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MRI Contrast-enhancement with superparamagnetic iron oxide nanoparticles amplify macrophage foam cell apoptosis in human and murine atherosclerosis

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Short title: Iron oxide nanoparticles cause plaque apoptosis

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ABSTRACT

Aims (Ultra) Small superparamagnetic iron oxide nanoparticles, (U)SPIO, are widely used as magnetic resonance imaging contrast media and assumed to be safe for clinical applications in cardiovascular disease. As safety tests largely relied on normolipidemic models, not fully representative of the clinical setting, we investigated the impact of (U)SPIOs on disease-relevant endpoints in hyperlipidemic models of atherosclerosis.

Methods and results RAW264.7 foam cells, exposed *in vitro* to Ferumoxide (dextran-coated SPIO), Ferumoxtran (dextran-coated USPIO), or Ferumoxytol (carboxymethyl dextran-coated USPIO) (all 1 mg Fe/ml) showed increased apoptosis and ROS accumulation for Ferumoxide and Ferumoxtran, whereas Ferumoxytol was tolerated well. Pro-apoptotic ($TUNEL^{+}$) and pro-oxidant activity of Ferumoxide (0.3 mg Fe/kg) and Ferumoxtran (1 mg Fe/kg) were confirmed in plaque, spleen, and liver of hyperlipidemic $ApoE^{-/-}$ (n=9/group) and $LDLR^{-/-}$ (n=9-16/group) mice that had received single IV injections compared to saline-treated controls. Again, Ferumoxytol treatment (1 mg Fe/kg) failed to induce apoptosis or oxidative stress in these tissues. Concomitant antioxidant treatment (EUK-8/EUK-134) largely prevented these effects *in vitro* (-68%, $P<0.05$) and in plaques from $LDLR^{-/-}$ mice (-60%, $p<0.001$, n=8/group). Repeated Ferumoxtran injections of $LDLR^{-/-}$ mice with pre-existing atherosclerosis enhanced plaque inflammation and apoptosis but did not alter plaque size. Strikingly, carotid artery plaques of endarterectomy patients who received Ferumoxtran (2.6 mg Fe/kg) before surgery (n=9) also showed 5-fold increased apoptosis (18.2 versus 3.7% respectively; $p=0.004$) compared to controls who did not receive Ferumoxtran. Mechanistically, neither coating nor particle size seemed accountable for the observed cytotoxicity of Ferumoxide and Ferumoxtran.

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Conclusions Ferumoxide and Ferumoxtran, but not Ferumoxytol, induced apoptosis of lipid-laden macrophages in human and murine atherosclerosis, potentially impacting disease progression in patients with advanced atherosclerosis.

Translational Perspective

Past and ongoing clinical trials with iron-based contrast agents in elderly, hyperlipidemic and/or cardiovascular patients should evaluate tissue apoptosis and monitor future cardiovascular complications well beyond the imaging timeframe. Safety studies of newly developed iron-based contrast agents should also be performed in hyperlipidemic settings.

Keywords: apoptosis, atherosclerosis, leukocyte, oxidative stress, iron oxide nanoparticles

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List of abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ApoE$^{-/-}$	Apolipoprotein E knockout
BAX	BCL2 Associated X, Apoptosis Regulator
CKD	Chronic kidney disease
DAB	3,3'-Diaminobenzidine
FDA	U.S. Food and Drug Administration
GTC	Guanidium isothiocyanate
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
hVLDL	Human very-low-density lipoprotein
IL-1β	Interleukin-1β
IV	Intravenous
LDLR$^{-/-}$	Low-density lipoprotein receptor knockout
MRI	Magnetic resonance imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide
NBT-BCIP	Nitro-blue tetrazolium chloride with 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
NLRP3	Nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
SPIO	Small superparamagnetic iron oxide nanoparticles
PET-CT	Positron emission tomography-computed tomography
TCA	Trichloroacetic acid
TEAC	Trolox equivalent antioxidant capacity
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labelling
USPIO	Ultrasmall superparamagnetic iron oxide nanoparticles
WTD	Western-type diet
XIAP	X-linked inhibitor of apoptosis protein

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INTRODUCTION

Functional imaging is widely employed to detect rupture-prone atherosclerotic plaques in coronary artery disease and carotid artery disease patients at risk of clinical symptoms [1]. Rupture-prone plaques are typified by extensive lipid deposition, inflammation, matrix degradation, and cell death. These factors eventually lead to fibrous cap rupture and subsequent formation of an atherothrombus [2, 3].

Several passive and active molecular imaging modalities have been considered to identify high-risk plaques, including ^{18}F-fluoro-deoxyglucose-guided positron emission tomography-computed tomography (PET-CT), marking plaque inflammation and contrast-enhanced magnetic resonance imaging (MRI), respectively. MRI offers the advantage of superior spatial resolution, especially when using small (50-150 nm; SPIO) and ultrasmall (15-30 nm; USPIO) superparamagnetic iron oxide nanoparticles to enhance signal contrast [4-10]. Upon systemic administration, both formulations are rapidly cleared by the reticuloendothelial system of lung and liver and by renal excretion, while accumulation in heart and brain is less pronounced, maximising signal to noise [11]. USPIO extravasation and uptake by macrophages are considerably increased in inflammation [12-14], and vascular macrophages in the atherosclerotic plaque, in abdominal aneurysm, and in the infarcted or inflamed heart show avid accumulation of these particles [4, 5, 15-20], rendering them useful for cardiovascular disease imaging.

Extensive toxicology studies led to the assumption that (U)SPIO are safe for clinical application [21-23]. However, this notion is merely based on studies in normolipidemic animal and cell culture models. In the hyperlipidemic setting of atherosclerosis, vascular macrophages will acquire a foam cell phenotype, with intracellular free cholesterol deposits, and increased production of reactive oxygen species (ROS) and susceptibility to apoptosis [24, 25]. Moreover, upon uptake USPIO will accumulate in endo-lysosomes, where their coating will be degraded and the entrapped iron oxide cargo released [26, 27]. Besides prolonging the imaging signal, [26, 27] this will also foster an oxidative stress response, which may well be detrimental to macrophage survival. Moreover, monocyte-derived macrophages were reported

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to enhance inflammatory cytokine secretion upon exposure to iron oxide particles [28]. This suggests that (U)SPIO toxicity data obtained in normolipidemic, inflammatory macrophages *in vitro* may not be representative of the clinical situation. This prompted us to study (U)SPIO in macrophage foam cells, and in murine and human atherosclerosis to elucidate potentially unfavourable effects under clinically relevant conditions.

METHODS

Cell culture

The RAW264.7 murine macrophage cell line was grown in DMEM, containing 10% foetal bovine serum (heat-inactivated for 30 min at 56 °C), 2 mmol/L L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from PAA, Cölbe, Germany), at 37 °C in a humidified atmosphere (5% CO_2).

Human VLDL (hVLDL) was isolated from human serum of healthy volunteers by discontinuous density gradient centrifugation (using KBr; 40,000 rpm for 22 h) [61]. The VLDL fraction was collected and dialyzed against PBS containing 1 mM EDTA. RAW264.7 cells were incubated with hVLDL (50 µg/ml) for 20 h and replaced by medium with or without Ferumoxide (100 µg Fe/ml, Guerbet) or two types of ultrasmall iron oxide nanoparticles (100 µg Fe/ml): Ferumoxtran (Guerbet, France), and Ferumoxytol (RIENSO, Takeda, EU tradename for Feraheme, AMAG Pharmaceuticals, USA). Cells were pre-treated with EUK-8 antioxidant 2 h prior to Ferumoxtran treatment (25 µM; Merck Chemicals Ltd., Nottingham, UK). Viability of RAW cells was assessed using MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide; Sigma). Apoptosis was quantified in ≥ 3 fields per well, 20x magnification after terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) (Roche Diagnostics, Basel, Switzerland), or by flow cytometry of Annexin V-Oregon green (120 ng/ml).

Iron content

Ferumoxtran (100 µg Fe/ml) uptake by RAW264.7 macrophages and foam cells was determined after incubation for 1 h at 37 °C. Quantitative determination of iron uptake by inductively coupled plasma

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atomic emission spectroscopy (ICP-AES; Optima 3300 RL, Perkin Elmer, Courtaboeuf, France) was carried out after mineralization of the pellet with HNO_3 (3 h at 80 °C).

Animals and Tissue Harvesting

All animal work was performed in compliance with the Dutch government and Directive 2010/63/EU guidelines and approved by national and local review boards (AVD1070020185705). Male low-density lipoprotein receptor knockout ($LDLR^{-/-}$) or Apolipoprotein E knockout ($ApoE^{-/-}$) mice (aged 12 weeks) were obtained from the local animal facility. Animals were housed in the laboratory animal facility of Leiden University under standard conditions. Food and water were provided *ad libitum* during the entire experiment. All animals were housed in individually ventilated cages (GM500, Techniplast) in groups of up to 5 animals per cage, with bedding (corncob, Technilab-BMI) and cage enrichment. Cages were changed weekly, reducing handling of the mice to one handling per week during non-intervention periods.

