Heme oxygenase-1 suppresses a pro-inflammatory phenotype in monocytes and determines endothelial function and arterial hypertension in mice and humans

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Aims
Heme oxygenase-1 (HO-1) confers protection to the vasculature and suppresses inflammatory properties of monocytes and macrophages. It is unclear how HO-1 determines the extent of vascular dysfunction in mice and humans.

Methods and results
Decreased HO-1 activity and expression was paralleled by increased aortic expression and activity of the nicotinamide dinucleotide phosphate oxidase Nox2 in HO-1 deficient Hmox1<sup>−/−</sup> and Hmox1<sup>+/−</sup> compared with Hmox1<sup>+/+</sup> mice. When subjected to angiotensin II-infusion, streptozotocin-induced diabetes mellitus and aging, HO-1 deficient mice showed increased vascular dysfunction inversely correlated with HO activity. In a primary prevention population-based cohort, we assessed length polymorphisms of the HMOX1 promoter region and established a bipolar frequency pattern of allele length (long vs. short repeats) in 4937 individuals. Monocytic HMOX1 mRNA expression was positively correlated with flow-mediated dilation and inversely with CD14 mRNA expression indicating pro-inflammatory monocytes in 733 hypertensive individuals of this cohort. Hmox1<sup>−/−</sup> mice showed drastically increased expression of the chemokine receptor CCR2 in monocytes and the aorta. Angiotensin II-infused Hmox1<sup>−/−</sup> mice had amplified endothelial inflammation in vivo, significantly increased aortic infiltration of pro-inflammatory CD11b<sup>+</sup>Ly6C<sup>+</sup> monocytes and Ly6G<sup>+</sup> neutrophils and were marked by Ly6C<sup>+</sup> monocytectosis in the circulation and an increased blood pressure response. Finally, individuals with unfavourable HMOX1 gene promoter length had increased prevalence of arterial hypertension and reduced cumulative survival after a median follow-up of 7.23 years.

Conclusions
Heme oxygenase-1 is a regulator of vascular function in hypertension via determining the phenotype of inflammatory circulating and infiltrating monocytes with possible implications for all-cause mortality.

Keywords
Heme oxygenase-1 • Angiotensin II • Hypertension/high blood pressure • Endothelial function • Population studies
**Translational perspective**

Heme oxygenase-1 (HO-1), a potent anti-inflammatory enzyme protecting from vascular oxidative stress is capable to suppress a pro-inflammatory phenotype of monocytes in mice and man leading to preservation of vascular endothelial function. An unfavourable length polymorphism in the HO-1 promoter region is related to a more than two-fold risk for arterial hypertension and is associated with increased all-cause mortality. Assessing HO-1 promoter genotype or HO-1 expression and activity in circulating cells might therefore contribute to clinical risk assessment of individuals at risk for cardiovascular events.

**Introduction**

Heme oxygenase-1 (HO-1, encoded by the Hmox1 gene in mouse and HMOX1 gene in humans) exerts beneficial antioxidant and anti-inflammatory effects, mainly by yielding the heme-breakdown products carbon monoxide (CO), biliverdin/bilirubin and stimulating expression of ferritin. Heme oxygenase-1 deficient cells are more susceptible to oxidative stress and cytotoxicity caused by heme and hydrogen peroxide. Activation of HO-1 or administration of CO has been shown to provide protection from many—in particular vascular—pathologies.

For instance, CO protected from restenosis and proliferative vascular disease in mouse models of vascular grafting and balloon injury. Induction of HO-1 was shown to stimulate vascular relaxation via soluble guanylyl cyclase and cyclic guanosine monophosphate and prevented smooth muscle proliferation in vascular injury, which was ascribed to CO bioactivity. High-fat diet fed Ldlr+/− mice transplanted with bone marrow from Hmox1+/− mice showed larger atherosclerotic lesions with greater macrophage content. Low density lipoprotein cholesterol-exposed macrophages isolated from HO-1 deficient mice secreted increased levels of interleukin-6 and monocyte chemoattractant protein-1 (MCP-1), indicating that HO-1 activity and expression might determine the macrophage phenotype in cardiovascular disease (CVD).

