# ONLINE SUPPLEMENTAL MATERIAL

**Sheep can be used as animal model of regional myocardial remodelling and controllable workload**

**SUPPLEMENTAL METHODS**

## **Pilot experiments**

### Pacing position

In a pilot experiment in one sheep, several temporary pacemaker electrodes were sutured to the RV in order to determine the stimulation site with the most pronounced mechanical dyssynchrony. Analysis of septal flash on M-mode echocardiography revealed that stimulating the RV free wall induces the best mechanical effect (**Supplemental Fig. 1**, arrows). Based on these findings, we aimed for implanting the ventricular lead in an RV free wall position in all subsequent animals.



**Supplemental Fig. 1** M-mode short-axis echocardiography images of the septal and lateral wall, recorded from a sheep during a pilot experiment to determine the optimal pacing position on the RV. Note the septal flash pattern during RV free wall pacing (arrows), which is not present during RV outflow tract pacing. Vertical dashed lines indicate valve timings. AVC: aortic valve closure; ECG: electrocardiogram; LV: left ventricle; MVC: mitral valve closure; RV: right ventricle; RVOT: right ventricular outflow tract.

### Duration of pacing

A pilot group of 4 sheep was paced for 16 weeks and the remodelling process was assessed by weekly echocardiography. Data analysis revealed that the differential changes in septal and lateral wall thickness plateaued after approximately 8 weeks although the LV diameter continued to increase. Because the study mainly aimed at inducing asymmetric remodelling in favour of dilatation, all subsequent animals underwent 8 weeks of rapid pacing.

## **Pacemaker implantation**

Animals were pre-medicated by intravenous administration of 4mg/kg ketamine, followed by anaesthesia induction with mask-supplied isoflurane in 3L O2. Analgesia was performed with 0.5 mg/kg intravenous meloxicam and 0.012 mg/kg buprenorofine, while 22.2 mg/kg intravenous cefazoline served as antibiotic prophylaxis. The animals were then mechanically ventilated through a cuffed endotracheal tube by which isoflurane anaesthesia was maintained. Monitoring of vital parameters (ECG, O2 saturation, CO2 output and arterial blood pressure) was performed during surgery. The animals were positioned in right lateral decubitus position on a specially designed table, allowing free access for the echocardiographic transducer to the right parasternum. After left thoracotomy via the third intercostal space, the pericardium was split at the right atrioventricular groove. An epicardial pacemaker lead (Capsure EPI; Medtronic, Heerlen, The Netherlands) was surgically sutured on to the RV free wall. After testing for pacing capture, we searched for signs of a pacing induced LBBB-like activation pattern such as QRS-width increase on the surface ECG as well as pre-ejection septal flash in the intra-operative transthoracic echocardiogram (**Supplemental Fig. 1**, arrows). If necessary, the RV electrode was re-positioned and the testing repeated until an optimal effect was achieved. Afterwards, the RA lead was surgically implanted and tested. The pacemaker leads were tunnelled and connected to the pacemaker (Adapta L DDDR; Medtronic), which was fixed in a subcutaneous pocket close to the left shoulder. The thoracotomy and pacemaker pocket were subsequently closed and the animal was allowed to recover. The pacemaker remained switched off during a post-operative recovery period of three days after surgery during which the animals were closely monitored. During the same period, analgesia was continued with subcutaneous injection of 0.5 mg/kg meloxicam.

## **Echocardiography**

A Vivid 7 ultrasound scanner with a 5S transducer (GE Vingmed Ultrasound, Horten, Norway) were used to record 2D grey-scale images from a right parasternal window. Three consecutive cardiac cycles were digitally stored for later offline analysis. All images were acquired at a frame rate of at least 70 frames/second to allow deformation analysis by speckle tracking echocardiography (STE). Closure artefacts in mitral and aortic valve spectral Doppler were used to determine ED and ES respectively [1].

All echocardiographic data were analysed offline with the use of EchoPAC BT13.0 software (GE Vingmed Ultrasound). Image analysis included qualitative motion pattern analysis from M-modes (septal flash), LV dimensions at ED and ES from 2D short axis images at the same level as the cardiac MRI, and regional circumferential deformation using STE at mid-ventricular level.