A pilot study was done with $ApoE^{-/-}$ mice (n=3/group) that received a single dose of saline, Ferumoxide (0.3 mg Fe/kg), or Ferumoxtran (1 mg Fe/kg) after 9 weeks on a western-type diet (WTD, 0.25% cholesterol,15% cocoa butter, SDS, Sussex, UK). Based on this, a sample size of 8 single mice per group was calculated by power analysis. No inclusion or exclusion criteria were set. Cages were randomly located on the racks. $LDLR^{-/-}$ (n=9/group) were fed a WTD for 3 weeks, after which they received weekly intravenous injections of saline or Ferumoxtran and fed a WTD for 5 consecutive weeks. $LDLR^{-/-}$ (n=8/group) fed a WTD for 14 weeks received either saline, Ferumoxtran, antioxidant EUK-134 (10 mg/kg, Cayman Chemicals, Ann Arbor, MI), or Ferumoxtran with EUK-134, whereby EUK-134 was administered intraperitoneally 1 h prior to the intravenous injection of Ferumoxtran. In a third experiment, male $LDLR^{-/-}$ (n=9/group) fed a WTD for 9 weeks received a single saline, or Ferumoxytol (1 mg Fe/kg) injection. In all experiments, 24 h after the final injection, mice were anaesthetised using a single dose of pentobarbital (100 mg/kg i.p.), subjected to blood sampling and *in situ* perfusion-fixation

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through the left cardiac ventricle. Aortic root, liver and spleen were collected for cryosectioning using a Leica CM 3050S Cryostat (Leica Instruments, Nassloch, Germany).

Human tissue collection

Atherosclerotic carotid arteries (n=18) were obtained at surgery from patients treated with Ferumoxtran (2.6 mg Fe/kg, single dose, n=9) and historic control patients (n=9) matched for age, sex and plaque type (Table 1) to analyse apoptosis in plaque sections [4]. Inclusion and exclusion criteria were described before [4]. Sample size was calculated based on interpatient MRI enhancement. Collection, storage, and use of tissue and patient data were performed in agreement with institutional ethical guidelines and the principles outlined in the Declaration of Helsinki, and approved by the Maastricht University Medical Center Medical Ethical Committee (MEC00-078b) [4]. Subjects gave informed consent prior to the inclusion and were enrolled consecutively between 2000 and 2002. Samples were processed and classified based on plaque morphology as described previously [62].

Histology and Morphometry

Aortic root cryosections (10 µm) were stained with Oil Red O (Sigma) and MoMa-2 (1:50; Serotec, Oxford, UK) to detect lipid deposits and macrophage content, respectively. Secondary antibody goat anti-rat IgG-AP (1:100; Sigma, St-Louis, MO, USA) and enzyme substrate nitro-blue tetrazolium chloride with 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (NBT-BCIP, DAKO, Glostrup, Denmark) were used for MoMa-2 visualization. The primary outcome of apoptotic cells in liver, spleen and atherosclerotic plaque was detected using TUNEL (Roche Diagnostics, Basel, Switzerland) and visualized using Nova-Red (DAKO) for mouse and AEC (Sigma) for human sections. Human atherosclerotic plaques were stained with 8OH-dG (Japan Institute for the Control of Aging, clone N45.1), TUNEL and cleaved caspase for apoptosis, CD68 to detect ROS, apoptosis, and macrophages, respectively. Quantitative morphometric analysis of Oil Red O, MoMa-2 and TUNEL was performed using Leica Qwin image analysis software and a Leica-DM-RE microscope (Leica Imaging Systems, Cambridge, UK). Cells were

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marked as apoptotic when double positive for TUNEL and DAPI. High-sensitive Perl's iron staining was performed to visualize iron nanoparticles. Following quenching of endogenous peroxidases by hydrogen peroxide (0.3% in methanol), slides were incubated for 90 min in 1:1 solution of 2% HCl + 2% potassium hexacyanoferrate Fe^{2+}. After washing, slides were incubated for 20 minutes in 3,3'-diaminobenzidine (DAB, DAKO). Slides were counterstained with nuclear fast red. Negative control sections incubated with DAB only were negative. Outcome and data analysis were done blindly.

Electron Microscopy

Tissue fragments of carotid endarterectomy specimens of ~1 mm^3 were fixed overnight in 2.5% glutaraldehyde (Ted Pella, Redding, CA), post-fixed in 1% osmium tetroxide solution, dehydrated and embedded in epoxy resin. Semi-thin (1 µm) serial sections were stained with toluidine blue to localize microvessels. Ultra-thin sections (70-90 nm) were mounted on Formvar (1595 E, Merck)-coated 75 mesh copper grids, and counterstained with uranyl acetate and lead citrate before analysis on a Philips CM100 transmission electron microscope.

Trolox equivalent antioxidant capacity

The trolox equivalent antioxidant capacity (TEAC) gives the concentration of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) radicals that can be scavenged by serum. It is a measure of antioxidant capacity. The TEAC was determined in serum that was deproteinated with a final concentration of 5% trichloroacetic acid (TCA) as described [63]. The samples were incubated with an ABTS radical solution for 5 min and subsequently, the reduction in absorbance at 734 nm, reflecting the extent of radical scavenging, was quantified. This is related to that of the reference antioxidant trolox and is expressed as µM trolox equivalents. The TEAC value gives the concentration of trolox that has the same capacity.

Uric Acid

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Uric acid was determined in serum that was deproteinated with a final concentration of 5% TCA, using HPLC. A Hypersil BDS C-18 end-capped column, 125 x 4 mm, particle size 5 µm (Agilent, Palo Alto, CA, USA), was used, with a mobile phase of 0.1% trifluoroacetic acid (v/v) in water. UV detection was performed at 292 nm.

IL1-β ELISA

IL1-β, secreted in supernatant medium of RAW cells, was measured as suggested by the manufacturer (Invitrogen, Breda, The Netherlands).

Quantitative RT-PCR

Quantitative RT-PCR analysis was performed to determine mRNA expression of apoptosis and oxidative stress related genes in RAW cells, foam cells and Ferumoxtran-treated foam cells. Total RNA extracts isolated using the guanidium isothiocyanate (GTC) method [64] were transformed into cDNA using RevertAid M-MuLV reverse transcriptase (Fermentas, Burlington, Canada) according to manufacturer's protocol. Quantitative gene expression analysis was performed with the SYBR-Green technology on a 7500 fast Real-Time PCR apparatus (Applied Biosystems, Foster City, CA). All Ct values were normalized to the stable-expressed reference gene hypoxanthine phospho-ribosyltransferase (HPRT). Supplementary table 1 shows a detailed overview of the different primer pairs (Eurogentec, Maastricht, The Netherlands), which were designed using NCBI primer blast.

Serum Cholesterol Analysis

Total cholesterol concentration in serum was determined using an enzymatic colorimetric assay (Roche Diagnostics). Precipath I (Roche Diagnostics) was used as an internal standard. Absorbance was measured at 490 nm. Cholesterol distribution over the different lipoprotein fractions was determined by fractionation of 30 µl serum using a Superose 6 column (3.2x300mm, Smart-system, Pharmacia, Uppsala, Sweden). Cholesterol content of the effluent was determined as described above.

Dextran uptake assay

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RAW264.7 cells were seeded at 75 00 cells per well in a 96-well black clear-bottom imaging microplate (Corning #353219) in DMEM medium with 4.5 g/L D-glucose and pyruvate (Gibco #31966-021) supplemented with 10% heat-inactivated (30 min at 56 °C) foetal bovine serum (SERANA S-FBS-SA-015) and 1% penicillin-streptomycin, and left to attach for 2 h (37 °C, 5% CO_2). Supernatant of cells was either replaced with 1, 2.4, or 4 µM TRITC-labelled dextran (ThermoFisher Scientific #D7139). After 1 h (37 °C, 5% CO_2), cells were washed with DMEM and nuclei were stained with Hoechst 33342 (Sigma #B2261) in DMEM for 10 min (37 °C, 5% CO_2). Cells were imaged using the BD Pathway 855 (BD Biosciences) and analysed with CellProfiler software 4.0.4 [65].

Chemicals for synthesis of iron particles

Ferric chloride anhydrous, ferrous chloride tetrahydrate, 1,10-phenanthroline, hydroxylamine hydrochloride, dextran and carboxymethyl (CM) dextran were purchased from Sigma-Aldrich (Munich, Germany). Trisodium citrate dihydrate, citric acid, hydrogen chloride, ammonium hydroxide (NH4OH) and sodium hydroxide were obtained from Carl Roth (Karlsruhe, Germany).

