



Original paper

## Investigation of the magnetic susceptibility properties of fresh and fixed mouse heart, liver, skeletal muscle and brain tissue

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## ABSTRACT

**Purpose:** Several magnetic resonance imaging (MRI) techniques exploit the difference in magnetic susceptibilities between tissues, but systematic measurements of tissue susceptibility are lacking. Furthermore, there is the question as to whether chemical fixation that is used for *ex vivo* MRI studies, affects the magnetic properties of the tissue. Here, we determined the magnetic susceptibility and water content of fresh and chemically fixed mouse tissue.

**Methods:** Mass susceptibility of brain, heart, liver and skeletal muscle samples were determined on a vibrating sample magnetometer at room temperature. Measurements at 50, 125, 200 and 295 K were performed to assess the temperature dependence of susceptibility. Moreover, we measured water content of fresh and fixed samples.

**Results:** All samples show mass susceptibilities between  $-0.068$  and  $-1.929 \times 10^{-8} \text{ m}^3/\text{kg}$ , compared to  $-9.338 \times 10^{-9} \text{ m}^3/\text{kg}$  of double distilled water. Heart tissue has a more diamagnetic susceptibility than the other tissues. Compared to fresh tissue, fixed tissue has a less diamagnetic susceptibility. Fixed tissue was not different in water content to fresh tissue and showed no consistent dependence of susceptibility with temperature, whereas fresh tissue shows a decrease to at least 125 K, indicative of a paramagnetic component.

**Conclusions:** Biological tissues are diamagnetic in comparison to water, where the heart is more diamagnetic than the other tissues, with paramagnetic contributions. Fixation rendered tissue less diamagnetic compared to fresh tissue. Our measurements revealed differences in tissue susceptibility between VSM and QSM, inviting more research to compare susceptibility-based MRI methods with physical measurements of tissue susceptibility.

## Introduction

Magnetic susceptibility is a fundamental electromagnetic property that measures the ability of a substance to become magnetized when placed in an external magnetic field. Many biological tissues exhibit either positive or negative susceptibility, and are termed paramagnetic or diamagnetic, respectively. The fact that different biological tissues and cellular structures have different magnetic susceptibilities has been exploited for contrast generation in magnetic resonance imaging (MRI). Using  $T_2^*$ -weighted gradient echo imaging, variations of tissue magnetic susceptibility typically lead to local signal cancellations in magnitude images and non-local changes in phase images due to the induced frequency shifts. The combination of magnitude and the phase information into a single image, the so-called susceptibility-weighted image, has been shown to significantly enhance image contrast qualitatively

between tissues of different magnetic susceptibility [1]. With the introduction of quantitative susceptibility mapping (QSM), a post-processing technique for gradient echo data, quantitative maps of bulk tissue magnetic susceptibility of the human brain and body can be obtained. QSM recovers the susceptibility distribution from the measured local field (i.e. phase) distribution [2–8]. A multitude of clinical and preclinical studies illustrated how QSM can be applied *in vivo*; for example, to detect vascular abnormalities, visualize blood products and calcifications; microstructural changes and lesions, or map abnormalities in iron metabolism [9–14]. These studies have collectively demonstrated that pathological alterations in the brain, heart, muscle and abdomen in various diseases are associated with changes of tissue magnetic susceptibility. For QSM, algorithms are required for the numerical solution of the field-to source inverse problem and susceptibility maps computed with different algorithm have shown substantial

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differences [15]. Thus, there is a general need for reference tissue values for interpreting QSM results.

Few studies have measured magnetic susceptibility of tissue itself [16,17], but rather measurements of magnetic susceptibility have been made with purified biochemical materials that are believed to constitute the main determinants of susceptibility (e.g., iron, myelin, lipids, heme protein etc.) in the tissue [16,18–21]. In general, biological tissues are diamagnetic. For example, human tissue has a volume susceptibility between  $-11.0 \times 10^{-6}$  to  $-7.0 \times 10^{-6}$  [16]. This value is within  $\pm 20\%$  of the value for water, which has a mass susceptibility of water at 37 °C (310 K) of  $-9.07 \times 10^{-9}$  m<sup>3</sup>/kg or a volume susceptibility of  $-9.05 \times 10^{-6}$  [16–18] and which is the major component of tissue. Iron in its most common oxidation states (Fe<sup>2+</sup>, Fe<sup>3+</sup>) and bound forms (e.g. heme proteins) is paramagnetic, which makes tissue containing iron less diamagnetic, whereas heavily myelinated tissue for example in the brain is more diamagnetic [16]. In addition, blood cells that perfuse tissue contribute to their susceptibility. The volume magnetic susceptibility of red blood cells depends on the oxidation state of iron in haemoglobin. Deoxygenated blood cells are more paramagnetic ( $-6.52 \times 10^{-6}$ ) when compared to oxygenated red blood cells ( $-9.19 \times 10^{-6}$ ) [16,22–24]. Thus, a systematic determination of magnetic susceptibility of tissues and organs is required for interpreting QSM results.

