Cadmium a Novel and Independent Risk Factor for Early Atherosclerosis

Mechanisms and *in vivo* Relevance

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**Figures**

**ONLINE DATA SUPPLEMENT Figure 1:**

Maximum far wall intima media thickness (IMT) was measured at predefined segments the proximal 10 mm of the internal (ICA) and the distal 40 mm of the common carotid artery (CCA) of both sides.
ONLINE DATA SUPPLEMENT Figure 2: Distribution of IMT levels measured at the common (CCA) and proximal internal carotid artery (ICA) in the ARFY Study (mean maximum IMT of both sides).

METHODS

Reagents

All reagents used were purchased from Sigma-Aldrich (Vienna, Austria) unless stated otherwise, and were of analytical grade quality. Cd and Zn were applied to cells by the dilution of stock solutions; (Stock solution of CdCl₂ 15 mM (Sigma-Aldrich, Vienna, Austria) and ZnCl₂ 10 mM (Merck, Darmstadt, Germany), both in A. bd.).

Association between Intima Media Thickness and Serum Metal Concentrations in Healthy Young Females - the ARFY-Study

The ARFY-study included 205 female participants from the Innsbruck School of Nursing aged 18–22 years. Classic vascular risk factors, lifestyle behaviours and family history were assessed by validated and standardized procedures¹. Characteristics of study participants are...
summarised in ONLINE DATA SUPPLEMENT Table 1. IMT, a marker of early atherosclerosis and fatty streak formation, was quantified by high resolution B-mode ultrasound (General Electrics, logiq 700, 10 MHz transducer) in two predefined segments. The maximum IMT was recorded on the far wall of the left and right distal 40mm of the common and proximal internal (10mm distal of the bifurcation) carotid arteries (see ONLINE DATA SUPPLEMENT Figure 1). Maximum IMT of each segment of both sides was averaged. High IMT was defined as an reading exceeding the 90th percentile of the distribution in one of the two examined segments\textsuperscript{1,2} (see ONLINE DATA SUPPLEMENT Figure 2). In addition, serum metal concentrations (aluminium, chrome, iron, nickel, copper, zinc, selenium, strontium, cadmium, barium and mercury) were measured by induced-coupled plasma mass spectrometric analyses (ICP-MS)\textsuperscript{3}. Fasting serum samples from 195 of the 205 study participants were available for assessment of metal concentration; all were stored in metal-free tubes.

The association between Cd and high IMT was analysed by means of multivariate logistic regression analysis adjusted for systolic blood pressure, active and passive smoking, fasting plasma glucose, insulin resistance, LDL and HDL cholesterol, waist circumference, social status, family history for hypertension, GOT, lipoprotein(a), C-reactive protein, homocysteine, T-cell reactivity against human Hsp60, soluble Hsp60 in serum, asthma and oral contraceptives. Differential effects of Cd on high IMT according to Zn levels were tested by inclusion of an appropriate interaction term.

\textit{Cell Culture}

The isolation and culture of human umbilical vein ECs (HUVECs) has been described elsewhere \textsuperscript{4}. In the long-term culture experiments culture medium (EBM 2, Lonza GmbH, Wuppertal, Germany) was replaced with fresh medium including the indicated compounds
every second day. In all experiments using Zn, cells were pre-incubated with Zn for 24 hours before the addition of Cd.

Quantification of Cell Death
The detection and quantification of cell death with the Annexin/PI method and light scatter analyses were performed as previously described \(^4\). For cell death analysis, 3 \( \times \) 10^5 HUVECs per well were seeded into gelatine-coated 6-well plates (Greiner Bio One, Kremsmünster, Austria). Inhibitor concentrations used were zVAD (Alexis Biochemical, San Diego, USA), 30\( \mu \)M; 3-Aminobenzamide (3-ABA, Sigma-Aldrich, Vienna, Austria), 2mM; Calpain inhibitor III, 50\( \mu \)M (Sigma-Aldrich, Vienna, Austria). Retroviral infection and expression of Bcl-xL in HUVECs was performed as detailed previously \(^5\).

Lactate dehydrogenase release assay
The amount of lactate dehydrogenase (LDH) released from cells was quantified using the LDH cytotoxicity kit II (Biovision, Mountain View, CA, USA) according to the manufacturer’s instructions.

Detection of DNA strand breaks
The detection and quantification DNA strand breaks was performed with the In situ cell death detection kit, POD (Roche, Vienna, Austria) according to the manufacturer’s instructions.

