Integration of multiple imaging platforms to uncover cardiovascular defects in adult zebrafish

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Aims
Mammalian models have been instrumental in investigating adult heart function and human disease. However, electrophysiological differences with human hearts and high costs motivate the need for non-mammalian models. The zebrafish is a well-established genetic model to study cardiovascular development and function; however, analysis of cardiovascular phenotypes in adult specimens is particularly challenging as they are opaque.

Methods and results
Here, we optimized and combined multiple imaging techniques including echocardiography, magnetic resonance imaging, and micro-computed tomography to identify and analyse cardiovascular phenotypes in adult zebrafish. Using alk5a/tgfbr1a mutants as a case study, we observed morphological and functional cardiovascular defects that were undetected with conventional approaches. Correlation analysis of multiple parameters revealed an association between haemodynamic defects and structural alterations of the heart, as observed clinically.

Conclusion
We report a new, comprehensive, and sensitive platform to identify otherwise indiscernible cardiovascular phenotypes in adult zebrafish.
1. Introduction

Cardiovascular diseases are a major cause of death worldwide. The understanding of their pathophysiology has relied on the identification and analysis of genes triggering the initiation and progress of cardiovascular defects. Several mammalian models including mice, rats, rabbits, pigs, sheep, and dogs, have been used to investigate various cardiovascular diseases, each with its own benefits and shortcomings. For instance, rodent and human hearts differ in their electrophysiology and oxygen consumption, while large animals have considerable limitations in terms of cost, handling difficulties, and genetic manipulation. Therefore, there is a need for additional animal models to investigate the mechanisms underlying cardiovascular diseases.

The zebrafish is a popular genetic model to study cardiovascular development, disease, and regeneration. External fertilization and optical transparency during embryogenesis allow for in vivo analysis of cardiovascular development at single-cell resolution. Previous studies have also highlighted the similarities between the mature human and zebrafish hearts, including comparable beating rates and action potential profiles. Importantly, mutating zebrafish orthologues of human disease genes can accurately model cardiovascular disorders, as observed with mutations leading to arrhythmogenic, dilated and Titin-associated cardiomyopathies. Unlike their mammalian counterparts, adult zebrafish hearts can regenerate, thereby providing a unique platform to understand mechanisms of cardiac regeneration. Furthermore, unlike their mammalian counterparts, adult zebrafish hearts can regenerate, thereby providing a unique platform to understand mechanisms of cardiac regeneration. Together, these studies indicate that adult zebrafish have the unique potential to provide high-throughput, translatable scientific knowledge for pre-clinical and clinical cardiovascular research. However, morphological and functional characterization of adult zebrafish is particularly challenging as they are no longer optically transparent and too small for most conventional pre-clinical imaging platforms. In addition, many cardiovascular disorders are associated with inherited genetic mutations, often leading to variable phenotypic expressivity. Therefore, to understand the cardiovascular phenotypes despite this variability between individuals, it is essential to extract all of the relevant information from a relatively large sample size, which is not easily done with adult specimens.

Due to the aforementioned imaging limitations, most studies of adult cardiac phenotypes in zebrafish rely on histological analyses of tissue sections, which prevent three-dimensional (3D) representation of the morphological features or assessment of cardiac function. In a clinical setting, diagnosing the onset and progression of cardiovascular diseases often requires a combination of multiple imaging analyses including echocardiography, magnetic resonance imaging (MRI), and computed tomography (CT). Recently, researchers have adapted light-sheet microscopy, MRI, and micro-computed tomography (µ-CT) to define the morphology of zebrafish organs. Others have used echocardiography in adult zebrafish to characterize the functional impact of cardiac disease or regeneration following injury. However, a systematic workflow to study cardiac and cardiovascular phenotypes in adult zebrafish has yet to emerge.

Here, we established new imaging protocols and combined functional analyses of the adult zebrafish heart using echocardiography and in vivo MRI with a morphological characterization of the cardiac compartments and adjacent vessels using µ-CT. As a proof-of-concept, we examined alk5a/tgfr1a variants in humans have been associated with aortic aneurysms and diseases of the great vessels, the diagnosis of which is most often recognized in young adults. We show that the use of this multimodal preclinical imaging strategy allows for a robust dissection of...
adult zebrafish cardiovascular phenotypes, even in the presence of high variability between samples with the same genotype at the disease locus.

2. Methods

2.1 Zebrafish husbandry and lines

All zebrafish husbandry was performed under standard conditions, and all experiments were conducted in accordance with institutional (MPG) and national ethical and animal welfare guidelines (Proposal number B2/1055). All procedures conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

All the zebrafish used in the study were in the Tg(kdrl:GFP)42363 background. The alk5α mutant (tgfr1a bns329) zebrafish carry a 2 bp insertion and a 6 bp deletion in exon 1. Unless otherwise noted, maternal zygotic alk5α–/– zebrafish were analysed and compared to WT zebrafish. When required, the mutants were genotyped as previously described, using high-resolution melt analysis (HRMA) (Eco-Illumina) and/or sequencing with the following primers: alk5α HRM fw: 5'-CTTCTGACAGACGCTGACA-3', alk5α HRM rv: 5'-GAAGGAGCCACTGAAAG-3'.

2.2 Confocal microscopy for live imaging of zebrafish larvae

For live confocal imaging, embryos and larvae were embedded in 1% low-melting agarose/egg water with 0.2% tricine (Sigma) to image stopped hearts. Larvae were imaged with a Zeiss LSM800 Axio Examiner confocal microscope with a W Plan-Apochromat 40×/1.0 or W Plan-Apochromat 20×/1.0 dipping lens. In order to image the heart, the larvae were placed in a supine position. All images were acquired using the ZEN Blue (Zeiss) software.

2.3 Histology and immunostaining

Adult hearts were fixed in 4% buffered paraformaldehyde for 1 h at room temperature, washed in 1× PBS and embedded as previously described. Briefly, the tissue was placed overnight at 4°C in a solution of 30% (w/v) sucrose prepared in 1× PBS, pre-embedded in 7.5% (w/v) porcine gelatin (Sigma)/15% (w/v) sucrose in 1× PBS at 37°C for 1 h and embedded with a new solution of gelatin. Tissue blocks were frozen in isopentane (Sigma) cooled in liquid nitrogen. Cryosections were cut at 10 μm using a Leica CM3050S cryostat (Leica) and kept at -20°C until further use. Prior to processing, the slides were thawed for 10 minutes at room temperature and the gelatin was removed in 1× PBS at 37°C.

For haematoxylin and eosin staining, the cryosections were stained with acidic hemalum (Waldeck) for 10 min, washed in running tap water for 2 min and rinsed in deionized water. The sections were then counterstained with eosin (Waldeck) for 6 min, dehydrated in 100% ethanol, cleared in xylene and mounted in entellan (Merck). The sections were imaged using a Nikon SMZ25 microscope.

