

1 MERCURY CONCENTRATIONS IN THE BLOOD OF HARBOUR
2 SEAL (*PHOCA VITULINA*) FROM THE NORTH SEA AND *IN*
3 *VITRO* EXPOSURE OF SEAL AND HUMAN PERIPHERAL
4 BLOOD MONONUCLEAR CELLS
5

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1

2 Abstract

3 Background

4 Methylmercury (MeHg) is highly immunotoxic and readily bioaccumulated in marine organisms.
5 Level of total Hg (T-Hg) was studied in 22 wild harbour seals (*Phoca vitulina*) from the German
6 North Sea. T-Hg is indeed a valuable proxy for MeHg exposure in the blood. Immunotoxic effects
7 of MeHg were studied in peripheral blood mononuclear cells (PBMCs) isolated from harbour seals
8 and human beings. Our investigations examined if some immune functions of harbour seals could
9 be impaired by low Hg level, leading to an increase susceptibility to infectious agents. Protein
10 expression affected *in vitro* by methylmercury exposure was also investigated in human PBMC by
11 mean of proteomic.

12 Methods

13 Functional tests (viability, proliferation, metabolic activity, RNA and DNA synthesis) were
14 performed on human (n = 9) and seal PBMCs (n = 5). The expressions of GAPDH, interleukin- IL-
15 2, IL-4 and TGF- β (Transforming Growth Factor- β) were investigated in seal PBMCs by RT-PCR
16 and real time quantitative PCR (n = 4). Proteome (proteins constituting a cell compartment or cell
17 type) was studied in human PBMCs to identify specific biomarkers (n = 3). Cytosolic proteins were
18 extracted and identified on 2D- DIGE gels followed by mass spectrometry.

19 Results

20 After exposure, the number of PBMCs, viability, metabolic activity, DNA and RNA synthesis were
21 reduced *in vitro* suggesting deleterious effects of MeHg in concentrations naturally encountered in
22 free-ranging seals. For human and seals, functional tests showed that a 1 μ M concentration was the
23 critical concentration above which lymphocyte activity, proliferation and survival were
24 compromised. IL-2 and TGF- β mRNA expression were weaker in exposed lymphocytes compared
25 to control cells (0.2 and 1 μ M). The differential proteomic analysis completed on the cytoplasmic
26 fraction of the human T-lymphocyte exposed to this MeHg concentration showed some variation in

1 the protein expression profile (*e.g.* vimentin). However, these potential biomarkers of MeHg
2 exposure are yet to be tested and validated on harbour seals.

3 Conclusion

4 To conclude, cell model revealed an *in vitro* immunosuppressive effect of MeHg even at
5 concentrations similar to that measured in free-ranging harbour seals.

6

7 Keywords: Mercury, *Phoca vitulina* – biomarkers- cytokine- PBMC –cell model

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1

2 1. BACKGROUND

3

4 Mercury is a widely present metal in the environment, with a major natural source of environmental
5 mercury pollution provided by degassing from the Earth's crust [1, 2]. Its environmental level has
6 also increased as a consequence of discharges from various industries, medical and scientific
7 wastes, and the processing of raw ores [1, 2]. Following the discovery in the early 1960s, of the
8 dangers to human health of mercury in the marine environment, there has been a steady reduction of
9 man-made inputs in the North Sea, partly through the imposition of strict controls on discharges of
10 wastes containing mercury, and partly through elimination of the use of mercury and mercurial
11 compounds [2, 3]. However, despite these regulations, Hg decrease in the biota is not obvious,
12 especially for marine mammals for which Hg levels may still raise in some species tissues due to
13 worldwide distribution of Hg [3-6].

14 Mercury concentrates in the marine environment, especially in deep ocean waters, which contain
15 approximately 74% of the global total [7, 8]. The cycling of mercury through the marine
16 environment involves different chemical forms [1]. A large portion of mercury in the ocean is
17 transformed to Hg^{2+} and becomes available for methylation, mainly occurring in sediments and
18 anoxic aquatic systems [2, 9, 10]. First described in 1969 [11], methylation of inorganic mercury
19 and demethylation of methylmercury (MeHg) in food webs are key processes influencing the
20 abundance of MeHg in food webs [9]. Rate of methylation and demethylation are affected by
21 several variables including temperature raising serious concerns in the light of global change and
22 increasing seawater temperature [12, 13]. MeHg has a high bioavailability, bioaccumulates and
23 biomagnifies at all trophic levels in the food web and has severe toxicological effects [14, 15]. This
24 justifies constant monitoring of levels and effects in wildlife. Usually, 80-95% of mercury found in
25 North Sea fish muscle tissue is MeHg [16]. Fish represents the major Hg source for human and

1 marine mammal population relying on this marine resource. Fish consumption is generally
2 correlated with total Hg and organic Hg in blood ([17, 18]. The highest Hg levels are found in
3 species, such as the harbour porpoise (*Phocoena phocoena*) and the harbour seal (*Phoca vitulina*),
4 displaying piscivorous habits and inhabiting polluted coastal waters of the southern North Sea ([19,
5 20]. Long-term Hg storage in liver and kidney of marine mammals has been described: Hg is
6 generally associated to Se under a non organic form (tiemannite or HgSe), believed to be less toxic
7 [21, 22]. However, the demethylation process is not “instantaneous” and before reaching these long-
8 term accumulation organs, mercury is assimilated from fish, within the blood stream and mainly
9 under its methylated form [14, 15]. A high percentage of blood mercury concentration is under a
10 methylated form (up to 90%), especially in human and marine mammal population relying on
11 seafood [14, 15, 23] and so may represent a threat towards blood cells, including immune cells.
12 Specific mechanisms of MeHg- induced immunotoxicity, however, remain unexplored.

13 Impairment of immune function is suggested to play a contributing role for the increasing incidence
14 of infectious diseases in marine mammals and adverse effects of environmental contaminants on the
15 immune system have often been suggested [24-27]. Information on the immune system of harbour
16 seals is quite well documented. This species has conveniently become the marine mammal of
17 choice for immunological studies [25, 26]. Interest in the harbour seal stemmed partly from earlier
18 captive studies on the reproductive toxicity of environmental contaminants using this species [28],
19 but more importantly as a consequence of recurrent PDV epizootics [29-31]. The development of *in*
20 *vitro* models for immunotoxicity testing increases the understanding of the mechanisms underlying
21 observations of immunotoxicity. This is of interest, particularly in humans and wildlife where
22 ethical limitations preclude *in vivo* experimentation [25, 32].

23 However, despite numerous studies involving *in vivo* and *in vitro* effects of persistent organic
24 pollutants [33, 34], effects of mercury on the marine mammal immune system and underlying
25 mechanisms remain scarce [35-37]. This might be caused by an underestimation of its toxicity

1 compared to organic pollutants. Mercury chloride (HgCl₂) exposure revealed immunotoxic
2 properties on seal leukocytes [35] but this non organic compound represents a minor percentage of
3 total mercury in the blood compared to the highly toxic methylmercury [15]. Developing grey seals
4 from the St Lawrence Estuary appeared vulnerable to *in vitro* MeHg exposure, especially during the
5 post-weaning period [36]. Several immune functions may be affected by methylmercury, including
6 cell proliferation and viability, protein synthesis and cell mediation through cytokine secretion [38].

7 Cell proliferation and viability can be easily studied through well established bioassays [35,
8 39] while proteomic appeared as a promising tool to identify protein expression consecutive to
9 pollutant exposure [40, 41]. Immune system cell such as macrophages and monocytes produce pro-
10 and anti-inflammatory cytokines and are responsible for the initiation and progression of immune
11 response. Cytokines are powerful biological mediators of the immune system [40, 42]. T helper
12 (Th) cell subpopulations are differentiated by their contrasting and cross-regulating cytokine
13 expressions [43]. Th1-cells produce proinflammatory cytokines such as interleukin (IL)-2 to
14 stimulate the cellular immune response [44], whereas Th2-cells produce anti-inflammatory
15 cytokines such as IL-4, which regulate the immune response [45]. Th3-cells are the source of
16 transforming growth factor (TGF)- β , a cytokine with strong anti-inflammatory characteristics
17 [46]. Cytokine expressions are modulated by various environmental pollutants [47-52] but specific
18 actions of methylmercury at environmental concentrations remain largely unknown.

19 In this pilot study, we used MeHg in an *in vitro* system to address the link between environmental
20 Hg levels measured in seal blood and lymphocyte function in harbour seal. Human lymphocytes
21 were also integrated in the study for an interspecies comparison and protocol establishment
22 (lymphocyte proliferation, protein, RNA and DNA synthesis). We propose here:

23 - (1) to assess total Hg levels in the blood of free-ranging seals from the North Sea and to evaluate
24 biotic factors influencing these levels (sex, length, body mass).

1 - (2) to isolate lymphocytes from seal and human and expose them *in vitro*, using various MeHg
2 concentrations reflecting levels encountered in wildlife. *In vitro* immunotoxicity of MeHg was
3 approached on DNA, RNA and protein levels.

4 - (3) to delineate biochemical pathways of methylmercury *in vitro* exposure on T-lymphocytes
5 using a first human PBMC model through the mean of proteomic.

