CHAPTER 2

The content and distribution of troponin I, troponin T, myoglobin and "-Hydroxy-Butyric acid Dehydrogenase (HBD) in the human heart.

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Summary

We investigated the content and distribution of the new heart-specific markers troponin I (measured with two methodologies), troponin T in relation to the conventional non-heart specific myoglobin and HBD in the hearts of 35 postmortem patients. Tissue was obtained from the right ventricle (RV), from the left ventricle (LV) areas posterior, lateral, anterior wall as well as the interventricular septum, from the right atrium (RA) and from the left atrium (LA). For troponin I ^{AxSYM} we found median values in µg per gram wet weight tissue (and per gram protein) of 2765 (50.0), 2453 (64.1), 1291 (33.9), and 1264 (31.4) respectively for the RV, LV, RA and LA locations. For troponin I^{Access} the median content in µg per gram wet weight tissue (and per gram protein) was 41 (0.88), 71 (1.54), 28, (0.66) and 26 (0.65) respectively for the various heart locations. For troponin T in µg per gram wet weight tissue (and per gram protein) we found 75 (1.77), 95 (2.06), 39 (0.99), and 39 (0.88) respectively for the examined heart locations. For myoglobin in mg per gram wet weight tissue (and per gram protein) the values per heart location were 1.90 (45.1), 2.40 (46.1), 1.00 (20.1), and 0.80 (20.9) respectively, and for HBD in U per gram wet weight tissue (and per gram protein) 116 (2.30), 140 (2.87), 50 (1.21), 52 (1.26) respectively. We conclude that there are statistically significant differences between the contents expressed per gram wet weight tissue in the right and in the left ventricle for the biochemical markers troponin I^{Access}, troponin T, myoglobin and HBD. The biochemical contents expressed per gram wet weight tissue as well as per gram protein in the right and in the left atrium are statistically significant lower for all examined biochemical markers than those in the right and in the left ventricle, whereas there are no statistically significant differences between the biochemical contents in the right and in the left atrium. Furthermore, within the left ventricle there is no statistically significant difference between the locations posterior-, lateral-, anterior wall and interventricular septum.

Introduction

The determination of cardiac markers in blood after myocardial infarction (AMI) is used for a long time as an estimation for the extent of myocardial tissue damage (1). Its relevance is indicated by the fact, that the prognosis of a patient after AMI is related to the amount of myocardial tissue loss (2). A frequently used conventional biochemical parameter for this purpose is hydroxy-butyric acid dehydrogenase (HBD) (3). However, this parameter is non-heart specific, since it is also elevated after hemolysis. An other marker, which can be used, is myoglobin. After AMI the increase in serum concentration of myoglobin is more rapid than the increase of HBD, whereas myoglobin concentration normalises also earlier. But like HBD, myoglobin is non-heart specific, because myoglobin is also a component of skeletal muscle tissue. Moreover, as myoglobin is cleared through the kidneys, the raise in serum concentration after AMI is more complicated than the raise of HBD, since the myoglobin clearence depends on the renal function of the patient.
Recently, more specific biochemical markers for myocardial tissue damage have been reported. These markers include the cardiac troponins (4,5). The troponins are components related to the actin-myosin complexes of the thin filaments of striated muscle. These complexes are involved in the process of muscle contraction and relaxation. Three isoforms of troponin have been described: troponin I, troponin C and troponin T. Each troponin has its own function. Troponin C binds calcium. This binding activates the muscle contraction. Troponin I inhibits troponin C by binding to

this component resulting in muscle relaxation, whereas troponin T binds the troponin I-troponin C complex to tropomyosin.
In contrast to troponin C, the amino acid sequences of troponin I and troponin T from skeletal muscle differ from those of myocardial muscle. In order to separate them immunologically, specific antibodies directed against the cardiac troponins I and T were developed. These antibodies form the basis of the techniques measuring cardiac troponin I and cardiac troponin T concentrations in serum. As the amino acid composition of troponin C from skeletal muscle equals that of myocardial muscle, no cardiac troponin C methodology can be developed to specifically measure myocardial troponin C.
In order to reliably relate the extent of myocardial tissue loss to the increase of cardiac troponin concentration in serum, the troponins should 1. be homogeneously distributed in the myocardium and 2. show inter-individually hardly any difference in myocardial content (6).
So far, a limited number of studies have been reported about the contents of cardiac markers in the heart (7-12). However, these studies concern a limited number of patients, or hearts of non human origin, whether only troponin I or troponin T is considered or only the contents of the ventricles are investigated. Furthermore, two different forms of content expression are used. These include expression per gram wet weight tissue or per gram protein.
The aim of this study is to investigate the distribution and the content (expressed per gram wet weight tissue as well as per gram protein) of cardiac troponin I (measured with two different troponin I methodologies) and cardiac troponin T in the human myocardium in relation to the distribution and the content of the more conventional parameters myoglobin and HBD.

