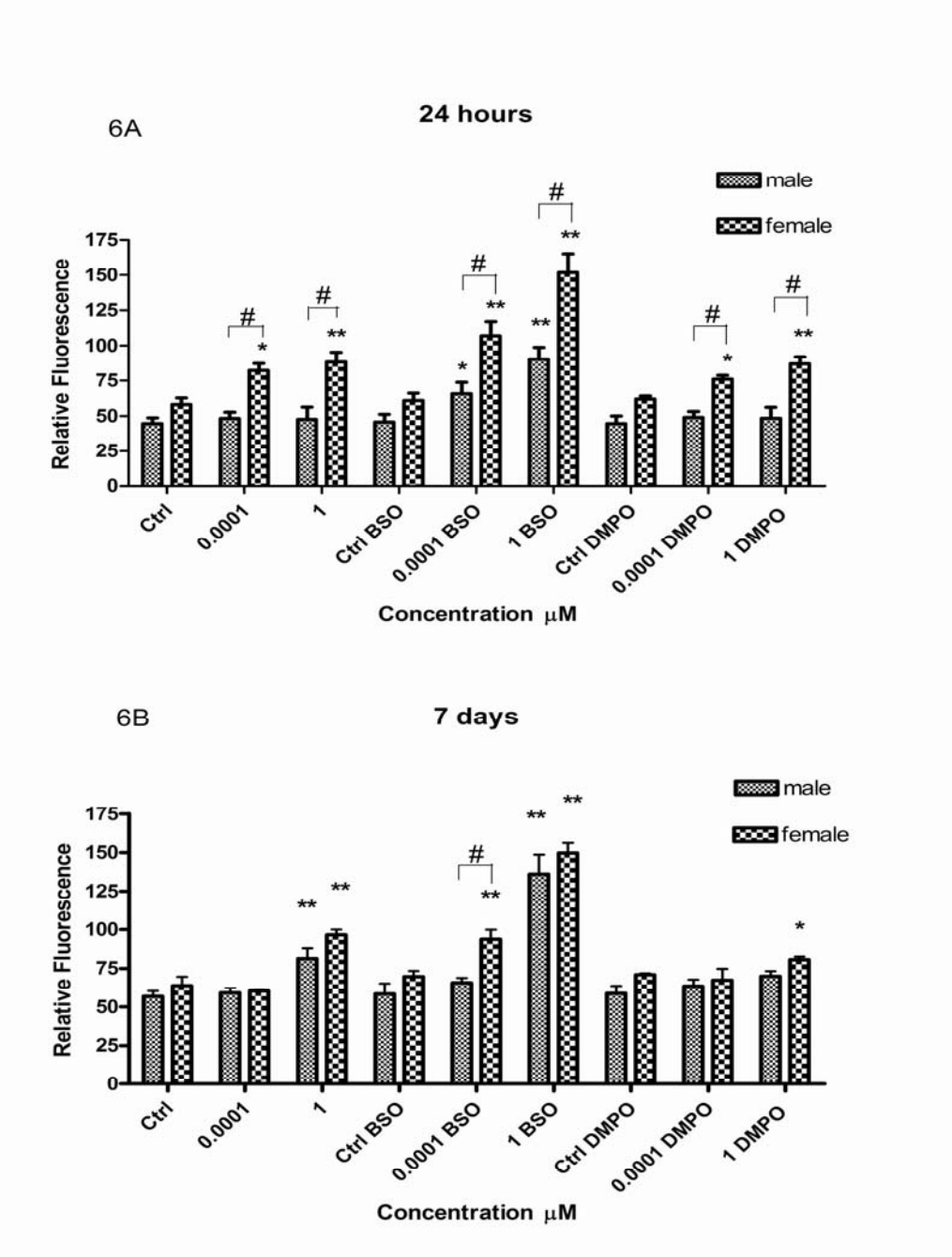


**Figure 6C. Fluorometric analysis of Reactive Oxygen Species after exposure to $iAs^{III}$ for 24 hours (A) and 7 days (B).** The fluorescence is expressed as relative fluorescence units. Concentrations are expressed in μM. Statistical significance between samples and controls is expressed as $^{*}p < 0.05$. High statistical significance between samples and controls is expressed as $^{**}p < 0.01$. Statistical significance between female and male is expressed as $^{\#}$ $p<0.05$.

### 3C.5. Discussion

The present study used a human cord blood cells *in vitro* model to elucidate mechanisms of arsenic toxicity on the hematopoietic system. The results revealed that the exposure to arsenic at environmentally relevant concentrations has profound effects on telomerase expression, telomere length, apoptosis and reactive oxygen species production (ROS). Moreover, these effects were also dependent on the sex of the cell donor.

At the low concentration of 0.0001µM, corresponding to the concentration at which arsenic has been shown to express stimulatory effects in female granulocytes-macrophages (GM) colonies (Ferrario et al., 2008), we found increased telomerase mRNA and protein expression, *myc* and *ras* oncogenes, and telomere length, with maintained cell viability. The concentration of 1 µM was chosen as the concentration able to inhibit the 50% of the colony forming unit GM for both sexes ($IC_{50}$ value). At this concentration, we found that arsenic inhibited the telomerase expression at mRNA and protein level, decreased telomeres length, and induced cell death in a ROS dependent manner in both genders.

Noteworthy, our results also demonstrate that arsenic acts in a different way in female and male human cord blood cells, the former being generally more sensitive to stimulation induced by low concentrations of arsenic. Indeed low concentrations of arsenic may have stimulatory properties, whereas higher concentrations may have apoptotic-like effects (Liao et al., 2004). Short exposures (24 hours or less) to very low arsenic concentrations were shown to significantly up-regulate both AP-1 and nuclear factor-κB (NF-κB) and DNA binding activity in human fibroblasts (Barchowsky et al. 1999; Hu et al., 2002). Furthermore, both NF-κB and AP-1 are implicated in the inducible expression of a wide variety of genes involved in oxidative stress and cellular response mechanisms (Allen and Tresini, 2000). Suppression of P21, G1 arrest, impaired DNA replication (Hainaut et al., 1995), enhanced cell proliferation and cell transformation, increased expression of oncogenes, such as epidermal growth factors (*egf*) and *c-myc* were also observed at low arsenic concentrations (Jiang et al., 1993; Trouba et al., 2000). Moreover, the oncogene *c-myc* has been shown to activate telomerase through a variety of sites (Wu et al., 1999), whereas the activation of *ras* and *myc* oncogenes and the over-expression of *htert* has been described as sufficient to drive normal human somatic cells into a tumorigenic state (Kendall et al., 2005). Zhang TC et al., 2003a performing a similar study showed that exposure to arsenic at the concentration of 0.1 µM increased the telomerase expression, with elongated telomere length, which in turn promoted cell proliferation, whereas higher concentrations of arsenic decreased telomerase, which promoted apoptosis in HL60 and HaCaT cells *in vitro*. The authors suggest that the carcinogenic effects of arsenic may be partly attributed to increase in

telomerase activity leading to promotion of cell proliferation and its anticancer effects by exerting oxidative stress and leading to telomeric DNA attrition and apoptosis. Moreover, Zhang Y et al., 2003 demonstrated that the increased cell senescence in response to arsenic is probably induced by an altered telomere state rather than by a loss of telomerase. However, in our experiments, we did not observe an increase in the proliferation rate of progenitor cells. The mechanism by which arsenic at low concentration induced increased telomerase expression and telomere length is still unclear. Some possible mechanism are the up-modulation of *ras* and *myc* oncogenes that in turn might up-regulate some others growth factors such as EGF or TGF-α that are known to be positive regulators of telomerase and telomere length (Yih et al., 2000). Our results may suggest that telomerase activation is not capable of increasing cell proliferation in primary cells, and other mechanisms are necessary to direct the cells towards "immortalised" growth properties.