6

1 2. MATERIALS AND METHODS

2 2.1. Total Hg analysis

3 Full blood was sampled from 22 harbour seals caught between 1997 and 2004 along the German
4 North Sea (Lorenzenplate, Germany, Table 1). Seals were physically restrained. Blood was drawn
5 from the extradural venous sinus into sterile evacuated blood collection tubes (serum tubes
6 Monovette®, Germany) and kept at -20°C until Hg analysis. Two grams (approximately 1.8 ml) of
7 blood were mineralized with 3ml HNO₃ 65% (Merck 456) and 1ml of H₂O₂ (MERCK 7210), using
8 a microwave oven (MLS-1200 Mega) for 17 minutes. Total Hg concentrations (in µg·g⁻¹ fw) were
9 determined by cold vapour atomic absorption spectrometry on a Perkin-Elmer Coleman Mas-50
10 Mercury Analyser (wavelength 253.7 nm). Quality control measurements for total mercury included
11 replicate analysis resulting in coefficients of variation <10% and analysis of certified material
12 (DORM-1, NRC, Canada). The Hg absolute detection limit was 10 ng corresponding to 0.13 µg·g⁻¹
13 fresh weight (fw) for an average of 1.5 g of sample analysed. Quality of the analyses was controlled
14 through participation in an intercalibration programme [53].

15

16 2.2. Cytokine detection

17 The expression of the house keeping gene glyceraldehyde-3-phosphate (GAPDH), interleukins- IL-
18 2 and IL-4 and the transforming growth factor (TGF)-β was investigated in control and
19 contaminated seal PBMCs.

20

21 Sampling - Blood from one captive female seal (Seal Station, Friedrichskoog) and 4 free ranging
22 males (Table 1) was sampled in EDTA tubes and kept at room temperature until PBMC isolation
23 (max duration 18h). Routine medical checks, blood counts, distemper antibodies, and bacterial
24 investigations showed no evidence of disease in these animals at the time of examination (Siebert,
25 personal communication).

1 The blood was diluted 1:2 with phosphate-buffered saline (PBS). Lymphocytes were
2 separated on a Ficoll gradient (PAA Laboratories GmbH, Pasing) and washed twice in PBS. Cells
3 were suspended in 20% medium (Minimum essential Medium, Eagle, Sigma-Aldrich containing
4 10% foetal calf serum (FCS, from PAA Laboratories). Phytohemagglutinin (PHA) mitogen (5
5 $\mu\text{g}\cdot\text{ml}^{-1}$) was diluted in culture medium (PHA; Sigma- Aldrich). Suspensions of PBMCs (200 000
6 cells) in a final volume of 300 μl per well were incubated for 72 h at 37°C with 5% CO₂ and
7 methylmercury (0.2 μM and 1 μM , Sigma-Aldrich Chemie GmbH). Control PBMCs were treated
8 with culture medium without with PHA (5 $\mu\text{g}\cdot\text{ml}^{-1}$). Viability and number of cells were determined
9 microscopically by trypan blue staining (Carl Roth GmbH, Karlsruhe, Germany).

10

11 Primer design - Primers for the PCR were designed using DNASTar™ software (GATC
12 Biotech, Konstanz, Germany). Primers for the detection of IL-2 and IL-4 were selected from
13 conserved nucleotide sequences of grey seal (*Halichoerus grypus*) and dog (*Canis familiaris*)
14 respectively (GenBank accession numbers AF072871, AF187322, AF104245). Primer sequences
15 for the amplification of GAPDH and TGF- β were selected from previously published sequences
16 ([52]; Table 2).

17

18 RT-PCR and Real time quantitative PCR - After the incubation period (72 h), total RNA
19 was isolated from exposed and control PBMCs (RNeasy® Mini Kit, Qiagen Sciences, Maryland,
20 USA) following manufacture recommendation. After DNase-treatment (DNA-free™, Ambion kit),
21 RNA was reverse transcribed with murine reverse transcriptase (RNA PCR Core Kit™, Applied
22 Biosystems, Weiterstadt, Germany) and the resulting cDNA served as a template for PCR following
23 the manufacturers protocol and using Thermocycler MX4000™ (Stratagene Europe). For real-time
24 quantification, the Brilliant SYBRGreen QPCR Master mix (Stratagen Europe) was used [54]. This
25 contained SYBRGreen I as a fluorescence dye, dNTPs, MgCl₂ and a hot start Taq DNA
26 polymerase. The fluorescence response was monitored in a linear fashion as the PCR product was

1 generated over a range of PCR cycles. For each cytokine, a standard curve was prepared using a
2 dilution series from 10^9 to 10^2 copies. The PCR started with an initial step at 95°C for 10 min,
3 followed by 40 cycles with denaturation at 95°C for 1 min, annealing temperature (table 1) for 30
4 sec and elongation at 72°C for 1 min. The fluorescence was measured at the end of the annealing
5 and at the end of the dissociation program at a wavelength of 530 nm. To exclude measurement of
6 non specific PCR products and primer dimers, and to determine true amplification, each PCR was
7 followed by a dissociation program for 1 min at 95°C, followed by 41 cycles during which the
8 temperature was increased in each cycle, starting at 55°C and ending at 95°C. Only PCR reactions
9 with one well-defined peak were used for analysis. All reactions were performed in duplicate and
10 two separate PCR reactions were performed. GAPDH was used as the control gene. To calculate the
11 cytokine expression GAPDH was used as calibration compound. Cytokine expression index (CI)
12 was calculated as follows:

$$CI = \text{Number of cytokine copies} / \text{Number of GAPDH copies}$$

14 2.4. Functional tests

15 Blood from 6 captive seals (4 females and 2 males from *Seehundstation*, Friedrichskoog, Germany)
16 was sampled in 9ml heparinized tube (Monovette® Statedt, Germany) and kept at room temperature
17 until PBMC isolation and exposure (Laboratory for Histology and Cytology, Liege University). The
18 Seal Rehabilitation Center keeps harbour seals on a regular basis for scientific and education
19 purposes. Routine medical checks, blood counts, distemper antibodies, and bacterial investigations
20 showed no evidence of disease in these animals at the time of examination (Siebert, personal
21 communication).

22

23 In parallel to seal samples functional tests were also realised on healthy 30-60 years old male
24 human PBMCs (n = 8). PBMC whole cells (collaboration with Croix Rouge de Belgique). PBMCs
25 were isolated from buffy coats by using standard Ficoll/Hypaque (Amersham) gradients

1 (centrifugation during 30 min at 700g, at 20°C). PBMCs were washed twice in phosphate buffer
2 saline solution (PBS) and the pellet was resuspended in the culture medium (RPMI supplemented
3 with 10% heat inactivated foetal bovine serum (In vitrogen), 1% L-glutamine, 10% penicillin-
4 streptomycin; Cambrex). 2×10^5 cells were seeded in 100 μ l and incubated in a 96-well
5 microculture plate (Falco, New Jersey, USA). Cell cultures were incubated without mitogen or in
6 the presence of phytohemagglutinin (PHA), a T-cell-specific mitogen in harbour seals (de Swart et
7 al. 1993). PHA was diluted in culture medium at optimal concentration of $1\mu\text{g}\cdot\text{ml}^{-1}$ for human
8 PBMCs (determined optimal concentration for 72hr culture) and $5\mu\text{g}\cdot\text{ml}^{-1}$ for seal PBMCs (Beineke
9 et al. 2004). Cell viability was assessed via trypan blue exclusion assay and viable cells were
10 counted microscopically using a hemocytometer.

11

12 After 72 hours of MeHg exposure (0.1, 0.2, 0.5, 1, 1.5 5 10 μ M), metabolic activity of cells
13 was quantified by a colorimetric microtiter plate MTS assay 3-(4,5-dimethylthiazol-2-yl)-5-(3-
14 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium, inner salt (MTS, Promega). MTS *in*
15 *vitro* cytotoxicity assay is a convenient method for assessing cell viability. 10 μ l of MTS solutions
16 prepared according to the manufacturer's instructions were added to the 100 μ l culture medium
17 containing the cells. MTS is chemically reduced by cells into formazan, which is soluble in culture
18 medium. After 2 hr in the dark and in the incubator, the measurement of the absorbance of the
19 formazan was carried out on a spectrophotometer (Powerwave X, Bio-Tek) at 492nm. Each
20 condition was realized in 5 wells.

21

22 Lymphoproliferation and protein synthesis were determined by measuring the amount of
23 incorporated radioactive precursors using a scintillation counter. 2×10^5 cells were seeded in 200 μ l
24 culture medium. During the last 24 h of culture, 1 μ Ci/ml of methyl- ^3H thymidine (specific
25 activity 20-40 Ci/mmmole), $^5,^3\text{H}$ uridine (specific activity 20-40 Ci/mmmole) or $^4,^5,^3\text{H}$ L-leucine
26 (specific activity 40-60 Ci/mmmole, MP Biomedicals) was added to each well and cellular
27 incorporation was determined. Cells were harvested on filter (Multiscreen HTS, Millipore) with a

1 millipore aspiration system as described by the manufacturer instructions. The following steps were
2 realized with an automatic dispenser (Precision power 2000): 100 µl PBS, 120 µl trypsin 10 min,
3 100 µl PBS, 100 µl ethanol 20 min, 3x 100 µl ethanol. The plates were dried overnight, and the
4 filters were collected in vials (Puncher Millipore). 400 µl sodium hypochlorite (1:10 v:v) were
5 added. After 30 min agitation, 4 ml scintillating liquid (Ready Safe, Beckman) were added and
6 quantification of the retained radioactivity by the cells was realised by liquid scintillation in a
7 counter (LS 6500 Scintillation system, Beckman). Results from triplicates (means of counts per
8 minute ± standard deviations) are expressed in percentages (control taken as 100%).