Monolayer Permeability Assay
For the analyses of endothelial monolayer permeability 1 \( \times \) 10^5 HUVECs were seeded into the upper chamber of gelatine-coated 24-well Transwell plates (Greiner Bio One, Kremsmünster, Austria). The experiment was started, i.e. the indicated compounds were added two days after
the seeding of cells. To determine monolayer permeability the medium in the bottom well was replaced with PBS after the indicated time of treatment, and 32 µg of horseradish peroxidase (HRP (Sigma-Aldrich, Vienna, Austria) was added to each upper well chamber. After various times of incubation at cell culture conditions, HRP enzyme activity was determined in the bottom well. To do so, 100 µl of the PBS solution in the bottom well were transferred into a 96-well plate and 100 µl of ABTS buffer containing 0.32 mg ABTS/ml was added. After an additional incubation time of 45 min (RT°C, dark), absorption at 560nm was analysed on an ELISA reader (Anthos Labtec HT2 Salzburg, Austria).

**Western Blotting**

Western Blotting was performed as previously described 4. Primary antibodies used were anti-phospho serine (1981)-ATM antibody (Cell Signaling, Danvers, MA, USA), anti-p53 antibody (BD Pharmingen, Rockville, MD, USA), anti-p21/CIP1/WAF1 antibody (BD Pharmingen, Rockville, MD, USA), anti-caspase-3 (BD Pharmingen, Rockville, MD, USA), anti-PARP antibody (Cell signalling, Danvers, MA, USA), and anti-BID antibody (Abcam, Cambridge, UK).

**Caspase 3 Activity Assay**

To analyse caspase-3 activity, 1 x 10^6 HUVECs per well were seeded in gelatine-coated petri dishes ((BD Pharmingen, Rockville, MD, USA)). Caspase-3 activity was performed as described elsewhere 6.
Analysis of Cell Proliferation

3 x 10^3 HUVECs were seeded in the 96-well plates (Greiner Bio One, Kremsmünster, Austria) and treated with Zn and various Cd concentration for the indicated times. Cell proliferation was determined using the XTT assay (Biomol GmbH, Hamburg, Germany).

Treatment of Animals and Assessment of Atherosclerotic Plaque Area

ApoE knockout (KO) mice were purchased from the Charles River Laboratories (USA) and kept in the Central Animal Testing Facility (ZVTA) of the Innsbruck Medical University at 24°C and a 12-h light/dark cycle. Animals received normal diet and drinking water ad libitum until the age of 8 weeks. Then, female ApoE KO mice were divided randomly into four groups. Group 1 received normal water; group 2, 100mg/l of CdCl₂ in drinking water; group 3, 400mg/l ZnCl₂; and group 4, 100mg/l CdCl₂ plus 400mg/l ZnCl₂. In addition to receiving the indicated compounds via drinking water, the mice were fed a western type diet (crude fat 21.2%; Ssniff, Soest, Germany). After 12 weeks of treatment, animals were anesthetized with xylasol/ketamine and weighed. Thereafter, the thorax was opened and blood samples were taken with a syringe from the vena cava, which were consequently subjected to serum preparation which was then stored at -80°C. In parallel to serum preparation, the aorta was excised between the aortic arch and the iliac bifurcation. The aorta was cleaned by removing connective tissue and fat, washed in PBS with 20µM BHT and 2µM EDTA (pH=7.4), opened longitudinally, pinned on silicone plates with acupuncture needles (0.20 x 15mm, asia-med, Suhl, Germany) and fixed overnight in 4% PFA, 5% sucrose, 20µM EDTA (pH 7.4). Fixed tissues were then incubated with Sudan IV (0.5% Sudan IV in 35% ethanol and 50% acetone) for 15 min to stain atherosclerotic plaques. After destaining of aortas with 75% ethanol, pictures were taken with a Samsung NV 10 camera, which were then subjected to image analysis using the Image ProPlus 5.1 software (plaque area).
A total of 18 sections of the aortic arch and descending aorta derived from 6 Cd-fed mice and 12 sections from 5 control mice were stained with haemotoxilin-eosin and Movat’s staining and graded according to AHA lesion types (I: initial change, II: minimal change, III: preatheroma, IV: atheroma, V: fibroatheroma, VI: hemorrhagic/thrombotic lesion, VII: calcific lesion, VIII: fibrotic lesion) by an external experienced rater blinded to mouse treatment and amount of atherosclerotic plaque area.

The treatment protocol has been approved by the Commission for Animal Testing of the Austrian Ministry for Science and Research.