Even though, most *in vitro* studies of arsenic have used supramicromolar concentrations, the use of non-cytotoxic, low concentrations better reflect the *in vivo* situation, and should be considered more relevant to chronic human exposure. Treatment with the concentration of 1 μM, in the range of arsenic blood levels measured in chronically exposed humans (Pi et al., 2000; Wu et al., 2003) decreased telomerase expression, and showed a loss of telomeric DNA, leading cells to apoptosis probably due to the increased production of oxygen species. The increase in ROS production has been shown to generate *in situ* inflammation, and to cause damage to telomeric DNA (NRC, 1999, Waris et al., 2006). Arsenic is known to generate reactive oxygen species, which in turn may cause DNA strand breaks, chromosome damage, and apoptosis (Ishibashi et al., 1998; Liu et al., 2003). We also found that the co-treatment of BSO, a well-known GSH synthesis inhibitor with arsenic increased arsenic-induced apoptosis with increased expression of reactive oxygen species. The reduction of GSH caused by BSO may decrease the capacity of the cells to repair the effects of oxidation, leading to cell membrane damage (Afzal et al., 2002), decreased cell viability and increased telomere attrition. Moreover, as observed in a recent study (Xu et al., 2008) an increase in the production of reactive species might decrease the methylating capacity of cells, resulting in increased potential health risk from arsenic exposure. The co-treatment of DMPO, an oxygen radical scavenger, with arsenic tended to protect the cells against arsenic-induced apoptosis at high concentration. These results suggest GSH as a fundamental antioxidant in our *in vitro* system, and furthermore that arsenic induced-apoptosis in a ROS dependent manner.

Telomere shortening might serve as important checkpoint to decrease or regulate cell proliferation, and when telomeres are shortened to a critical length, the signal for senescence or

apoptosis is activated. The expression of human Telomerase Reverse Transcriptase (hTERT) is detected in germ cell tissues, stem cells and often, but not always in human tumours, and plays an important role in tumorigenesis (Slijepcevic et al., 1995, Holt et al., 1999, McEachern et al., 2000). Tumour cells are able to bypass this checkpoint by activation of telomerase, (Holt et al., 1999), since studies demonstrated that telomerase activation is necessary for the growth of most human tumours and immortalised cell lines (Holt et al 1999; Shay et al., 2001). It has been reported that telomere loss is an early event of DNA damage-induced apoptosis (Ramirez et al., 2003), and for this reason has been considered as a tumour suppressor mechanism (de Magalhaes et al., 2004). Possibly for this reason, arsenic at the concentration of about 1 μM has been shown to be active in the treatment of several cancer, inducing a high rate of clinical remissions in patients suffering from acute promyelocytic leukaemia (APC) (Shen et al., 1997; Chen et al., 1997; Au et al., 2003). Notably, numerous cell doublings are required for telomere attrition, thus longer arsenic exposure should be performed than in our study. For this reason, we speculate that parallel mechanisms besides telomere shortening and ROS formation might have contributed to the increase in apoptotic cells after exposure to the high concentration of arsenic. In fact, as already observed (Chen et al., 1998; Maeda et al., 2001) an increase in the formation of oxygen species after exposure to 1 μM arsenic may cause telomere attrition also through the activation of caspases, induction of protein P53, or down-modulation of bcl-2.

Interestingly, at low concentration of arsenic, that is generally considered safe for human exposure (WHO, 2001), we observed a gender dimorphism in response to arsenic, females being more sensitive to low concentrations stimulation, which is also consistent with our previous study (Ferrario et al., 2008). We speculate that low arsenic concentrations might have a stronger effect on female progenitors, acting through the up-modulation of oncogenes, such as *ras, myc*, or hTERT mRNA and protein expression, and probably also by interfering with the expression of estrogens receptor (ERs) as already observed (Davey et al., 2007). In our previous study we reported that arsenic was able to interfere with ERs expression in murine bone marrow cells (Cimino-Reale et al., 2008), whereas other studies showed that arsenic can modulate basal expression of steroid receptors (Stoica et al., 2000; Chen et al., 2002; Chow et al., 2004b). Liu et al., (2007) demonstrated that transplacental exposure to arsenic in mice significantly alters the expression of various genes encoding for estrogens signalling and steroid synthesis. However, in this study we did not address this hypothesis. For this reason, a deeper investigation on the modulation of ERs and their signalling on haematopoietic cells after exposure to arsenic should be a research priority.

Differences in sensitivity to arsenic exposure between females and males were already observed in human population and in experimental animals (Vega et al., 2004: Waalkes et al., 2006; Lindberg et al., 2007). Evidence suggests that sex steroid hormones may also be good candidates as physiological regulators of *htert* expression and *c-myc* (Dubik et al., 1992). Nevertheless, arsenic may be able to interact through a possible modulation of genes involved in cell cycle progression, that are known to interfere with regulation of telomerase (Dong et al., 2005), or through the activation of growth factors and cytokines, expressed differently between the two genders as we have observed (manuscript in preparation). It is noteworthy that in our experiments we observed that telomerase activity is differently expressed in progenitors at different stages of maturation in both genders. In fact, telomerase activity was found in the floating, less differentiated progenitor cells, whereas very low activity was found in the adherent, mesenchymal committed cells. These results suggest that the down-regulation of hTERT may cause loss of pluripotency and differentiation to hematopoietic and immune lineages. Moreover, it is likely that hTERT has an important role of human cord blood cells cell cycle regulation, and *in vitro* differentiation capacity.

Together with our previous study, the present results might suggest that arsenic at micromolar concentration (1 μM) which is relevant of arsenic blood levels measured in exposed population can target the human hematopoietic system, inducing immunosuppression by increasing apoptotic pathways and telomerase inhibition in a ROS dependent manner. These findings may help to explain the effective value of arsenic as therapeutic agent for treatment of leukaemia, but also indicate that hematopoietic cells also constitute sensitive targets for arsenic induced-apoptosis. This toxicity to haematopoiesis might have strong side effects on immune system differentiation and development, causing a decrease in the 'self-renewing' and replacement of progenitor cells. More attention should be given on submicromolar concentrations exposure of arsenic since the increase of the telomerase expression, and telomere length, together with the up-modulation of oncogenes observed might increase the proliferative rate of cells, leading to increased risk of developing cancer. Furthermore, the gender dimorphism observed in our study after exposure to low arsenic concentration is consistent with the hypothesis that arsenic acts differently between male and female, suggesting a possible role of arsenic in the stimulation of pathways that deserve a deeper investigation.

In conclusion, since the developing immune system represents a sensitive target of arsenic exposure, and the safety limits of xenobiotic exposure are established based on adult exposure, our *in vitro* model using umbilical cord blood cells may represent a good model for developmental immunotoxicity.