Immunostaining started with a wash in 0.1M glycine (Sigma) followed by permeabilization for 7 minutes at -20°C in pre-cooled acetone. The sections were incubated in a blocking solution of PBDX (1% (w/v)
Table 2 Inter-observer measurements for outflow tract outer wall and luminal expansion

| Fish | Sample mean | Observer 1 | Observer 2 | Sample mean | Observer 1 | Observer 2 |
|------|-------------|------------|------------|-------------|------------|------------|
| WT 1 | 6.4         | 5          | 3          | 11.5        | 14         | 7          |
| WT 2 |             | 4          | 8          |             | 12         | 9          |
| WT 3 |             | 3          | 8          |             | 11         | 6          |
| WT 4 |             | 7          | 6          |             | 11         | 8          |
| WT 5 |             | 11         | 4          |             | 12         | 15         |
| WT 6 |             | 14         | 3          |             | 13         | 13         |
| WT 7 |             | 5          | 3          |             | 21         | 15         |
| WT 8 |             | 12         | 8          |             | 7          | 7          |
| WT 9 |             | 10         | 5          |             | 14         | 8          |
| WT 10|             | 3          | 5          |             | 12         | 14         |
| Mean |             | 7.4        | 5.3        |             | 12.7       | 10.2       |
| MUT 1| 2           | 2          | 4          | 34          | 2          | 27         |
| MUT 2| 7           | 7          | 6          | 41          | 41         | 15         |
| MUT 3| 14          | 14         | 10         | 34          | 33         | 35         |
| MUT 4| 5           | 5          | 12         | 30          | 30         | 45         |
| MUT 5| 8           | 8          | 6          | 38          | 40         |           |
| MUT 6| 2           | 2          | 6          | 38          | 40         |           |
| MUT 7| 7           | 7          | 5          | 34          | 33         |           |
| MUT 8| 5           | 5          | 3          | 31          | 38         |           |
| MUT 9| 9           | 9          | 2          | 46          | 30         |           |
| MUT 10|          | 7          | 9          | 44          | 36         |           |
| MUT 11|         | 6          | 9          | 44          | 31         |           |
| Mean | 6.8         | 6.9        | 6.8        | 35.0        | 33.2       |           |

Bovine Serum Albumin, 1% (w/v) DMSO, 1% (w/v) Triton-X100 in PBS with 15% (v/v) goat serum for a minimum of two hours at room temperature. Incubation with the following primary antibody was performed overnight at 4°C: anti-GFP chicken (Aves Technology; 1:400), anti-Elastin2 rabbit (1:100). The slides were washed several times with PBDX and incubated with the corresponding secondary antibodies (1:500) overnight at 4°C: anti-chicken AlexaFluor 488 (ThermoFisher), anti-rabbit AlexaFluor 647 (ThermoFisher). For all incubations, the slides were covered with a piece of Parafilm-M to ensure homogenous distribution of the solution. The slides were washed a minimum of 3 times for 15 minutes each in a solution of 0.3% (v/v) Triton-X100 in PBS (PBST) and counterstained with 0.0002% (w/v) DAPI (Merck) in PBST for 10 minutes. The slides were then washed a minimum of 3 times for 15 minutes each in PBST and mounted with DAKO Fluorescence mounting medium (Agilent). Elastin2 antibody was purified from the previously described serum stock. The sections were imaged using a Zeiss LSM700 microscope, and the ZEN software (Black edition, Zeiss).

2.4 Transmission Electron Microscopy (TEM)

Adult hearts were dissected and fixed in 4% PFA with 2.5% glutaraldehyde in 0.05 M HEPES buffer (pH 7.2) for 2 hours at room temperature, and subsequently stored at 4°C. The samples were rinsed three times in 0.05 M HEPES buffer (pH 7.2) and post-fixed in 1% (w/v) OsO4 for 1 hour. After washing three times with distilled water, the blocks were stained with 2% uranyl acetate for 1 hour. The samples were dehydrated through a graded series of ethanol washes, transferred to propylene oxide, and embedded in Epon according to standard procedures. Tissue semi-thin sections (900 nm thick) were obtained in a Ultracut E microtome (Reichert-Jung, Leica) and stained with Richardson staining solution. Ultra-thin 70 nm sections were then collected on copper grids. After post-staining with uranyl acetate and lead citrate, the ultra-thin sections were examined with a JEM-1400 Plus transmission electron microscope (Jeol, Japan), operated at an accelerating voltage of 120 kV. Digital images were recorded with an EM-14800Ruby Digital CCD camera unit.

2.5 Body volume measurements

Total body volume was determined by submerging each animal in a 2 mL syringe filled with water and determining water displacement (1 mL water displacement corresponds to 1 cm³ body volume).

2.6 Doppler echocardiography

Zebrafish were anaesthetized in system water with 0.016% buffered Tricane. Zebrafish were placed in a supine position in a bed made of modelling clay, adjustable to the size of the zebrafish, and submerged in anaesthesia solution to ensure the propagation of the ultrasound signal. A Vevo2100 Imaging System (VisualSonics) and the VisualSonics Ultrasound Imaging Software (Version 1.6.0) were used for echocardiography. Since the required ultrasound frequency depends on the size of the sample, we used a high-frequency MicroScan transducer (MS700 v3.0) at a frequency of 40 MHz, as opposed to the 2–15 MHz used for...
Figure 1 alk5a−/− adult zebrafish display variable cardiac phenotypes without gross morphological defects. (A) Schematic of Alk5a WT and mutant proteins depicting each domain and the site of the mutation (star). (B, C) Brightfield images of 9 mpf WT (B) and alk5a−/− (C) zebrafish. (D, E) Brightfield images of alk5a−/− hearts (E) which occasionally exhibit a dilated outflow tract (OFT) lumen compared to WT (D). (F–I) Cryosections of WT (F, H) and alk5a−/− (G, I) hearts immunostained for Tg(kdrl:eGFP) expression (endothelial cells) and Elastin2 (F, G), and stained for haematoxylin-eosin (H, I) showing the expanded OFT lumen (Lu, dashed line) in alk5a−/− zebrafish. (J–L) Quantification of age (J), standard length (K), and body volume (L) of WT (n = 10) and alk5a−/− (n = 12) zebrafish used in the subsequent analyses. Plots show the values for each individual and the mean ± SD; P-values were determined by unpaired t-test (J, L) or Mann–Whitney test (K). The colour of each dot refers to the same zebrafish across all graphs. The dot adjacent to the number symbol (⋆) identifies the individual zebrafish mentioned in the text. Scale bars: 2 mm (B, C), 200 μm (D, E, H, I), 400 μm (F, G). A, atrium; EC, extracellular; GS, glycine–serine rich; SP, signal peptide; STK, serine–threonine kinase; TM, transmembrane; V, ventricle.
humans. The transducer was oriented along the longitudinal axis of the zebrafish and the images were acquired with the anterior side of the zebrafish to the left (Supplementary material online, Figure S2A). Blood flow imaging and quantification of the haemodynamic parameters were performed in Pulsed-wave (PW) Colour Doppler mode. For optimal quality, imaging was acquired with a field of view of 6.00 × 6.73 mm.