Besides *in vivo* examinations, *ex vivo* MR susceptibility imaging of autopsy or biological samples is also often carried out [25–28]. *Ex vivo* studies allow for high spatial resolution MRI [29] and can guide pathological examinations, or can be combined with histopathology or other biochemical methods to identify underlying differences in tissues [25,30–32]. Tissues that undergo *ex vivo* MRI are usually subjected to chemical fixation, using (para-)formaldehyde (PFA) solution for tissue preservation [33,34]. The fixation procedure has many effects on the chemical composition and biophysical properties of tissue. A previous QSM study has shown no effects of chemical fixation on magnetic susceptibility of human brain tissue [28]. Thus, an independent validation of this observation and a more systemic investigation of the other tissue types has so far not been attempted, but remains important for comparing *in vivo* and *ex vivo* MR susceptibility imaging studies.

In the current study, we determined the mass susceptibility of fresh and fixed heart, brain liver and skeletal muscle tissue of mice, that are organs and tissue most often investigated with MR susceptibility imaging with a vibrating sample magnetometer (VSM). Measurements of water content of tissues was performed to establish influence on magnetic susceptibility. Susceptibility was obtained from measuring induced magnetization as a function of applied magnetic field. We have further observed the temperature dependence of susceptibility, which will indicate if the tissue is purely diamagnetic or if there is a contribution arising from a paramagnetic component.

## Methods

### Animals and sample preparation

All experiments were performed in accordance with the Swiss Federal Act on Animal Protection and approved by the Cantonal Veterinary Office Zurich (permit number: ZH044/19). Ten C57Bl6/J mice (Janvier, France) were used that had a weight range from 20 to 25 g and an age range from 12 to 20 weeks. Animals were housed in individually ventilated cages inside a temperature-controlled room, under a 12-hour dark/light cycle. Pelleted food and water was provided *ad-libitum*.

Mice were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (100 mg/kg body weight). To remove blood from circulation, mice were perfused intracardially with 10 ml of 0.1 M phosphate buffered saline (PBS, pH 7.4). The heart, liver, thigh muscles of both hind legs and the brain were subsequently removed from each animal. Tissues and organs were divided (except the heart for measurement of magnetic mass susceptibility, which would have rendered samples too small). One half was immersed in 4% PFA in 0.1 M PBS (pH

7.4) for 24 h. The other half was placed in a container, frozen on dry ice and kept frozen at  $-20$  °C until further use.

### Magnetic mass susceptibility

Volume magnetic susceptibility ( $\chi$ ) is a material constant that reflects the relationship to the induced magnetization ( $M$ ) that a material acquires in a weak applied field ( $H$ ):

$$M = \chi H \quad (1)$$

Both  $M$  and  $H$  are measured in A/m such that  $\chi$  is without units. Magnetic mass susceptibility ( $\chi_{\text{mass}}$ ) was calculated by:

$$\chi_{\text{mass}} = \chi / \rho \quad (2)$$

where densities ( $\rho$ ) of the tissue were taken from [35]. The units for  $\chi_{\text{mass}}$  are in m<sup>3</sup>/kg.