9

10 *2.5. Study of the proteome*

11

12 The preliminary proteomic analysis was realized on human PBMC whole cells. Human PBMCs
13 were isolated from buffy coats by using standard Ficoll/Hypaque (Amersham) gradients
14 (centrifugation during 30 min at 700g). PBMCs were washed twice in phosphate buffer saline
15 solution (PBS) and the pellet was re-suspended in the culture medium (2×10^6 cells/ml). 20 ml were
16 dispersed in each T75 flasks (Susp Cell Ven green, Sarsted).

17 After a 72 h of MeHg exposure, cells were collected by centrifugation and washed with PBS.
18 Cytosolic proteins were extracted in a hypotonic buffer (10mM HEPES, pH 7.4; 10mM NaCl;
19 KH_2PO_4 1mM, NAHCO_3 5mM, EDTA 5mM, EDTA 5mM, $\text{CaCl}_2 (\text{H}_2\text{O})_2$ 1mM, $\text{MgCl}_2 (\text{H}_2\text{O})_6$ 0.5
20 mM and anti-proteases Roche) followed by two centrifugation steps at 6300g and 4000g for 10 and
21 5 min respectively. A protein assay was carried out on the extracted fractions by using the DC RC
22 Protein Assay Kit (according to the manufacturer's instructions). The different extracts were
23 purified using the 2D clean-up Kit (according to the manufacturer's instructions). After this
24 purification step, each sample was redissolved in the DIGE buffer (urea 7M, thiourea 2M, Tris-HCl
25 pH 8.8 30 mM, Chaps 1.5%, ABS-14 1.5%). After a second assay, allowing us to know the exact
26 concentration of our extracts, they are diluted to a protein concentration of $5 \mu\text{g}/\mu\text{l}$ and to pH 8,

1 using DIGE buffer. Mowse score (MOlecularWeightSEarch) was calculated. Mowse scoring is
2 based on peptide frequency distribution [55].

3

4 2.5.1. Labelling

5

6 After extraction, the variations in protein abundance between the treated (with 1 μ M of MeHg) and
7 non-treated samples were measured using the 2D DIGE technique (Two Dimension Difference Gel
8 Electrophoresis).

9 The labelling of both the control fraction (12.5 μ g), and the exposed fractions (12.5 μ g) are carried
10 out with 100 pmol of Cy5 and of Cy3 respectively. The internal standard consists of a mixture of
11 each sample (12.5 μ g/sample) labelled with 1200 pmol of Cy2. This step is then followed by
12 incubation on ice, in the dark, for 45 minutes. The eventual excess of Cy5 is eliminated by the
13 addition of 10^{-4} mol of lysine on ice for 15 minutes in the dark. The labelled samples forming a
14 same gel are pooled. In addition 150 μ g of unlabelled protein are added to achieve a sufficient
15 quantity of unlabelled protein required for analysis by mass spectrometry. A volume of buffer 2X
16 (urea 7M, thiourea 2M, chaps 1.5%, ASB-14 1.5%, destreak reagent 12.5 μ l/ml, DTT 10 mM,
17 ampholyte 1%) is added in equivalent quantities to the previously pooled samples. The final volume
18 of the samples is brought up to 450 μ l with IEF buffer (urea 7M, thiourea 2M, chaps 1.5%, ASB-14
19 1.5%, destreak reagent 12.5 μ l/ml, ampholyte 0.5%, bromophenol blue 0.5%).

20

21 2.5.2. 2D-PAGE

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23 The first dimension of the gel is carried out on gel strips with a fixed pH gradient (Immobiline
24 DryStrip) in an electrical focalisation cell (Protean IEF cell, Bio-Rad). After active rehydration for 9
25 hrs with the sample, the isoelectric focalisation is carried out up to 50 000 Vh over night. Before the
26 second dimension, reduction buffer is added for 15 minutes (DTT 130 mM, urea 6M, Tris-HCl pH

1 8.8 0.373M, glycerol 20%v/v, SDS 2%w/v) followed by the addition of an alkylation buffer for 15
2 minutes (iodoacetamide 135 mM, urea 6M, Tris-HCl pH 8.8 0.373M, glycerol 20%v/v, SDS
3 2%w/v).

4 The second dimension is carried out overnight after having deposited the strips on a 12.5%
5 polyacrylamide gel (Tris-HCl pH 8.8 1.5M, SDS 0.4%w/v, acrylamide/bisacrylamide 40%,
6 ammonium persulfate 10%w/v, TEMED 0.03%v/v, MilliQ water). The gels are then placed on non-
7 fluorescing (Bind-Silane) plates (Ettan-DALT Casting Cassette « Low Fluorescence », Amersham
8 Biosciences). After migration, the gels are scanned at the three different wave lengths of the
9 cyanines (Cye2 520 nm bandwidth 40, Cye3 580 nm Bp 30, Cye5 670nm Bp 30) using a Typhoon
10 9400 (Amersham Biosciences), with a resolution of 100 μ m. The images obtained are analysed
11 using a program (Decyder 6.0, Amersham Biosciences). Only the regulations above ± 1.5 the
12 standard deviation were taken into account and compared with a T Student value at 95% confidence
13 level.

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15 2.5.3. Protein identification

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17 The spots of interest, selected using the software program were excised from the gel, by a spot
18 picker (Ettan Spot Picker, Amersham Biosciences), then placed in 96 well plates for enzymatic
19 digestion (Proteineer dp automated digester, Bruker). The peptides resulting from the digestion
20 were spotted onto a prespotted MALDI plate (AnchorChips, Bruker). The proteins were identified
21 using an Ultraflex MALDI TOF/TOF (Bruker). The mass values from the mass fingerprints, and
22 from the MS/MS spectra were processed with in different data bases (Sprot, NCBI, MSDB). The
23 search engine used was MASCOT, using the following parameters: the mass tolerance for peptides
24 was set at ± 60 ppm, there charge state at 1+ and the maximum number of missed cleavages at 1.
25 Taxonomy for data base search was Homo sapiens, Mammals. Extension of the data base search to
26 mammals (and not only to humans) enables us to identify contaminants, such as keratin. The mass

1 tolerance for peptides was set at ± 60 ppm, there charge state at 1+ and the maximum number of
2 missed cleavages at 1.

3 Trypsin was used for protein digestion into peptides. Carbamidomethylation of cystein residues
4 were set as fixed modifications and oxidation of methionine as variable modifications.

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3. RESULTS

3.1. Hg levels in blood of free-ranging seals

Hg levels in full blood varied from 0.04 to 0.56 $\mu\text{g}\cdot\text{g}^{-1}$ fw (43 to 611 $\mu\text{g Hg}\cdot\text{L}^{-1}$) with a mean concentration of 0.16 $\mu\text{g}\cdot\text{g}^{-1}$ fw (table 3). Hg concentrations were similar between males and females (T-Student, $p > 0.5$) and individuals were regrouped. Hg concentration in blood was significantly correlated to the length ($r_p = 0.6$ and $p < 0.001$) and body mass of the seals ($r_p = 0.6$ and $p < 0.001$, figure 1).

3.2. Proliferative response of controls and exposed human and seal PBMCs

Control seal and human lymphocyte proliferation, determined by cell counts, was stimulated for 72 hr in the presence of 5 and 1 $\mu\text{g}\cdot\text{ml}^{-1}$ respectively (figure 2). After 72 hr, decrease of proliferation was already noticeable at 5 and 10 μM of MeHgCl. Cytotoxicity was moderate at 1 and 1.5 μM MeHgCl concentrations for harbour seal PBMCs (figure 3).

3.3. Functional tests

DNA, RNA and protein synthesis were evaluated through ^3H -thymidine, ^3H -uridine and ^3H -leucine incorporation by mitogen-stimulated PBMCs. A decrease of DNA, RNA and protein synthesis was observed even at low MeHg concentrations (0.2 and 1 μM) whereas inhibition of synthesis was clearly noticed at 10 μM (figure 4). DNA, RNA and protein synthesis showed a similar profile but nucleic acid synthesis seemed more inhibited compared to proteins. After 48h, the concentration inhibiting 50% of DNA and RNA synthesis the two species were around 1 μM and 1.5 μM while 50% protein synthesis inhibiting concentration was around 1.5 μM .

1 The MTS assay (water soluble tetrazolium salts) reflects cell proliferation and the metabolic activity
2 of the mitogen-stimulated lymphocytes. Metabolic activity was significantly reduced compared to
3 the controls around 1 μ M both for human and seal cells (figure 5).

4 3.4. Detection of housekeeping gene and cytokines

5 In this set of experiments, seal PBMCs were cultured in the presence of non-cytotoxic doses of
6 MeHg (0.2 and 1 μ M) to examine mRNA expression. GAPDH, IL-2, IL-4 and TGF- β mRNA were
7 successfully detected both in controlled and exposed PBMCs (figure 6). As a general feature,
8 change in mRNA expression occurred after exposure: the number of copies decreased at 0.2 μ M,
9 except for IL-4. For a quantitative analysis of mRNA expression, comparison between samples can
10 be made by relating gene expression levels to housekeeping gene expression, the latter regarded as
11 not being affected by exposure conditions. The large dispersion of data between seals (coefficient of
12 variation \leq 68%) indicated high grade inter-individual variability in cytokine mRNA
13 responses. Cytokine index were strikingly low for IL-2 and TGF-beta even at 0.2 μ M (figure 6a and
14 6b) while an increasing trend was observed for IL-4 (figure 6c).