### Table 3 Description and abbreviation terms for each parameter

| Parameter                                      | Abbreviation | Description                                                                 |
|------------------------------------------------|--------------|-----------------------------------------------------------------------------|
| Standard length                                | Std. length  | Length from the anterior tip of the snout to the anterior part of the caudal fin |
| Body volume                                    | Body vol.    | Three-dimensional space occupied by the fish body                            |
| Outflow tract velocity time integral           | OFT VTI      | Area within the Doppler curve (integral). It indicates how far blood travels during the flow period and it is a measure of cardiac systolic function and cardiac output. |
| Outflow tract mean velocity                    | OFT mean velocity | Average of the instantaneous velocity from the outflow curve for each heartbeat, measured from all cardiac cycles across 10 s |
| Outflow tract mean gradient                    | OFT mean gradient | Average of the instantaneous pressure gradients over the entire outflow Doppler curve, measured from all cardiac cycles across 10 s |
| Outflow tract velocity peak velocity           | OFT peak velocity | Maximum velocity of the blood passing from the ventricle to the outflow tract, \( V_{\text{max}} \) |
| Outflow tract velocity peak gradient           | OFT peak gradient | Pressure gradient calculated from the peak velocity with the Bernoulli equation: \( 4(V_{\text{max}})^2 \) |
| Ejection time                                  | Ejection time | Period of blood flow from ventricle to the outflow tract                     |
| Heart rate                                     | Heart rate   | The speed at which the heart beats measured in beats per minute               |
| Area aortic flow                               | Area aortic flow | Amount of blood (in a 2D plane) detected in the aorta                        |
| Area inflow                                    | Area inflow  | Amount of blood (in a 2D plane) going from the atrium to the ventricle       |
| Area outflow                                   | Area outflow | Amount of blood (in a 2D plane) going from the ventricle to the outflow tract |
| Regurgitation fraction in the atroventricular canal | Regurg. fraction AVC | Relative amount of blood (in a 2D plane) that returns to the atrium during ventricular contraction (systole); inflow area retrograde flow area |
| Regurgitation fraction in the outflow tract    | Regurg. fraction OFT | Relative amount of blood (in a 2D plane) that returns to the ventricle after ventricular contraction (systole); outflow area retrograde flow area |
| Percentage ventricular luminal expansion       | % Vent. lum. exp. | Lumen displacement between minimum and maximum ventricular contraction in a cardiac cycle |
| Percentage ventricular outer wall expansion    | % Vent. outer wall exp. | Outer wall displacement between minimum and maximum ventricular contraction in a cardiac cycle |
| Percentage outflow tract luminal expansion     | % OFT lum. exp. | Lumen displacement between minimum and maximum outflow tract expansion in a cardiac cycle |
| Percentage outflow tract outer wall expansion  | % OFT outer wall exp. | Outer wall displacement between minimum and maximum outflow tract expansion in a cardiac cycle |
| Heart volume                                   | Heart vol.   | Three-dimensional space occupied by the heart—calculated by the sum of the volumes of each cardiac compartment |
| Ventricular volume                             | Vent. vol.   | Three-dimensional space occupied by the ventricle—including the lumen       |
| Percentage ventricular volume                  | % Vent. vol. | Relative volume of the ventricle in relation to the entire heart             |
| Atrial volume                                  | Atrial vol.  | Three-dimensional space occupied by the atrium—including the lumen           |
| Percentage atrial volume                       | % Atrial vol. | Relative volume of the atrium in relation to the entire heart                |
| Outflow tract volume                           | OFT vol.     | Three-dimensional space occupied by the outflow tract—including the lumen   |
| Percentage of outflow tract volume             | % OFT vol.   | Relative volume of the outflow tract in relation to the entire heart         |
| Outflow tract luminal volume                   | OFT lum. vol. | Three-dimensional space occupied by the outflow tract lumen                  |
| Percentage outflow tract luminal volume        | % OFT lum. vol. | Relative volume of the outflow tract lumen in relation to the entire outflow tract |
| Outflow tract wall volume                      | OFT wall vol. | Three-dimensional space occupied by the outflow tract wall; OFT volume—OFT lumen volume |
| Aortic diameter                                | Aortic diam. | Maximum diameter of the aorta measured in the most proximal linear portion of the aorta |
| Aortic volume                                  | Aortic vol.  | Three-dimensional space occupied by the aorta anteriorly to the heart         |
Doppler gain of 34 dB and 2D Gain of 48 dB, half line density and low persistence. Videos were recorded for 10 seconds and across different 2D planes, spanning the atrium, ventricle and OFT. At least two videos per area were acquired and used to determine an average of all measurements and increase reproducibility. Image acquisition was completed within 5 minutes of sedation to avoid cardiac function aberrations. After imaging, the zebrafish were returned to system water and observed until recovery. All zebrafish recovered after imaging.

To minimize variability between individuals, all conditions including anaesthesia concentration and temperature were maintained constant throughout the measurements. Image acquisition was completed using the Vevo Lab™ software package v.1.7.0 (VisualSonics) and refer to the curves highlighted in Supplementary material online, Figure S2C,C’.

### Table 4 Biological features

| Parameters               | Wild type (WT) | alk5a−/− |
|--------------------------|----------------|----------|
|                         | Median | Minimum | Maximum | Inter-quartile range (IQR) | Median | Minimum | Maximum | Inter-quartile range (IQR) | P-value |
| Age (months)             | 8.5    | 6.0     | 14.0    | 6.0–13.3                     | 12     | 6.0     | 14.0    | 6.0–14.0                     | 0.3766  |
| Std. length (mm)         | 3.2    | 2.9     | 3.5     | 3.1–3.3                      | 3.3    | 2.6     | 3.5     | 3.2–3.3                      | 0.6054  |
| Body vol. (mm³)          | 0.68   | 0.45    | 0.75    | 0.56–0.74                    | 0.65   | 0.40    | 1.10    | 0.56–0.83                     | 0.0578  |

### Table 5 Echocardiography parameters

| Parameters               | Wild type (WT) | alk5a−/− |
|--------------------------|----------------|----------|
|                         | Median | Minimum | Maximum | Inter-quartile range (IQR) | Median | Minimum | Maximum | Inter-quartile range (IQR) | P-value |
| OFT VTI (mm)             | 12.3   | 6.9     | 28.5    | 11.2–19.4                    | 11.8   | 1.8     | 20.3    | 9.2–16.4                     | 0.2997  |
| OFT mean velocity (mm/s) | 77.4   | 44.7    | 132.0   | 64.4–94.9                    | 75.2   | 17.6    | 123.5   | 59.6–95.3                     | 0.5757  |
| OFT mean gradient (mm Hg)| 0.024  | 0.008   | 0.070   | 0.017–0.036                  | 0.022  | 0.001   | 0.062   | 0.014–0.037                   | 0.5903  |
| OFT peak velocity (mm/s) | 110.4  | 59.7    | 186.1   | 86.6–128.9                   | 98.9   | 25.6    | 171.8   | 82.4–129.4                    | 0.5278  |
| OFT peak gradient (mm Hg)| 0.050  | 0.014   | 0.139   | 0.030–0.067                  | 0.040  | 0.003   | 0.119   | 0.027–0.066                   | 0.5229  |
| Ejection time (s)        | 0.182  | 0.136   | 0.246   | 0.159–0.200                  | 0.157  | 0.103   | 0.237   | 0.137–0.181                   | 0.1947  |
| Heart rate (bpm)         | 107.8  | 58.4    | 131.5   | 87.4–118.9                   | 98.4   | 61.0    | 143.5   | 84.8–112.3                    | 0.8138  |
| Area aortic flow (×10³ mm²) | 0    | 0       | 0.67    | 0                             | 0      | 0       | 3.79    | 0–0.161                      | 0.1729  |
| Area inflow (×10³ AU)    | 0.81   | 0.45    | 1.24    | 0.67–0.97                    | 0.52   | 0.04    | 3.69    | 0.38–0.82                     | 0.1583  |
| Area outflow (×10³ AU)   | 6.40   | 3.27    | 11.75   | 5.22–6.89                    | 4.12   | 0.56    | 10.09   | 3.47–5.90                     | 0.1447  |
| Regurg. fraction AVC (%) | 0      | 0       | 11.2    | 0–1.2                        | 2.6    | 0       | 84.0    | 0–27.0                        | 0.1732  |
| Regurg. fraction OFT (%) | 0      | 0       | 24.4    | 0                             | 6.0    | 0       | 55.1    | 0–16.0                        | 0.0847  |

#### 2.6.1 Sequential echocardiography measurements and isoprenaline treatments

In order to follow the progression of the phenotype over time, WT and alk5a−/− zebrafish were analysed with pulsed-wave colour Doppler echocardiography (as described above), at time 1 (t1), 3 weeks after t1 (t2), and 2 months after t1 (t3). Prior to each echocardiography session, two groups of zebrafish (7 WT and 7 alk5a−/−) were treated with isoprenaline (+ISO, Sigma, Cat. # I5627), a β-adrenergic agonist analogue of adrenaline, in order to increase heart rate and potentially aggravate the cardiovascular phenotype.69–72 Isoprenaline powder was dissolved in water to create a stock solution (10 mM) and kept in the dark until use (within 6 hours). Zebrafish were immersed in system water containing 5 μM isoprenaline (diluted from stock) for 30 minutes prior to echocardiography. The zebrafish were subsequently returned to system water without isoprenaline to recover from the anaesthesia and kept in standard conditions until follow up.