The magnetic susceptibility of the samples is determined from the slope of the induced  $M$  of the sample in the applied DC field [36] when using a VSM. VSMS have been previously shown to yield accurate and reliable values of susceptibility of mineral inclusions, nanoparticles and biological tissue [37–39]. Measurements on tissue samples from 5 mice were made on a VSM (Model 3900, Princeton Measurements Corporation, PCM) at room temperature (295 K) using a 250 ms averaging time and 2.55 kA/m sample interval between  $\pm 637$  kA/m. A full magnetization curve was obtained within 500 s. The instrument was calibrated with a Yt-Fe-garnet that was certified by the U.S. National Institute of Standards (NIST). For measurements, samples were inserted into a plastic straw (length 19.8 cm, diameter 0.6 cm) and gently pressed into the straw (6 mm) so that the height of sample was on the order of the width to preserve a dipole signal. The straw was sealed at the bottom end with hot glue. [Supplementary Table 1](#) provides a list of tissue samples. Each holder was measured before the samples was inserted, so that its contribution to the total magnetization could be removed. The empty sample holder consisted of a plastic straw in which one end was sealed with glue. Frozen samples were allowed to thaw and reach room temperature for measurements. Note that the tissue samples were not measured in any particular order with respect to tissue type or whether the tissue was fresh or fixed tissue. A magnetization curve was also measured for three samples of double-distilled water and two samples of the PFA solution for comparison with the tissue samples. In this case, a glass capillary tubes with ca. 6 mm length and 5 mm diameter that were sealed on one end with epoxy was used for the liquid samples.

To assess the behavior of tissue susceptibilities, magnetization curves were measured on the VSM with a PCM cryostat at 50, 125, 200 and 295 K. The same measurement parameters were used for the low temperature measurements. For low temperature, two samples of both fresh and fixed tissue from the brain, liver and muscle and one fresh and fixed heart samples were cooled to temperature until thermocouple showed that the region immediately below the sample had reached the set temperature. If there was any thermal drift, the magnetization was repeated. Note that the temperature within the cryostat varied by  $\leq 1.0$  K during the measurement. Diamagnetic susceptibility is independent of temperature [40], but an empty holder was also measured to confirm that there was no change in susceptibility as a function of temperature.

### Measurement of water content

We assessed fresh and fixed tissue samples from 5 mice. Samples were weighted (wet weight) after preparation and placed in an oven at 120 °C for 50 h. Samples for weighted again (dry weight). Water content (%) was calculated as the percentage difference between wet and dry weights.

### Statistics

Statistical analysis was performed using SigmaPlot Version 13

(Systat Software Inc., CA, USA). A two-way ANOVA with Holm-Sidak’s post-hoc analysis was used for comparison of magnetic susceptibilities of fresh and fixed tissues. The difference between groups was considered significant (\*) at p value < 0.05.

**Results**

*Biological tissue shows diamagnetic susceptibility*

All holders showed a weak paramagnetic magnetization, where slight variations between holders were due to small differences in the length of the straws and the amount of glue. For this reason, each holder was measured before the samples was inserted, so that its contribution to the total magnetization could be removed. The frozen samples were allowed to thaw during sample preparation. Therefore, initial magnetization measurements were made at room temperature for both the fresh and fixed tissue. The magnetization for all fresh and fixed samples is weak, but shows a linear relation of their magnetization as a function of field (Fig. 1a). The magnetization curve of each sample was measured again after approximately 24 h to monitor for any change in susceptibility over time (data not shown). All measurements were reproducible which showed that the measurement of  $\chi_{\text{mass}}$  was repeatable. Based on the measurements the magnetic mass susceptibility for each sample was calculated (Supplementary Table 1) All samples show diamagnetic susceptibility between  $-0.068$  and  $-1.929 \times 10^{-8} \text{ m}^3/\text{kg}$ . The sample of double distilled water has a diamagnetic susceptibility of  $-9.338 \times 10^{-9} \text{ m}^3/\text{kg}$  (Table 1, Fig. 1b). Heart tissue has a higher susceptibility (i.e., more diamagnetic) than the other tissue samples.

**PFA fixation increases mass susceptibility**

We tested the effect of fixation on magnetic mass susceptibility (Table 1, Fig. 1b). Generally, less fixed than fresh tissue was available. PFA solution (4% in 0.1 M PBS) has a susceptibility of  $-4.966 \times 10^{-9} \text{ m}^3/\text{kg}$ . Fixation made the sample less diamagnetic for the heart tissue. For all other organs there were not significant differences in magnetic mass susceptibility between fresh and fixed tissue. Notwithstanding, when we pooled values of organs we observed that the fixed tissue was less diamagnetic compared to fresh tissue.