15 3.4. Study of the proteome

16 Preliminary proteomic analysis was realized on human PBMCs. We focus this part of the study on
17 cytoplasmic fraction (soluble proteins). Cells were treated with 1 μ M MeHgCl in the presence of
18 PHA for 72h. Quantitative analysis was realized on 2D DIGE gels in two steps (figure 7 and 8).
19 First, individuals were treated separately (n = 3) to apprehend interindividual variability (figure 7,
20 gel for one individual) and secondly, individuals were pooled in order to obtain sufficient amount of
21 proteins to realize the gel (figure 8). Most of the proteins had a I_p between 3 and 10. Mowse score
22 was calculated for each identification, which is considered as significant above 54 (table 2). Low
23 doses of organic mercury (1 μ M) affected cytoplasmic protein expression. Proteins are generally less
24 expressed in treated gels. Volume ratio (expression of protein in treated gel compared to control) is
25 positive (over-expression) for spots 1142, 1452 but negative (lower-expression) for spots 1319

1 (vimentin), 897 and 923 (table 2). Preliminary results showed that identified proteins are involved
2 in many cellular functions such as cell proliferation (SYW), cytoskeleton (VIME), protein
3 degradation (PRS10), melatonin biosynthesis as well as transduction pathways (GBLP, AN32A)
4 (table 4).

6 4. DISCUSSION

7 4.1. Hg levels in wild seals

8 Mercury levels can reach high concentration in the liver and kidney collected on harvested or
9 stranding marine mammals [20, 22, 56]. Fewer studies described Hg concentrations in the blood of
10 pinnipeds [57-59] (table 3). Total Hg levels measured in the blood of harbour seals caught in the
11 North Sea are higher than that previously described for caught in North Atlantic or in Arctic regions
12 (table 3). Interestingly, the level of T-Hg in blood of harbour seals from the North Sea is not lower
13 than that encountered in other seal species 30 years ago. Similarly, Hg level have not decreased in
14 Arctic biota despite the recent emission reductions in North America and Western Europe [4-6].
15 Obviously decreasing trends previously described for invertebrates in the North Sea [60] is not
16 reflected in marine mammals. Ecosystem-scale simulations suggested that a ~50% reductions in
17 mercury inputs would be required to ensure safe exposure levels for community oriented towards
18 fish and marine mammal diet (Boot and Zeller, 2005).

19 However, Hg concentration in the blood is strongly correlated to length and body mass on the seals
20 (figure 1). This correlation reflects daily Hg intake and thus, the amount of fish ingested which
21 differ according the body mass of animals. Adult harbour seals eat 5% to 6% of their body weight
22 per day, up to 7 kg for big individuals [61, 62].

23 The measurement of the concentration of total Hg in blood is generally a good surrogate for the
24 concentration of MeHg in blood in human populations with high fish consumption [15, 17]. Median
25 cord blood concentration in a human cohort with high fish consumption in the Faroe Islands was 24

1 $\mu\text{g.L}^{-1}$ [63]. Methylmercury in humans is believed to have a blood half-life of 44 days with a low
2 urine excretion [64]. The interpretation of Hg concentrations in the blood is always difficult in term
3 of toxicity. A commonly used reference interval for human beings is 0.6 – 59 $\mu\text{g.L}^{-1}$ [64, 65]. Clear
4 signs of mercury toxicity develop in most individuals only at some point much higher than the
5 upper reference limit. Environmental Protection Agency recommended that blood mercury levels
6 should not be higher than 5.8 $\mu\text{g.L}^{-1}$ at least for the more sensitive individuals such as pregnant
7 women [18]. Obviously harbour seals from the North Sea displayed higher concentrations (from 43
8 to 611 $\mu\text{g.L}^{-1}$). It should be quoted that an adult seal may eat up to several kilograms of fish per day
9 [61], resulting in elevated MeHg ingestion and assimilation. Even if interspecies comparison brings
10 limited information on potential toxicity of these environmental concentrations, question arises
11 about biochemical effects of these MeHg levels on harbour seal immune cells.

12 4.2. *Proliferative response to MeHg exposure*

13 Mercury is often considered as a “paradigm” [38], because of its rather unique range of
14 immunotoxic properties. Exposure to low concentrations of mercury can depress or stimulate the
15 immune system and even induce autoimmune disease in various animal species, through
16 mechanisms that are still poorly understood [38].

17 Exposure of B-lymphocytes *in vitro* to MeHg has been shown to inhibit mitogen-induced DNA
18 synthesis, cell proliferation, and antibody synthesis.

19

20 Here, we exposed harbour seal and human lymphocytes *in vitro* to organic mercury (MeHgCl) and
21 we examined the effects on cell-mediated immunity: cell mortality, synthesis of DNA, RNA and
22 protein and metabolic activity. Although the *in vitro* approach utilized in this investigation
23 represents an extreme reductionism relative to the complex situation in the intact organism, it can
24 provide insight into specific effects of model agents. Low Hg concentrations reflecting Hg levels
25 measured in harbour seal blood were chosen (200 $\mu\text{g.L}^{-1}$, around 1 μM). Cytotoxicity was reduced

1 in the interval 0.1- 1 μ M (figure 3) both for human and seal PHA –stimulated PBMCs suggesting
2 limited mortality for T-lymphocytes until 1 μ M. However, despite the variability associated with
3 our samples, a clear suppressive effect of MeHg on DNA, RNA and protein synthesis in seal and
4 human PBMCs was present, even at low concentrations. Protein synthesis seemed less affected,
5 probably due to their longer response time. Proliferation and metabolic activity reflected by MTS
6 assay confirmed that 1 μ M was a critical concentration with significantly reduced *in vitro* activity of
7 human and seal PBMCs relative to controls. Up to 90% of cells exposed to 1 – 1.5 μ M of MeHg
8 were alive whereas metabolic activity was lower relative to controls, as well as DNA, RNA and
9 protein synthesis. This could be related to a cytostatic effect. At higher concentrations, 5- 10 μ M,
10 75% of cells were still alive, but metabolic activity dropped to very low levels related to this
11 cytotoxic effect.

12 No striking difference appeared between human and seal *in vitro* resistance to MeHg.

13

14 4.3. Cytokine expression in MeHg-exposed seal PBMCs

15

16 Among the earliest detectable cellular response to antigen recognition and T-Cell receptor signaling
17 is the secretion of cytokine. Cytokines mRNA quantification is widely used in immunological
18 research to dissect the early steps of immune responses or pathophysiological pathways. IL-2 and
19 TGF- β seemed highly sensitive to MeHg *in vitro* exposure in regard to their drastic decrease gene
20 expression at 0.2 μ M compared to controls (figure 6) reflecting a high sensibility of these cytokine
21 to methylmercury exposure. However, large inter-individual variability was observed and cytokine
22 expression in seal PBMCs vary rapidly following MeHg exposure duration [66]. This cytokine
23 modulation is supported by real-life studies of harbour seals in which PBMC IL-1 and IL-2
24 expressions were also decreased relative to controls following exposure to BaP and CB-169 [50].

25 IL-2, the growth factor for antigen-stimulated T-cells, is responsible for T-cell clonal expansion
26 after antigen recognition, primarily through autocrine activity. IL-2 also increases synthesis of other

1 cytokines in T cells, promotes the proliferation and differentiation of NK cells, and acts as a growth
2 factor and stimulus for antibody synthesis in B cells. The pro-inflammatory cytokine IL-2 is
3 generally produced at the site of infection and tissue damage [44]. Its presence in blood can precede
4 changes in the concentrations of acute phase proteins and neutrophilia associated with the
5 inflammatory response [44]. TGF- β is often considered as an anti-inflammatory cytokine [67, 68].
6 TGF- β is a powerful mediator of immune cell phenotype and function. A variety of murine models
7 provide strong evidence that eliminating TGF- β or disrupting its downstream signaling cascade
8 leads to inflammatory disease [46]. For example, deletion of the TGF- β 1 gene causes systemic
9 inflammation and early death [69]. Expression of a dominant negative TGF- β type II receptor leads
10 to CD4+ T cell hyperactivity and autoimmunity [70].

11

12 On contrast, cytokine index of IL-4 showed an increasing trend from control to 1 μ M. *In vitro*
13 studies of culture lymph node cells showed that PCBs increased IL-4 [71]. IL-4 induces
14 differentiation of naive helper T cells (Th0 cells) to Th2 cells. Upon activation by IL-4, Th2 cells
15 subsequently produce additional IL-4 [45].

16 Our findings are consistent with those of previous researchers working with human and rodent
17 systems and support the hypothesis of contaminant-altered lymphocyte function mediated by
18 cytokine production.