Fixation and Scanning Electron Microscopy of Mouse Aortas

For scanning electron microscopic (SEM) analyses of the vascular endothelium of mouse aortas, animals were treated and anesthetized as described above. After anaesthesia the thorax was opened, the left heart ventricle was cannulated and the right atrium was perforated, followed by rinsing of the circulation with 0.9% NaCl for 2-3 min through the cannula in the left ventricle. Aortas were then fixed by changing the perfusate to 2.5% glutaraldehyde in PBS (pH = 7.4) solution and rinsing for 5-6 minutes. After careful removal of the aortas, tissues were dehydrated in a graded ethanol series (70%, 90%, 100%, 100%, and 100% aceton), desiccated by critical point drying (Balzers Union Med 030, Liechtenstein), mounted, sputtered with gold-palladium (Balzers Med 080; Liechtenstein), and examined with a Zeiss DSM 982 Gemini scanning electron microscope. The images were taken from the central non-plaque containing parts of the aortas. The in vivo fixation procedure has been approved by the Commission for Animal Testing of the Austrian Ministry for Science and Research.
SEM Analysis of Cultured Cells

For SEM analyses 1 x 10⁵ HUVECs were grown on gelatine-coated round glass coverslips (Menzel Gläser, Thermo Fischer Scientific, USA) in 24-well plates, and treated as indicated. After the incubation, cells were fixed by replacing the medium with 2.5% glutaraldehyde (in PBS). Dehydration, desiccation etc. were performed as described above.

Analysis of Serum Lipid Profiles

Lipoprotein profiles were analysed by FPLC employing two Superose-6 columns (Amersham, Braunschweig, Germany) connected in series as described elsewhere.

Statistical analysis

In cell culture and animal experiments (where applicable) the distribution of values of all groups were tested for a Gaussian distribution (Kolmogorov-Smirnov test) and equality of variances (Levene’s test). Further analyses were performed using ANOVA / Bonferroni adjustment, followed by t-test comparisons of the groups.

Reference List


### FIGURE LEGENDS

**Figure 1** – The balance between serum cadmium and zinc levels defines the risk for increased intima-media thickness in healthy young adults.
The histogram in figure 1 shows an analysis of the risk for increased IMT at the internal and common carotid arteries in relation to serum Zn and Cd levels. The study population was split into tertiles according to serum Cd and Zn concentrations (i.e. low (1), medium (2) and high (3) levels) and the odds ratio (OR) for increased IMT of the groups was determined. Since the subgroups with low and medium Zn concentrations showed comparable findings the groups were pooled to increase group sizes and statistical power.

**Figure 2 – Cadmium increases the permeability of vascular endothelial monolayers in vitro – protection by zinc.**

The left histogram of figure 2 shows an analysis of time- and dose-dependent changes of endothelial monolayer permeability in vitro with or without the application of the indicated concentrations of Cd and/or 60 µM Zn to the luminal side of the cells. The right histogram shows the effects of an abluminal addition of Cd to the monolayers for 48 hours. Mean values + S.D. of a representative experiment performed in quadruplicates are shown. Asterisks indicate significant differences (i.e. p < 0.05) compared to the corresponding controls.

The images in figure 2 show scanning electron microscopic analyses of vascular endothelial cells in vitro. The cells were treated for 96 hours as follows: A…control; B…15 µM Cd; C…60 µM Zn; D…60 µM Zn plus 15 µM Cd. Representative images are shown. In all experiments, Zn was added 24 hours prior to the addition of Cd.

**Figure 3 – Cadmium inhibits the proliferation of endothelial cells and causes a necrotic form of cell death**

Figure 3A shows the effects of an application of 15 µM and 100 µM of Cd on endothelial cells +/- the addition of 60 µM of Zn on the number of viable cells in vitro determined by the XTT assay. Mean values +/- S.D. of a representative experiment performed in quadruplicates...
are shown. Asterisks indicate significant differences (i.e. $p < 0.05$) compared to the corresponding controls.

In Figure 3B, Cd effects on cell death induction, analysed by annexinV-FITC staining and FACS analyses as well as by lactate dehydrogenase release, are depicted. Mean values $\pm$ S.D. of a representative experiment performed in triplicates (annexinV staining) and quadruplicates (LDH assay) are shown. Asterisks indicate significant differences (i.e. $p < 0.05$) compared to the corresponding controls.

$\ldots$specific LDH release = % of LDH released by the treatment of total LDH present in cultures – % of released LDH of controls of total LDH present in controls. $\ldots$% of cell death induced by treatment - % of cell death in controls.

Figure 3C shows a scanning electron microscopic analysis of a cell treated with 15 µM of Cd for 96 hours. A representative image is shown.