# 4. Summarising Discussion

Toxicology testing of chemicals required by regulatory agencies all around the world to implement appropriate risk-assessment and risk-management actions is mostly based on *in vivo* approaches. Yet, the time and cost of such *in vivo* testing, in addition to ethical consideration on the extensive use of animals, is driving the development of alternative testing methodologies, utilizing *in vitro* mammalian system. In the European Union, the recently implemented REACH program (Registration, Evaluation, Authorisation of Chemicals) calls for the testing of ~30,000 existing substances for which adequate information on possible adverse effects on human health is still lacking. In an effort to reduce costs and animal usage, the European Commission has advocated the use of alternative approaches. In this direction *in vitro* toxicology generally refers to the study of toxicological phenomena in non whole animal models.

Specifically in this thesis, *in vitro* haematotoxicology provides the opportunity to study the effects of toxicants directly on relevant human target tissues, reducing toxicological uncertainties due to animal/human extrapolation, supporting the knowledge and experience necessary for applying this kind of models to other continuously renewing tissues in the body. Damage to blood forming tissues is a major side-effect of anticancer drugs, and of several environmental pollutants. Validated *in vitro* systems for evaluating the effects of candidate medicines on the various blood lineages would improve drug development, refining in the meantime the accuracy of the estimate of the maximum permissible exposure limit (PEL) in the risk assessment of food contaminants, additives and industrial chemicals.

In this field, a Colony Forming Unit Granulocyte-Macrophages (CFU-GM) assay for predicting acute neutropenia in humans has been validated, to assist the pre-clinical dosage finding for clinical trials of some highly toxic drugs used in chemotherapy for cancer, as a substitute to using a second species, such as the dog in preclinical studies. Using bone marrow culture from mice and cord blood cells from humans, this test could decrease the risk of a lethal overdose in the first cohort of patients to which anticancer drugs are administered, a risk that cannot be identified during current preclinical testing strategies. International studies have shown that this new test can provide more accurate predictions than testing on animals, so the new method will not only reduce the number of animals needed, but also increase the safety of patients. The optimized assay can now also be applied to *in vitro* toxicology studies.

The increasing evidence showing that inorganic arsenic exposure can cause immunosuppressive effects both in animal models as well as in humans (Patterson et al., 2004; Soto-Pena et al., 2006), suggests giving priority to the evaluations of such effects on this tissue. Moreover, it has been reported that arsenic exposure can produce both inhibition and induction of proliferative

responses in human cells depending on the concentration administered. Very low arsenic concentrations (nM range) can induce lymphocyte proliferation (Vega et al., 1999; Meng and Meng, 2000), whereas higher concentrations (μM range) inhibit the proliferative responses of lymphocytes (Gonsenbatt et al., 1992, 1994; Meng and Meng, 2000). The major limitation of immunotoxicity risk assessment has been the lack of human data (Descotes, 2006). In fact, usually the immunosuppression has been investigated in animal models (Selgrade, 2004), and uncertainties remain about the use of animal data for predicting human risk. Furthermore, recently, concern over immunotoxicity has been increased by the assumption that the developing immune system may be more sensitive to immunotoxic chemicals than the adult one (Luebke et al., 2006). For this reason, modifications to adult testing have been proposed, since adult exposures may not adequately predict the risk of prenatal and early postnatal exposure (Dietert and Piepenbrink, 2006).

Starting from this assumption, the rationale of this thesis was to evaluate the toxic effects of inorganic arsenic and its metabolites on the developing haematopoietic and immune systems. Then, since several studies suggest that health effects of arsenic are manifested differently between male and female (Vega et al., 2004; Vahter et al., 2006) we also aimed to compare the toxicity between genders. Finally, we compared the toxicity of arsenic between species, by using both human cord blood cells and murine bone marrow cells. Eventually, considerations on the applicability of human cord blood cells in developmental toxicity testing have been discussed.

The cord blood cells contain a population of multipotent hematopoietic stem cells, capable of *in vitro* self-renewal as well as limited differentiation toward the lymphoid stem cells or myeloid multipotent stem cells. The myeloid progenitor is the precursor of granulocytes and macrophages of the immune system (Morrison et al., 1997). The lymphoid progenitors give rise to two major types of lymphocytes known as B or T cells. The bone marrow cells also contain multipotent stem cells that can differentiate into a variety of hematopoietic, mesenchymal, and endothelial cell types. Haematopoietic stem cells *in vitro* can give rise to the three classes of blood cells that are found in the circulation: white blood cells (leukocytes), red blood cells (erythrocytes), and platelets (thrombocytes). Following infections or blood loss, the hematopoietic system must have the capacity to respond quickly to an increased demand of mature differentiated cells. In addition, both immature and committed cells circulating into the blood stream are usually exposed to higher concentration of xenobiotic than any other internal cells type. Xenobiotics are well known to interfere with complex regulation pathways that regulate differentiation and proliferation of hematopoietic cells. These characteristics make the hematopoietic and immune progenitors particularly sensitive xenobiotics targets, and for this reason it is considered a very

attractive system for *in vitro* testing under well defined culture conditions (Pessina et al., 1992, 2002, 2005). Generally, *in vitro* models of hematopoiesis consist of short-term cloning assays for various hematopoietic progenitor cells, such as colony forming units granulocyte-macrophages (CFU-GM), erythroids (CFU-E), and megacaryocytes (CFUMK). These models have been used to investigate haematotoxicity in preclinical safety studies on candidate drugs (Deldar and Stevens, 1993; Deldar, 1994; Deldar and Parchment, 1997). These tools are also useful for determining the relative sensitivities of various animal species to haematotoxic effects and for studying synergistic and antagonistic effects of several compounds (Du et al., 1990). Usually, the most frequent *in vitro* studies on hematotoxicity investigate the acute effects of toxicants on bone marrow progenitors, such as granulocyte-macrophages (CFU-GM), erythroids (CFU-E), and megacaryocytes (CFU-MK), which is quantified from the number of surviving progenitors as a function of exposure level under maximal stimulatory cytokine concentrations (Metcalf, 1984). These *in vitro* models complement *in vivo* animal testing and have been shown to be predictive for hematotoxicity associated with anticancer and antiviral agents in humans.

Inorganic arsenic is a common element of the earth crust, which millions of people are exposed to high levels in daily life. Chronic exposure has been widely reported in many areas of the world, and usually the exposure occurs by consumption of arsenic contaminated water (Smith et al., 2000; NRC 2001; IARC 2004). A number of epidemiological studies have shown association between arsenic exposure and several diseases, including skin cancer, and others internal organs such as bladder, kidney and liver, and other non-cancer diseases (IARC 2004). Immunosuppressive activity of arsenic exposure has also been observed both in humans and animals (Patterson et al., 2004). Arsenic and its metabolites methylarsonic acid (MMA) and dimethylarsinic acid (DMA) are easily transferred to the foetus through the placental barrier both in human beings and other mammals (Lindgren et al., 1984; Concha et al., 1998), compromising the normal immune development of the unborn. In spite of the large number of studies on the health effects of arsenic, few studies have focused on the potential immune developmental effects. The health effects are mostly documented in adults, and few information exists on the variation in susceptibility depending on age and gender. Thus, there is a strong need for more accurate studies on the variation in susceptibility to arsenic of the immune system development, and on the effects of combined exposure that are likely to occur in real life (Vahter et al., 2008).