19 It is unclear how mercury triggers an activation of T cells and an upregulation of cytokine
20 production in genetically susceptible animals [72]. Mercury and other heavy metals can have
21 mitogen-like effects on cells and are strong activators of the immune system [73-75]. However,
22 most of the available evidence, including the presence of self-reactive and metal-specific cells as
23 well as the specificity of the autoantibody response, suggests that mercury induces also a specific
24 antigen-driven response [72]. These preliminary *in vitro* results suggest that MeHg could induce a
25 differentiation of naïve cells (increase of IL-4) while T-lymphocyte clonal expansion is inhibited

1 (decrease of IL-2); Increase of inflammatory response as suggested by TGF- β decreases would
2 require a polynuclear cell model.

3 4 4.4. Study of the proteome and biochemical mechanism of toxicity

5 Proteomic (study of the proteome) has been attempted on human PBMCs exposed to environmental
6 levels of MeHg. Proteomic facilitate the identification of new biomarkers of chemicals exposures
7 and studies of mechanisms by which protein modification contribute to the adverse effects of
8 environmental exposures [41, 76]. Proteome of T-lymphocytes is well known [77-79]. However,
9 expression of proteins in MeHg-exposed T-lymphocytes has not been described yet. As a general
10 feature, many spots are underexpressed in exposed gel, reflecting the inhibition of protein synthesis
11 linked to MeHg toxicity. As for cytokine mRNA expression, high variability between individuals
12 was evidenced contrasting to functional tests displaying weaker inter-individuals variations. This
13 feature raises several perspectives in the framework of individual susceptibility to pollutants. To our
14 knowledge, proteomic analysis on MeHg exposed cells has been rarely investigated merely on
15 neurons [76].

16 Preliminary results showed that identified proteins are involved in many cellular functions such as
17 cell proliferation (SYW), cytoskeleton (VIME), protein degradation (PRS10), melatonin
18 biosynthesis (ASML) and transduction pathways (GBLP, AN32A). Human lymphoid cells are an
19 important physiological source of melatonin which could be involved in the regulation of the human
20 immune system [80]. BTB1 belongs to the BTB domain, a large family whose members function in
21 a variety of biological processes. This evolutionarily conserved protein is often found at the N-
22 terminus of developmentally regulated zinc-finger transcription factors, as well as in some actin
23 associated proteins bearing the kelch motif [81]. In humans, BTB domain has been shown to
24 mediate transcriptional repression through the local control of chromatin conformation [81].

1 MeHg exposure is known to induce a rapid and sustained increase in intracellular calcium levels
2 [82, 83]. Shenker and co-authors [84] investigated the mechanisms by which MeHg chloride
3 induces human T-cell apoptosis. They reported that the earliest detectable event following MeHg
4 exposure was the level of mitochondria. Exposure of T-Cells to MeHg chloride caused a decrease in
5 the overall size of mitochondria and changes in the structure of the cristae. [84, 85]. After 16h of
6 treatment with MeHgCl, expression and activation of different caspases was observed [85].
7 Caspases are cysteine proteases that are essential for executing apoptosis and degrade vimentin [86,
8 87]. The lower vimentin expression found in exposed MeHg lymphocyte agrees with this caspase
9 activation. *In vitro* effect of methylmercury (60nM) on protein expression realised on human
10 neurons suggest different protein targets linked to neurotoxicity [76]. Prolonged exposure to
11 nanomolar concentrations of methylmercury reduced the amount of the expression of the
12 mitochondrial enzyme 3-ketoacid-coenzyme A transferase I, a key enzyme in the catabolism of
13 ketonic bodies and therefore in the synthesis of neural lipids [76].

14 In this work, we found that exposure for 72 hours *in vitro* to 1 μ M affect not only cell proliferation
15 but also many cellular functions such as the cytoskeleton, melatonin biosynthesis, signal
16 transduction pathway and transcription. This variability in affected cellular functions suggests
17 various toxicity pathways, depending on duration of exposure, MeHg concentration, cell type and
18 individual susceptibility.

19

20 Summary and conclusions

21 This study is to our knowledge, the first to investigate effects of *in vitro* MeHg exposure on cell
22 function and cytokine expression in marine mammals, using low MeHg concentration reflecting
23 environmental levels measured in harbour seals from the North Sea. The level of total mercury
24 analysed in the blood of wild harbour seal from the German North Sea are high compared to
25 previous studies on marine mammals and human beings. The level observed reflects both their

1 piscivorous habits and the contamination of the North Sea. Our cell model revealed an *in vitro*
2 immunosuppressive effect of MeHg even at low concentration (0.2 and 1 μ M). The present study
3 showed association between observed blood levels in wild seals and *in vitro* effects of these levels
4 on T-lymphocytes which suggest that mercury could-be an additional cofactor in this pollutant
5 cocktail and therefore raise the possibility of additional additive effects. Continued investigation of
6 the cellular and molecular pathways of MeHg-induced immune dysfunction in marine mammals
7 will enhance our understanding of individual and population health.

8

9 **Competing interests**

10 The authors declare that they have no competing interests.

11 **Author's contributions**

12 KD conceived of the study and participated in its design and coordination, provided expert advice
13 on mercury exposure and drafted the manuscript. US participated in the study design, coordinated
14 the sample collection and provide expert advice on seals. AG and AD contributed to sample
15 preparation and data acquisition. SF contributed to the study design and provided expert advice on
16 cytokines. GM and EDP provided expert advice on proteomic. MCDPG participated in the study
17 design, provided expert advice on cell culture, functional tests and contributed to the preparation of
18 the manuscript.

19

20 All authors read and approved the final manuscript.

21

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23

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8

9

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Figures

Figure 1. Relationship between body mass (kg) of harbour seals and Hg concentrations ($\mu\text{g}\cdot\text{g}^{-1}$ fw) in blood

Figure 2 - Response of control PBMCs to PHA (5 and 1 $\mu\text{g}\cdot\text{ml}^{-1}$ for harbour seal and human respectively) after 72h of stimulation.

Results are expressed as a percentage expressed relative to the initial $2\cdot 10^6$ cells per ml of culture medium.

Figure 3 - Percentage of surviving cells estimated by Trypan blue coloration after 72 hours of MeHgCl exposure ($p < 0.05$; **, $p < 0.01$ relative to control PBMCs 100%)*

5 wells per condition for both species

Humans: $n = 8$ for 0.1 μM and 1 μM ; $n = 3$ for other concentrations.

Harbour seals: $n = 5$ for 1 μM and $n = 2$ for other concentrations

Figure 4 - Effects of increasing MeHgCl concentration on DNA (^3H -thymidine), RNA (^3H -uridine) and proteins (^3H -leucine) in human (A) and seal (B) PBMCs.

Results from quadruplets (means of counts per minute \pm standard deviation, $n = 4$) are expressed in percentages (control taken as 100%)

Figure 5 - MTS activity of human and seal PBMC relative to MeHgCl exposure ($p < 0.05$; **, $p < 0.01$ relative to control PBMCs 100%)*

For humans: and $n = 7$ for 0.1 μM and 1 μM ; $n = 3$ for other concentrations

For harbour seals: $n = 5$ for 1 μM and $n = 2$ for other concentrations

5 wells per condition for both species

Figure 6: Cytokine Index of (A) IL-2, (B) TGF- β , (3) IL-4 mRNA in function of MeHgCl medium exposure.

Results are expressed in mRNA copy numbers per GAPDH mRNA copies. The mean and standard deviation on the mean of four individuals and carried out in duplicate are shown

Figure 7: 2D-Dige Gel with cytoplasmic proteins extracted from one human being control and treated PBMCs (IPG strip pH 3to 10).

Each of the 3 individuals were analysed separately. O: excised spot for further protein identification

Figure 8: 2D-Dige Gel with cytoplasmic proteins extracted from human control and treated PBMCs (IPG strip pH 3to 10). Cytoplasmic proteins extracted from 3 individuals were pooled for this gel. O: excised spot for further protein identification

Tables

Table 1 - Table 1 : Sampling description of harbour seal Phoca vitulina

Table 2 - Primer sequences used for the amplification of cytokine and housekeeping gene transcripts in lymphocytes

bp = base pair ; S = sense; AS = antisense; IL = interleukin; TGF = transforming growth factor

1 *Table 3 - Total Hg concentrations in blood of pinniped species*
2 mean ± standard deviation, n = number of samples analysed, nd not determined
3 ...