Synthesis of citrate-coated iron particles

Citrate-coated iron particles were prepared via the standard co-precipitation technique. Briefly, 8 mmol of ferric chloride was dissolved in 10 ml of deionized water (from here on water) and mixed for 5 min under mechanical stirring and nitrogen atmosphere. Subsequently, 4 mmol of ferrous chloride tetrahydrate was added to the solution and mixed for a further 5 min at room temperature. The pH of the solution was adjusted to 11.0 by adding 80 ml of 1 M aqueous ammonia solution drop-wisely and vigorously stirred at room temperature for 30 min. The formed black-coloured iron oxide nanoparticles were decanted using a permanent magnet and washed at least three times with 500 ml of water. Afterwards, 20 ml of 0.1 M hydrochloric acid was added to the particles and sonicated for 10 min. Following, 2.5 g trisodium citrate dihydrate in 10 ml of water were added to the mixture and was stirred at 80 °C for 2 h. The citrate-coated polydisperse particles were separated using a permanent magnet

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and then resuspended in 35 ml of water. Finally, the suspension was passed through a 0.2 µm filter to remove larger particles.

This highly polydisperse crude batch was subjected to five sequential rounds of centrifugation to obtain monodispersed iron particle subfractions. As depicted in Supplementary Fig. 1, the supernatant obtained after 20 min of centrifugation at 14,000 rpm is referred to as Citrate S (USPIO size). The precipitate was resuspended in water and centrifuged again at progressively lower speed. The monodisperse batch obtained after 5 centrifugation cycles is referred to as Citrate L (SPIO size).

Synthesis of dextran coated iron particles

Dextran-coated iron particles were prepared via the standard co-precipitation technique, under a nitrogen atmosphere. Briefly, 1 mmol of ferric chloride was dissolved in 2 ml of water and mixed for 5 min under mechanical stirring and nitrogen atmosphere. Subsequently, 0.5 mmol of ferrous chloride tetrahydrate in 1 ml of water was added to the solution and mixed for 5 min at room temperature after which pH was adjusted to 11.0 (1 M aqueous ammonia). The suspension was stirred at 0 °C for 1 or 30 min to obtain differently sized particles and an aqueous solution of dextran (1 kDa or 10 kDa; 1 gr) or CM-dextran (10-20 kDa, 1 gr), filtered over a 200 nm syringe filter, was added. The temperature was slowly increased to 80 °C and was kept at that temperature for 60 min. Afterwards, the solution was cooled down to room temperature. The formed black-coloured iron oxide particles were sonicated for 20 min. Following, the solution was dialyzed for 24 h against 5 l of water (25 kDa cutoff, SnakeSkin™ dialysis membrane, ThermoFisher Scientific, Massachusetts, USA). Finally, the suspension was passed through a 0.2 µm filter to remove the aggregates. Also here, the highly polydisperse starting batch was fractionated by size via two sequential rounds of centrifugation as described above (14,000 rpm, 20 min (S batch) and 7,000 rpm, 10 min (L batch)).

Characterisation of synthesized iron particles

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Particle size and size distribution, Zeta potential, average hydrodynamic diameter (Dh) and polydispersity index (PDI) of the particles were measured by dynamic light scattering using a Zetasizer Nano-ZS instrument (Malvern Instruments, Malvern, UK) at 25 °C. The machine was equipped with a 633 nm He–Ne laser and a detector at angle of 173°. The samples were diluted and sonicated in a water bath prior to size analysis. Iron concentration was measured using 1,10-phenanthroline assay as described previously [59]. Finally, the absorbance was detected at 510 nm using an Infinite M200 Pro TECAN reader (TECAN, Germany).

Apoptosis assay

RAW264.7 cells were seeded at 6000 cells per well in a 96-well black clear-bottom imaging microplate (Corning #353219) in DMEM medium with 4.5 g/L D-glucose and pyruvate (Gibco #31966-021) supplemented with 10% heat-inactivated (30 min at 56 °C) foetal bovine serum (SERANA S-FBS-SA-015) and 1% penicillin-streptomycin, and left to attach for 24 h (37 °C, 5% CO_2). Supernatant of cells was either replaced with 50 µg/ml hVLDL in DMEM or fresh DMEM. After 20 h (37 °C, 5% CO_2), cells were incubated with iron particles (100 µg Fe/ml) small (S) or large (L) in size with different coatings (citrate, dextran, or carboxymethylated dextran) in DMEM or fresh DMEM for 1 h (37 °C, 5% CO_2). Nuclei were stained with Hoechst 33342 (Sigma #B2261) in DMEM for 10 min (37 °C, 5% CO_2). After washing with Annexin binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$; pH 7.37), cells were incubated with 2.5 ng/µl Annexin V for 15 min (37 °C, 5% CO_2). Cells were imaged using the BD Pathway 855 (BD Biosciences) and analysed with CellProfiler software 4.0.4 [66].

Statistical Analysis

Data are expressed as mean ± SEM. Normal distribution was assessed by Shapiro-Wilkes normality test, followed by Student's t-test or ANOVA for normal distributed data or a non-parametric Mann-Whitney U test or Kruskal-Wallis to compare two or more groups. Sidak or Dunn's multiple comparison test

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followed significant changes demonstrated by ANOVA or Kruskal-Wallis respectively. Results were considered statistically different when $p < 0.05$.

RESULTS

Iron oxide nanoparticles induce macrophage foam cell apoptosis *in vitro*, plaque, liver and spleen *in vivo*

RAW264.7 foam cells were formed by preloading with 50 µg/ml human very-low-density lipoprotein (hVLDL) for 20 h. Ninety-minute treatment with the dextran-coated USPIO Ferumoxtran (100 µg Fe/ml) increased apoptosis of foam cells compared to normolipidemic RAW macrophages (Fig. 1a) in a time- and hVLDL concentration-dependent manner (Fig. 1e). Specifically, foam cells showed apoptotic morphology (blebbing and shrinkage) and 4-fold enhanced TUNEL positivity (Fig. 1a-c), despite similar iron uptake as normolipidemic macrophages (Fig. 1d). Together, these data indicate that Ferumoxtran preferentially induced apoptosis in lipid-laden foam cells.

These findings were confirmed *in vivo* in $ApoE^{-/-}$ mice fed a western-type diet (WTD) for 9 weeks before receiving a single dose of the SPIO platform Ferumoxide (0.3 mg Fe/kg), of Ferumoxtran (1 mg Fe/kg), or saline. Even these subclinical doses of Ferumoxide or Ferumoxtran markedly enhanced TUNEL-positive apoptosis in atherosclerotic plaque compared to saline controls (Fig. 2a-d). As expected from the short follow-up time, plaque size was not affected 24 h after treatment (Fig. 2e), also excluding that observed effects were biased by differences in plaque stage.

Superparamagnetic iron oxide nanoparticles induce apoptosis in murine liver and spleen *in vivo*

Notably, apoptosis was not only limited to atherosclerotic plaques, as macrophage-rich liver and spleen of $ApoE^{-/-}$ mice also showed significant 2.5- to 4-fold increases in TUNEL-positive cells after only a single dose of Ferumoxide or Ferumoxtran treatment (Fig. 2f, g). TUNEL-positive cells in spleen were exclusively localised in the red pulp area (Supplementary Fig. 2), which harbours mainly marginal

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metallophilic macrophages. These data clearly show that Ferumoxide and Ferumoxtran treatment both enhance apoptosis in atherosclerotic lesions and other macrophage-rich tissue in hyperlipidemic mice.

Repeated iron oxide injection show smaller, but less stable plaques

The effect of Ferumoxtran on atherosclerotic lesion progression was studied in LDLR$^{-/-}$ mice, fed a WTD for 3 weeks to develop initial lesions and then subjected to weekly Ferumoxtran injections (1 mg Fe/kg) for another 5 weeks. Ferumoxtran-treated mice again showed a 5.4-fold increase in TUNEL-positive plaque cells compared to controls (Fig. 2h-j). Most apoptotic cells were foam cells, and few endothelial and smooth muscle cells, as inferred from cell morphology and location. Repeated Ferumoxtran treatment prevented weight gain, but increased serum levels of total, VLDL, and LDL cholesterol (Supplementary Fig. 3). These findings are in agreement with a study showing that selective depletion of monocytes/macrophages in circulation and peripheral tissue results in increased (V)LDL-derived cholesterol levels with reduced atherogenesis [29]. Indeed, atherosclerotic plaque size was reduced in Ferumoxtran-treated mice (Fig. 2k), despite the moderate serum cholesterol increase. This observation concurs with known effects of enhanced apoptosis on early atherosclerosis [2]. Notwithstanding increased apoptosis, plaque macrophage content was increased in Ferumoxtran-treated mice (Fig. 2l), possibly reflective of influx of new phagocytes upon apoptotic eat-me signals.