## **Cardiac magnetic resonance imaging**

We used a 3T Magnetom Trio unit (Siemens Medical Solutions, Erlangen, Germany), with a phased-array surface receiver coil, ECG triggering and dedicated cardiac software. The protocol included cine sequences in short axis, horizontal, vertical and radial long axis projections. Cine imaging was performed using retrospectively gated fast low-angle shot gradient echo sequences with slice thickness of 5 mm, and 1 mm gap. Post-processing analysis included assessment of LV septal and lateral wall thicknesses, LV diameter, LV volumes, LV ejection fraction (LVEF), left atrial (LA) volume, and degree of mitral valve regurgitation. Wall thicknesses and diameter were measured in short axis at the level of the mitral valve leaflet tips. LV volumes, LVEF and cardiac output (CO) were assessed using delineation of the endocardial short axis stack at end-diastole (ED) and end-systole (ES). LA volume was calculated using the area-length method. LV myocardial mass was calculated from the difference in volume between the epi- and endocardial contours, multiplied by the myocardial density index 1.05 g/ml. Mitral valve regurgitation (MR) was graded visually on the cine images as no (score 0), minimal (0.5), mild (1), moderate (2), moderate to severe (3), severe (4) [2]. Semi-quantitative grading of MR on MRI images was chosen over Doppler echocardiography, due to the limited echocardiographic windows in sheep. Mitral valve and aortic valve closure were used to determine ED and ES, respectively. All MRI data were analysed offline using custom software (RightVol, KU Leuven, Belgium, [3]). All morphologic measurements were obtained with intrinsic LV conduction (AAI pacing).

## **Hemodynamic measurements**

### Animal preparation for invasive hemodynamic assessment

Anaesthesia and standard pacing settings (i.e. a fixed rate of 110 bpm) were applied as described above and a bolus of 10000 IU heparin was given prior to instrumentation. For arterial access, the left femoral artery was punctured and a 9F sheath was placed in Seldinger technique. This access was used to position an 5F micromanometer-tipped pressure-volume catheter (Ventri-Cath 507, Millar Instruments, Houston, TX, USA) in the LV under fluoroscopic guidance. The catheter was connected to the MPVS Ultra Pressure-Volume Loop system (Millar Instruments), and data were recorded using Labchart 7 Pro (ADInstruments Europe, Oxford, UK). Before introduction, the catheter was pressure calibrated in a 37°C water tank. Calibration was re-checked after removal of the catheter. MRI data were used for volume calibration of the catheter signal.

**Histological analysis**

After sacrifice each heart was quickly removed, and blocks of transmural tissue were taken from the septal and lateral wall at a mid-ventricular level. These blocks were immersion-fixed in paraformaldehyde 4%, embedded in paraffin, and sliced in 5 µm thick sections. Microscopic analysis was performed with a Zeiss Axio Imager ML light microscope (Zeiss, Oberkochen, Germany).

Myocyte hypertrophy was measured on Haematoxylin-Eosin (H&E) stained sections, by using the mean linear intercept method [4,5]. The number of cardiomyocytes transsected by a reference line of 500µm at x200 magnification was counted on 10 fields per sample and then averaged. The number of transsected cardiomyocytes correlates with the inverse of myocyte volume and thus represents myocyte hypertrophy.

Modified Sirius Red stained samples were used to determine the collagen-positive area for fibrosis. A 9-point grid with an area of 0.25 mm² at x200 magnification was projected at 10 quadrants. The proportion of reference points hitting cardiomyocytes, connective tissue or vessels equals the volume occupied by this compartment according to the principle of Delesse [5,6]. The percentage of cardiomyocytes, collagen and vessels was expressed as fraction of the total area examined. The values from the 10 quadrants were then averaged.

All histological measurements were performed while the observer (E.V.) was blinded for the experimental group and the wall section from which the tissue was taken.

# SUPPLEMENTAL RESULTS

## **LV remodelling by MRI**

Progressive LV remodelling was also documented by MRI at both baseline and after 8 weeks of rapid pacing. Within 8 weeks, the septal wall thickness had decreased –24% in ED and –24% in ES (from 9.4±0.68 mm to 7.1±0.74 mm, p<0.0001 and from 10.8±1.0 mm to 8.2±1.0 mm, p<0.0001, respectively) (**Supplemental Fig. 2a**). The lateral wall thickness was increased by +20% in ED and +17% ES (from 9.3±0.66 mm to 11.2±0.92 mm, p<0.0001 and from 11.2±0.63 mm to 13.1±0.99 mm, p<0.0001, respectively) (**Supplemental Fig. 2a**). At the same time, the LV cavity diameters increased by +18% in ED and +25% in ES (from 38.3±2.62 mm to 45.2±4.78 mm, p<0.0001 and from 33.1±2.64 mm to 41.3±4.57 mm, p<0.0001, respectively) (**Supplemental Fig. 2b**). Wall thicknesses and diameter measurements by echocardiography and MRI correlated very well (r=0.99, p<0.0001) and showed no bias [ICC 0.995, 95%CI(0.993-0.996), p<0.0001] (**Supplemental Fig. 2c**). LV myocardial mass increased with +9% from 75.7±10.8 g to 82.2±13.7 g (p=0.009), corresponding with the global LV enlargement.