4 *Table 4 - Expression of identified proteins after spot excision (pooled from 3 individuals)*
5 *spot number after figure 9

6
7

8 **Additional files**

9 **Additional file 1 – The harbor seal Phoca vitulina**

10 Picture taken by A. Dupont

11

12 **Additional file 2 – Another sample additional file title**

13 Additional file descriptions text (including details of how to view the file, if it is in a non-standard
14 format).

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Fig 1

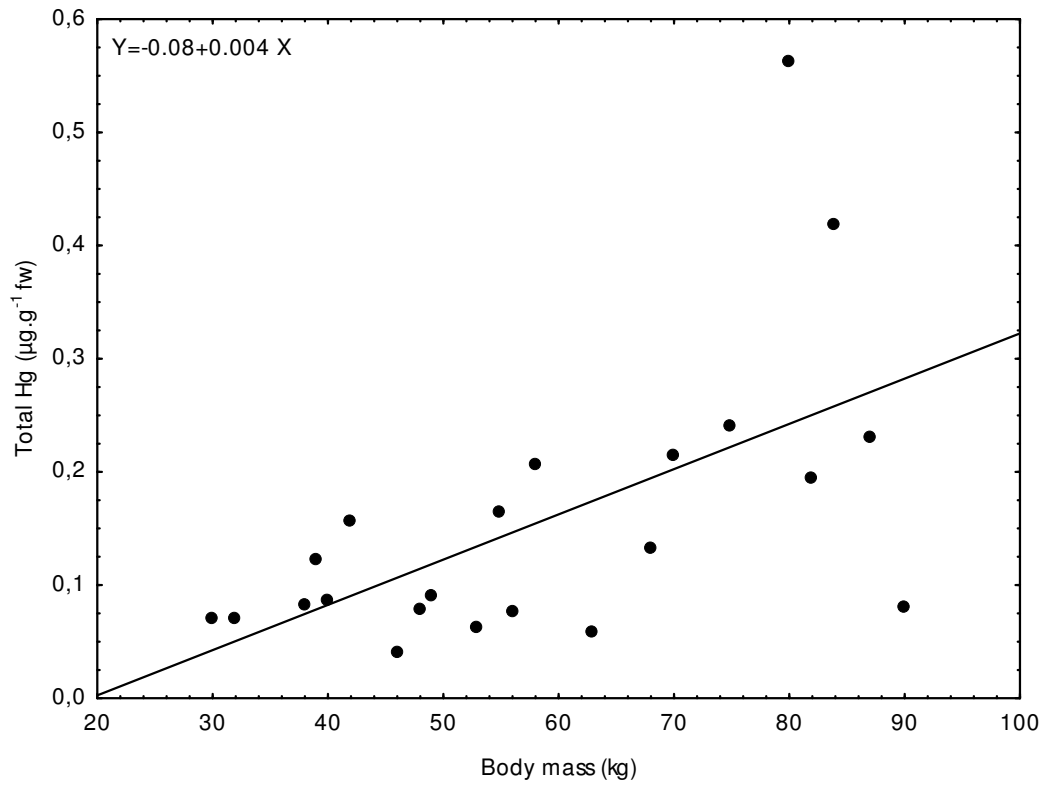


Fig 2

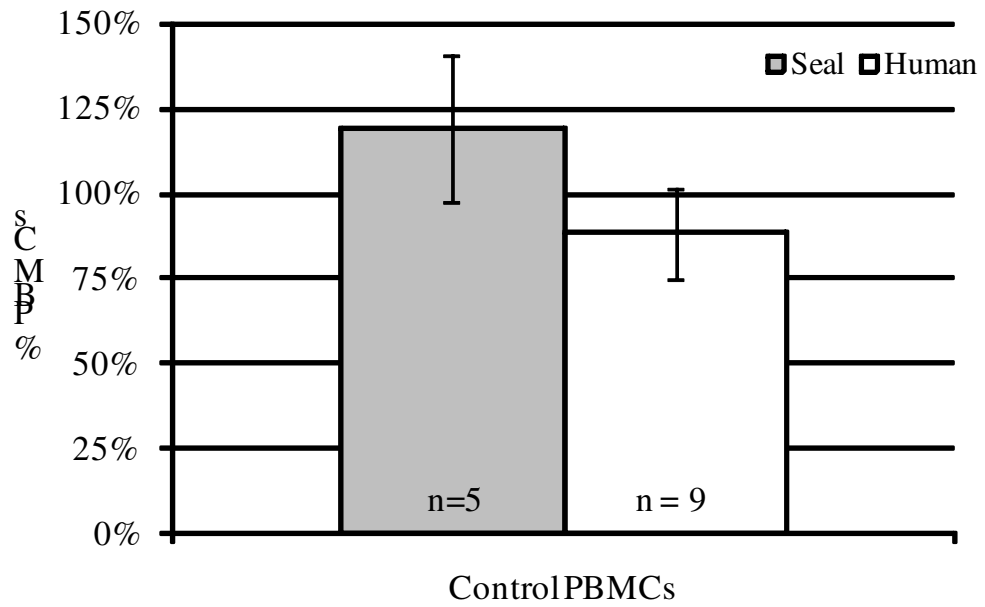


Fig 3

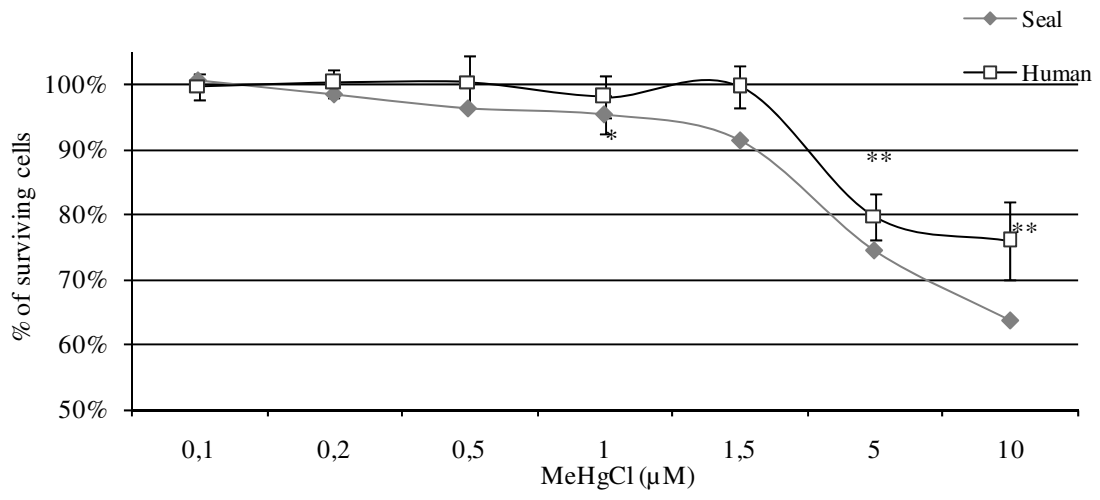
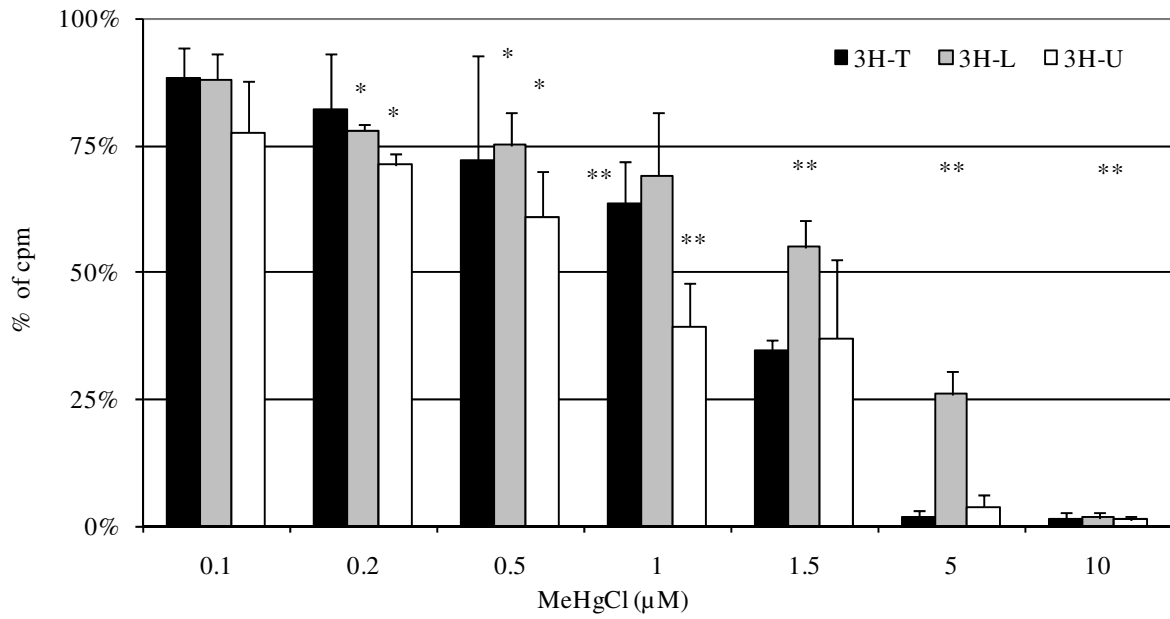
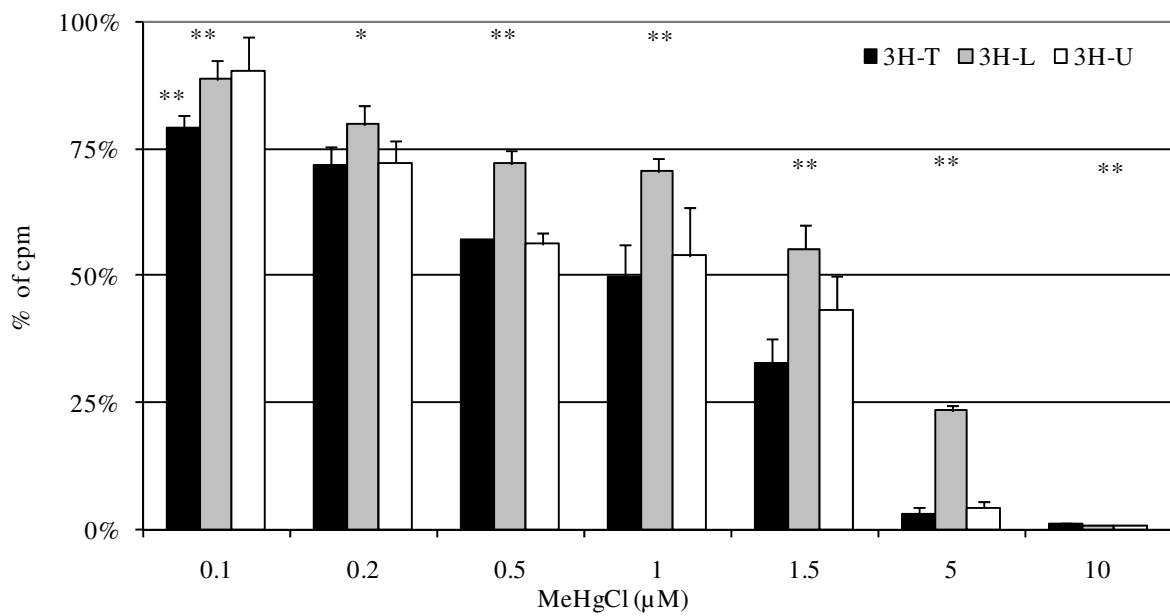