Antioxidant treatment prevents iron oxide nanoparticle-induced apoptosis *in vitro* and *in vivo*

As iron oxide metabolism has been suggested to lead to oxidative stress [26, 27], we investigated whether this could explain Ferumoxtran-induced apoptosis. Twenty-four hours after Ferumoxtran injection, serum antioxidant levels were increased as shown by increased uric acid concentration with a consequential increase in Trolox equivalent antioxidant capacity (TEAC) (Fig. 3a, b), a common response to oxidative stress exposure [30]. Uric acid is an established oxidative stress marker. Although uric acid was previously shown to display moderate antioxidant activity, this seems outweighed by its pro-oxidant and pro-inflammatory effects [31]. In addition, plaque oxidant damage (8OH-dG) showed a trend to increase

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(data not shown), and quantitative RT-PCR of Ferumoxtran-treated and control foam cells revealed a strong upregulation of oxidative stress-related p22phox (also known as neutrophil cytochrome b light chain), and the pro-apoptotic BCL2 Associated X, Apoptosis Regulator (Bax) and X-linked inhibitor of apoptosis (XIAP) genes (Fig. 3c). Although nanoparticle-induced reactive oxygen species (ROS) have been shown to activate the NLR Family Pyrin Domain (NLRP3) inflammasome [32], Ferumoxtran did neither enhance serum interleukin-1β (IL-1β), nor mRNA expression of IL-1β and NLRP3 in liver and spleens *in vivo* (data not shown).

Importantly, Ferumoxtran-augmented apoptosis could be prevented by EUK-8 and its more lipophilic O-methyl derivative EUK-134, both antioxidants with superoxide dismutase, catalase, and oxyradical scavenging properties [33]. Antioxidant treatment resulted in normalisation of Ferumoxtran-induced apoptosis *in vitro* (Fig. 3d), as well as in $LDLR^{-/-}$ mice with advanced atherosclerosis (Fig. 3e-j).

Superparamagnetic iron oxide nanoparticles associated with enhanced human plaque apoptosis

As Ferumoxtran has been widely used as contrast agent for MRI detection of inflammatory human atherosclerosis [4, 5], the potential impact of Ferumoxtran treatment on human disease was studied in carotid endarterectomy samples. Samples were collected from symptomatic patients that had received Ferumoxtran (2.6 mg Fe/kg I.V., n=9) 2-11 days prior to surgery, and from control patients (n=9), matched for sex, age and plaque phenotype (Table 1) [4]. All patients were eligible, gave informed consent, and completed all steps of the protocol, and were thus included in the analysis. Notably, the dose used in our mouse model experiments was almost 3 times lower than that used for the clinical study. Electron microscopy detected Ferumoxtran mainly in macrophages and smooth muscle cells (Fig. 4a-c). The percentage of TUNEL-positive apoptotic cells (Fig. 3d-j; 3.7±1.4 (95% CI of mean: 0.5-6.9) versus 18.2±5.3 (95% CI: 5.9-30.5) for the control and Ferumoxtran group, respectively), as well as the number of apoptotic cells per plaque area (33.4± 11.1 (95% CI: 7.9-59.0) versus 191.2 ±48.0 (95% CI: 80.5-301.9) for the control and Ferumoxtran group, respectively) was increased 4-fold in atherosclerotic

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plaque from Ferumoxtran-treated compared to untreated patients. Most apoptotic cells were macrophage foam cells (Fig. 4k, l). TUNEL-positive cells co-localised with CD68-positive macrophages (Fig. 4e, h) and activated caspase-3 (Fig. 4f, i). Thus, the use of Ferumoxtran in patients with cardiovascular disease aggravated plaque apoptosis, and possibly subsequent plaque instability.

New USPIO formulation does not enhance macrophage apoptosis *in vitro* or *in vivo*

The newly developed carboxymethyl dextran-coated USPIO Ferumoxytol has recently been approved by the United States Food and Drug Administration (FDA) to treat anaemia in chronic kidney patients [34]. In light of the initial controversy regarding acute side-effects at time of injection [35] and its use in clinical trials of several cardiovascular diseases [9, 15-19, 36], this new generation USPIO was also tested *in vivo* and *in vitro*. In contrast to prior USPIO formulations, Ferumoxytol did not enhance apoptosis in plaques or liver of hypercholesterolemic $LDLR^{-/-}$ mice (Fig. 5a-g). In line, Ferumoxytol did not enhance foam cell apoptosis *in vitro*, despite positive Perl's iron staining (Fig. 5h-j). This supports a favourable safety profile of Ferumoxytol for cardiovascular imaging, in line with recent safety reports [37], and in contrast to Ferumoxtran and Ferumoxide, the use of which may have side effects on atherosclerotic plaque stability.

Dextran coating and particle size are not associated with enhanced macrophage apoptosis

Since Ferumoxide and Ferumoxtran particles are coated with dextran, while Ferumoxytol particles are coated with negatively charged carboxymethylated dextran, the effect of iron particle coating on macrophage apoptosis was investigated. Dextran was avidly taken up by RAW264.7 macrophages (Fig. 6a, b) but, compared to the untreated control, no significant induction of apoptosis in both foam cells and normolipidemic macrophages was observed (Fig. 6c, d). Moreover, no significant difference in apoptosis was observed in both normolipidemic and hVLDL-laden RAW264.7 cells incubated with iron nanoparticles coated with citrate, dextran, or carboxymethylated dextran (Supplementary Fig. 4). Particle size itself also did not appear to be a major determinant of USPIO-induced apoptosis

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(Supplementary Fig. 4). Taken together, these findings suggest that neither particle size nor coating accounts for the difference in apoptosis induction found between Ferumoxtran and Ferumoxytol.

DISCUSSION

Collectively, our findings indicate that administration of previous formulations of superparamagnetic iron oxide nanoparticles enhances apoptosis in murine and human atherosclerosis. Despite the large body of evidence supporting the safety of superparamagnetic iron oxide nanoparticles [22, 23], concern is growing that (U)SPIO uptake may lead to intracellular release of iron ions, generation of oxidative stress, and DNA damage, and that it will promote thrombogenicity in the heart [26, 38, 39]. Our study shows that Ferumoxide and Ferumoxtran induce apoptosis and ROS in lipid-laden macrophages and that its effects can be prevented by antioxidant treatment *in vivo* and *in vitro*, pointing to iron oxide-induced oxidative stress as causative factor for USPIO-induced apoptosis. In support of this finding, bare USPIO was seen to induce endothelial cell apoptosis *in vitro,* which was also mediated by USPIO-related ROS, and reversed by antioxidant treatment [40, 41]. In fact, we observed occasional endothelial apoptosis in atherosclerotic plaque after treatment with dextran-coated Ferumoxide (SPIO), and to a lesser extent Ferumoxtran (USPIO). The apparent preference for foam cell apoptosis may be explained by the increased susceptibility to apoptosis of lipid-laden foam cells subjected to a second stressor [24].

Moreover, repeated Ferumoxtran treatment of mice with early atherosclerosis not only led to enhanced plaque apoptosis but also increased plaque macrophage accumulation. Interestingly, this did not translate in progressive plaque growth. Although the jury is still out on this notion, increased macrophage apoptosis has indeed been suggested to impede plaque progression in early-stage atherosclerosis as phagocytic clearance of apoptotic cells is fully operative at this stage [29, 42, 43]. In contrast, in advanced plaques with compromised efferocytosis [44], accumulating apoptotic cell debris and secondary necrosis will directly promote plaque progression by expansion of the necrotic core and inflammation [2, 45]. As Ferumoxtran exacerbated plaque apoptosis in mice and patients with advanced

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atherosclerosis, this raises concerns regarding potentially deleterious effects on plaque progression and destabilisation. This is especially true considering the prolonged residence time of USPIO platforms in tissue. In fact, in porcine heart, USPIO persisted for several months after intracardiac injection of USPIO-labelled stem cells [46]. However, our CVD cohort, with its limited group size, is not fit to draw any conclusions on USPIO treatment-associated clinical adverse events.

Considering the widespread cardiovascular and non-cardiovascular clinical use of USPIO, our findings may have broader impact. USPIO-based imaging has been used for the diagnosis of tumour metastases, autoimmune diseases, rheumatoid arthritis, for targeted stem cell transfer to the infarcted heart, and for treatment of anaemia in patients with chronic kidney disease (CKD) [23, 46-48]. Although Ferumoxtran has been discontinued in 2010 [49], several trials have been started to test the potential of similar USPIO platforms for diagnosis of prostate cancer [50, 51], head and neck squamous cell carcinoma [52], and aortic dissection [53]. Most applications involve elderly patient populations, expected to suffer from moderate to advanced atherosclerosis. Nevertheless, Ferumoxytol, a new generation USPIO equipped with a carboxymethyl dextran coating, appears to display a safer profile for cardiovascular disease. Biodistribution, macrophage uptake route and speed, and intracellular release of USPIO entrapped iron are dependent on particle and iron core size and composition, coating chemistry and charge and will define the particle's pro-oxidant and pro-apoptotic activity. Dextran-coated SPIOs Ferumoxide and Ferumoxtran, while differently sized, both acted pro-apoptotic, suggesting that coating (chemistry) may be critical (Table 2) [54-56]. However, it has previously been shown that blocking CD206 or CD11b does not reduce SPIO uptake by macrophages, indicating that interaction of the dextran coating with carbohydrate receptors does not notably contribute to SPIO uptake [57]. Our data show that dextran polymers of similar size and molecular concentration as the derived SPIO were completely inert, suggesting that particle charge or subtle factors in the SPIO production process may be critical. Net macrophage uptake (and thus gross iron oxide availability) of Ferumoxide was highest, followed by

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Ferumoxytol and Ferumoxtran [54, 58], which underpins that particle intrinsic factors are decisive. Ferumoxytol uptake, while higher than that of Ferumoxtran, did not enhance apoptosis. As Ferumoxytol and Ferumoxtran differ in coating chemistry and charge (negatively charged carboxymethyl dextran versus non-ionic dextran [56]), but not in particle size and core composition, this suggests that the former factors are indeed instrumental in its toxicity. The lack of toxicity of citrate- versus dextran- versus carboxymethyl dextran-coated USPIO particles [59] of similar size, suggest however that the driving determinant may be even more subtle, and relate to differences in coating density or heterogeneity. Nevertheless, our study may reassure recent investigators and their patients on safe use of Ferumoxytol [9, 16, 17, 23, 36, 37, 58].