Patients and methods

Postmortem fresh myocardial tissue was obtained from seven different locations in the heart and from the psoas muscle in 35 patients. Care was taken that all tissue samples were free of scarring and necrosis. The heart locations include right ventricle (RV), left ventricle (LV) posterior wall (LV1), LV lateral wall (LV2), LV anterior wall (LV3), LV interventricular septum (LV4), right atrium (RA), and left atrium (LA). Directly after collection, the samples were weighed and frozen in liquid nitrogen. Subsequently, they were destructed in liquified nitrogen using a Micro-dismembrator U (Braun Biotech International, distributed by Salm and Kipp, The Netherlands) at 2000 rpm for one minute. After finishing this procedure, five ml modified PBS buffer (pH=7.0) was added. These solutions were frozen and stored at -20°C until analysis of the biochemical markers troponin I (troponin I^{AxSYM} and troponin I$^{Access)}$, troponin T, HBD, and myoglobin and the protein concentrations.
Troponin I^{AxSYM} was measured on an AxSYM analyser (Abbott Diagnostic Division, Hoofddorp, The Netherlands) according to the instructions of the manufacturer (13). The coefficients of variation (c.v.'s) at three different levels were determined using control sera. These were 6.8 %, 5.2 %, and 5.8% respectively for the levels 2.9, 7.6, and 29.0 µg/l.
An Access Analyser was used to determine the troponin I^{Access} (Beckman, Mijdrecht, The Netherlands) according to the instructions of the manufacturer (14). The c.v.'s of this assay methodology were 8.9%, 8.2% and 7.4% respectively for the levels 0.22, 5.88 and 25.4 µg/l.
Troponin T measurements were performed with an Elecsys 2010 analyser (Roche-Boehringer Mannheim, Almere, The Netherlands). For these measurements the 'third' generation troponin T

reagent was used. This reagent is calibrated with standards of human origin and uses a one amino acid modified signal antibody, which result in a better linearity than the ’second’ generation troponin T reagent, which uses standards of bovine origin (15).
Myoglobin was measured using a BNII-nephelometer (Dade-Behring, Leusden, The Netherlands) according to the instructions of the manufacturer.
HBD activities were measured at 37°C using HBD reagent (product nr 1489267, manufacturer Roche-Boehringer Mannheim, Almere, The Netherlands) on a Mega analyser (Merck, Amsterdam, The Netherlands).
For all these methodologies the linearities were validated by using various dilutions of different tissue homogenate-solutions. Furthermore, all assay-methodologies show no blank reaction with the modified PBS-buffer.
The protein measurements were performed using the pyrogallol red methodology on a Mega analyser. The pyrogallol red reagent (product nr. A01217) was manufactured by Biotrol Diagnostic (Chennevières les-Louvres, France) and was distributed by Merck. The instructions of the manufacturer were modified by using 3 µl homogenate-solution and 250 µl reagent. This modification resulted in an enhancement of the linearity up to 4 g/l. The imprecision was determined using two controls. The mean value of control 1 was 0.78 (standard deviation 0.04) g/l and that of control 2 was 2.98 (s.d. 0.11) g/l. The measured concentrations of the tissue homogenate-solutions were between 1 and 3 g/l.
All biochemical results were expressed as µg (for both troponin I methodologies and for troponin T), mg (for myoglobin) and Units (for HBD) per gram wet weight myocardial tissue and per gram protein.
Statistics. Differences of the biochemical markers between the sampled locations were tested by the Friedman test. If significant differences were found with the Friedman test, the Wilcoxon Signed Ranks test was used as post hoc test between the individual

Table 1. Patient characteristics.