A pro-inflammatory phenotype of monocytes and macrophages has been proposed to be mechanistically important in the progression of atherosclerosis as well as in some of the most relevant disease states that drive CVD such as arterial hypertension and vascular dysfunction in diabetes mellitus. Administration of heme induced HO-1 expression and prevented arterial hypertension in spontaneously hypertensive rats. Heme oxygenase-1 is upregulated in response to angiotensin II (ATII) as an endogenous mechanism to protect from ATII-induced nicotinamide dinucleotide phosphate (NADPH) oxidase activity, and HO-1 induction has been shown to attenuate blood pressure increase in response to ATII. Likewise, HO-1 induction protected from vasculopathy in streptozotocin (STZ) induced experimental diabetes in rats.

The (GT)n length polymorphism in the promoter region for the HMOX1 gene by fragment analysis. The study protocol and sampling design were approved by the local ethics committee. Heme oxygenase activity in isolated mouse fibroblasts was measured by a calorimetric assay. In the mouse studies, we assessed blood pressure non-invasively by tail-cuff measurements, vascular endothelial inflammation by intravital microscopy, vascular function by isometric tension studies, vascular reactive oxygen species formation by oxidative fluorescent microtopography, protein expression by western blot, mRNA expression by quantitative real-time PCR, and vascular and circulating immune cell composition by flow cytometry. Five thousand individuals of the Gutenberg Health Study (GHS), a community-based, prospective, observational single-centre cohort study, were examined for flow-mediated dilation (FMD) and other parameters of vascular function, bilirubin levels, and myelomonocytic cells impacts on endothelial function and potentially on patient outcomes.

We therefore aimed to test the hypothesis that HO-1 expression and activity protects from vascular dysfunction and oxidative stress by guiding the polarization of monocytes and suppressing vascular inflammation.

**Material and methods**

The complete Materials and methods can be found in Supplementary material online. In brief, male Hmox1+/+, Hmox1+/−, and Hmox1−/− mice (3, 6, and 12 month of age) on a 129sv_BALB/c background were used. In selected experiments, mice were infused subcutaneously with ATII (0.1 or 1 mg kg⁻¹ d⁻¹ for 7 days) via mini-osmotic pumps (model 1007D, ALZET, Cupertino, CA, USA) vs. sham-operated mice. Hyperglycaemia due to absolute insulin deficiency was induced by streptozotocin injections (three repetitive i.v. injections of STZ, 60 mg kg⁻¹). Animal treatment was granted by the Mainz University local Ethics Committee. Heme oxygenase activity in isolated mouse fibroblasts was measured by a calorimetric assay. In the mouse studies, we assessed blood pressure non-invasively by tail-cuff measurements, vascular endothelial inflammation by intravital microscopy, vascular function by isometric tension studies, vascular reactive oxygen species formation by oxidative fluorescent microtopography, protein expression by western blot, mRNA expression by quantitative real-time PCR, and vascular and circulating immune cell composition by flow cytometry. Five thousand individuals of the Gutenberg Health Study (GHS), a community-based, prospective, observational single-centre cohort study, were examined for flow-mediated dilatation (FMD) and other parameters of vascular function, bilirubin levels, and (GT)n repeats in the promoter region of the HMOX1 gene by fragment analysis.

Transcriptome-wide gene expression profiles in monocytes were assessed using Illumina HT-12 v3 BeadChips. The study protocol and sampling design were approved by the local ethics committee and by the local and federal data safety commissioners. In animal studies, data were expressed as mean ± SD and statistically tested for significance by one- or two-way analyses of variance. Results of regression analyses are reported as betas with their 95% confidence intervals (CIs). All statistical analyses within GHS were performed using R version 2.14.2 software.