In conclusion, iron-based contrast agents, such as Ferumoxide and Ferumoxtran increase apoptosis in human and murine atherosclerotic plaque, an effect that can be prevented by antioxidants. Their administration to patients with advanced lesions may result in plaque destabilization. Although Ferumoxytol appears to have a safe cardiovascular profile, our findings indicate that caution should be exercised when applying other iron-based contrast agents in patients with clinical atherosclerosis or other inflammatory disorders that involve lipid-laden macrophages.

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Data availability statement

The data underlying this article are available in the article and in its online supplementary material.

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Conflicts of interest

The principal investigator (E.A.L.B.) has received financial support for this work from Guerbet.

Author contributions

F.S., T.vB. and E.B. were responsible for conception and design. All authors were involved in analysis and interpretation of data. F.S., A.R., I.B., T.vB., B.H., J.S., and E.B. drafted the manuscript, or revised it critically for important intellectual content. All authors gave final approval of the submitted manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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FIGURE LEGENDS

Figure 1 Ferumoxtran exposure increased apoptosis of RAW foam cells compared to normolipidemic macrophages

(a) RAW264.7 cells were transformed into foam cells (20 h incubation with hVLDL 50 µg/ml) and incubated with Ferumoxtran (USPIO, 100 µg Fe/ml). (b) Fluorescent microscopic pictures of DAPI and TUNEL-stained foam cells incubated with or without Ferumoxtran for 1.5 h. (c) RAW264.7 control macrophages (circles) and foam cells (squares) were harvested at different time points and cytospins were analysed for TUNEL in 3 random fields of view (magnification 20x; 412 ± 104 cells/cytospin were analysed). (d) The ability of macrophages and foam cells to take up iron-based contrast media was quantitatively analysed using an ICP-AES assay. (e) Cell viability after Ferumoxide (100 µg Fe/ml, black circles) or Ferumoxtran treatment (white circles) of RAW264.7 cells with different lipid loading were determined using a cytotoxic MTT assay. Data are mean ± SEM (n=3), 4-12 technical replicates, and representative of three independent experiments. Statistics: unpaired student's t-test, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to control conditions. CON: control.

Figure 2 Ferumoxide and Ferumoxtran increased apoptosis in atherosclerotic lesions in hyperlipidemic ApoE$^{-/-}$ and LDLr$^{-/-}$ mice

(a) ApoE$^{-/-}$ mice with advanced atherosclerosis were injected once with NaCl 0.9% (n=3), Ferumoxide (0.3 mg Fe/kg; n=3) or Ferumoxtran (1 mg Fe/kg; n=3). TUNEL-positive cells in the atherosclerotic plaques of the aortic root were quantified and normalised to total cell count. * indicates p-value <0.05 for Kruskal-Wallis across three groups, not significant changes in Dunn's post-hoc testing between individual groups. (b) Representative image of TUNEL staining of plaques in control, (c) Ferumoxide or (d) Ferumoxtran-treated mice. Scale bar in (b) to (d) corresponds to 100 µm. (e) Plaque area of ApoE$^{-/-}$ was determined by computer-assisted morphometric analysis of Oil red O-stained section. (f) The percentage of TUNEL-positive cells was quantified in liver and (g) spleen of ApoE$^{-/-}$ mice controls and mice receiving a single Ferumoxide or Ferumoxtran dose. * indicates p-value <0.05 for Kruskal-Wallis across three groups, # p-value <0.05 in Dunn's post-hoc testing versus control. (h) LDLR$^{-/-}$ mice with initial plaques

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were fed a high cholesterol diet and received weekly intravenous injections with NaCl 0.9% (control group, n=8) or Ferumoxtran (n=8). The percentage of TUNEL-positive cells was quantified in the atherosclerotic plaques of the aortic root. Unpaired student's t-test, * p<0.05 and *** p<0.001 compared to control conditions. (i) Representative image of TUNEL staining of plaques in control, and (j) Ferumoxtran-treated $LDLR^{-/-}$ mice. (k) Plaque area (Oil red O) and (l) the percentage of macrophages (MoMa-2-positive) cells were quantified in plaques of control $LDLR^{-/-}$ and mice repeatedly treated with Ferumoxtran. Statistics: unpaired student's t-test, * p<0.05 and *** p<0.001 compared to control conditions. Data are mean ± SEM. CON: control.

Figure 3 Ferumoxtran triggered ROS-induced apoptosis *in vitro* and *in vivo*

(a) Serum uric acid and (b) Trolox equivalent antioxidant capacity were increased in serum samples of $LDLR^{-/-}$ mice 24 h after Ferumoxtran injection compared to control, a common response to oxidant stress exposure. Statistics: unpaired Student's T-test. Data are mean ± SEM. (c) Gene expression levels of apoptosis- and oxidative stress-related genes of RAW untreated macrophages (white bars), hVLDL–treated foam cells (yellow bars), and hVLDL–treated foam cells exposed to 100 µg/ml Ferumoxtran (yellow dotted bars). (d) RAW264.7 cells transformed into foam cells with hVLDL (yellow bars) were incubated with Ferumoxtran (dotted filling) to induce apoptosis. Pre-treatment of hVLDL foam cells with antioxidant (grey bars) prior to Ferumoxtran reduced apoptosis. Untreated RAW264.7 cells were used as a control (white bars). Cells were harvested, cytospins were made and apoptotic cells quantified by TUNEL analysis in 4 randomly chosen field of views (20x magnification; average 93 ± 27 cells/fov analyzed). Data are mean ± SEM, include 3-6 technical replicates, and are representative of three independent experiments. Statistics: * p<0.05, and p<0.001 versus control (Kruskal-Wallis; Dunn's multiple comparison). (e) $LDLr^{-/-}$ mice on a high cholesterol diet for 6 weeks received a single treatment with either saline (n=8), saline with antioxidant EUK-134 (10 mg/kg, n=8), Ferumoxtran alone (1000 µg Fe/kg, n=9), or Ferumoxtran with EUK-134 (n=9). The percentage of TUNEL-positive cells was quantified

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in the atherosclerotic plaques of the aortic root. Statistics: * $p<0.05$, and $p<0.001$ versus control (ANOVA; Sidak's multiple comparison). (f) Plaque area (Oil red O) was quantified in plaques of $LDLR^{-/-}$ treated once with saline, EUK-134, Ferumoxtran, or Ferumoxtran with EUK-134. (g) Representative image of TUNEL staining of plaques in saline, (h) EUK-134, (i) Ferumoxtran, or (j) Ferumoxtran with EUK-134 treated $LDLR^{-/-}$ mice. Data are mean ± SEM. CON: control.

Figure 4 Ferumoxtran increased apoptosis in human carotid atherosclerotic lesions

a) Electron microscopy shows human carotid atherosclerotic plaque with accumulation of superparamagnetic iron oxide nanoparticles, scale bar corresponds to 10 µm. Boxed region shows particle-laden macrophages, represented in (b) high power view of macrophage with intracellular Ferumoxtran (arrows). Scale bar corresponds to 2 µm. (c) Smooth muscle cell with numerous mitochondria (*) also showing intracellular accumulation of Ferumoxtran nanoparticles (arrow). Insert shows origin of smooth muscle cell. Scale bar corresponds to 0.5 µm. (d) Atherosclerotic lesions obtained from symptomatic patients undergoing carotid endarterectomy and receiving no injection (n=9, d-f) or a single dose of Ferumoxtran (2.7 mg/kg IV, n=9, g-i) prior to surgery. Sections were stained with TUNEL (AEC red precipitate, d, g). Apoptotic TUNEL-positive cells co-localise with macrophages and activated caspase-3 on serial sections stained respectively with CD68 (red precipitate; e, h), (f) activated caspase 3 (red precipitate; f, i). Scale bars in (d) to (i) corresponds to 100 µm. (j) Apoptosis was quantified as TUNEL-positive cells per total cell count. (k) Arrows indicate TUNEL-positive nuclei in endothelial cells lining an intraplaque microvessel and (l) smooth muscle cells. Scale bars in (k) and (l) correspond to 100 µm. Data are mean ± SEM. Statistics: ** p=0.004 (Mann-Whitney compared to control). CON: control.