Fig 4
A – Human PBMCs



B – Harbour seal PBMCs



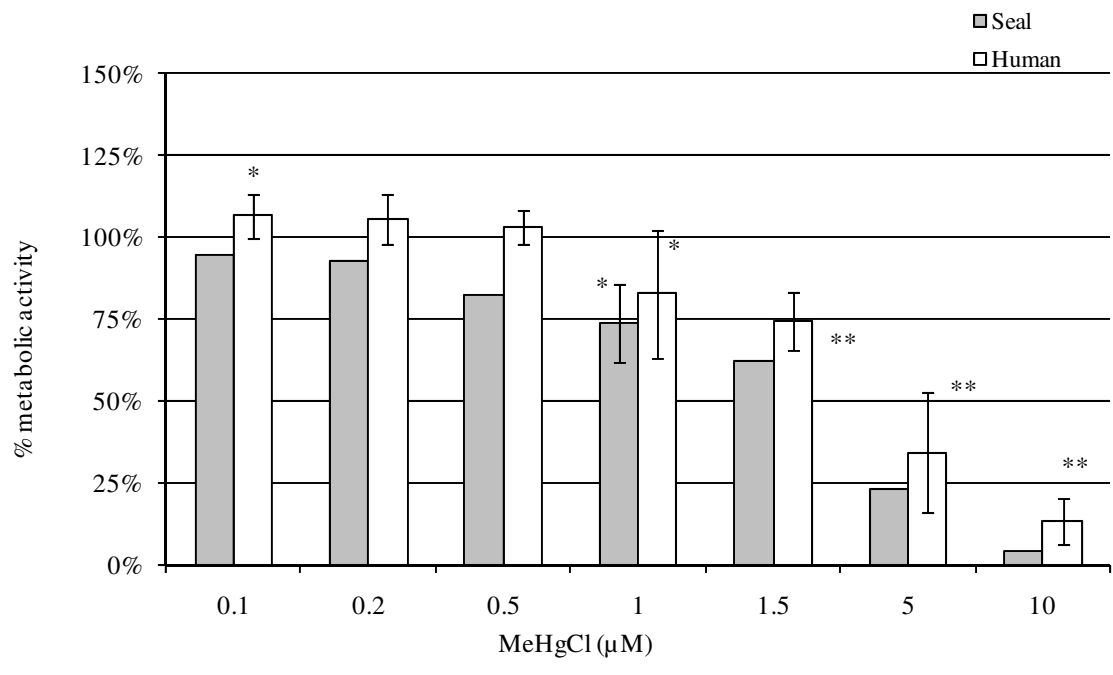
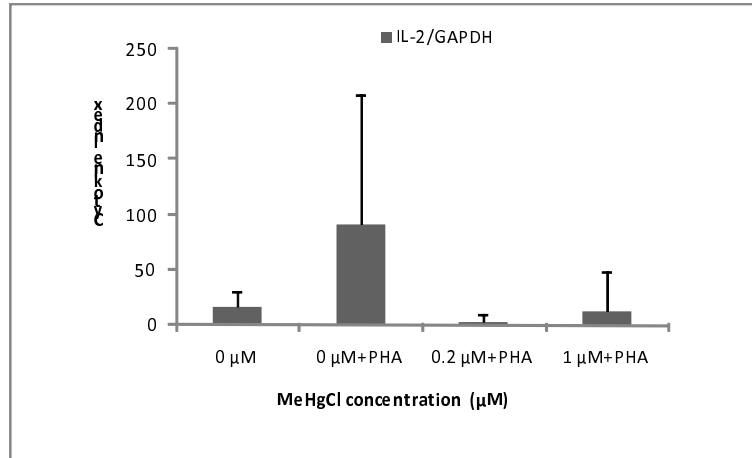
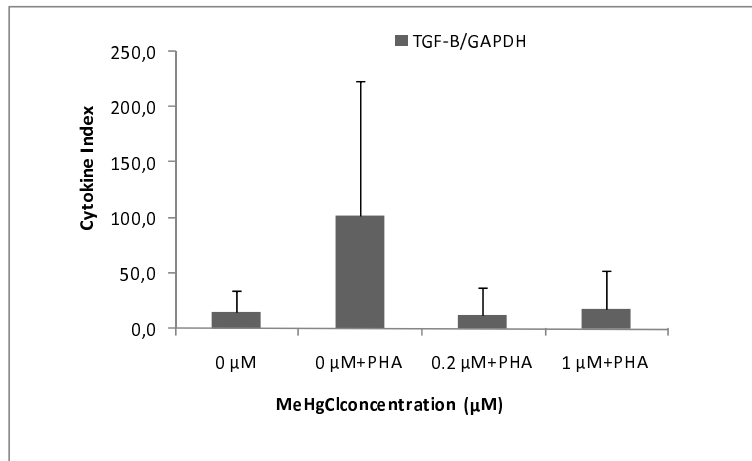