For the reasons described above, in the first study we assessed the toxicity of arsenic and its metabolites dimethylarsinic acid ($DMA^V$), monomethylarsonic acid ($MMA^V$) and monomethylarsonous acid ($MMA^{III}$) at relevant environmental concentrations on male and female human cord blood cells and murine bone marrow CFU-GM colonies *in vitro*. Then, we

also assessed potential molecular mechanisms of arsenic toxicity on the telomere length, telomerase expression, apoptosis, gene expression and formation of reactive oxygen species in human cord blood cells. In addition, we investigated and compared the immunotoxic effects on CFU-GM murine colonies formation after *in vivo* arsenic exposure and co-exposure of arsenic with atrazine.

This study confirmed that granulocytes-macrophages progenitor cells are a sensitive target for arsenic toxicity, as already observed by other studies (Sakurai et al., 2006). In addition, the effects of inorganic arsenic exposure on the proliferation of granulocytes-macrophages colonies were biphasic, producing either inhibition or induction of proliferative responses depending on the concentration used. In fact, the chemical at the concentration of 1 μM produced immunosuppressive effects on granulocytes-macrophages colonies, whereas at very low concentrations (around 0,0001 μM) produced an increase in the number of such colonies. Notably, the concentration of 1 μM is very close to the blood arsenic concentration found in exposed populations which suffer of immune dysfunction (Pi et al., 2000, Wu et al., 2003). Both human cord blood and murine bone marrow cells, were shown to be sensitive to the arsenic toxicity on granulocytes-macrophages to about the same extent and without differences between sexes (IC50 of 1.34 $\pm$ 0.43 μM and 1.22 $\pm$ 0.13 μM for male mice and male human respectively, and 0,95 $\pm$ 0.26 μM μM and 1,34 $\pm$ 0.43 μM for female mice and female human respectively). Others have already observed toxicity of arsenic at about the same concentrations (Schwerdtle et al., 2003; Shi et al., 2004; Lemarie et al., 2006; Ramadan et al., 2009), and they concluded that the toxicity of arsenic may be caused by genetic damage, inducing cell apoptosis, chromosome aberration, and possibly by increasing reactive oxygen species production. In our study, the results observed for the $IC_{50}$ values might lead to a possible conclusion that the mechanisms of arsenic toxicity in both genders are conserved between species. We also observed that $MMA^{III}$ was the most toxic compound among all the arsenicals. In fact, $MMA^{III}$ was about five times more toxic than arsenic on granulocytes-macrophages colonies formation (IC50 of 0.21 $\pm$ 0.03 μM and 0.13 $\pm$ 0.02 μM for males and females respectively). This observation of the higher rate of toxicity of $MMA^{III}$ also confirms previous studies on arsenic metabolites (Hirano et al., 2004; Kligerman et al., 2005). We also confirm that $DMA^{V}$ and $MMA^{V}$ are considerally less toxic than other arsenicals (Petrick et al. 2000; Styblo et al. 2000; Vega et al. 2001; Schwerdtle et al. 2003; Kligerman and Tennant 2006), at least on hematopoietic progenitor cells. In fact, both the pentavalent metabolites did not exert either toxicity or increased the proliferative rate of male and female human granulocytes-macrophages progenitors up to the maximum concentration tested (50 μM). What it is not well defined is why $MMA^{III}$ is much more toxic than arsenic,

however several mechanisms have been proposed. The higher toxicity caused by $MMA^{III}$ is probably due to the increased oxidative stress, and damage to the DNA structure (Nesnow et al., 2002), or to its capacity to bind with more affinity to the tissues (Lindberg et al., 2007). It has also been suggested that $MMA^{III}$ is likely to be more membrane permeable to cord blood cells, and the higher uptake of the trivalent methylated arsenicals with respect to arsenic may be responsible for the reported greater cytotoxic effects of this compound (Schwerdtle et al., 2003; Dopp et al., 2004; 2005; Yamanaka et al., 2004). In addition, both trivalent arsenic and metabolites are highly reactive, and are able to inhibit numerous enzymes (NRC, 1999) such as DNA repair enzymes (Hartwig et al 2003), and methyltransferases (Wu et al., 2006). However, this work did not address all these mechanisms, thus further investigations are needed in order to confirm or not these hypotheses. We only observed that $MMA^{III}$ was able to decrease the mRNA expression of Glutathione-S-transferase-omega (GSTO-1) whereas both arsenic and the other metabolites did not cause modulation in the GSTO-1 expression. During the biotransformation of inorganic arsenic, GSTO-1 catalyzes the reduction of arsenate, $MMA^{V}$ and $DMA^{V}$ to the more toxic trivalent arsenic species (Chowdhury et al., 2006). It could be concluded that the down-modulation of GSTO-1 enzyme observed after exposure to $MMA^{III}$ might be a possible defensive mechanism of the cell, trying to reduce the formation and accumulation of the most toxic trivalent metabolite. In addition, the mRNA expression of arsenic +3 methyltranferase (AS3MT), one of the methyltranferase responsible for arsenic methylation was almost undetectable in cord blood cells, suggesting that cord blood cells are not capable of arsenic methylation *in situ*.

Surprising was the capacity of arsenic at very low concentrations, to increase the granulocytes-macrophages colonies' number only for female donors, observed in both species. That low arsenic concentrations have some stimulatory effects was already observed by other studies. Germolec et al. (2003) observed increased mRNA transcripts and secretion of keratinocyte growth factors, including granulocyte macrophage-colony stimulating factor (GM-CSF) and transforming growth factor-alpha (TGF-alpha) and the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-alpha) in primary human epidermal keratinocytes cultured in the presence of low micromolar concentrations of sodium arsenite. Total cell numbers, as well as c-myc expression were also elevated in keratinocyte cultures treated with sodium arsenite. Meng and Meng, (1994) also observed that very low arsenic concentrations enhanced DNA synthesis in human blood lymphocytes, whereas higher concentrations inhibited DNA synthesis.

Our findings support that the toxicity of arsenic always assumed as a linear dose response curve, is likely to be non-linear at very low concentrations (Calabrese and Baldwin 2003b; Schoen, et

al., 2004). However, this modulation at low concentrations has not yet been well elucidated. A study by Brown and Kitchin (1996) observed that DNA damage was reduced (although not statistically significant) in the liver and lungs of rats exposed to low doses of arsenic in drinking water compared to controls. It has also been proposed that these proliferative effects might be consistent with induction of DNA repair mechanisms, glutathione related genes, different mechanisms of response to oxidative stress, as well as increased telomerase activity (Barnes et al., 2002; Droge at al., 2002; Andrew et al., 2003; Zhang et al., 2003).