#### 2.7 Magnetic Resonance Imaging (MRI)

Cardiac in vivo MRI measurements were performed on a 7.0 T Bruker PharmaScan (Bruker Biospin, Ettlingen, Germany) small animal MRI system. The instrument was equipped with a 780 mT/m gradient system, a cryogenically cooled 4 channel phased array element 1H receiver-coil (CryoProbe™, Bruker Biospin) and a 72 mm room temperature volume coil.
Figure 2. alk5α<sup>−/−</sup> adult zebrafish display variable haemodynamic defects. (A–D) Parameter quantification obtained with echocardiography analyses of WT (n = 10) and alk5α<sup>−/−</sup> (n = 12) adult zebrafish, including heart rate (A), area of the aortic flow (B), and regurgitation fraction in the AV (C) and OFT (D) canals. Plots show the values for each individual and the mean ± SD; P-values were determined by unpaired t-test (A) or Mann-Whitney test (B–D). (E, E') WT zebrafish exhibit unidirectional blood inflow (red, E) and outflow (blue, E') without signs of regurgitation. (F–G') Examples of alk5α<sup>−/−</sup> zebrafish exhibiting a detectable aortic flow (F) and retrograde blood flow (F'–G'). The colour of each dot refers to the same zebrafish across all graphs and images. The dot adjacent to the number symbol (#) identifies the individual zebrafish mentioned in the text. Scale bars: 1 mm (E–G).
Zebrafish were anaesthetized in water with 0.016% buffered Tricaine (Sigma) and placed in a supine position in a bed made of modelling clay. Due to reduced imaging quality using a container with water flow, we decided to analyse our specimens without water flow and reduce the imaging time to less than 20 minutes to ensure animal survival.

Localizer images were acquired using a spin-echo sequence and correction of slice orientation was performed when necessary. In addition, RARE (Rapid Acquisition with Relaxation Enhancement) sequences (TR = 2500 ms, TE = 36.7 ms, slice thickness = 0.3 mm) in a sagittal orientation were used to determine the correct coronal plane for imaging of the ventricle or OFT (Supplementary material online, Figure S3 A–B). The imaging was performed with the following parameters: IntraGate 2D FLASH: TE/TR: 4.2/8.2 ms, flip angle 10°, slice thickness: 0.3 mm, in-plane resolution: 59 × 59 or 47 × 47 μm². Navigator based self-gating was used to determine the cardiac rate and to perform cine retrospective reconstruction. Similar to the values observed with echocardiography, heart rates used for reconstruction had a median of 122 and 125 bpm, and minimum and maximum values of 62–172 and 72–200 bpm for the WT and alk5a⁺⁻ zebrafish, respectively. With the help of the profile images and the navigator signal, we were also able to detect and exclude sudden movements of the anaesthetized zebrafish as well as shifts in resting position (Supplementary material online, Figure S3 C–C†).

The evaluation of the functional heart data of adult zebrafish, following MRI, was performed with the Qmass MR 8.1 (Medis Medical Imaging Systems) software. The percentage of OFT and ventricular expansion was calculated by the software after manually defining the lumen (or wall) outline during maximum and minimum chamber dilation. The OFT and ventricular measurements were performed in different recordings with different orientations selected to show the cross-section of the desired chamber. All the values represent the average of two independent analyses of the same data performed by two different scientists. Inter-observer variability in parameter quantification is presented in Tables 1 and 2. Despite the variability between inter-observer measurements, both observers obtained similar sample mean values.
2.8 Micro-CT

After the MRI recordings, the zebrafish were euthanized with an overdose of anaesthetic in water (0.16% Tricaine in system water, buffered to pH 7.0–7.5), fixed in ice-cold 4% PFA and kept in PFA over-night at 4°C. As previously described, a solution of 2.5% phosphomolybdic acid (PMA) prepared in demineralized water was used to stain each zebrafish individually for a period of 6 days. Samples were then gradually transferred to a 70% ethanol solution where they were preserved at room temperature until imaging.

All samples were scanned using a μ-CT scanner model Skyscan 1276 (Bruker). X-ray parameters for the imaging were: Source voltage = 55 kV; source current = 72 μA; image pixel size = 2.965 μm; filter = Al 0.25 mm; rotation step = 0.25° to 180°. For image reconstruction, we used the NRecon (version 1.7.1.0) and InstaRecon (version 2.0.3.7) soft-wares. Resulting reconstructed images were 3856 × 2248 pixels, with a pixel size of 2.965 μm.

All the morphological measurements were performed with the Imaris (Bitplane) software. The atrium, ventricle, OFT, and aorta were manually segmented across the entire 3D volume. The lumen of the OFT was also separately segmented. The volumes of each compartment were calculated with the ‘Surface tool’ in Imaris and, when required, normalized to the heart volume (Supplementary material online, Figure S4C–E) or total OFT volume (Figure 4H). The OFT wall volume was calculated by subtracting the OFT luminal volume from the total OFT volume. The diameter of the aorta was measured in the most proximal third of the aorta.

2.9 Electrocardiography (ECG)

Adult zebrafish were anaesthetized in 0.016% of Tricaine for 5 min, and placed dorsal side up in a wet sponge. Anaesthesia was maintained during the recording through oral perfusion with zebrafish tank water containing 0.016% Tricaine using a roller pump (ISM827B, ISMATEC Germany) at a 2 mL/min speed. Two custom-made stainless-steel electrodes were used to record the ECG. One electrode was placed above the cardiac region using micromanipulators (Marzhauser MM33, Marzhauser, Germany). The second electrode was placed as a reference electrode in the sponge submerged in recording solution. The electrodes were connected to a differential AC amplifier (A-M Systems, WA, USA) with the following filter settings: Low cut-off filter = 10 Hz; high cut-off filter = 1 KHz; Gain = × 100. Signals were digitized in a PowerLab 4/30 (AD instruments, USA) and recorded at 10 k/s in LabChart 7.0 (AD instruments, USA) using a digital low pass filter (cut-off frequency = 50 Hz; active input amplifier = 5 V range; low pass filter = 200 Hz; Mains filter = active). The zebrafish were recorded for minimum of 5 minutes, and the ECG was analysed in 30 consecutive beats using LabChart 8 (ADInstruments, version 8).

2.10 Data analyses and statistics

All statistical analyses were performed in GraphPad Prism (Version 6.07) and illustrations were done in Inkscape (XQuartz X11).

For comparison of WT vs \(alk5α^{−/−}\) samples, a Gaussian distribution was tested for every sample group using the D’Agostino-Pearson omnibus normality test. When both samples passed the normality test, \(P\)-
Figure 4 μ-CT analyses of cardiovascular morphology in alk5a−/− adult zebrafish reveal OFT defects. (A, B) μ-CT scans showing a sagittal plane of the anterior region of WT and alk5a−/− adult zebrafish. Boxed area is shown in A’ and B’. Dashed lines outline cardiac compartments. (C–D’’’) 3D reconstructions of the cardiac compartments in WT (C–C’’) and alk5a−/− (D–D’’) zebrafish, including the aorta (C’, D’) and OFT lumen (Lu; C’’, D’’’). (E–J) Quantification of morphological parameters for each cardiac compartment in WT (n = 10) and alk5a−/− (n = 12) zebrafish. Plots show the values for each individual and the mean ± SD; P-values were determined by unpaired t-test (G–J) or Mann–Whitney test (E, F). The colour of each dot refers to the same zebrafish across all graphs and images. The dot adjacent to the number symbol (#) identifies the individual zebrafish mentioned in the text. A, atrium; Ao, aorta; OFT, outflow tract; V, ventricle. Scale bars: 1 mm (A, B), 500 μm (A’, B’), 300 μm (C, D’), 400 μm (C’’–D’’’).