Figure 5 Ferumoxytol did not enhance apoptosis *in vivo* or *in vitro*

a) Plaque area at sacrifice of $LDLR^{-/-}$ mice with early atherosclerosis which were fed a high cholesterol diet for 9 weeks after single weekly intravenous injections with NaCl 0.9% (control group, n=8) or

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Ferumoxytol (n=8). Representative images of apoptotic cells detected by TUNEL in atherosclerotic plaques of the aortic root of $LDLr^{-/-}$ mice injected with saline (control, b) or Ferumoxytol (c), with corresponding quantification (d). Quantification (e) and representative images of apoptotic cells detected by TUNEL in liver of $LDLr^{-/-}$ mice injected with saline (control, f) or Ferumoxytol (g). (h) Perl's iron staining of RAW cells, incubated with hVLDL and Ferumoxytol, detects iron accumulation. (i) Oil red O staining of RAW cells, incubated with hVLDL and Ferumoxytol, confirmed massive lipid accumulation. (j) The percentage of apoptotic, Annexin V-positive cells wasquantified by flow cytometry of RAW cells incubated with or without hVLDL, in the absence or presence of Ferumoxytol (n=5-8 technical replicates per group). Data are mean ± SEM. * $p<0.05$, (unpaired Mann-Whitney test or Kruskal-Wallis with Dunn's multiple comparison test, compared to control). CON: control.

Figure 6 Dextran does not increase apoptosis in RAW foam cells compared to normolipidemic macrophages

(a) Fluorescent microscopic pictures of RAW264.7 incubated with 1 µM, 2.4 µM or 4 µM TRITC-dextran for 1 h or untreated RAW264.7 (CON) and stained with Hoechst 33342. Per well, 9 fluorescent images were taken and merged, corresponding to around 2700 cells imaged per well, with 3 technical replicates per condition. Scale bars correspond to 50 µm. (b) Quantification of (a), percentage of cells positive for TRITC-dextran. (c) Fluorescent microscopic pictures of RAW264.7 transformed into foam cells with hVLDL (yellow bars) or normolipidemic cells (white bars) exposed to 2.4 µM or 4.8 µM dextran or fresh DMEM for 1 h. Untreated cells served as control (CON), RAW264.7 cells treated with 1 µM staurosporine for 20 h served as positive control (POS). Cells were stained with Annexin V and Hoechst 33342. 9 fluorescent images were taken and merged per well, corresponding to around 4400 cells imaged per well, with 3 (POS) or 7-8 technical replicates per conditions. Scale bars correspond to 100 µm. (d) Quantification of (c), percentage of cells positive for Annexin V. Data are presented as mean ± SEM and representative of three independent experiments (c, d). Statistics: * $p<0.05$ (Kruskal-Wallis with Dunn's multiple comparison test).

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Table 1 Patient characteristics

Patient characteristics		Control (n=9)	Ferumoxtran (n=9)
Gender	% male; male/female	89%; 8/1	89%; 8/1
Age	years+/- SD	64.6±2.9	63.9±3.2
Plaque type	thin/thick cap fibroatheroma	67% (n=6)	67% (n=6)
	intraplaque/luminal thrombus	33% (n=3)	33% (n=3)
Clinical stage	% symptomatic	100%	100%
Stenosis	percentage patients with >70%	100%	100%

No significant p-values.

Table 2 Nanoparticle characteristics

Feature	Ferumoxide	Ferumoxtran	Ferumoxytol
SPIO/USPIO	SPIO	USPIO	USPIO
Particle size (nm)	120-180	31±5	28±4
Crystal core size (nm)	5	6	6.7
Polydispersity index	n.a.	0.374±0.019	0.252±0.018
Half-life (h)	2-3	30	15
Coating	Dextran	Dextran	Carboxymethyl dextran
Zeta potential ζ (mV)	n.a.	-27±7.1	-43.9±8.4
Macrophage uptake (pg Fe/cell)*	5.0-7.0	0.5-1.8	1.0-1.5
Mechanism uptake	SRA1	SRA1	SRA1
Clinical dose mg/kg	0.6	4	2.7

Integrated data from [54-56, 60] and own measurements; *THP-1 with 200 µg Fe/ml for 24 h; SRA1: scavenger-receptor A mediated; n.a.: information not available