*Water content of fresh and fixed tissue*

To assess the relation between water content and magnetic

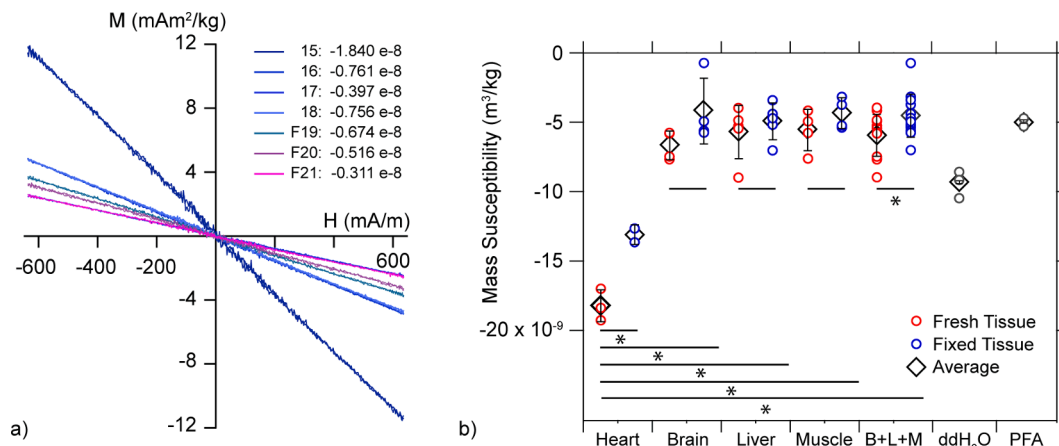
susceptibility of the tissue, we measured water content in fresh and fixed tissue samples (Table 2). For comparison we have also summarized water content for dog [41], rat [42,43], and human tissue [44–46]. Values for water content in mouse tissue was is in good agreement with literature values from the tissues of the other species. We also assessed the effect of chemical fixation on water content. Fixation changed water content by  $-3.0, 0.5, 3.9$  and  $3.3\%$  in the heart, brain, liver and muscle respectively. We did not find statistically significant differences in the water content between fresh and fixed samples of all tissues.

*Paramagnetic contribution to magnetic mass susceptibility in fresh tissue*

Selected fresh and chemically fixed samples were measured at low temperature to establish if their susceptibility shows a temperature dependence. Note that diamagnetic susceptibility does not show a temperature dependence, whereas paramagnetic susceptibility, according to Curie’s law, will show an inverse relationship on a first order [40]. Fresh samples of all tissue types show a small systematic change in susceptibility between 295 K and 125 K that makes it less diamagnetic, but a slight decrease at 50 K (Fig. 2). This behavior indicates a partial paramagnetic contribution to the mass susceptibility. Chemically fixed tissue does not show a systematic dependence of susceptibility with temperature.

**Discussion**

We determined the mass magnetic susceptibility of different mouse organs and skeletal muscle tissue with VSM. All measured tissue samples show diamagnetic susceptibility between  $-0.0675$  and  $-1.929 \times 10^{-8} \text{ m}^3/\text{kg}$  and points to the predominance of diamagnetic constituents in tissue. Heart tissue was found more diamagnetic to what has been reported in the literature, whereas for the other tissues values were slightly below what has been reported [16,40]. However, in one study [16] it was not clear how the reported values were measured. While water is diamagnetic and a major constituent of most biological tissues, the susceptibility of the tissue is not equal to the susceptibility of water [16,18]. We have not seen a relation between magnetic mass susceptibility and water content in fresh tissue. The heart tissue showed a more diamagnetic response compared to water and the other tissues showing a higher susceptibility compared to water, while also the brain has a similar water content than the heart. The more diamagnetic behavior of the heart tissue was noted in the unprocessed magnetic data before normalization by the sample mass, as seen from the steeper slope of the



**Fig. 1.** Magnetic mass susceptibility of fresh and chemically fixed mouse tissues. a) Example of magnetization as a function of applied field for tissue samples from animal 277,305 with their mass susceptibility. b) Mass susceptibility for fresh (red) versus PFA fixed (blue) tissue samples, water and 4% PFA solution. Values are presented as scatter plot with the diamond showing the mean and the whisker shows the standard deviations. Statistical analysis compared magnetic mass susceptibility between fresh tissues, and fresh and fixed condition were compared for each tissue. B + L + M are combined susceptibilities for the brain, liver and muscle tissue together. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 1**  
Magnetic mass susceptibility  $\chi_{\text{mass}}$  of fresh and fixed tissues measured at  $295.1 \pm 0.6$  K ( $22.1 \text{ }^\circ\text{C}$ ).