**Supplemental Fig. 2** LV remodelling during 8 weeks of dyssynchronous rapid pacing in all animals (n=13). (a): Changes in LV septal and lateral wall thickness, and (b) LV internal diameter, as measured by cardiac MRI. Solid lines on the upper panels represent the lateral wall, while dashed lines represent the septal wall measurements. Red coloured markings are end-systolic, while blue coloured markings are end-diastolic measurements. (c): Correlation plot of linear LV measurements between echocardiography and MRI (r=0.99, p<0.0001).

## **Global LV function**

### Hemodynamic data

**Supplemental Table 1:** Hemodynamic data of acute pacing switch after 8 weeks of rapid A-V pacing.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **AAI** | **DDD** | **p-value** |
| Heart rate (bpm) | 110 | 110 | p=NS |
| ED LV pressure (mmHg) | 8±7.3 | 9±7.2 | p=NS |
| CO (L/min) | 3.4±1.0 | 3.3±1.0 | p=NS |
| MAP (mmHg) | 56±14.7 | 54±13.4 | p=NS |
| SVR (dynes\*sec\*cm-5) | 1369±433 | 1365±455 | p=NS |

Values are mean with SD. CO: cardiac output; ED: end-diastolic; LV: left ventricle; MAP: mean arterial pressure; SVR: systemic vascular resistance.

### Pressure-volume loops



**Supplemental Fig. 3** Representative loops of changes in LV pressure-volume loop area (stroke work) between AAI pacing (a) and DDD pacing (b). Note the S-shaped morphology of the DDD pacing loop during the isovolumic contraction phase, caused by the aberrant pre-ejection septal motion (= septal flash).

**Histological analysis**

### Cardiomyocyte hypertrophy

After 8 weeks of rapid A-V pacing, the H&E stained histological samples displayed hypertrophy of the lateral wall, compared to the septal wall (**Supplemental Fig. 4a and 4b**). The number of transsected cardiomyocytes in the septal wall and the lateral wall was on average 19.9±3.3 µm and 19.2±3.4 per 500 µm, respectively (p=0.03) (**Supplemental Fig. 4c**), indicating a relative hypertrophy of the lateral wall in the remodelled state.

### Percentage of cardiomyocytes, collagen and vessels

On visual inspection, no difference was found in the cardiomyocyte, collagen and vessel content between the septal and lateral wall (**Supplemental Fig. 4d and 4e**). This was confirmed by the similar percentage of cardiomyocytes, collagen and vessels in the septal and lateral wall (all p=NS) on quantitative assessment. See **Supplemental Fig. 4f**.

**Supplemental Fig. 4:** Representative histological characteristics of septal and lateral wall myocardial samples after 8 weeks of rapid A-V pacing. (a+b): H&E stained samples of both septal and lateral wall. Note the hypertrophied appearance of the lateral wall (b) versus the septal wall (a). (c): Number of transsected cardiomyocytes along a 500 µm reference line in the septal and lateral wall (n=10). The lateral wall shows a lower number of transsected cells, indicating hypertrophy. Dotted lines connect measurements from the same animal. (d+e): Sirius Red stained samples of both septal and lateral wall, with collagen coloured red/brown. No difference can be appreciated in collagen content between septal (d) and lateral wall (e). (f): Percentage of cardiomyocytes, collagen and vessels averaged over 10 reference regions of septal and lateral wall (n=10).

# SUPPLEMENTAL DISCUSSION

### LV conduction

**Supplemental Table 2:** Comparative table on the major differences between LBB ablation, RV pacing, and A-V pacing.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **LBB ablation** | **RV pacing** | **A-V-pacing** |
| Similarity to true LBBB | + | +/- | +/- |
| Simple acute reversibility | - | + | + |
| Simple switch to normal ventricular conduction at same heart rate | - | - | + |
| Difficulty of procedure | ++ | ++ | + |
| Allowing rapid heart rate to accelerate remodelling | - | + | + |

# SUPPLEMENTAL REFERENCES

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