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Figure 1 Segers et al

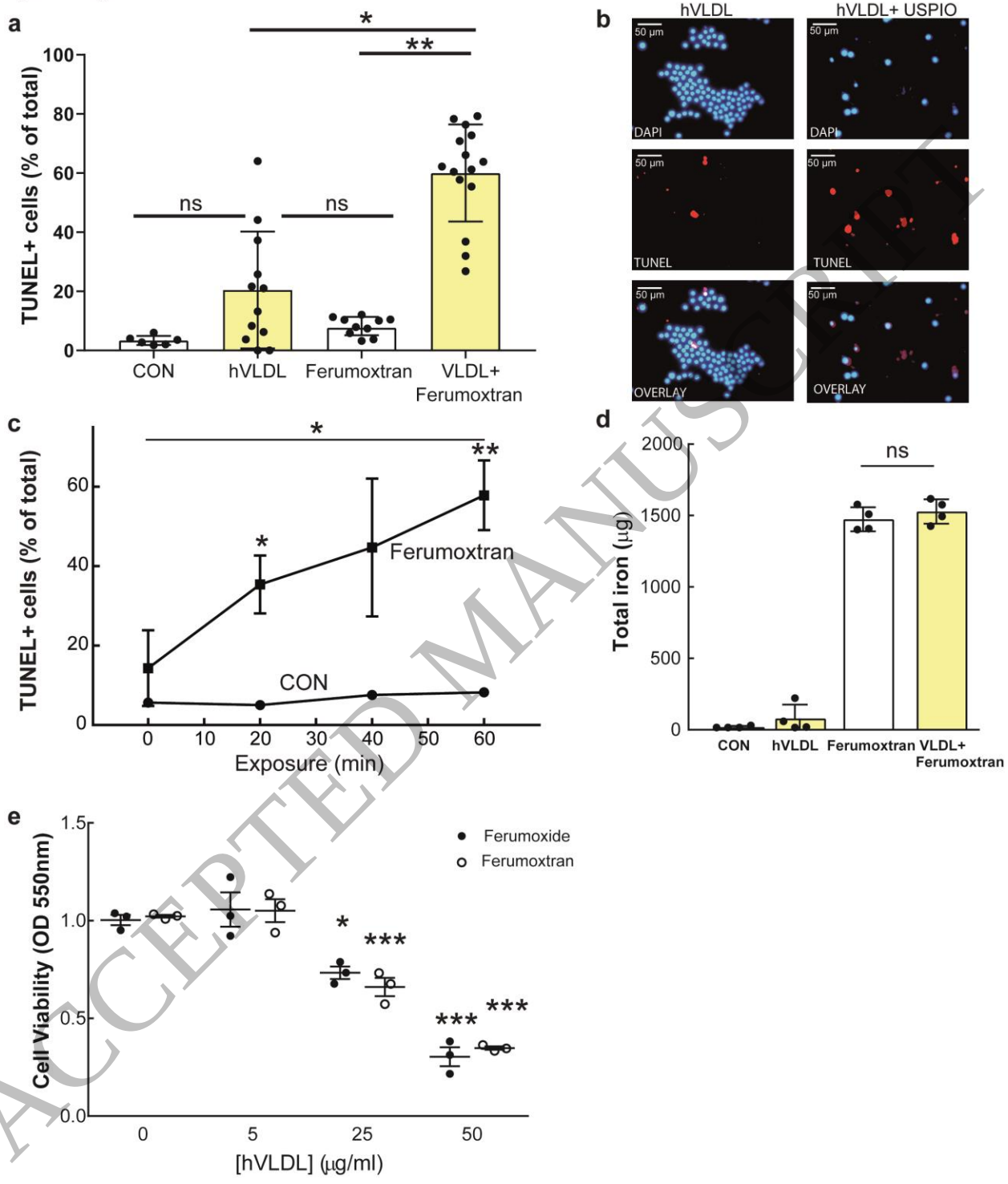

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Figure 2 Segers et al

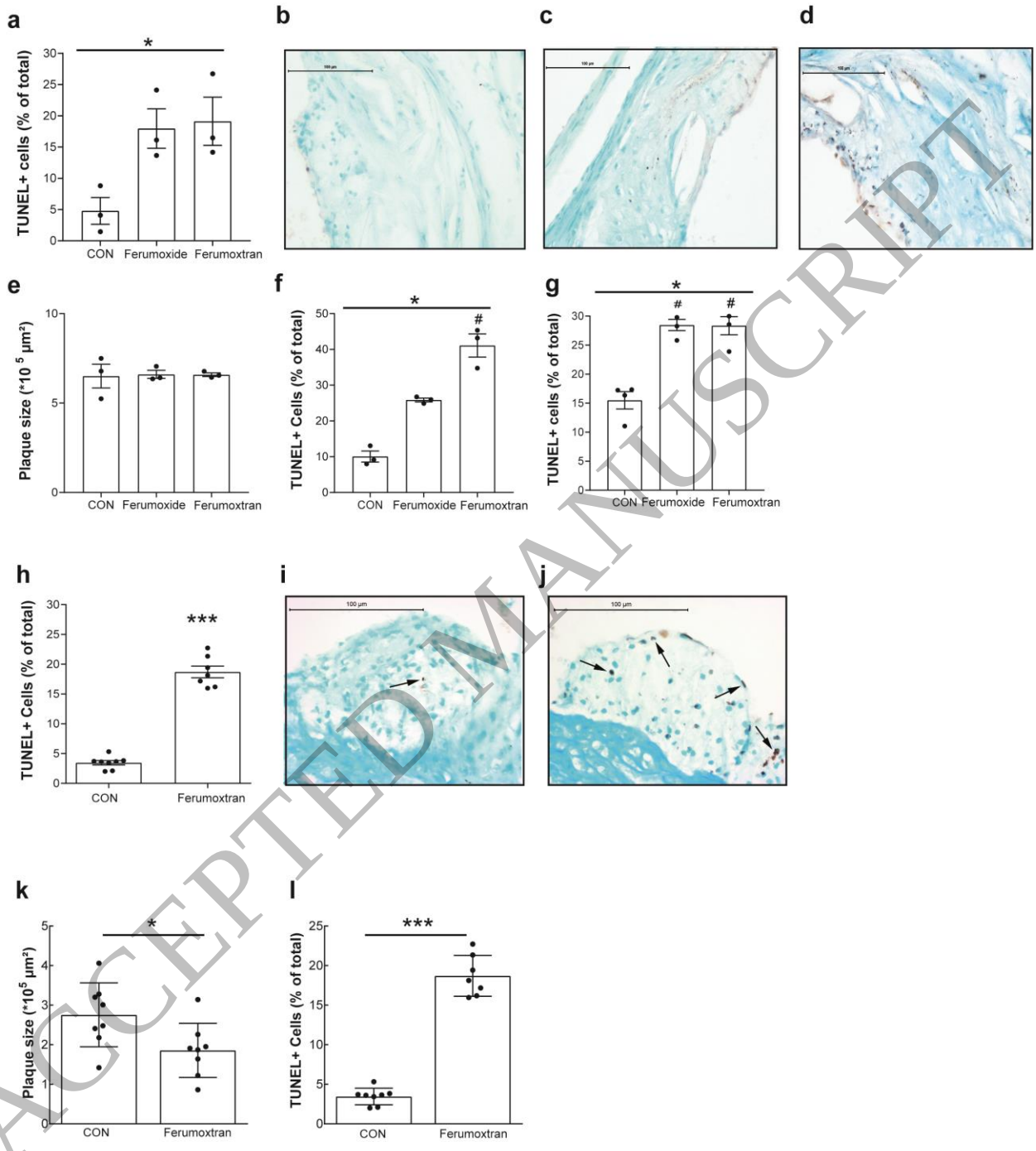

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Figure 3 Segers et al

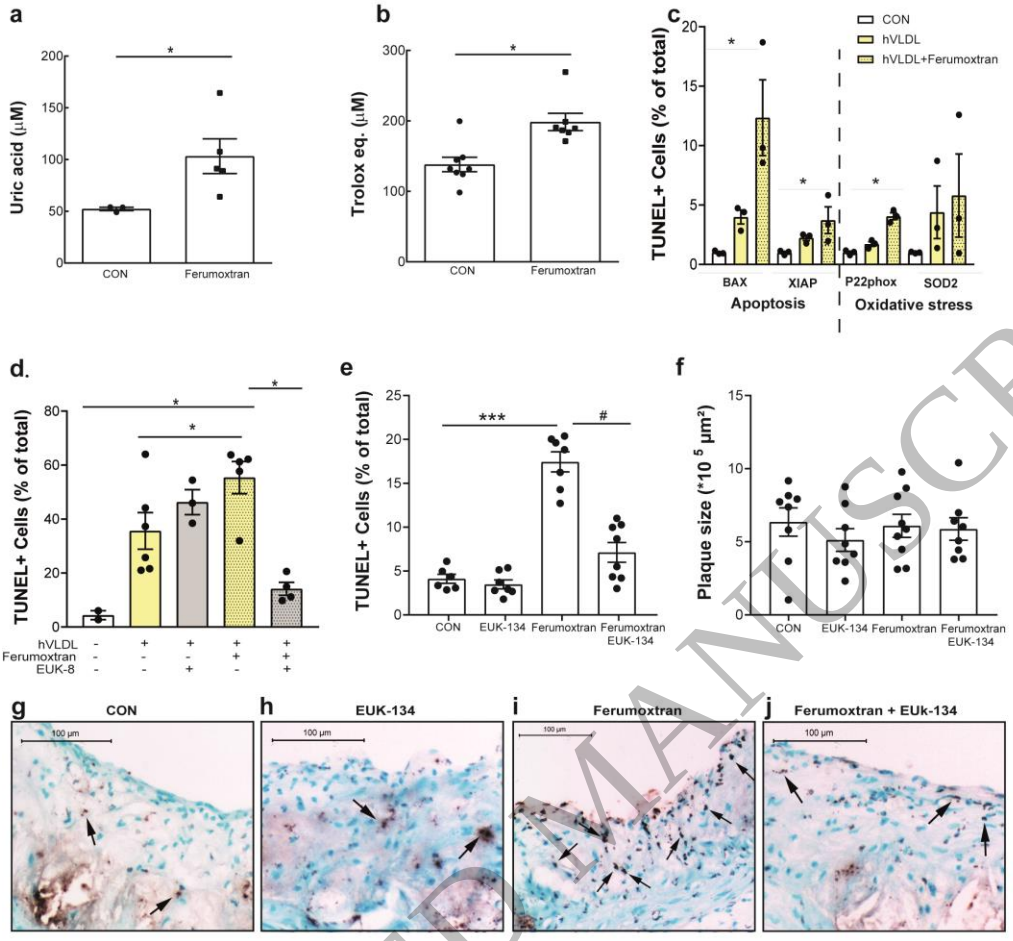