Therefore, to better understand the possible molecular mechanism behind the biphasic effects of arsenic, in the second study we decided to evaluate the activity of arsenic on telomerase expression (hTERT), telomere length, oncogene expression, apoptosis and oxygen species production on human cord blood cells. We chose two concentrations for this purpose. The higher one used was causing toxicity on progenitor cells in the first study (1 μM), whereas the second one was the concentration at which we observed increased proliferative effects on the same progenitor cells (0.0001 μM). At the higher concentration, arsenic decreased telomerase expression and telomere length, induced apoptosis rather than cell death, possibly through the increased production of reactive oxygen species. These observations might partially explain the toxicity of arsenic observed in the CFU-GM assay. Moreover, since the higher concentration used in this study is close to the total blood arsenic levels of the Bangladesh population that ranged from 0.05 to 1.2 μM (Snow et al., 2005), our results could be helpful to better understand the possible arsenic developmental immunotoxicity in highly arsenic contaminated countries. Previous studies have already observed that arsenic at low concentrations (≤ 1 μM) increased telomerase activity/expression and induced cell proliferation in human epidermal keratinocytes and leukemia cells *in vitro*, whereas at higher concentrations (> 1 to 40 μM) decreased telomerase activity/expression inducing cell apoptosis (Zhang et al. 2003). However, the discrepancy between the concentrations could be due to the different responses to arsenic cytotoxicity by different cell types, the primary haematopoietic cells may be more sensitive to arsenic toxicity than other cells. Particularly interesting was the capacity of arsenic to increase the expression of telomerase both at mRNA and protein level in female donors. Multiple mechanisms exist to regulate telomerase transcription, resulting in repression or activation of telomerase activity in cells (Horikawa and Barrett 2003). Studies have shown that arsenic induced a number of gene expression alterations, including DNA repair response, oxidative stress, and signal transduction pathways by direct action on regulatory molecules (Germolec et al. 1996; Liu et al. 2001). Decreased telomerase expression may be associated with an increased DNA damage induced by the production of reactive oxygen species, that in turn could activate

apoptotic pathways. On the other hand the capacity of arsenic to induce oxygen radicals and increased apoptosis might also explain the well known arsenic activity as anticancer drugs, used in the treatment of some leukemia (Chen et al., 1996; Shen et al., 1997).

In this study, it has been observed that arsenic activity might be gender related, as already suggested in other investigations (Vega et al., 2004, Vather et al., 2007). At very low arsenic concentrations, female cord blood cells were more sensitive than male ones to arsenic induced telomerase expression stimulation, with maintained telomere length and cellular growth, possibly related to the increased expression of *ras* and *myc* oncogenes. This increased proliferative pathway observed in female donors might also partially explain the increased proliferation of GM colonies observed in females exposed at the same concentration in the first study. In addition, the gender differences observed at low concentrations might be the results of arsenic interaction with sex hormones. Very low arsenic concentrations were already described to enhance hormone gene transcription, whereas higher concentrations were suppressive (Bodwell et al., 2006). The telomerase gene promoter is also a target of hormone carcinogenesis in humans. There is a putative estrogen response element in the telomerase promoter (Nanni et al. 2002). It is possible that in females very low arsenic concentration can increase the expression of estrogen receptor α, that can bind to this element in telomerase promoter and then activate telomerase transcription as observed by others (Kyo et al. 1999; Misiti et al. 2000). Another possible mechanism, such as the increased cytokines production, and above all GM-CSF (granulocyte/macrophage colony-stimulating factor), as already shown by other authors in different *"in vitro"* model (Germolec et al., 1996, 1998; Vega et al., 2001) might be differently expressed and modulated between genders. However, this field deserves further investigations, since our findings are only partially conclusive. It has also been described that arsenic can interact with epigenetic regulation, mainly interfering with DNA methylation (Chen et al., 2004). Arsenic causes reduction of methylation, possibly by the reduction of DNA methyltranferases (Cui et al., 2006). DNA methylation is an important mechanism of the foetal programming (Langley-Evans, 2006), and the activity of arsenic-induced changes in DNA methylation may have severe consequences for the development both during gestation and after birth. Arsenic also interact with the complex mechanism of biotransformation, inhibiting several other methyltransferases (Wu et al., 2006), responsible for xenobiotics detoxification. As expected, in our study AS3MT was not expressed, therefore this possibility cannot be assessed in our model, since one of the major limitations in the employment of *in vitro* methods is the lacking of a reliable biotrasformation system. For this reason we cannot conclude that the differences

observed in the genders after exposure to low arsenic concentration might be due to differences in biotransformation.

In conclusion, this comparison provides evidence that exposure to arsenic and $MMA^{III}$ at μM concentrations is associated with immunosuppression "*in vitro*". Notably, arsenic at very low concentrations increased the proliferative rate of female donor progenitors, supporting the fact that different mode of action of arsenic in the two genders at low concentrations does exist. These results suggest that immunosuppression is not the only risk associated with modulation of immune system. In fact, also the immune stimulation resulting in enhanced risks of allergic and autoimmune disease is also a concern. More attention should be given at low arsenic concentration exposure, above all in genders differences in health effects, since the proliferative pathways observed in female donors may in turn lead to compromised immune functions, such as the increased incidence of asthma, cardiovascular diseases, and atherosclerosis, as observed in population exposed to arsenic (Soto-Pena et al., 2006). For this reason, it would be highly recommended to perform tests for developmental immunotoxicity that should be predictive of both immunosuppression and increased risk of immunostimulation. In this regard, assessment of *in vitro* human cord blood cells might be used as predictive model, with the potential to avoid interspecies differences in toxicity testing.

We also investigated the effects of arsenic exposure on the clonogenic activity of CFU-GM progenitor cells of murine bone marrow. In addition, as in real life people are usually exposed to mixtures of chemical contaminants and heavy metals (ATDSR, 2004), we assessed if the co-exposure of arsenic with atrazine (a widely used chloro-S-triazine herbicide) was able to modulate, and even increase the immune toxicity of arsenic. Surprisingly, at the concentration tested, arsenic was not found to exert toxicity on immune progenitor cells in both genders. We only observed a gender differences in response to arsenic health effects after the co-exposure with atrazine, where in female donors the percentage of CFU-GM significantly increased leaving male completely unaffected by the same treatment. We postulated that these results might have been due to the activity of gender hormones or estrogen receptors, as already documented by other studies (Kio et al., 1999), so we investigated the expression of estrogen receptors alpha (ERα) and beta (ERβ). ERα was not modified by the treatments in both genders. On the contrary, ERβ was modulated. In males, ERβ was significantly up-modulated after exposure to arsenate, atrazine, and co-exposure. In females we still observed an increase in ERβ gene expression, even though the interpretation of results was less clear, due to the high variability among the individuals, and only the co-exposure caused a significant up-modulation of ERβ. These results seem in contradiction with other animal studies that have shown that mRNA levels of ERα were

3.1-fold higher in liver of arsenic-exposed mice than in those of controls (Waalkes et al. 2000, 2004). However, it is possible that differences in the estrogen modulation exist between different cell types. In addition, previous observations (Shim et al., 2006) suggested a new unknown role for ERβ in regulating the differentiation of pluripotent hematopoietic progenitor cells, and a modulation in this receptor might then lead to a compromised immune response. However, it should be noted that the degree of ERβ expression was much lower than that of ERα, as already observed (Lim et al., 1999) and for this reason we cannot conclude that the modulation observed in ERβ alone can play a key role in regulation of immune response in our system. Other studies have shown ERβ is expressed in immune cells (Koehler et al., 2005; Ulziibat et al., 2006); ERβ functions early in the bone marrow to regulate proliferation of the progenitor cells and loss of in ERβ in $ER\beta^{-/-}$ mice leads to myeloproliferative disease (Shim et al., 2003). Moreover, ERβ has been shown to have an anti-inflammatory action, inhibiting the proliferation of breast cancer cells (Lazennec et al., 2001; Strom et al., 2004). For this reason, we speculate that the over expression of this receptor in our study might be considered as a defensive mechanism of the organism in order to prevent immune dysfunctions. Nevertheless, we also observed gender differences in response to arsenic toxicity in the results from microarrays spotted with genes related to cancer. In fact, arsenic modulated the expression of several genes only in female donors. The functional role exerted by the modulated genes after treatment in females, was related to cell adhesion, and biosynthesis of chemokines and cytokines. The increased expression of either cell adhesion and cytokine related genes might suggest that even if the arsenic concentration was not capable to exert overt immunotoxicity on GM colonies, however, was able to initiate inflammation in female donors, by modulating genes related to immune response. Major significant changes on the gene expression resulted following the co-exposure to arsenic and atrazine in both male and female.