Integrated cardiovascular imaging in adult zebrafish
3. Results

3.1 Selecting a mutant to test the sensitivity of the imaging platform

In order to test the power of multiple imaging techniques to detect subtle adult phenotypes, we first needed to select a suitable model. Many homozygous mutant zebrafish exhibiting cardiovascular defects at early developmental stages have been reported,76–79 and some animals heterozygous for mutations display subtle defects in adult stages.25,80 Other zebrafish mutants do not display embryonic phenotypes but exhibit pericardial oedema at adult stages (Figure 1B,C),81 indicating cardiac function defects. We elected to investigate alk5a mutants (Figure 1A), a third type of mutant, i.e. one that does not exhibit embryonic phenotypes or pericardial oedema at adult stages but can potentially develop cardiac phenotypes. alk5a; alk5b double mutants exhibit severe pericardial oedema at early developmental stages, due to a defective outflow tract (OFT).64 In contrast, alk5a single mutant larvae do not exhibit any obvious defects (Supplementary material online, Figure S1A,B), including in the cardiovascular system (Supplementary material online, Figure S1 C–D), and they survive to adulthood. Similarly, alk5a–/– zebrafish at 9 months post-fertilization (mpf) display no gross morphological defects or pericardial oedema (Figure 1B,C). However, analysis of dissected hearts including histological sections suggested that 3/6 alk5a–/– adult zebrafish have an expanded OFT lumen when compared with wild type (WT; Figure 1D–I). The amount of Elastin2, a major component of the ECM in the cardiac OFT, appears to be slightly reduced in the alk5a–/– OFT wall (Figure 1F and G). However, as histological sections do not yield a 3D representation of morphological features, and the size of the OFT lumen depends on the plane of the section, it was not possible to conclude whether the OFT lumen was indeed dilated in alk5a–/– zebrafish. Moreover, due to the opacity of adult zebrafish, one cannot use live microscopy to investigate the functional consequences of morphological defects in the cardiovascular system. Therefore, we aimed to adapt and optimize cardiovascular preclinical imaging techniques to collect multiple cardiac parameters (Table 3) and analyse adult zebrafish phenotypes. We randomly selected 10 WT and 12 alk5a–/– zebrafish, ranging from 6 to 15 mpf (Figure 1J, Table 4), and tracked each individual throughout all the analyses. To account for any body size differences between the two groups, we measured standard length (Figure 1K) and body volume (Figure 1L), and observed no significant differences between WT and mutant groups.

3.2 Echocardiography reveals variable haemodynamic defects in alk5a–/– adult zebrafish

Echocardiography, the fastest (approximately 5 minutes per animal) and least invasive imaging technique, records haemodynamic parameters in live animals.82–87 We analysed cardiac function from sagittal views of the heart (Supplementary material online, Figure S2A), and through Doppler echocardiography were able to assess a range of features including the direction, amount, and velocity of blood flow (Figure 2A–D and Supplementary material online, Figure S2B–K, Table 5). Due to the intrinsic variability between individuals, the heart rate ranged from 58 to 143 beats per minute (bpm), with no statistically significant difference between the two genotypes (Figure 2A). Likewise, previous studies have reported heart rate values for WT adult zebrafish ranging from 68 to over 175 bpm.48,51,53–55 Regarding haemodynamics, as previously described by our group,11,86 we also aimed to assess the presence of blood regurgitation. Therefore, we measured the area of blood inflow and outflow on a two-dimensional (2D) plane and noticed that while most zebrafish exhibited only unidirectional inflow (red; 7/10 in WT and 6/12 in alk5a–/–; Figure 2C and E, Supplementary material online, Video S1) and outflow (blue; 8/10 in WT and 5/12 in alk5a–/–; Figure 2D and E, Supplementary material online, Video S1), some alk5a–/– zebrafish (7/12) also presented blood flow regurgitation in the atrioventricular canal (AVC; Figure 2C, F,G, Supplementary material online, Videos S2 and S3) and/or in the OFT canal (Figure 2D,C, Supplementary material online, Video S3). In addition, while most zebrafish exhibited a flow signal exclusively in the heart, a few displayed a signal rostral to the OFT, in the region of the ventral aorta, hereafter referred to as aortic flow (Supplementary material online, Video S2). The presence of aortic flow was detected more frequently in alk5a–/– zebrafish (1/10 in WT and 4/12 in alk5a–/–; Figure 2B,F), suggesting an increase in blood flow in their aorta. Nonetheless, the relative inflow and outflow areas in a 2D plane, as well as most haemodynamic parameters including blood flow velocity and ejection time, were highly variable and not significantly different between WT and alk5a–/– zebrafish (Supplementary material online, Figure S2D–K). In contrast, by determining several parameters in each animal, we identified one alk5a–/– zebrafish (indicated by # in all plots) with a large inflow area (Supplementary material online, Figure S2J) that also displayed severe blood regurgitation in its AVC and OFT (Figure 2CD), as well as reduced blood flow velocity through its OFT (Supplementary material online, Figure S2D, E, G).

In summary, despite the great variability obtained with echocardiography, this technique provides important information on cardiac function, particularly regarding haemodynamics in the heart and adjacent vessels.

3.3 In vivo MRI analyses of beating hearts reveal the expansion of most alk5a–/– outflow tracts

To achieve higher resolution of cardiac morphology and performance, we used in vivo MRI to study the same 22 zebrafish. MRI is the gold standard technique for clinical analyses of cardiac morphology and function due to its precision and high reproducibility.57–95 Although MRI has been increasingly used in small animal models including mice,95–98 in zebrafish it has been mostly restricted to ex vivo samples or static images of the regenerating heart.32,33,43,44,96 without presenting videos of beating hearts. In rodent models, MRI permits the reconstruction of beating hearts,73,97 but this type of analysis is challenging in adult zebrafish due to the small size of their heart. Hence, we developed a protocol for adult...
Correlation analysis of all the measured parameters reveals previously undetected cardiovascular phenotypes. (A, B) Correlograms of all the parameters analysed including biological features, as well as echocardiography, MRI and μ-CT analyses across all the WT (A; \(n=10\)) and \(\text{alk}5\alpha^{-/}\) (B; \(n=12\)) zebrafish. Positive and negative correlations are shown in blue and red, respectively; dot size represents Pearson’s correlation coefficient. (C, D) Significant correlations (\(P<0.05\)) in WT (C) and \(\text{alk}5\alpha^{-/}\) (D) zebrafish.

Figure 5 Correlation analysis of all the measured parameters reveals previously undetected cardiovascular phenotypes. (A, B) Correlograms of all the parameters analysed including biological features, as well as echocardiography, MRI and μ-CT analyses across all the WT (A; \(n=10\)) and \(\text{alk}5\alpha^{-/}\) (B; \(n=12\)) zebrafish. Positive and negative correlations are shown in blue and red, respectively; dot size represents Pearson’s correlation coefficient. (C, D) Significant correlations (\(P<0.05\)) in WT (C) and \(\text{alk}5\alpha^{-/}\) (D) zebrafish.
Figure 6 Combined imaging analyses facilitate the selection of specific phenotypes for high resolution morphological characterization. (A–C) Regurgitation fraction as obtained by echocardiography (A, B) and OFT luminal expansion as obtained by MRI (C) for two zebrafish (orange and purple, n = 2) plotted against the previously measured zebrafish (WT, black, n = 10; alk5a−/− grey, n = 12). Plots show the values for each individual and the mean ± SD; P-values were determined by Mann–Whitney test. Purple dot identifies the zebrafish selected for subsequent analysis with transmission electron microscopy (TEM). (D–E’) alk5a−/− zebrafish showing mild (orange, D, D’) and severe (purple, E, E’) regurgitation fraction in the AVC and OFT regions. (F, G) Semi-thin sections of WT and alk5a−/− OFTs stained with Richardson staining solution. Dashed line outlines the OFT lumen (Lu). (H–I”) TEM images of WT and alk5a−/− OFTs, showing the OFT lumen in blue, lined by ECs in green (H, I) and the OFT wall (H’, H”, I’, I”), including SMCs (red) and ECM (asterisk). Scale bars: 1 mm (D–E’), 200 μm (F, G), 5 μm (H), 10 μm (H’–I”).
zebrafish MRI to assess cardiac function in live animals more accurately (see Materials and Methods).