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Figure 4 Segers et al

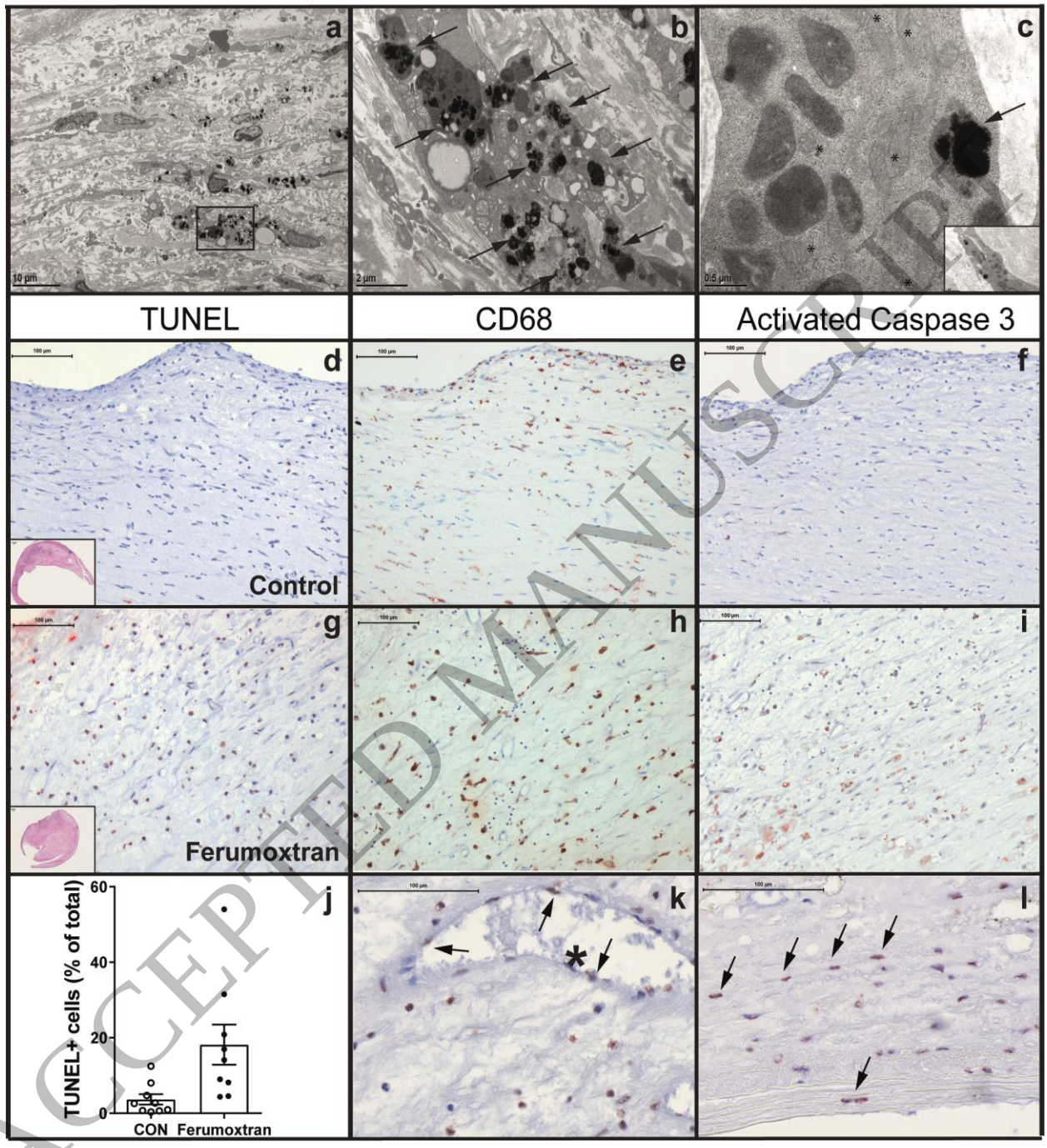

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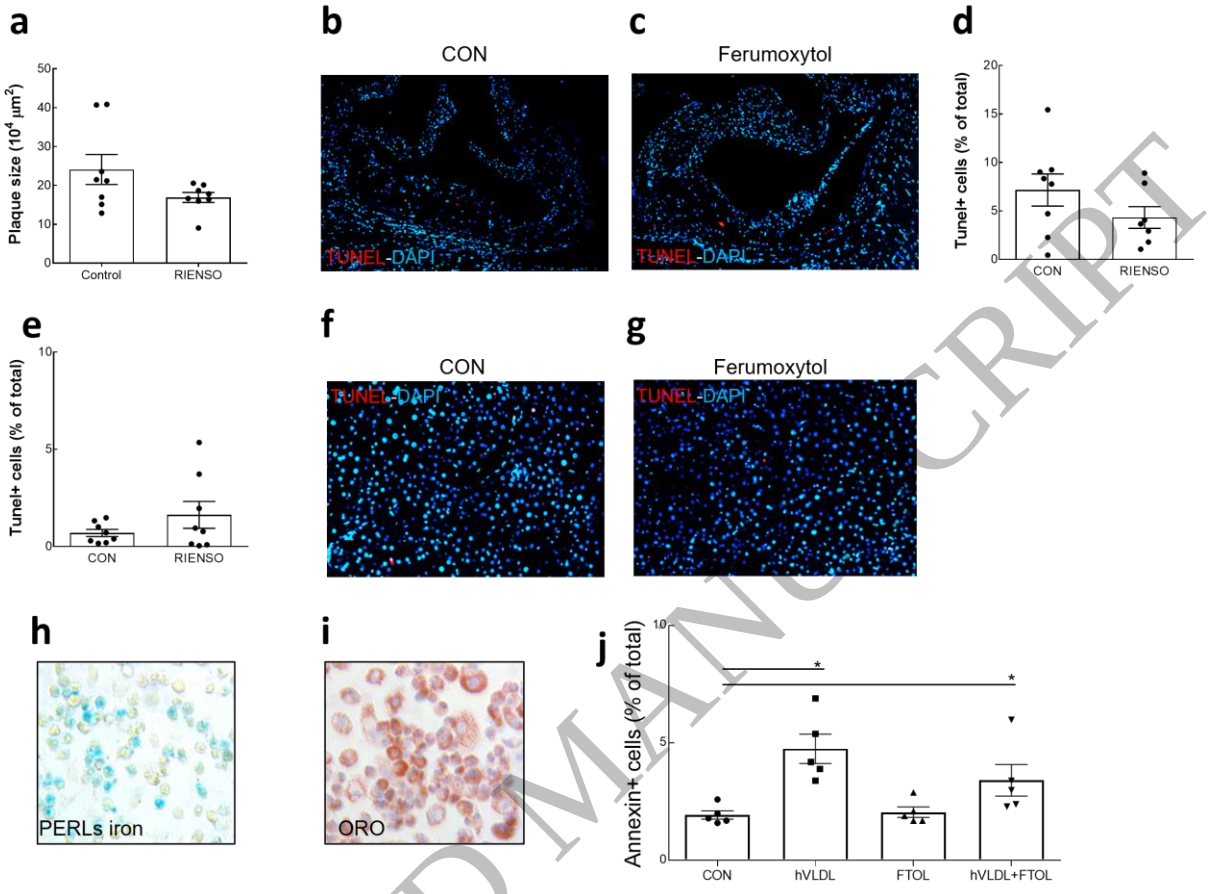
Figure 5 Segers et al



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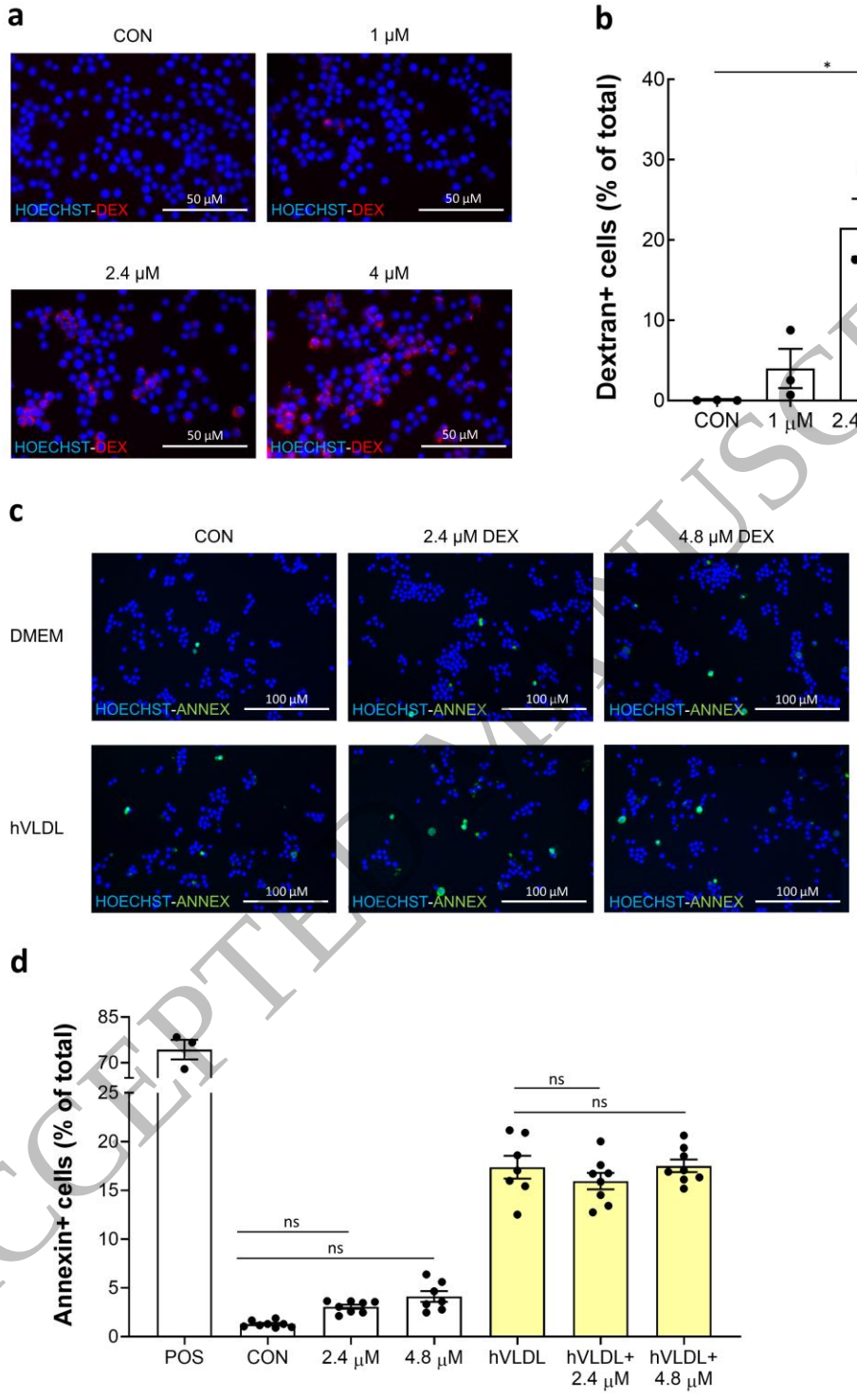

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