**Results**

**Heme oxygenase-1 activity and expression preserve endothelial dysfunction and protect from vascular oxidative stress**

Primary fibroblasts from Hmox1+/+, Hmox1+/−, and Hmox1−/− mice subjected to an amplified bilirubin fluorescence assay revealed
decreased HO activity (459.3 ± 14.1 vs. 291.5 ± 21.7 vs. 114.2 ± 11.9 pmol h⁻¹ mg⁻¹, respectively) according to their genotype. Recombinant HO-1 fully restored total HO activity in Hmox1⁺/⁻ samples (Figure 1A), indicating that the majority of HO activity can be attributed to HO-1 and not HO-2. In vascular relaxation studies, isolated aortic rings of Hmox1⁺/⁺, Hmox1⁺/⁻, and Hmox1⁻/⁻ mice showed a stepwise and gradually impaired response to the endothelium-dependent vasodilator acetylcholine (Figure 1B). This direct link of HO-1 deficiency to endothelial dysfunction was not sufficiently counteracted by compensatory upregulation of HO-2 protein expression (Supplementary material online, Figure S1A). Stepwise increased ROS formation was abrogated by the flavin-dependent oxidoreductase inhibitor diphenyliodonium and significantly blunted by both the specific NADPH oxidase inhibitor Vas2870 and the protein kinase C inhibitor chelerythrine (Figure 1C). This implies an NADPH oxidase activation in HO-1 deficiency (see also Supplementary material online, Figure S1B). Decreasing HO activity was paralleled by decreasing aortic HO-1 protein expression in HO-1 deficient mice, whereas aortic expression of Nox2 showed a stepwise increase in Hmox1⁺/⁻ and Hmox1⁻/⁻ mice when compared with Hmox1⁺/⁺ controls. Aortic protein levels of the constitutively expressed smooth muscle predominant NADPH oxidase isoform Nox1 were not significantly different between groups (Figure 1D). These data are compatible with a specific activation of the phagocyte-type Nox2 containing NADPH oxidase.

We then subjected Hmox1⁺/⁺, Hmox1⁺/⁻, and Hmox1⁻/⁻ mice to three different disease models of vascular dysfunction to test the relevance of HO-1 deficiency for CVD. Activation of the renin–angiotensin–aldosterone system is of central importance in the pathogenesis of CVD like arterial hypertension or coronary artery disease. ATII infused at subpressor doses (0.1 mg kg⁻¹ d⁻¹ for 7 days) has been used to uncover increased susceptibility for

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**Figure 1** Impaired heme oxygenase-1 activity and expression is linked to vascular endothelial function and oxidative stress in mice. (A) Heme oxygenase activity of isolated murine fibroblasts in the presence of biliverdin reductase as assessed by breakdown of heme to bilirubin in a photometry colorimetric assay. n = 4–10 mice/group, one-way analysis of variance with Bonferroni post hoc test. (B) Concentration–relaxation curve in response to acetylcholine (endothelium dependent) of isolated aortic segments of Hmox1⁺/+ and Hmox1⁻/⁻ mice. One-way analysis of variance with Bonferroni post hoc test of maximal relaxation, n = 5–8 animals/group. (C) Oxidative fluorescence microtopography. Left: Representative photomicrographs of isolated aortic segments incubated with dihydroethidium (1 μM, 30 min at 37°C) in the absence or presence of diphenylene iodonium (10 μM), Vas2870 (25 μM), or chelerythrine (1 μM). Green: Laminae (autofluorescence), red fluorescence: superoxide formation. E, endothelium, M, media, A, adventitia. Right: Densitometric analysis. One-way analysis of variance with Bonferroni post hoc test, n = 4–6 animals/group. (D) Protein expression analysis of Nox1, Nox2, and heme oxygenase-1. Top: representative western blots. Bottom: densitometric analysis. One-way analysis of variance with Bonferroni post hoc test; n = 8–12 animals/group.
endothelial dysfunction in mice that are prone to oxidant, metabolic, or inflammatory stress. This regimen caused endothelial dysfunction in Hmox1−/−, while it did not significantly alter endothelial function in Hmox1+/+ (Figure 2). Additionally, STZ-induced diabetes mellitus and aging caused vascular endothelial dysfunction that was directly correlated with the HO-1 genotype in these mice and most pronounced, when the Hmox1 gene was completely absent (Supplementary material online, Figure S2A and B).