In conclusion, even though we demonstrated that some molecular mechanisms were differently expressed and regulated in female donors at low concentrations, the results on the estrogen receptors modulation were not convincingly demonstrated and remain controversial. Further investigations of the effects of estrogens on the arsenic toxicity are strongly needed in order to confirm or not this hypothesis. Moreover, also the results of *in vivo* exposure of mice to arsenic were unexpected, and in contrast with other publications. In fact, several similar *in vivo* studies have already demonstrated the toxic role of arsenic on bone marrow cells, even if at higher arsenic concentrations (Hong et al., 1989; Biswas et al., 2007). It could be concluded that in our experiments arsenic was not able to exert toxicity on GM colonies, probably because the dose was not enough to exert overt immune toxicity on GM progenitors. Another explanation might

be that since mice have been already documented to be very good arsenic methylators in comparison with other mammals, humans included (Vahter and Marafante, 1983), arsenic was probably easily converted and expelled from the body, resulting in much less toxicity on the GM progenitors than expected. Nonetheless, in female donors arsenic was able to exert modulation in genes involved in inflammation, suggesting that also for gene modulation differences between gender exist, and female is supposed to be the most sensitive one to arsenic toxicity.

Taking into consideration the overall result of our study, the methods we applied showed advantages and disadvantages when used to test hematotoxicity and immunotoxicity. The *in vitro* colony-forming assays above described can give information on the hematopoietic and immune function. In fact, by using *in vitro* cell cultures it is now possible to predict *in vivo* hematotoxicity and immunotoxicity. These systems are particularly sensitive and powerful in toxicology when the *in vitro* assay employs the actual target cell of the xenobiotic *in vivo*, and above all when the concentrations and the time of exposure used are relevant for prediction of the *in vivo* scenario. Using these tools, in theory, it is possible to test the toxicity of a single compound on different hematopoietic lineages. In fact, hematopoietic progenitors when stimulated with appropriate cytokines can produce different colonies (such as granulocyte-macrophage CFU-GM, erythroid-burst-forming units BFU-E, and others). Moreover, humanised *in vitro* tests may provide tools to detect inter-species and inter-individual differences. Although many of these assays have been used in the last decades, only one (CFU-GM) has been validated by the European Centre for Validation of Alternative Methods (ECVAM) (Pessina et al., 2003).

On the other hand, *in vitro* tests still have some limitations. They do not provide information on the absorption, distribution, metabolism, and excretion of the substances, making it challenging to extrapolate from an effective *in vitro* concentration to an effective *in vivo* dose. Although the possible combination of *in vitro* tests with *in vitro* metabolic systems may overcome the limitations of the lacking metabolism in the future (Bremer and Hartung, 2004), information on the parameters of pharmacokinetics cannot be obtained *in vitro*. In addition to this, *in vitro* tests can only give prediction on primary effects on the cell type in consideration, whereas secondary effects will be probably not be predicted. Moreover, cell cultures still grow in stressful conditions, given by the presence of oxygen, and perturbations of temperature.

The use of murine bone marrow cells is an excellent tool to detect the interaction between chemicals and the differentiation and proliferation processes into a specific target tissue. Moreover, they could also give some information on the possible metabolites formation and biotransformation. However, as these cells are murine, it is likely that interspecies differences exist, and for this reason the results obtained in mice cannot be used to predict human toxicity.

Moreover, it might be that differences exist both in permeability of the placental barrier as well as in the biotransformation systems between species.

In conclusion, our investigation demonstrated that arsenic has a biphasic effect on the immune progenitors depending on the concentration used: immunotoxicity was observed at micro-molar concentration, whereas immune stimulation at sub micro-molar concentrations. Our data also suggest gender differences especially for low arsenic exposures in both species. Despite several proposed mechanisms, it is not clear how arsenic exerts these effects, although there is reason to believe that sex hormones may play a role in these effects. More research in this direction is highly warranted in particular concerning the potential low arsenic concentration activity on genders, and the mechanisms behind such effects.

Finally, in order to prevent inter-species differences humanised *in vitro* cell systems should be favoured, although often *in vitro* tools cannot completely replace animal testing. It is reasonable to use *in vitro* methods at least to reduce the number of animals in toxicology screening. Efforts still have to be invested in order to increase and ameliorate *in vitro* test systems.

# 5. Summary

The development of the haematopoietic system proceeds through a well-defined sequence of events, many of which are restricted during the prenatal or early postnatal period. For this reason it is not surprising that critical windows of vulnerability in the *in utero* and early postnatal immune system development do exist. This possibility makes this system an extremely sensitive target organ to environmental toxicants exposure. The major manifestations of immune toxicity include: inflammatory responses, immune cells loss, infections, immune-related diseases, and eventually cancer. It should be mentioned that for several developmental immunotoxicants, marked sex-based differences in toxic outcomes have been well documented.

Inorganic arsenic and its metabolites are transplacentally active metalloids that have been observed to exert immunosuppressive effects both on humans and animals. Several of such diseases resulting from alteration of immune response may not have been previously correlated to the arsenic exposure, for this reason a probing into this field is required. Hematopoietic cells coming both from human cord blood or murine bone marrow offer an excellent tool for monitoring the immunotoxic effects of arsenic *in vitro*. In fact, pluripotent hematopoietic cells have the capacity to differentiate *in vitro* into many highly specialized circulating blood cells. This characteristic makes the pluripotent hematopoietic cells attractive for *in vitro* testing using well-defined culture conditions. However, the assessment of immunosuppression is often restricted to animal models to predict human adverse effects, and *in vivo* rodent tests employing the use of bone marrow cells represent a good predictive model for immunotoxicants.

In this study the effects of inorganic arsenic, its metabolites dimethylarsinic acid ($DMA^V$), monomethylarsonic acid ($MMA^V$) and monomethylarsonous acid ($MMA^{III}$), and the co-exposure with another contaminant (atrazine) have been assessed on the capacity of hematopoietic progenitors to give rise to granulocyte-macrophage colonies (CFU-GM). This evaluation was performed in both sexes, both on human cord blood cells and murine bone marrow cells. The results suggest an immunosuppressive role of arsenic at micro-molar concentration (1μM) on CFU-GM colonies *in vitro*, without any gender or inter-species differences in sensitivity to arsenic toxicity. On the contrary, at sub micro-molar concentration (0.0001μM) arsenic exerted a proliferative activity on CFU-GM colonies *in vitro* depending on the sex tested. In fact, only female CFU-GM colonies were modulated. Both $DMA^V$ and $MMA^V$ did not exerted toxicity, whereas $MMA^{III}$ was at least five times more toxic to the CFU-GM compared to arsenic. In addition, mRNA expression of two enzymes involved in the biotrasformation of arsenic, Arsenic Methyltranferase (AS3MT) and Glutathione S-transferase omega 1 (GSTO1) were investigated. AS3MT mRNA expression was not induced in human cord blood cells after arsenic exposure.