We imaged the heart in two different planes to obtain coronal views of the OFT and ventricle and observe the expansion of these compartments during the cardiac cycle (Supplementary material online, Figure S3 A–B). Additionally, using self-gating signals,33,97 we were able to monitor the cardiac rate of the animal (Supplementary material online, Figure S3 C–C′) to accurately reconstruct the cardiac cycle. Then, we calculated the percentage of the cardiac chambers’ expansion, measuring both the outer wall and the lumen (Figure 3A–D′′ and Supplementary material online, Figure S3 D–G′, Table 6). We found that the expansion of the ventricular outer wall and lumen during the cardiac cycle was comparable between WT and alk5a−/− zebrafish (Supplementary material online, Figure S3D,E, Supplementary material online, Videos S4 and S5). In contrast, we observed that alk5a mutants displayed a severe and consistent increase in OFT luminal dilation (Figure 3B, C–D′′, Supplementary material online, Videos S6 and S7), whereas the OFT outer wall expansion was comparable with that of WT (Figure 3A C–D′′). Also, when analysing the results for the alk5a mutant with severe haemodynamic defects detected with echocardiography (#), we observed that it had one of the highest OFT luminal expansion levels (Figure 3B).

In summary, by optimizing MRI for beating adult zebrafish hearts, we were able to obtain unique and valuable data on cardiac performance in vivo. Moreover, we identified the OFT as the main cardiovascular compartment affected in alk5a−/− zebrafish.

### 3.4 Micro-CT analyses reveal outflow tract defects in alk5a−/− adult zebrafish

X-ray-based ex vivo CT is considered the most powerful technique to provide high spatial resolution of small structures.98–101 Here we use µ-CT, which is capable of volumetric analysis with a voxel size <20 μm,102–104 for a novel characterization of the 3D morphology of the adult zebrafish cardiac compartments and adjacent vessels. Due to the exposure to radiation, the requirement for a contrast agent, and for long imaging periods to acquire high-resolution images, we performed this imaging on fixed samples. Individual X-ray projections were used to reconstruct cross-section images, providing views of the tissues in multiple planes (Supplementary material online, Figure S4A–A′).

After manual segmentation and volumetric surface rendering (Figure 4A–D′′, Supplementary material online, Videos S8–S11), we could determine the volume of each cardiac compartment, as well as the diameter and volume of the aorta (Figure 4E–J and Supplementary material online, Figure S4 B–G, Table 7, Supplementary material online, Videos S12 and S13). Consistent with the measurements obtained with the other techniques, we did not observe any statistically significant differences between WT and alk5a−/− zebrafish regarding the volume of the entire heart (Figure 4E), atrium (Figure 4F, Supplementary material online, Figure S4D), or ventricle (Supplementary material online, Figure S4B and C). Moreover, while the total volume of the OFT appeared to be lower in alk5a−/− zebrafish, this difference was not statistically significant (Figure 4G and Supplementary material online, Figure S4E). However, alk5a−/− zebrafish displayed a statistically significant increase in OFT luminal volume (relative to OFT volume: Figure 4H), and a decrease in OFT wall volume (Figure 4I). Despite the observation of aortic flow in alk5a−/− zebrafish suggesting higher aortic volumes, alk5a−/− zebrafish exhibited only a slight increase in aortic diameter compared with WT (Figure 4C′′,D′′,J, Supplementary material online, Videos S14 and S15), while displaying an unaltered volume (Supplementary material online, Figure S4G).

It is important to note that the µ-CT analyses provided sufficient resolution to visualize smaller structures such as the ventricular trabeculae (Figure 4A–B and Supplementary material online, Figure S4A′) and the atrioventricular valve (Figure 4B′). Moreover, by tracking

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**Table 8 Regurgitation fraction values obtained in the sequential echocardiography measurements (related to Figure 7)**

|                | Zebrafish | WT (%) | alk5a−/− (%) |
|----------------|-----------|--------|--------------|
|                |          | t1     | t2 | t3 | t1 | t2 | t3 | t1 | t2 | t3 | t1 | t2 | t3 | t1 | t2 | t3 | t1 | t2 | t3 |
| Regurgitation fraction OFT |          | 1      | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 1.9 | 11.6 |
|                |          | 2      | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 7.9 | 51.9 |
|                |          | 3      | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
|                |          | 4      | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 90.7 | 100.0 |
|                |          | 5      | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0.0 | 23.9 |
|                |          | 6      | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0.0 | 44.9 |
|                |          | 7      | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0.0 | 2.2  |
| Regurgitation fraction AVC |          | 1      | 1.2 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 3.3 | 0  | 0.0  | 0.1 |
|                |          | 2      | 1.4 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 2.0 | 0  | 0.8  | 1.4 |
|                |          | 3      | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0.0  | 56.7 |
|                |          | 4      | 15.3 | 0  | 0  | 12.4 | 0  | 0  | 12.5  | 100.0 | 100.0 | 100.0 | 0.0  | 39.4 | 40.7 |
|                |          | 5      | 0  | 0  | 0  | 0  | 45.2 | 7.2  | 1.5  | 0.0  | 0.0  | 0.0  | 0.0  | 0.0  | 56.7  | 39.2 |
|                |          | 6      | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 2.1  | 30.7  | 4.1  | 55.4  | 0.0  | 0.0  | 76.3 |
|                |          | 7      | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0.0  | 6.9  | 0.0  | 0.0  | 3.0  | 0.5  |

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individual zebrafish with the different techniques, we observed that the zebrafish displaying the most severe functional phenotype as assessed by echocardiography and MRI (#) also presented severe morphological defects when analysed with μ-CT. In particular, this individual exhibited the largest OFT luminal volume (Figure 4H), one of the largest aortic diameters (Figure 4J), but the smallest OFT wall volume (Figure 4I). This zebrafish also displayed an abnormally large atrial volume (Figure 4F), in line with the high regurgitation fraction in the AVC identified by echocardiography.

Thus, with the help of μ-CT imaging, we were able to obtain precise structural information about all the cardiac compartments and further resolve the OFT defects identified using the other platforms.

Table 9 Summary of the cardiovascular defects identifiable with each imaging technique

| Imaging Technique | Extracted data | Strengths | Weaknesses | Phenotypes |
|------------------|----------------|-----------|------------|------------|
| Histological sections | Tissue morphology and cellular organization | High resolution, Identification of different cell types, Marker analyses (e.g. immunostaining), Identification of tissue alterations | Static images, 2D assessment, Need to sacrifice the animal, Loss of anatomical information due to tissue extraction | Alterations at the cellular level (e.g. cell specification, cell death, cell proliferation) |
| Echocardiography | Haemodynamic parameters (blood velocity and direction), 2D assessment of ventricular size, Heart rate | In vivo data recording, Monitoring the progression of phenotypes | Low tissue resolution, 2D assessment | Altered blood flow due to impaired cardiac pumping capacity, Cardiac valve malfunction leading to blood regurgitation, Abnormal blood flow in the great vessels, Severe alterations of the ventricular size—assessment in 2D, Severe arrhythmia |
| In vivo MRI | Contraction/expansion of cardiac compartments, 2D assessment of ventricular size, Heart rate | In vivo data recording, Sufficient resolution to assess cardiac chambers expansion, Monitoring the progression of phenotypes | Low tissue resolution, 2D assessment, Heart rate based on indirect measurement, Slow imaging acquisition, Complex and expensive set up | Altered contraction/expansion of cardiac compartments, Gross morphological alterations of the cardiac compartments—assessment in 2D, Severe arrhythmia |
| Ex vivo μ-CT | Volume of the cardiac compartments, Spatial organization of the heart, Gross morphological features of the heart, connecting vessels and cardiac valves | Preserves heart integrity—no need for organ extraction, High resolution at the tissue level, Provides spatial information of the heart and surrounding tissues (3D), Data on morphology of connecting vessels | Static images, Ex vivo analyses—need for animal sacrifice, Time-consuming analysis, Expensive | Altered volumes of cardiac compartments (static) — assessment in 2D and/or 3D, Morphological alterations of the great vessels, Gross morphological alterations of the myocardium and cardiac valves, Abnormal spatial position/or- ganization of the cardiac compartments and accessory vessels |
| ECG | Electrophysiology of the heart, Heart rate, Heart rhythm | Monitoring the progression of phenotypes | No tissue observation | Altered cardiac conduction, Arrhythmia |
3.5 Correlation analysis between all parameters reveals previously undetected phenotypes in alk5a–/– adult zebrafish