Fig 6



B



C

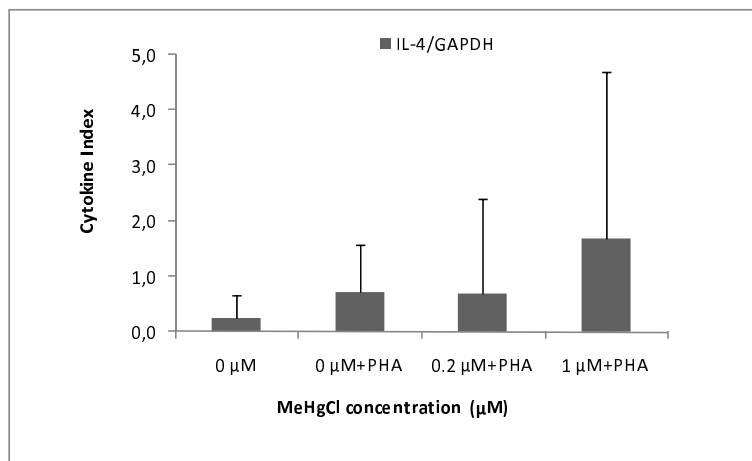


Fig 7

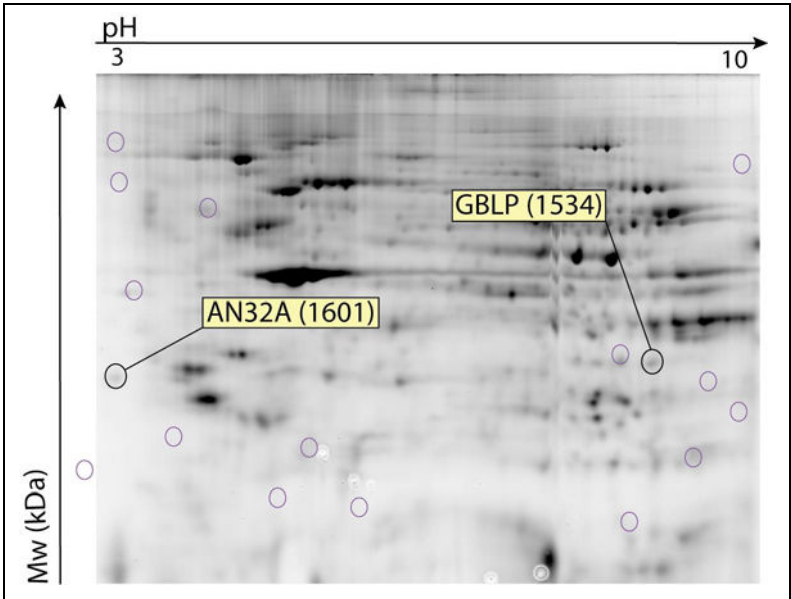


Fig 8

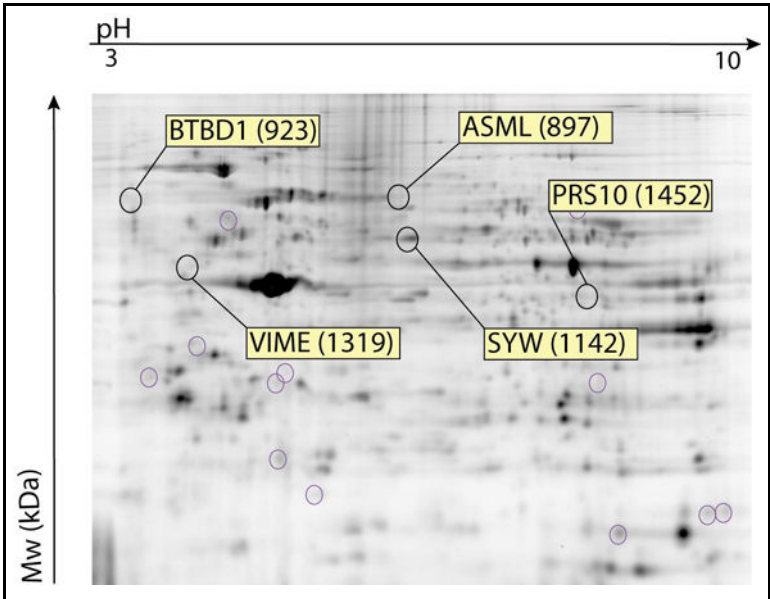


Table 1 : Sampling description of harbour seal *Phoca vitulina*

| ID | Date | Sampling site | Sex | Length (cm) | Body mass (kg) | |
|---------|--------------------------|----------------|-----|-------------|----------------|------------------|
| Pv 455 | 20/10/97 | Lorenzensplate | M | 117 | 32 | T-Hg |
| Pv 456 | 20/10/97 | Lorenzensplate | F | 104 | 30 | T-Hg |
| Pv 460 | 20/10/97 | Lorenzensplate | M | 133 | 49 | T-Hg |
| Pv 2257 | 09/04/03 | Lorenzensplate | M | 144 | 68 | T-Hg |
| Pv 2258 | 09/04/03 | Lorenzensplate | M | 148 | 63 | T-Hg |
| Pv 2259 | 09/04/03 | Lorenzensplate | F | 129 | 38 | T-Hg |
| Pv 2260 | 09/04/03 | Lorenzensplate | F | 143 | 48 | T-Hg |
| Pv 2261 | 09/04/03 | Lorenzensplate | F | 158 | 56 | T-Hg |
| Pv 2262 | 09/04/03 | Lorenzensplate | F | 132 | 46 | T-Hg |
| Pv 2263 | 09/04/03 | Lorenzensplate | F | 148 | 55 | T-Hg |
| Pv 2265 | 09/04/03 | Lorenzensplate | F | 148 | 55 | T-Hg |
| Pv 2687 | 25/08/04 | Lorenzensplate | M | 170 | 84 | T-Hg |
| Pv 2688 | 25/08/04 | Lorenzensplate | M | 170 | 82 | T-Hg |
| Pv 2689 | 25/08/04 | Lorenzensplate | M | 175 | 80 | T-Hg |
| Pv 2690 | 25/08/04 | Lorenzensplate | M | 175 | 90 | T-Hg |
| Pv 2691 | 25/08/04 | Lorenzensplate | F | 150 | 58 | T-Hg |
| Pv 2692 | 25/08/04 | Lorenzensplate | F | 165 | 70 | T-Hg |
| Pv 2694 | 25/08/04 | Lorenzensplate | M | 160 | 75 | T-Hg |
| Pv 2695 | 25/08/04 | Lorenzensplate | M | 175 | 87 | T-Hg |
| Pv 2697 | 25/08/04 | Lorenzensplate | F | 135 | 42 | T-Hg |
| Pv 2699 | 25/08/04 | Lorenzensplate | M | 140 | 40 | T-Hg |
| Pv 2700 | 25/08/04 | Lorenzensplate | M | 125 | 39 | T-Hg |
| Kirsa | 28/02/05 | Seal station | F | nd | 28 | Cytokine |
| Pv 2883 | 12/04/05 | Lorenzensplate | M | 174 | 93 | Cytokine |
| Pv 2885 | 12/04/05 | Lorenzensplate | M | 168 | 81 | Cytokine |
| Pv 2887 | 12/04/05 | Lorenzensplate | M | 180 | 96 | Cytokine |
| Pv 2893 | 12/04/05 | Lorenzensplate | M | 164 | 71 | Cytokine |
| Lümmen | 31/03/2006 30/07/2006 | Seal station | M | nd | 105 | Functional tests |
| Hein | 31/03/2006 30/07/2006 | Seal station | M | nd | 86 | Functional tests |
| Deern | 31/03/2006 | Seal station | F | nd | 70 | Functional tests |
| Lilli | 28/04/2006 | Seal station | F | nd | 54 | Functional tests |
| Mareike | 28/04/2006 | Seal station | F | nd | 66 | Functional tests |
| Kirsa | 28/04/2006 | Seal station | F | nd | 35 | Functional tests |

Table 2:

Primer sequences used for the amplification of cytokine and housekeeping gene transcripts in lymphocytes

| Gene | Primer sequence (5'-3') | Sens | Annealing temperature | Nucleotide position | Calculated length of amplicon | Sequences used for primer pair selection (accession number, NCBI) |
|--------------|---|---------|-----------------------|------------------------|-------------------------------|---|
| IL-2 | TTT AAG TTT TAC ACG CCC AAG TGT TTC AGA TCC CTT TAG TTTC | S AS | 55°C | 218-400 | 183 pb | AF072871 |
| IL-4 | ACT CAC CAG CAC CTT TGT CCA TCC TTA TCG CTT GTT CTT TG | S AS | 49°C | 48-200 | 153 pb | AF187322, AF104245 |
| TGF- β | TTC CTG CTC CTC ATG GCC AC GCA GGA GCG CAC GAT CAT GT | S AS | 57°C | 826-845 1218-1199 | 393 pb | Beineke et al., 2004 |
| GAPDH | GCC AAA AGG GTC ATC ATC TC GGG GCC ATC CAC AGT CTT CT | S AS | 57°C | 1225-1244 1452-1433 | 228 pb | Beineke et al., 2004 |

bp = base pair ; S = sense; AS = antisense; IL = interleukin; TGF = transforming growth factor

Table 3. Total Hg concentrations in blood of pinniped species (mean \pm standard deviation, n = number of samples analysed, nd not determined)

| Species | Location, year | Condition | Reference | Concentration | |
|--------------------------------|--|--|--------------------------|---------------------------------------|--|
| | | | | $\mu\text{g}\cdot\text{L}^{-1}$ | $\mu\text{g}\cdot\text{g}^{-1}$ fw |
| <i>Phoca vitulina</i> | North German Sea. (1997-2004) | free ranging seals | this work | 172 \pm 143 (43 - 611) n = 22 | 0.16 \pm 0.13 (0.04 – 0.56) n = 22 |
| <i>Phoca groenlandica</i> | NW Atlantic | Captive seal (before MeHg exposure) | Ronald et al., 1977 | 80 \pm 40 (n = 6) | nd |
| <i>Phoca groenlandica</i> | Gulf of St Lawrence (1976 - 1978) | Free-ranging adult seals | Ronald et al., 1984 | nd | Males: 0.15 \pm 0.02 n = 2 Females: 0.07 \pm 0.04 n = 48 |
| <i>Phoca groenlandica</i> | Front ice off Newfoundland – Labrador (1976-1978) | Free-ranging adult seals | Ronald et al., 1984 | nd | Males: 0.04 \pm 0.02 n = 3 Females: 0.02 n = 1 |
| <i>Leptonychotes weddellii</i> | Antarctic 1980 | Free ranging seals | Yamamoto et al., 1987 | nd | 0.02 n = 2 |
| <i>Callorhinus ursinus</i> | Alaska, 1972 | Wild nursing cows | Kim et al. 1974 | nd | 0.099 n = 2 |

Table 4 : Expression of identified proteins after spot excision (A: pooled from 3 individuals and B from 3 individuals separately)

A

| Spot number * | Ref | Name | Abundance | Volume ratio | Mw (Da) | Cellular function | Mowse score |
|---------------|--------|---|-----------|--------------|---------|---|-------------|
| 1142 | P23381 | SYW : Tryptophanyl-tRNA synthetase | ↑ | 1.52 | 53474 | Negative modulation of cell proliferation ; protein biosynthesis | 170 |
| 1319 | P08670 | VIME : Vimentine | ↓ | -1.67 | 53545 | Cytoskeletal protein | 286 |
| 1452 | P62333 | PRS10 : Proteasome 26S subunit ATPase 6 | ↑ | 1.63 | 44430 | Involved in the degradation of ubiquitineous proteins (ATP dependant). | 139 |
| 897 | O95671 | ASML : N-acetylserotonin O-methyltransferase-like protein | ↓ | -1.53 | 69526 | Melatonin biosynthesis and methyltransferase activity | 55 |
| 923 | Q9H0C5 | BTBD1 : BTB/POZ domain-containing protein 1 | ↓ | -1.55 | 53365 | Interact with topoisomerase 1 and isoform Delta of the ring finger protein TRIM5. | 55 |

*spot number after figure 8

B

| Spot number | Ref | Name | Abundance | Volume ratio | Mw (Da) | Cellular function | Mowse score |
|-------------|--------|--|-----------|--------------|---------|-----------------------------|-------------|
| 1534 | P63244 | GBLP : Receptor of activated protein kinase C 1 | ↓ | -1.51 | 35380 | Signal transduction | 118 |
| 1601 | P39687 | AN32A :Acidic leucine-rich nuclear phosphoprotein 32 family member A | ↓ | -1.93 | 28682 | Signal transduction pathway | 62 |

*spot number after figure 7