The mRNA expression of one enzyme responsible for arsenic reduction, GSTO1 decreased after $MMA^{III}$ treatment, whereas it was not modulated by other arsenical compounds. In addition, some possible molecular mechanisms modulated by arsenic exposure were also investigated. Especially the telomere length, the telomerase expression, the reactive oxygen species production, the cell viability and apoptosis, as well as the expression of two oncogenes, such as *ras* and *myc*, known to be involved in arsenic toxicity were evaluated after exposure to arsenic. It was shown that at sub micro-molar concentration, arsenic was able to increase the telomerase mRNA and protein expression maintaining both telomere length, cell viability, possibly through the increasing expression of *ras* and myc oncogenes. Female donors were the more sensitive gender to the modulation of these molecular pathways, after low micro-molar arsenic exposure. At micro-molar concentration, in accordance with the decreased CFU-GM colonies described above, we observed a decrease in the cell viability of human cord blood cells, together with a decreased telomere length, possibly due to increased reactive oxygen species production. Buthionine sulfoximine (BSO), an inhibitor of glutathione (GSH) synthesis fundamental for arsenic detoxification, markedly increased the percentage of apoptotic cells. The reactive oxygen species (ROS) scavenger, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), was shown to exert protection from arsenic toxicity.

Finally, *in-utero* and juvenile exposure of mice to arsenic at the concentration of 1mg/l did not cause modulations on the CFU-GM colonies proliferation in both genders. Nevertheless, a strong modulation in cancer-related gene expression of female donors has been observed. A dramatic increase in the GM proliferation was observed in female donors after co-exposure of arsenic with atrazine. An increase in the expression of estrogen receptor beta was observed for both genders, even if more significant for male donors.

Taking into account our results, it can be concluded that arsenic exerts a double effect depending on the concentration on haematopoietic and immune progenitors. In fact, at micro-molar concentration it exerts an immunosuppressive activity on immune progenitor cells both on cellular and molecular endpoints, whereas at low sub micro-molar concentration is capable of immune stimulation. Although the mechanisms by which arsenic induces adverse immune effects have not clearly elucidated, some possible mechanisms are observed and discussed in this thesis, such as the increased apoptotic pathways and oxygen species production, down regulation of the telomerase expression, and decreased telomere length. The possible stimulatory effects of low arsenic concentrations is a relatively new finding, suggesting that arsenic toxicity, always assumed to follow a linear dose response curve, is likely to be non-linear at very low concentrations. In addition, gender differences in the toxic outcome of arsenic do exist,

especially for low arsenic exposures, even if the mechanisms related to these differences have not been fully understood. Therefore, we propose that the use of *in vitro* methods employing human umbilical cord blood cells taken from both sexes might avoid the interspecies variations in developmental immunotoxic studies, and should be preferable to better understand the possible mechanism behind arsenic immunosuppression and immunostimulation after early life exposure. On the other hand major limitations still exist in the use of *in vitro* system, such as the lack of a reliable metabolic system. For this reason, using well designed *in vitro* and *in vivo* methods together to predict immunotoxicity, may contribute to reduce the number of animals used in toxicological screening, and may help to refine safety margins by reducing uncertainties due to interspecies extrapolations. This would provide a more rational basis for calculating clinical dosages and setting human exposures limits.

# 6. Zusammenfassung

Die Entwickung des hämatopoetischen Systems erfolgt in einer wohldefinierten Folge von Ereignissen, von denen sich viele auf eine prä- oder postnatale Periode beschränken. Aus diesem Grund überrascht es nicht, dass es kritische Fenster der Vulnerabilität *in utero* und nach der Geburt für die Entwicklung des Immunsystems gibt. Diese Möglichkeit macht dieses System ein extrem sensitives Zielorgan für Umweltexpositionen zu Giften. Die wichtigsten Manifestationen von Immuntoxizität umfassen: Entzündliche Prozesse, Immunzellverlust, Infektionen, immunvermittelte Erkrankungen und letztlich Krebs. Bemerkenswerterweise wurden für verschiedene Gifte, die die Entwicklung des Immunsystems beeinflussen, starke Geschlechtsunterschiede in der Literatur beschrieben.

Anorganisches Arsen und seine Metaboliten sind plazentagängige Metallverbindungen, für die immunsuppressive Effekte in Mensch und Tier beobachtet wurden. Verschiedene solcher Erkrankungen, die durch eine Veränderung der Immunantwort hervorgerufen werden, mögen früher nicht mit Arsen-Exposition in Zusammenhang gebracht worden sein, weshalb Studien in diesem Bereich nötig sind. Hämatopoetische Zellen aus menschlichem Nabelschnurblut oder Knochenmark der Maus bieten ein ausgezeichnetes Werkzeug, immuntoxische Effekte von Arsen *in vitro* zu studieren. Tatsächlich haben pluripotente hämatopoetische Zellen die Fähigkeit, *in vitro* in die verschiedenen hochspezialisierten zirkulierenden Blutzellen zu differenzieren. Diese Eigenschaft macht die pluripotenten hämatopoetischen Zellen attraktiv für *in vitro*-Tests unter wohldefinierten Kulturbedingungen. Allerdings ist die Erfassung von Immunosuppression oft auf Tiermodelle angewiesen, um schädliche Wirkungen auf den Menschen vorherzusagen, und *in vivo*-Nagetiertests unter Verwendung von Knochenmarkzellen stellen ein recht prädiktives Modell für Immuntoxine dar.

In der vorgelegten Arbeit wurden die Effekte von anorganischem Arsen, seinen Metaboliten Dimethylarsinsäure ($DMA^V$), Monomethylarsonsäure ($MMA^V$) und Monomethylarsoniger Säure ($MMA^{III}$), und die Coexposition mit einem anderen Umweltgift (Atrazin) untersucht in Bezug auf die Fähigkeit von hämatopoetischen Vorgängerzellen, sich zu Granulozyten-Makrophagen-Kolonien (CFU-GM) zu entwickeln. Diese Untersuchung wurde mit beiden Geschlechtern sowohl mit menschlichen Nabelschnurblutzellen als auch Maus-Knochenmarkszellen durchgeführt. Die Ergebnisse geben Hinweise auf eine immunsuppressive Rolle von Arsen bei micromolaren Konzentrationen (1µM) auf CFU-GM-Kolonien *in vitro*, ohne Geschlechts- oder Interspeziesunterschied in Bezug auf die Sensitivität für Arsentoxizität. Im Gegenteil dazu hatte Arsen bei submicromolaren Konzentrationen (0.0001µM) eine proliferative Aktivität auf CFU-