Tissue	Condition	Density $\text{kg/m}^3$ from [35]	Mass magnetic susceptibility, $\chi_{\text{mass}}$ , $\text{m}^3/\text{kg}$ (Volume magnetic susceptibility, $\chi$ )	% Change after fixation	Number of specimen
Heart	Fresh	1060	$-1.823 \pm 0.115 \times 10^{-8}$ ( $-1.932 \times 10^{-5}$ )	28.1	3
	Fixed	1060	$-1.310 \pm 0.071 \times 10^{-8}$ ( $-1.389 \times 10^{-5}$ )		2
Brain	Fresh	1040	$-6.662 \pm 1.039 \times 10^{-9}$ ( $-6.928 \times 10^{-6}$ )	37.0	4
	Fixed	1040	$-4.197 \pm 2.375 \times 10^{-9}$ ( $-4.365 \times 10^{-6}$ )		4
Liver	Fresh	1060	$-5.708 \pm 1.918 \times 10^{-9}$ ( $-6.050 \times 10^{-6}$ )	13.5	6
	Fixed	1060	$-4.938 \pm 1.326 \times 10^{-9}$ ( $-5.234 \times 10^{-6}$ )		4
Muscle	Fresh	1040	$-5.559 \pm 1.488 \times 10^{-9}$ ( $-5.781 \times 10^{-6}$ )	22.0	4
	Fixed	1040	$-4.334 \pm 1.107 \times 10^{-9}$ ( $-4.507 \times 10^{-6}$ )		4
Double distilled water		1000	$-9.338 \pm 0.026 \times 10^{-9}$ ( $-9.338 \times 10^{-6}$ )		3
4% PFA solution		1000	$-4.966 \times 10^{-9}$ ( $-4.966 \times 10^{-6}$ )		2

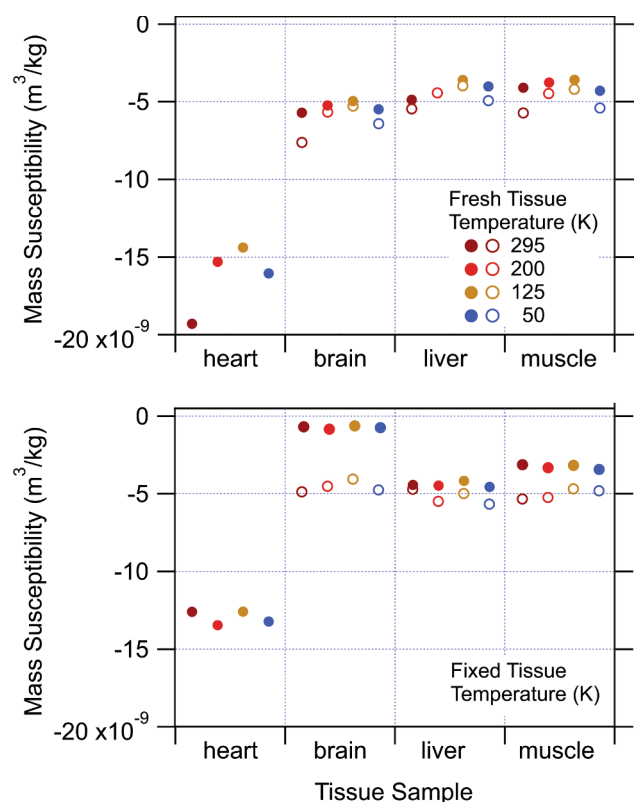
**Table 2**  
Water content of fresh and fixed tissues (five specimens each condition) and comparison with the literature.