Patient	Characteristics.				
No.	Age(y)	Gender	Cause of death	weight	timelag[a]
1	86	female	Heart failure	380	8 hours
2	56	male	Heart failure	620	20 hours
3	91	male	Pneumonia	145	9 hours
4	64	female	Amyloidosis, LVH	400	18 hours
5	75	female	Cardiomyopathy	475	11 hours
6	60	male	Intracranial bleeding	400	9 hours
7	67	male	Metastatic lung cancer	250	6 hours
8	62	male	Pulmonary embolism	450	13 hours
9	52	male	Pulmonary embolism	555	12 hours
10	52	female	Metastatic endometrial cancer	270	16 hours
11	91	female	Resp insuff. in interst lungdisease	185	34 hours

12	64	female	Metastatic endometr. ca	275	12 hours
13	73	male	Sepsis and multi organ failure	520	14 hours
14	47	female	Respiratory insufficiency	350	6 hours
15	87	male	Bronchopneum, Wegener disease	350	24 hours
16	40	female	Encephalitis	340	24 hours
17	56	female	Meningitis	260	10 hours
18	34	female	Sudden death, right ventricular dyspl	240	48 hours
19	76	female	Pneumonia	600	10 hours
20	54	male	Metastatic lung cancer	450	24 hours
21	29	female	Metastatic breast cancer	250	10 hours
22	68	female	Pneumonia	280	48 hours
23	66	male	Intracranial bleeding	600	24 hours
24	70	male	Aortic aneurism	600	12 hours
25	60	male	Pulmonary embolism	304	24 hours
26	63	male	Septic shock	384	12 hours
27	49	female	Metastatic pancreatic cancer	286	48 hours
28	61	male	Pancreatitis	554	12 hours
29	59	male	Pulmonary embolism	396	24 hours
30	53	female	Haemochromatosis	410	4 hours
31	67	male	AMI	540	6 hours
32	71	female	aneurisma abdom.	700	48 hours
33	74	male	Pancreatitis, ARDS	380	6 hours
34	74	female	Cerebral infection	450	48 hours
35	81	female	Metastatic cancer unknown primary	420	48 hours

[a] time between death and autopsy.

sampled locations using Bonferroni as correction method. Differences in heart weight were considered by testing differences in content of biochemical markers for the sampled locations in 'normal' (heart-weight 450 gram or less) and hypertrophic (heart-weight over 450 gram) hearts using the Mann-Whitney U-test. P values# 0.05 were considered to be statistically significant different. According to this methodology also gender dependency was considered.

Results

The patient characteristics are depicted in table 1. These include age, gender, cause of death, heart-weight and the time between death and autopsy. From table 1 it can be seen that eighteen females were included. The mean age of these patients was 62 (range 29-91) y, the mean heart-weight was 365 (range 185-700) gram and the mean time between death and autopsy was 26 (range 4-48) h. Also seventeen males were included. The mean age of these patients was 66 (range 52-91) y, the mean heart-weight of these patients was 441 (range 145-600) gram, and the mean time between death and autopsy was 15 (range 6-24) h.
Table 2 shows the content of the examined biochemical markers in the different parts of the left ventricle. This table shows that within the left ventricle there is no statistically significant difference

between the sampled areas posterior-, lateral-, and anterior wall, as well as the interventricular septum. Therefore, the mean value of the left ventricle samples will be used in the remaining. In figure 1a the ranges of the contents expressed per gram wet weight tissue of the examined biochemical markers per myocardial location and of the skeletal muscle tissue are shown by the use of a box-plot. In figure 1b this is shown for the contents expressed per gram protein. From figure 1a and figure 1b it can be seen that the contents of troponin I^{Access}, troponin T, myoglobin, and HBD per gram wet weight myocardial tissue of the right ventricle are significantly lower than those of the left ventricle overall. In contrast, there is no difference, if the contents per gram protein are compared. Furthermore, the contents per gram wet weight tissue as well as per gram protein of all examined markers in the right and in the left ventricle are significantly higher than those in the right and in the left atrium. The contents of biochemical markers in the right atrium do not statistically significant differ from those in the left atrium. For all sampled locations we observe lower results of the troponin I^{Access} methodology compared with that of the troponin I^{AxSYM}. The contents of the examined troponins are at least 500-1000 times lower in the skeletal muscle than in the various heart locations. The measured values of the skeletal muscle may be explained by a-specific binding. The content of HBD in skeletal muscle is 1.5 - 5 times lower than in the considered heart locations, whereas the content of myoglobin in skeletal muscle is 1.5 - 4 times higher than in heart muscle tissue. In Table 3 the median contents per gram wet weight myocardial tissue and per gram protein are summarised per sampled location (right ventricle, left ventricle overall, right atrium,