**Monocytic HMOX1 mRNA expression is associated with endothelial dysfunction in humans with arterial hypertension**

To study the relevance of HO-1 for vascular function in humans, we used the cross-sectional data in a study sample of n = 5,000 individuals from the GHS, a primary prevention cohort study (Study sample A2, Supplementary material online, Table S1). We analysed the length of the HMOX1 promoter (GT)n, repeat. In our cohort, we established a bimodal distribution of allele length frequency of 4937 individuals (Supplementary material online, Table S2), that matches well with the reported allele frequencies in other cohorts of individuals of Caucasian ethnicity (Figure 3A and B). We then analysed, whether the (GT)n, repeat length correlated with FMD in these individuals or with serum levels of bilirubin, a well-reported surrogate parameter of HO-1 bioactivity. As known from the literature and shown in our cohort, reproducibility of FMD under standardized conditions is good. As published before, FMD was significantly different between groups (Supplementary material online, Table S3 and Figure S3). We therefore decided to directly investigate HMOX1 mRNA expression levels in individuals from the GHS. Systematic transcriptomics analysis was performed in a subgroup of all GHS participants (n = 1467) by mRNA array using mRNA isolated form venous blood monocytes as reported. We found that monocytic HMOX1 mRNA expression had a Gaussian distribution in the GHS population (Figure 3C). When performing linear regression analysis, we found that monocytic HMOX1 mRNA expression levels were positively associated with FMD and with serum bilirubin levels in individuals with arterial hypertension (Table 1).
Figure 3  Distribution (GT)$_n$ repeats and monocytic HMOX1 mRNA and correlation of monocytic HMOX1 and CD14 mRNA expression in humans. (A) Semiautomated pyrosequencing analysis of cDNA of 4937 individuals of the Gutenberg Health study was analysed. n(GT), number of GT repeats. (B) Box plot of the distribution of the GT-allele length in the GHS sample. Genotyping results were in Hardy–Weinberg equilibrium. (C) Expression of HMOX1 mRNA isolated from circulating monocytes was analysed by array and by qPCR. (D) Regression analysis of the association of HMOX1 mRNA expression with CD14 mRNA in 1467 individuals of the Gutenberg Health Study, of which 734 were non-hypertensive and 733 hypertensive (see Tables 1 and 2).

Table 1  Effect of monocytic HMOX1 mRNA expression on flow-mediated dilation and serum bilirubin levels

<table>
<thead>
<tr>
<th>HMOX1 mRNA</th>
<th>$\beta$</th>
<th>SD</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) FMD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All individuals ($n = 1467$)</td>
<td>0.539</td>
<td>0.440</td>
<td>$-0.324$ to $1.402$</td>
<td>0.22</td>
</tr>
<tr>
<td>Non-hypertensive ($n = 734$)</td>
<td>$-0.178$</td>
<td>0.703</td>
<td>$-1.558$ to $1.203$</td>
<td>0.8</td>
</tr>
<tr>
<td>Hypertensive ($n = 733$)</td>
<td>1.132</td>
<td>0.539</td>
<td>$0.073$ to $2.191$</td>
<td><strong>0.04</strong></td>
</tr>
<tr>
<td>(b) Bilirubin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All individuals ($n = 1467$)</td>
<td>0.047</td>
<td>0.031</td>
<td>$-0.013$ to $0.107$</td>
<td>0.12</td>
</tr>
<tr>
<td>Non-hypertensive ($n = 734$)</td>
<td>$-0.007$</td>
<td>0.047</td>
<td>$-0.099$ to $0.084$</td>
<td>0.88</td>
</tr>
<tr>
<td>Hypertensive ($n = 733$)</td>
<td>0.088</td>
<td>0.041</td>
<td>$0.008$ to $0.168$</td>
<td><strong>0.03</strong></td>
</tr>
</tbody>
</table>

Multivariable adjusted linear regression analysis of the association of HMOX1 mRNA expression with command variable FMD (a) and serum levels of bilirubin (b) in 1467 individuals of the GHS. Models were adjusted for sex, age, diabetes, smoking, arterial hypertension, dyslipidemia, body mass index, family history of myocardial infarction and history of coronary artery disease, myocardial infarction, peripheral artery disease, chronic heart failure, stroke, atrial fibrillation, chronic kidney disease, chronic liver disease, chronic obstructive pulmonary disease and cancer; additionally for bilirubin (a) and FMD (b) and stratified into hypertensive ($n = 733$) and normotensive ($n = 734$) individuals. Significant P-values ($P < 0.05$) are highlighted in bold.
The Hmox1 gene (Figure S5E and F). This enhanced inflammatory phenotype was associated with increased ATII-induced arterial hypertension in Hmox1 deficient mice (Figure S5G).