Organ	Species	Condition	Water content %	References
Heart	Mouse	Fresh	$79.0 \pm 1.4$	[41] [44]
	Mouse	Fixed	$76.6 \pm 3.5$	
	Dog	Fresh	$78.3 \pm 0.4$	
	Human	Fresh	80.0	
Brain (grey and white matter)	Mouse	Fresh	$78.5 \pm 1.2$	[42] [43] [44]
	Mouse	Fixed	$78.9 \pm 0.4$	
	Rat	Fresh	75.8	
	Rat	Fresh	77.2–78.6	
	Human	Fresh	77.5	
Liver	Mouse	Fresh	$66.3 \pm 3.4$	[42] [44]
	Mouse	Fixed	$68.9 \pm 1.5$	
	Rat	Fresh	77.3	
	Human	Fresh	71.1	
Skeletal muscle	Mouse	Fresh	$72.0 \pm 0.5$	[44] [45] [46]
	Mouse	Fixed	$74.4 \pm 2.5$	
	Human	Fresh	79.2	
	Human	Fresh	$76.2 \pm 1.1$	
	Human	Fresh	$77 \pm 2$	

For measurements of water content, 5 specimens were investigated for each condition.

heart samples with respect to the sample mass, in comparison to other tissue samples (Supplementary Fig. 1). Because the heart samples were measured between measurements with other tissue samples, indicates that the difference is real and not an artefact of the experimental procedure.

In biological tissues, other biochemical constituents than water contribute to the net bulk susceptibility in a complex way. For example, it is known that lipids and structural proteins such as actin, collagen and myelin are diamagnetic [16,18–21]. But lipids are less diamagnetic than water and proteins [47]. They are highly abundant in some tissues e.g. 70% as myelin in the white matter of the brain [48], but less abundant in others, e.g. 12% in skeletal muscle [49]. However, as we have not investigated the determinants of magnetic susceptibility other than water for each tissue, we do not know which constituent contributes to the diamagnetic property of tissue. Moreover, anisotropy of magnetic susceptibility of proteins like collagen and myoglobin [50,51] would make VSM sensitive to the distribution of constituents in tissue of similar composition but different architecture [52]. However, these



**Fig. 2.** Assessing temperature dependency of magnetic mass susceptibility. Magnetization curves were measured with a) fresh and b) chemically fixed tissue samples at 50, 125, 200 and 295 K. Individual values are presented.

contributions are expected to be very small particularly because the tissue was not placed in the holders that would lead to a preferential orientation of proteins or myoglobin. More research would be needed with physically aligned elements, to understand the exact contribution of the microarchitecture to the tissue susceptibility.

The temperature dependence of susceptibility to at least 125 K in fresh tissue suggests that there is a paramagnetic contribution to the mass susceptibility. Ions, atoms, and molecules with unpaired spins provides a paramagnetic contribution to tissue susceptibility. The

transition elements known to be present in biological tissue are iron, copper, manganese, nickel, chromium [20]. At physiological temperatures, paramagnetism is much more powerful than diamagnetism on an atom by atom basis [20]. A single paramagnetic molecule can cancel the diamagnetism of hundreds or thousands of water molecules [16]. We have removed blood, which would be mainly paramagnetic deoxygenated blood [16,22–24] in *ex vivo* tissue, by PBS perfusion prior dissection, thus have eliminated circulating sources of iron. However, iron is stored in tissue predominately in myoglobin and ferritin [53]. Myoglobin is largely found in skeletal and cardiac muscle [54], but under physiological conditions is has not be found in the brain [55]. Deoxymyoglobin is paramagnetic [50]. Thus, in the absence of hemoglobin, we think that we have paramagnetic contributions of these proteins to the magnetic susceptibility of the tissue. However, despite their strong effects paramagnetic elements have a comparably low concentration in tissue, thus rendering the tissue overall diamagnetic. This applies even to organ that are iron stores like the liver and spleen, where we found liver tissue to be diamagnetic. Even with excessive storage of iron in tissues that occurs in hereditary hemochromatosis the liver becomes only weakly paramagnetic [10]. Taken together, all tissues are diamagnetic, but are less diamagnetic than water due to the presence of other constituents with higher susceptibility, such as any paramagnetic constituent.