Figure 4 – DNA strand breaks and DNA damage response precede caspase-independent, BclXL-inhibitable cadmium-induced cell death.

In order to detect potential DNA strand breaks in response to Cd treatment of endothelial cells in vitro and the interference of Zn with Cd effects TUNEL assays were performed. Figure 4A shows mean value ratios of TUNEL-positive cells/total cells $\pm$ S.D. of a representative experiment performed in triplicates. Asterisks indicate significant differences ($p < 0.05$) compared to the control. The Western blots in Figure 4B show a time course expression analysis of classical DNA damage response proteins and caspase-target proteins in the presence or absence of Cd and Zn. Representative blots are shown. p-ATM…phosphor-ATM (on serine 1981). Figure 4C summarises the results of experiments where the effect of different inhibitors of cell signalling processes were studied for their potential to inhibit Cd-induced cell death. All experiments were performed in triplicates and were repeated at least
three times. Mean values +/- S.D. are shown. Asterisks indicate significant differences (p < 0.05) compared to the controls. The Western blot in Figure 4D shows an analysis of caspase-3 activation (pro-enzyme at 32 kD; active fragments at 20 and 17 kD) 72 hours after Cd addition (Zn was added 24 hours prior to Cd addition). A representative blot is shown. An effector-caspase activity assay (caspases 3, 6, and 7) is depicted in Figure 4E. Means +/- S.D. of a representative experiment performed in triplicates is shown. All groups, except the Zn and the 15µM Cd + Zn groups differed significantly. Figure 4F shows the surface of Cd-treated endothelial cells at 5000 x magnification (treatment 96 hours). A representative image is shown.

**Figure 5 – Cadmium causes endothelial damage, alterations in lipid profiles, and accelerated plaque formation in Apo E knockout mice in vivo.**

After the administration of Cd and/or Zn via drinking water (control group, no additives in drinking water) to Apo E KO mice for 12 weeks, scanning electron microscopic images of the aortic endothelium were taken. Representative images are shown for control-treated animals (A) and Cd-treated animals (B, C, D). In image C, arrows indicate contracted endothelial cells at different stages of contraction. The number (1, 2, 3, 4) indicates the potential sequence of events. Intercellular gaps (C) and necrotic endothelial cells (D) which were not present in the controls are also indicted by arrows. The aortic endothelium of Zn-treated animals did not differ from the controls, and the endothelium of Zn plus Cd-treated animals did not differ from Cd-only treated animals. An analysis of the lipid profiles of the above treatment groups and WT control animals is given in Figure 5E. In this experiment VLDL particles had an EV of ~ 15; LDL ~ 18, and HDL ~ 27. An analysis of the atherosclerotic plaque area of the aorta of the animals is given in Figure 5 F. (n per group = 7).
**TABLE 1**

**Table 1: Characteristics of the ARFY population (all-female) expressed as mean ± standard deviation, median (interquartile range) or number (percentage).**

<table>
<thead>
<tr>
<th>VARIABLES</th>
<th>mean±SD, Median(IQR), n(%)</th>
</tr>
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<tbody>
<tr>
<td>Age – years</td>
<td>20.6±1.0</td>
</tr>
<tr>
<td>Body-mass index – kg/m²</td>
<td>21.9±2.9</td>
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<tr>
<td>Waist – cm</td>
<td>84.8±6.9</td>
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<tr>
<td>Systolic blood pressure – mmHg</td>
<td>112.2±8.7</td>
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<tr>
<td>Diastolic blood pressure – mmHg</td>
<td>68.9±7.2</td>
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<tr>
<td>Hypertension (office) – no.(%)*</td>
<td>10(4.9)</td>
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<tr>
<td>Family history, hypertension – no.(%)</td>
<td>52(25.4)</td>
</tr>
<tr>
<td>Fasting glucose – mmol/l</td>
<td>4.6±0.4</td>
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<tr>
<td>Total cholesterol – mmol/l</td>
<td>4.7±0.9</td>
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<tr>
<td>HDL-cholesterol – mmol/l</td>
<td>1.9±0.4</td>
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<td>LDL-cholesterol – mmol/l</td>
<td>2.7±0.8</td>
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<tr>
<td>Triglycerides – mmol/l‡</td>
<td>1.1(0.7-1.5)</td>
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<tr>
<td>HOMA – Insulin resistance-index</td>
<td>1.9±1.0</td>
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<tr>
<td>AST – U/l</td>
<td>20.4±6.3</td>
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<td>Lipoprotein (a) – mmol/l‖</td>
<td>0.4(0.1-1.0)</td>
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<td>Homocysteine – µmol/l‖</td>
<td>8.7 (7.5-10.1)</td>
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<tr>
<td>C-reactive protein – mg/l‖</td>
<td>2.0(1.0-4.0)</td>
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<tr>
<td>Anti-mHsp60 antibody – titer</td>
<td>2.9±1.2</td>
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<tr>
<td>Soluble hHsp60 – µg/µl‖</td>
<td>3.3 (0.2-21.7)</td>
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<tr>
<td>hHsp60 stimulation-index‡</td>
<td>28.7(8.1-73.8)</td>
</tr>
<tr>
<td>Asthma – no.(%)</td>
<td>13(6.3)</td>
</tr>
<tr>
<td>Smoking – no.(%)</td>
<td>82(38.4)</td>
</tr>
<tr>
<td>Pack-years of smoking‡</td>
<td>0.0 (0.0-0.8)</td>
</tr>
<tr>
<td>ETS (calculated cumulative exposure)§</td>
<td>0.6 (0.0-5.0)</td>
</tr>
<tr>
<td>Oral contraceptive intake – no.(%)</td>
<td>154(75.1)</td>
</tr>
<tr>
<td>Low social status – no.(%)</td>
<td>99(48.3)</td>
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<tr>
<td>IMT internal carotid arteries – mm</td>
<td>0.52±0.12</td>
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<tr>
<td>IMT common carotid arteries – mm</td>
<td>0.40±0.08</td>
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</table>