From the 32 parameters measured with the different imaging platforms, only three showed a P < 0.05 between WT and alk5a–/– samples after bulk analysis. The lack of statistical significance in group comparisons is due to the intrinsic variability between individuals of the same genotype, a factor which needs to be considered when analysing heterogeneous samples. However, when one follows a single individual (e.g. #), it becomes clear that a higher severity in functional phenotypes is accompanied by stronger morphological alterations. Therefore, we analysed how variables were linked by determining the pairwise Pearson’s correlation coefficients across all the WT zebrafish and across all the alk5a–/– zebrafish, herein represented as correlograms (WT, Figure 5A, and alk5a–/–, Figure 5B).

As expected, we observed strong correlations between measurements obtained within the same platform, but also several interesting inter-platform correlations. As seen in humans where cardiovascular parameters strongly correlate with gender, age and ethnicity,38,92,105–107 we noticed within the WT group a positive correlation between age and the volume of the OFT relative to the entire heart (% OFT volume; Figure 5C). Furthermore, retrograde OFT flow, which was present in two WT zebrafish, correlated with atrial enlargement.

In addition to the 11 correlations observed in the WT zebrafish, we identified 37 new correlations in the alk5a mutants (Figure 5B and D), suggesting a disruption of the morphology and/or function of the mutant heart. For example, we were able to address whether defects in OFT morphology affect cardiac performance and haemodynamics and vice versa. Indeed, we identified a negative correlation between OFT wall volume and heart rate, as well as between OFT luminal volume relative to the OFT (% OFT luminal volume) and OFT velocity time integral (VTI), suggesting that OFT morphology is linked with cardiac performance and blood velocity. We also observed a negative correlation between OFT volume and OFT wall volume with the regurgitation fraction in the AVC, suggesting that a more pronounced AVC retrograde flow is linked with defective (smaller and thinner) OFTs. Furthermore, there was a positive correlation between % OFT luminal volume and regurgitation fraction in the OFT, suggesting that larger OFT lumens are associated with higher retrograde flow in the OFT. When analysing whether these morphological OFT defects were influenced by age or body size, we found that OFT total and wall volumes were negatively correlated with standard length and body volume, suggesting an aggravation of these phenotypes in older and bigger zebrafish.

Regarding other cardiac compartments, the extent of retrograde flow across the AVC was associated with a smaller ventricle and bigger atrium, consistent with the consequences of valve regurgitation which leads to a higher amount of blood stalling in the atrium.108 Interestingly, alk5a–/– zebrafish—but not WT—with a higher regurgitation fraction in the OFT also displayed higher regurgitation fraction in the AVC, suggesting that these defects are closely associated, at least in the alk5a mutants.

These correlation analyses helped us to identify links between morphological and functional phenotypes, even when statistical tests comparing WT and mutant groups failed to provide significance due to the high variability of the defects.

Additionally, in order to better understand the cardiovascular phenotypes, we tested whether alk5a–/– adult zebrafish displayed electrophysiological defects using electrocardiography (ECG) analysis (Supplementary material online, Figure S5). Because we lacked the required expertise in house, these studies were performed in a different site and with a different set of zebrafish. We were able to identify arrhythmia in four of the six alk5a–/– zebrafish analysed while no alk5a+/+ siblings exhibited any obvious defects (Supplementary material online, Figure S5A–B). There were no statistically significant differences in P-wave duration, PR interval (representing atrial depolarization), or QRS interval (representing ventricular depolarization and contraction) between alk5a+/+ and alk5a–/– zebrafish (Supplementary material online, Figure S5A–E). However, alk5a mutants exhibited higher variability in P-wave duration and PR interval in the same individual (Supplementary material online, Figure S5A”,B”) and between individuals (Supplementary material online, Figure S5CD), suggesting alterations of the conduction system and setting the ground for future studies.

3.6 Combined imaging analyses facilitate the selection of specific functional phenotypes for downstream morphological characterization

Having observed that alk5a–/– zebrafish frequently display retrograde blood flow and increased expansion of their OFT lumen, we analysed additional animals using echocardiography and MRI to perform further analyses (Figure 6A–E). We chose an alk5a–/– zebrafish displaying severe retrograde blood flow through its AV canal (Figure 6A,D,E), OFT blood regurgitation (Figure 6B,D,E), as well as an unusually large expansion of its OFT lumen (Figure 6C). To analyse the morphological and ultrastructural defects in this animal, we performed light microscopy (Figure 6F,G) and transmission electron microscopy (TEM; Figure 6H–I’) on tissue sections. Semi-thin sections for light microscopy showed a dilated lumen and thinner wall in the OFT (Figure 6F,G). TEM analysis revealed that the lumen of the WT OFT was barely detectable and lined with flattened or cuboidal endothelial cells (ECs; Figure 6H). We also observed that the WT OFT wall was composed of both spindle-shaped and cuboidal smooth muscle cells (SMCs) surrounded by a dense ECM (Figure 6H’). In contrast, the mutant OFT displayed an enlarged lumen lined by rounded ECs (Figure 6I). Notably, many cells lining the lumen in alk5a–/– OFT appeared empty or detached, suggesting cell death.

Despite a thinner wall and a sparser and less dense ECM in the alk5a–/– OFT, there were no obvious alterations in the SMCs (Figure 6I”).

Overall, these data indicate that non-invasive imaging techniques such as echocardiography and in vivo MRI represent useful platforms to identify individuals displaying functional phenotypes (Table 9) for further downstream high-resolution analyses.

3.7 Characterizing phenotypic progression using sequential in vivo measurements

In order to determine whether we could analyse the progression of the alk5a–/– phenotype in the same zebrafish, we decided to use echocardiography, as the least invasive technique, to follow the same zebrafish in three measurements at 0 (t1), 3 (t2), and 8 (t3) weeks (Figure 7; Table 8).

Interestingly, we observed that some zebrafish with OFT retrograde flow consistently presented this defect at all three time-points, while others never did, further illustrating the robustness of the method.

Additionally, we treated both WT and alk5a–/– zebrafish with isoprenaline to increase the heart rate,69–72 in order to determine whether we could aggravate the phenotype, or accelerate its progression in alk5a–/– zebrafish.
zebrafish (Figure 7A). We observed that in WT zebrafish the regurgitation fraction in the OFT and AVC was very low, or none, at all time-points, and even after isoprenaline treatment; however, in some alk5a⁻/⁻ zebrafish, we could observe an increase in the regurgitation fraction from t1 to t3 (Figure 7B–F; Table 8). Most of the alk5a⁻/⁻ zebrafish treated with isoprenaline (5/7) exhibited no detectable functional phenotype at the beginning of the experiment (t1, Figure 7B,D–F), but several of them (3/5) progressively started to display blood regurgitation in the OFT at t2 and t3 (Figure 7E,F). Notably, at t3, only 2/6 of the untreated alk5a⁻/⁻ zebrafish exhibited OFT regurgitation, while 5/7 of the isoprenaline-treated alk5a⁻/⁻ mutants did (Table 8).