Tissue composition can vary considerably, depending on the species, organ and subregions, age, gender, genetics, and environmental factors (diets etc.) etc. We opted to use mouse tissue and not human autopsy tissue for the measurement where factors like age, gender, housing conditions as well as the timing and methods of tissue collection can be better controlled. It is widely assumed that biochemical and cellular composition in the mouse is highly conserved with humans, and thus the current findings might be extrapolated to human tissues [56]. It may be interesting to study effects of different factors such as age on susceptibilities of different tissues. For example, it is known that concentrations of paramagnetic iron in tissue can vary considerably with ageing in most tissues [57]. In the brain, iron concentration increases during early childhood and during ageing and are pathologically altered in neurodegenerative diseases [58,59]. Also the concentration of diamagnetic constituents such as myelin in the brain change during ageing and under pathological conditions [60]. To validate the diagnostic utility of use susceptibility-based MRI samples from pathological tissues should be investigated as a variety of diseases is associated with changes in tissue composition [10,58–60].

There are differences in volume magnetic susceptibility of mouse tissues measured *ex vivo* with VSM and values reported previously from QSM *in vivo* studies. For the brain volume magnetic susceptibilities between  $-0.003$  to  $0.2 \times 10^{-6}$ , depending on the brain region, were reported [6,27,61], which are lower than  $-6.393 \times 10^{-6}$  obtained with VSM. A volume magnetic susceptibility  $-9.11 \times 10^{-6}$  for the forearm muscle was reported, which is more diamagnetic than the value of  $-5.781 \times 10^{-6}$  obtained here. Differences in values are likely, in addition to differences in samples (*in vivo* vs *ex vivo*, location, etc.) due to differences in measurements of susceptibility. With a VSM, the induced magnetization is measured that arises from the material that is being vibrated in an applied DC field. In QSM, bulk susceptibility is inferred from the phase evolution of the MRI signal [2–8]. While QSM is supposed to yield a physical tissue property, susceptibility maps calculated with different algorithm from the same dataset have shown substantial differences [15]. Moreover, while there is a good correlation between the observed tissue susceptibility on susceptibility maps with the concentrations of tissue constituents with strong magnetic moments such as iron [27,28], the contribution of many other tissue constituents to the computed tissue susceptibility is largely not known and may not only rely on the electromagnetic properties of its components, but also their distribution in the tissue and the microarchitecture of the tissue [47,62–65]. Thus, there is a general need to better understand how susceptibility of tissue components give rise to variations in

susceptibility maps in MRI.

Besides examinations *in vivo*, *ex vivo* MR susceptibility imaging of autopsy or biological samples is also often carried out [25–28]. Chemical fixation with PFA solution is ideally suited to prevent or arrest tissue autolysis, to maintain cellular and subcellular architecture of tissue and the local chemical composition, and to allow for long-term preservation of tissue [33]. In chemical fixation cross-links in and between proteins, in and between nucleic acids and between nucleic acids and proteins are formed. The process causes non-uniform shrinkage of tissue [66] and changes water diffusion properties [67–71]. It addition, chemical fixation shortens the  $T_1$ ,  $T_2$  and  $T_2^*$  relaxation times of tissues compared to their *in vivo* state [70–74]. A previous QSM study has observed no differences in the magnetic susceptibility of fresh and fixed human brain tissue [28], but validation of this finding and systematic measurements of the effect of fixation on magnetic susceptibility on other tissues are lacking.

We used immersion fixation of all tissues and found that the magnetic mass susceptibility of fixed tissue was less diamagnetic than the fresh tissue. This was more obvious for the heart and differences were smaller for the other tissues, and were only significantly different upon pooling of values. To test if these changes are due to effects of changes in water content, we measured water content in fresh and fixed tissue samples. Interestingly, we did not find significant changes in water content between fresh and fixed tissue. Similar to the fresh tissue, magnetic susceptibility was not mirrored by the water content of the fixed tissue. Paraformaldehyde has both a paramagnetic and diamagnetic contribution [74,75]. A paramagnetic effect of formalin was reported previously reported [74]. Here, we found that 4% PFA solutions is less diamagnetic than water. Thus, it is likely that the presence of paraformaldehyde has led to the observed small shift in the susceptibility of tissue upon fixation. Moreover, tissue fixation is accompanied by changes such as shrinkage, swelling and hardening of tissue components, and thus affecting the magnetic microarchitecture. Effect of fixation manifests differently in different tissues. Pathological studies and studies using diffusion tensor imaging have shown profound changes in morphology and microstructure (e.g. shifts in fiber orientation) in the heart upon chemical fixation with PFA solution [67–69]. For the brain mixed results on the effect of fixation on microstructural changes have been reported [70,71,76–78]. Thus, cross-linking of proteins and changes in microstructure might have additional effects on tissue susceptibility. We have not tested for fixation intervals longer than 24 h. Given the speed of fixation of 1 mm tissue in one hour [33] and the tissue thickness of approximately 3–5 mm, all tissue was expected to be fixed completely in the chosen interval. However, long-term storage in the fixative can lead to dissolution and/or leaching of transition elements [79,80], which may significantly alter tissue susceptibility.