* Hypertension was defined as systolic ≥140, diastolic blood pressure ≥90 mmHg or intake of antihypertensive drugs.

‡ Due to a non-Gaussian distribution variable levels are expressed as medians (interquartile range).
§ Environmental tobacco smoke (ETS): in analogy to the pack-years of smoking, cumulative exposure was calculated with the formula, ‘hours of exposure per day’ x ‘years of exposure’.
Online supplement: Assessment of Vascular Risk Factors in the ARFY Study

Participants' risk factors were assessed according to standardised protocols validated and used previously in the Bruneck\(^1\) and ARMY\(^2\) studies.

Waist was measured at the narrowest point between the costal margin and the iliac crest (on the naked abdomen) and hip circumference was recorded over the widest diameter of the buttocks (with underwear). Subjects were categorized as ‘smokers’ if they reported regular consumption of at least 1 cigarette per week and lifetime consumption exceeded 40 cigarettes. Cigarette pack-years were calculated by multiplying the years of smoking by the packs smoked per day.

Subjects were given an automated blood pressure monitor (Omron, Mannheim, Germany) and advised to measure BPs at 7 a.m. and 7 p.m (on both arms). Hypertension was defined as a mean systolic BP $\geq$140 and/or a mean diastolic BP $\geq$90 mmHg. No regular use of antihypertensive drugs was reported. A family history of hypertension was considered positive if at least one first-degree relative was taking or took hypertension and BP-lowering medication.

Insulin resistance was estimated according to the HOMA-Index\(^4\). Social status was deduced from the parents' occupation, i.e. rated low for an unskilled worker and high for academics and skilled or supervisory workers. Asthma was self-reported physician-diagnosed. In analogy to pack-years of active smoking, cumulative exposure to environmental tobacco smoke (ETS) was computed by multiplying the ‘hours per day in ETS’ and the ‘years of exposure’.

Blood samples were drawn after an overnight fast and abstention from smoking and glucose, triglycerides, total, LDL- and HDL-cholesterol determined with standard colorimetric assays (ModularP, Roche Diagnostics, Mannheim, Germany), C-reactive protein (CRP) with a latex-enhanced immunologic assay (ModularP, Roche Diagnostics, Mannheim, Germany), lipoprotein(a) (Lp(a)) with immunoturbidimetry (ModularP, Roche Diagnostics, Mannheim,
Germany), insulin with an enzyme-linked chemiluminescent immunosorbent-assay (ModularE170, Roche Diagnostics, Mannheim, Germany), homocysteine with an automated fluorescence-polarization immunoassay (Axsym, Abbott, Wiesbaden, Germany) and AST with a UV-test standardised according to the International Federation of Clinical Chemistry (ModularP, Roche Diagnostics, Mannheim, Germany). All samples (except those for lymphocyte proliferation) were immediately cooled to 4°C and centrifuged within 90 min. An enzyme-linked immunosorbent assay was used to determine antibody titers to recombinant mycobacterial and human HSP60 and the serum concentration of soluble human Hsp60 (sHsp60). The use of peripheral blood mononuclear cell (PBMC) proliferation assays to determine T-lymphocyte reactivity to various antigens in vitro has also been described before¹.

Reference List