Interestingly, when analysing histological sections of the alk5a⁻/⁻ zebrafish with the most severe increase in OFT blood regurgitation during the isoprenaline experiment (zebrafish 2, Figure 7G–I), we noticed...
that only some of the sections showed a dilated OFT lumen, highlighting the importance of a comprehensive analysis.

4. Discussion

In this study, we developed new protocols and combined multiple cardiovascular imaging modalities including in vivo echocardiography and MRI along with ex vivo μ-CT to analyse the adult zebrafish heart. For each animal, we measured 32 different cardiovascular parameters in WT and alk5a–/– samples. Bulk analyses of alk5a–/– vs WT, as well as intra-individual correlations between these parameters led to the identification of a primary OFT phenotype.

4.1 Optimization of preclinical cardiovascular imaging analysis in zebrafish

While during development it is possible to use microscopy for single-cell resolution imaging of the zebrafish internal organs, as the animals grow, the tissues become opaque forcing researchers to rely on tissue sections. Previous publications have reported the optimization of preclinical imaging techniques such as echocardiography, MRI, and μ-CT for adult zebrafish. However, these studies have not (i) characterized haemodynamics such as blood regurgitation using echocardiography; (ii) shown functional MR images of reconstructed beating hearts in living animals; (iii) quantified the volumes of the cardiac compartments using μ-CT; and (iv) analysed the integrated data obtained from different techniques.

Here, taking advantage of the echocardiography Colour Doppler Mode, we were able to gather data on the haemodynamics within the heart and aorta of adult zebrafish. We observed blood flow perturbation in some alk5a–/– zebrafish including blood regurgitation in the AV and OFT canals, and increased aortic flow.

With our new in vivo MRI protocol, we were able to observe the contracting heart with considerably improved image contrast and resolution, compared with echocardiography. This technique allowed us to assess cardiac performance in adult zebrafish, and we observed that 11/12 alk5a–/– zebrafish exhibited a severely expanded OFT.

We complemented this functional analysis with ex vivo μ-CT imaging to examine the morphology of the adult zebrafish heart at high resolution. With a few exceptions,30,45,46,109,116 this technique has been mainly used for ex vivo samples, and focusing on the skeleton.34,111–113 Three dimensional (3D) rendering of the cardiac compartments and aorta allowed the identification of alterations specifically in the OFT, atrial volume, and diameter of the aorta in some alk5a–/– zebrafish. Since this imaging modality does not require tissue dissocation, the preservation of tissue integrity and organ positioning brings additional information, allowing the investigation of phenotypes affecting the great vessels and other connecting structures. Developing the use of μ-CT for in vivo imaging will have to take into consideration the exposure to radiation and the need to immobilize the sample during data acquisition. This approach, also combined with an intra-venous contrast agent, would be especially powerful to study aneurysm development and the progression of conditions affecting cardiac performance, including during cardiac regeneration.

Moreover, much like clinicians strongly rely on reference values to define a healthy heart considering the gender, age and ethnicity of the patients,38,92,105–107 we define some reference values for each technique (Tables 5–7). We also summarized the information that can be extracted, and the phenotypes that can be analysed, with each technique, highlighting their strengths and weaknesses, in order to help select the optimal approach(es) (Table 9).

4.2 Multimodal approach to identify cardiovascular phenotypes

When designing studies with adult specimens, the reduced sample size and the high individual variability need to be considered. Moreover, the same disease-causing mutation can lead to a phenotype in only some of the mutant individuals (incomplete penetrance), or lead to different phenotypic severity (variable expressivity).44 In our analyses, we observed high phenotypic variability between individuals, particularly related to haemodynamic measurements. Overall, we observed that few of the parameters measured with each technique were significantly different (defined as a P-value <0.05) between WT and alk5a–/–. Nevertheless, it was evident that some alk5a–/– zebrafish presented cardiovascular defects. Therefore, in order to investigate this variability between individuals, we subjected the same zebrafish to multiple imaging techniques to increase the data collected per individual and decrease the number of specimens required. We used these techniques to select specimens with specific features for high resolution approaches including TEM. This approach is similar to that proposed by clinicians who have advocated the use of multimodal imaging tools for a more accurate diagnosis of complex and variable cardiovascular diseases.39,114–116

In zebrafish thus far, only a few studies have integrated different modalities for phenotypic characterization, for example combining μ-CT and electron microscopy.112,117 Here, we analysed parameters that encompass haemodynamics, cardiac performance, and morphology in the same zebrafish. To highlight the power of a sensitive and comprehensive imaging platform, we selected to study a model with a relatively mild cardiovascular phenotype. In fact, only 50% of alk5a–/– zebrafish exhibit defects as observed on histological sections, making the study of the phenotype with solely morphological techniques inconclusive. Accordingly, with our imaging platform, we identified some zebrafish exhibiting clear functional defects (as assessed by echocardiography and/or MRI), but lacking obvious morphological defects (as assessed by μ-CT). ECG analysis suggests that alk5a–/– adult zebrafish also display electrophysiological defects, including cardiac arrhythmia and high variability in atrial depolarization; and, it will be interesting to integrate the ECG into our multimodal platform to complement the image analysis with electrophysiology. Moreover, by correlation analysis of each parameter, in addition to bulk analysis of WT vs mutant samples, and by selecting individuals with specific functional phenotypes, we achieved a better understanding of the cardiovascular phenotypes in alk5a–/– zebrafish.

4.3 Clinical perspective of the alk5a mutant cardiovascular phenotype

Overall, our data indicate that while the loss of alk5a alone does not cause embryonic defects,64 it can cause cardiovascular defects which often remain asymptomatic even at adult stages. Our data also identify the OFT (orthologous to the mammalian adult aortic root) as the main compartment affected in alk5a–/– zebrafish. Many of the phenotypes we observed are similar to those associated with aortopathies in clinical settings, pathologies often derived from mutations in TGF-β pathway genes.59–61 At the morphological level, we observed that alk5a–/– zebrafish present a thinning of the OFT outer wall, similar to patients affected by aortic aneurysms prone to dissection.118,119 It is also interesting to
noted that the volume of the OFF wall decreased according to the age and size of the zebrafish, similar to the decrease in aortic wall thickness in older patients. In the functional level, we observed that increased OFF dilation was linked with retrograde blood flow, as often reported in patients presenting aortic dilation and regurgitation. In addition, the increased aortic flow phenotype in adult zebrafish is challenging due to the limited resolution of the current techniques. However, future implementation of in vivo μ-CT with intra-venous contrast agents, and/or the integration of flow measurements with MRI in higher resolution, might help with the detection of such phenotypes.

In summary, we propose a combinatorial imaging and data analysis platform to detect phenotypes that are too variable or subtle to identify with conventional histological analyses. This study further illustrates the importance of the zebrafish model to help clarify adult phenotypes potentially translatable to the clinic.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding authors.

Authors’ contributions

Conceptualization, A.B.B., G.L.M.B. and D.Y.R.S.; Methodology, A.B.B., G.L.M.B., A.W., R.R., P.R.L., A.N., and C.M.; Validation, A.B.B., G.L.M.B., and J.C.S.; Formal Analysis, A.B.B., G.L.M.B., and J.C.S.; Investigation, A.B.B., G.L.M.B., A.W., R.R., P.R.L., and J.P.; Resources, C.S.M.H.; Writing—Original Draft, A.B.B. and G.L.M.B.; Supervision, A.B.B. and D.Y.R.S.; Project Administration, A.B.B. and D.Y.R.S.; Funding Acquisition, D.Y.R.S.

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**Translational Perspective**
This study further illustrates the importance of the zebrafish model to investigate cardiovascular phenotypes including morphological and functional alterations as observed in human disease settings.