In the current study, we used VSM to determine magnetic mass susceptibility in biological tissue samples from the mouse. One advantage to measurements on the PCM VSM is the rapidity of measurement, compared to other systems that measure induce magnetization in an applied magnetic field. This has the advantage that less temperature variation and chemical alteration can occur within the (fresh) tissue during the measurement. Moreover, VSM has a high sensitivity and can be used to determine weak susceptibilities of tissues and solution with a high reproducibility. The instrument accuracy was verified from the determination of  $\chi_{\text{mass}}$  of distilled water, which is  $< 3\%$  difference with the theoretical value of pure water of  $-9.07 \times 10^{-9} \text{ m}^3/\text{kg}$  [16,18]. Note that this difference is on the order of the sensitivity range of the instrument, therefore any slight variation in the susceptibility of the sample holder between measuring the empty state and filling, or a slight difference in positioning could account for the small variation.

The current study has a few limitations. 1) We measured the mass magnetic susceptibility of fresh tissue *ex vivo*, which may differ from the susceptibility of tissue *in vivo*. Susceptibilities of heme proteins in tissue depend on oxygen saturation [16,22–24,50] and might thus differ

between *in vivo* from *ex vivo* conditions, where proteins are deoxygenated. *In vivo* VSM measurements are not feasible and notwithstanding these limitations, *ex vivo* VSM investigations have provided reference values for magnetic susceptibilities of different tissues. 2) We have removed blood prior dissection of the tissue by extensive PBS perfusion. We reasoned that the fixation procedure might remove blood from the tissue in an inhomogeneous way, introducing variability. Furthermore, we would not have the same amount of hematocrit in fresh and fixed samples. However, the removal of blood affects vascular organs with a high blood volume like the liver or heart more strongly than organs that have a lower blood volume such as the brain. Thus, we do not know the contribution of blood to the tissue susceptibility under *in vivo* conditions. 3) The time lapse between animal sacrifice and tissue immersion in PFA solution was insignificant, and tissue autolysis was prevented. For the fresh tissue some tissue degradation may have started with thawing of the sample and the effects on the measurements are not known. 4) We measured mass magnetic susceptibilities of organs and tissues at room temperature as it was not possible to heat the sample to body temperature i.e.  $36.5 \pm 0.5$  °C. As paramagnetic susceptibility is dependent on temperature there might be differences between susceptibility of fresh tissue and tissue *in vivo*, but due to the small difference in temperature between the two conditions and the low concentration of paramagnetic elements in tissue, this difference is expected to be small. 5) We used densities for fresh tissue for the calculation of volume magnetic susceptibilities, though fixation might alter tissue densities. 6) The low sample sizes for same tissues like the heart, and the measurement of the temperature dependency of susceptibility. 7) Lastly, we only measured bulk magnetic mass susceptibility of organs and tissue. Brain tissue but also skeletal muscle are known to have susceptibility variations across the tissue. However, the measured sample volumes were on the limit in terms of signal strength, i.e., trying to isolate individual parts of organs e.g. specific brain regions or certain muscle fibers would leave too little material for reliable measurement of susceptibility values. Future studies could investigate regional variations in tissues e.g. the brain, determine biochemical underpinnings, and study oriental effects of tissue magnetic susceptibility.

## Conclusions

We showed with VSM data that biological tissues are diamagnetic in comparison to water with paramagnetic contribution, where the heart is more diamagnetic than the other tissues. Fixation rendered tissue less diamagnetic compared to fresh tissue. The magnetic susceptibility of fresh and fixed tissues is not mirrored by its water content. VSM measurements revealed differences in tissue magnetic susceptibility of biological tissue obtained with QSM *in vivo*, inviting more research to compare susceptibility-based MRI methods with physical measurements of tissue susceptibility.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmp.2021.06.014>.

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