Long (GT)$_n$ repeats are associated with arterial hypertension and increased all-cause mortality

Association of (GT)$_n$ repeats and HO-1 expression differences have been primarily described in collectives with induced promoter activity$^{23}$; hypertension is consistently associated with FMD.$^{17}$ Based on our findings in animal models, we performed multiple logistic regression analyses and found that interaction of (GT)$_n$ repeat length in both alleles had an odds ratio $> 1$, implying a small but statistically significant adverse effect with regards to the prevalence of arterial hypertension (Supplementary material online, Table S6). Individuals with long (GT)$_n$ repeats (cutoff: 30/30) on both alleles had a more than two-fold increased risk for the prevalence of arterial hypertension (odds ratio: 2.166) compared with the protective reference short/short (GT)$_n$ repeat length (i.e. $< 23/23$). Intriguingly, the cutoff of 30 on both alleles being the most frequent (GT)$_n$ repeat (Figure 3A) still was protective, whereas the risk of arterial hypertension drastically increased in individuals with very long (GT)$_n$ repeats on both alleles (Table 3).

To assess whether our findings would have implications for clinical practice, we performed Kaplan–Meier analysis for

Table 2 Effect of monocytic HMOX1 mRNA expression on monocytic CD14 mRNA expression

<table>
<thead>
<tr>
<th>HMOX1 mRNA</th>
<th>$\beta$</th>
<th>SD</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All individuals ($n = 1467$)</td>
<td>-0.109</td>
<td>0.014</td>
<td>-0.137 to -0.082</td>
<td>$1.1 \times 10^{-14}$</td>
</tr>
<tr>
<td>Non-hypertensive ($n = 734$)</td>
<td>-0.105</td>
<td>0.020</td>
<td>-0.144 to -0.066</td>
<td>$1.6 \times 10^{-7}$</td>
</tr>
<tr>
<td>Hypertensive ($n = 733$)</td>
<td>-0.115</td>
<td>0.020</td>
<td>-0.153 to -0.076</td>
<td>$1.1 \times 10^{-7}$</td>
</tr>
</tbody>
</table>

Regression analysis of the association of HMOX1 mRNA expression with CD14 (a, b) in 1467 individuals of the GHS. Two probes capture CD14 mRNA expression on Illumina HT12 BeadChips: ILMN_2396444 (a) and ILMN_1740015 (b), representing for total CD14 mRNA expression. The models were adjusted for sex, age, diabetes, smoking, arterial hypertension, dyslipidaemia, body mass index, family history of myocardial infarction and history of coronary artery disease, myocardial infarction, peripheral artery disease, chronic heart failure, stroke, atrial fibrillation, chronic kidney disease, chronic liver disease, chronic obstructive pulmonary disease and cancer and stratified into hypertensive ($n = 733$) and normotensive ($n = 734$).

Significant $P$-values ($P < 0.05$) are highlighted in bold.
cumulative survival. Individuals with long (GT)$_n$ repeats (cutoff: 30/30 on both alleles) had higher all-cause mortality compared with carriers of short (GT)$_n$ repeats (cutoff: 23/23 on both alleles; Figure 6).

**Discussion**

Here, we present evidence that HO-1 activity and expression correlate with improved endothelial function and attenuated arterial hypertension in mice and humans.
phagocyte-type NADPH oxidase (Nox2)-mediated oxidative stress in mice. We demonstrate that HO-1 deficiency in mice amplifies vascular infiltration of CD11b^+Ly6C^hi myelomonocytic cells, proposing a mechanism that links HO-1 function with inflammatory monocytes and with vascular (dys)function in arterial hypertension (see scheme in Figure 7).