GM-Kolonien *in vitro* abhängig vom untersuchten Geschlecht. Tatsächlich wurden nur weibliche CFU-GM-Kolonien beeinflusst. Sowohl $DMA^V$ als auch $MMA^V$ hatten keinen toxischen Effekt, während $MMA^{III}$ mindestens fünf mal toxischer auf CFU-GM war als Arsen. Zusätzlich wurde die mRNA-Expression von zwei Enzymen untersucht, die in die Biotransformation von Arsen involviert sind, Arsenic-Methyltranferase (AS3MT) und Glutathion-S-Transferase omega-1 (GSTO1). AS3MT-mRNA-Expression wurde in menschlichen Nabelschnurblutzellen nicht induziert. Die mRNA-Expression eines Enzymes verantwortlich für Arsenreduktion, Glutathione S-transferase omega 1 (GSTO1), nahm nach $MMA^{III}$-Behandlung ab, während sie durch andere Arsenverbindungen nicht beeinflusst wurde. Weiterhin wurden einige mögliche molekulare Mechanismen der Veränderungen bei Arsenexposition untersucht. Speziell wurden die Telomerenlänge, die Telomeraseexpression, die Bildung von reaktiven Sauerstoffspezies, die Zellvitalität und -apoptose, als auch die Expression von zwei Onkogenen untersucht, nämlich *ras* und *myc*, von denen bekannt ist, dass sie in die Arsentoxizität involviert sind. Es wurde gezeigt, dass bei submicromolaren Konzentrationen Arsen in der Lage war, die Telomerase-mRNA und Proteinexpression zu steigern und sowohl die Telomerenlänge als auch die Zellvitalität zu erhalten, möglicherweise durch eine gesteigerte Expression der Onkogene *ras* und *myc*. Weibliche Spender waren das sensitivere Geschlecht für die Modulierung dieser molekularen Signalwege nach niedrigen micromolaren Arsenexpositionen. Bei micromolaren Konzentrationen, in Übereinstimmung mit der reduzierten Bildung von CFU-GM-Kolonien wie oben beschrieben, beobachteten wir eine Abnahme der Zellvitalitat der menschlichen Nabelschnurblutzellen, zusammen mit einer reduzierten Telomerenlänge, möglicherweise aufgrund gesteigerter Bildung von reaktiven Sauerstoffspezies. Buthioninsulfoximin (BSO), ein Inhibitor der Glutathion(GSH)-synthese fundamental für die Arsenentgiftung, steigerte deutlich den Prozentsatz von apoptotischen Zellen. Der Hemmer von reaktiven Sauerstoffspezies (ROS), 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO), zeigte Schutzwirkungen gegen die Arsentoxizität.

Letztlich, bei *in-utero* und iuveniler Exposition von Mäusen mit Arsen bei einer Konzentration von 1mg/l hatte keinen modulierenden Effekt auf das CFU-GM-Koloniewachstum in beiden Geschlechtern. Trotzdem wurde eine starke Modulation der Krebs-assoziierten Genexpression bei weiblichen Spendern beobachtet. Eine dramatische Steigerung des GM-Wachstum wurde bei einer Koexposition weiblicher Spender mit Arsen und Atrazin beobachtet. Ein Anstieg der Expression des Estrogenrezeptor-beta wurde in beiden Geschlechtern beobachtet, sogar ausgeprägter für männliche Spender.

Angesichts dieser Resultate kann man auf einen doppelten Effekt von Arsen auf hämatopoetische und Immunvorläuferzellen abhängig von der Konzentration schliessen.

Tatsächlich übt es bei micromolaren Konzentrationen eine immunsuppressive Aktivität auf Immunvorläuferzellen sowohl auf zelluläre als auch molekulare Endpunkte aus, während es bei niedrigen submicromolaren Konzentrationen zur Immunstimulation befähigt ist. Obwohl die Mechanismen, wie Arsen seine schädlichen Immuneffekte entfaltet, noch nicht eindeutig identifiziert wurden, wurden einige mögliche Mechanismen in dieser Arbeit beobachtet und diskutiert, wie zum Beispiel der Anstieg von Apoptose-Signalwegen und die Produktion von reaktiven Sauerstoffspezies, eine Runterregulation der Telomeraseexpression und eine reduzierte Telomerenlange. Der mögliche stimulierende Effekt von kleinen Arsenkonzentrationen ist ein relativ neuer Befund, der darauf hinweist, dass die Arsentoxizität, die immer als lineare Dosis-Wirkungskurve angesehen wurde, bei niedrigen Konzentrationen vermutlich nicht linear ist. Weiterhin bestehen Geschlechtsunterschiede in der toxischen Wirkung von Arsen, speziell bei niedriger Arsenexposition, selbst wenn die Mechanismen dieser Unterschiede noch nicht völlig verstanden sind. Daher schlagen wir die Verwendung von *in vitro*-Methoden unter Verwendung von menschlichen Nabelschnurblutzellen von beiden Geschlechtern vor, um Interspeziesvariationen bei Entwicklungsimmuntoxizitatsstudien zu vermeiden, die bevorzugt zum besseren Verständnis der möglichen Mechanismen der Immunsuppression und Immunstimulation nach Arsenexposition in frühen Lebensphasen verwendet werden sollten. Andererseits bestehen weiter wesentliche Limitationen für die Verwendung von *in vitro*-Systemen, wie das Fehlen von verlässlichen metabolisierenden Systemen. Aus diesem Grund sollten gut zugeschnittene *in vitro*- und *in vivo*-Methoden zusammen zur Vorhersage von Immuntoxizität dazu beitragen können, die Zahl von Versuchstieren bei toxikologischen Reihenuntersuchungen zu reduzieren, und möglicherweise helfen, die Sicherheitsmargen aufgrund geringerer Unsicherheit bei der Interspeziesextrapolation zu reduzieren. Dies würde eine rationalere Basis für die Berechnung von klinischen Dosierungen und das Setzen von menschlichen Expositionslimits bieten.

# 7. Aufstellung der eigenen und fremden Anteile an der Promotion

Daniele, Ferrario
Via Valle del Sole, 1
21020 Taino (VA)
Italy
September 2009

**Aufstellung der eigenen und fremden Anteile an der Promotion**

Am 02.04.2009 wurde ich als Doktorand im Fach Biologie angenommen. Mein Doktorvater ist Prof. Dr. T. Hartung ausserdem wurde ich Dr. Laura Gribaldo betreut. Der Titel der Arbeit ist

**In vitro Assessment of Arsenic Immune Toxicity using Human Cord Blood and Murine Bone Marrow Cells**

**In vitro Studien zur Immunotoxizität von Arsen unter Verwendung Menschlicher Nabelschnurblutzellen und Knochenmark der Maus.**

Anteile, die von mir und anderen Personen im Rahmen der Dissertation übernommen wurden:

**1. Introduction: Arsenic Exposure and Immunotoxicity: A Review of the Influence of Age and Gender.**

Review publication — own work

Supervision: Thomas Hartung, Laura Gribaldo

**2. Aims of the study** — own work

**3. Manuscripts:**

**3A. Toxicity of inorganic arsenic and its metabolites on haematopoietic progenitors "in vitro": comparison between species and sexes.**

Experimental publication

Biological part: own work

Experimental assistance: Cristina Croera

Supervision: Laura Gribaldo, Marie Vahter

**3B. Combined in utero and juvenile exposure of mice to arsenate and atrazine in drinking water modulates gene expression and clonogenicity of myeloid progenitors.**

Experimental publication

Biological part**:** Daniele Ferrario, Graziella Cimino

Experimental assistance: Roberta Brustio, Angelo Collotta

Supervision: Laura Gribaldo, Erminio Marafante

**3C. Arsenic induces telomerase expression and maintains telomere length in human cord blood cells.**

Experimental publication

Biological part**:** own work

Experimental assistance: Angelo Collotta, Gerard Bowe, Maria Carfì

Supervision: Laura Gribaldo

| | |
|---|---|
| **4. Summarising discussion** | own work |
| **5. Summery** | own work |
| **6. Zusammenfassung** | German translation of Summary by Thomas Hartung. |
| **7. References** | own work |

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