Our data strengthen the concept that HO-1 may counteract NADPH oxidase activity and expression (especially in phagocytic cells) by controlling and reducing the pool of available heme necessary for proper gp91phox (Nox2) biosynthesis and function.23 Consequently, HO-1 deficient mice were more susceptible to vascular injury induced by ATII, hyperglycaemia and aging that cannot be compensated by HO-2. In western blot analysis of aortic tissue, we found evidence for an increase of eNOS activity, presumably to compensate for lack of endogenous CO, the sGC-activating breakdown product of heme (Supplementary material online, Figure S1C). However, these counterregulatory changes are obviously not sufficient to prevent the development of endothelial dysfunction in mice deficient in HO-1.

Increased monocytic HMOX1 mRNA expression was associated with reduced markers of inflammatory polarization of monocytes and with vascular (dys)function in arterial hypertension (see scheme in Figure 7).

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higher levels of pro-inflammatory monocytes and disturbed endothelial function, a prognostic indicator of future cardiovascular events.\textsuperscript{24,25} ATII-infused HO-1 deficient mice had increased levels of Ly6C\textsuperscript{hi} monocytes in the circulation, paralleled by an increased endothelial inflammation \textit{in vivo} and infiltration of the vasculature with CD45\textsuperscript{hi} leukocytes, inflammatory Ly6C\textsuperscript{hi} monocytes, and Ly6G\textsuperscript{hi} neutrophils. Infiltration of the vasculature with these cells has been shown to be essential for the development of vascular dysfunction and hypertension in mice.\textsuperscript{6} Attenuation of the vascular infiltration by these cells via direct depletion,\textsuperscript{6} interruption of pro-inflammatory functions of T-cells\textsuperscript{26} or natural killer cells,\textsuperscript{27} knockout of macrophage colony-stimulating factor\textsuperscript{28} or inflammatory signals like IL-17\textsuperscript{29} or the MCP-1 receptor CCR2\textsuperscript{30} has been shown to prevent vascular endothelial dysfunction and partially also blood pressure increase in response to ATII. Induction of HO-1 by hemin was shown to prevent the capability of ATII to increase ROS formation, CCR2 expression and chemotactic activity of human monocytes, an effect that was mimicked by CO and bilirubin.\textsuperscript{30} Vice versa, shortage of HO-1 can induce a pro-inflammatory phenotype of myelomonocytic cells with increased CCL2 (e.g. MCP-1) release.\textsuperscript{3} In our study, monocytes and the vasculature of Hmox\textsuperscript{1}\textsuperscript{-/--} mice had sharply increased levels of Ccr2 mRNA expression, thereby permitting enhanced influx of monocytes into the vessel wall in response to ATII.

A protective role of certain HO-1 deficient resident F4/80\textsuperscript{hi} Ly6M\textsuperscript{hi} macrophages in metabolic syndrome was recently shown.\textsuperscript{31} These cells can develop independently of bone marrow-derived cells.\textsuperscript{32} Heme oxygenase-1 deficiency did not have a major impact on the levels of vascular F4/80\textsuperscript{hi} macrophages; these findings points towards the multifaceted and divergent roles of inflammation in different disease states and highlight the impact of different myeloid cell lineages for CVD.

\textit{HMOX1} expression on monocytes was strongly associated with both FMD and markers of vascular inflammation in our study. It was recently shown that GT\textsuperscript{(*)} repeats > 30 on both alleles (L/L genotype) are associated with higher incidence of CVD and progressive atherosclerosis in a population-based cohort of 812 individuals.\textsuperscript{33} Interestingly, we have found, that this unfavourable \textit{HMOX1} gene promoter length polymorphism potentially increases the risk of arterial hypertension (Table 3) and is associated with increased all-cause mortality (Figure 6). Current guidelines on the management of arterial hypertension put little emphasis on the use of biomarkers for risk stratification.\textsuperscript{34} Therefore, development of clinically applicable assays to routinely assess HO-1 expression or activity or \textit{HMOX1} gene promoter polymorphisms in circulating cells might result in useful biomarkers. Future studies are needed to test whether such an approach would improve risk stratification for primary or secondary prevention of CVD events, in particular in patients with arterial hypertension.

### Supplementary material

Supplementary material is available at European Heart Journal online.

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### Conflict of interest: none declared.

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