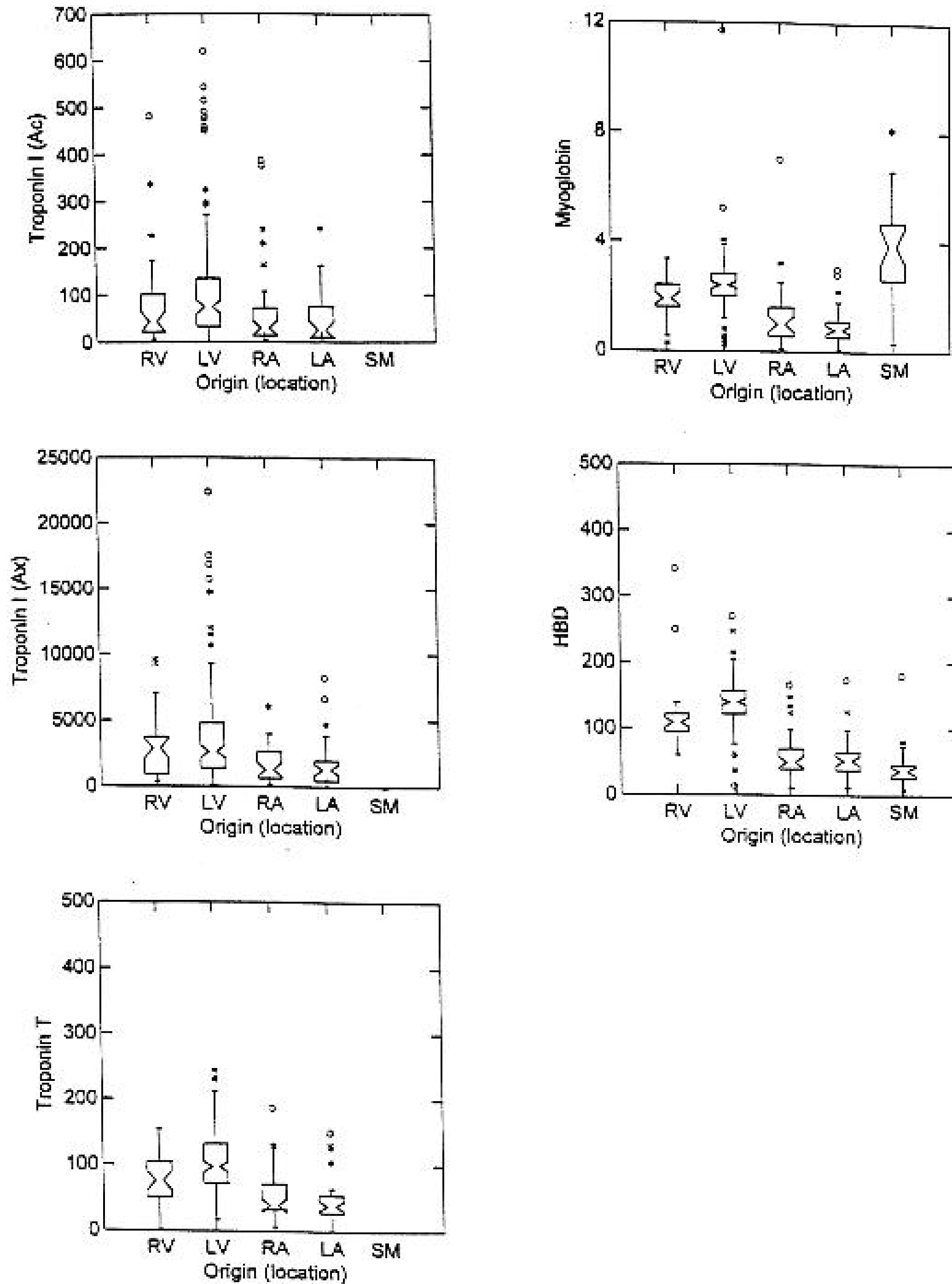

Figure 1. The ranges of contents expressed per gram wet weight myocardial tissue for several cardiac markers sampled at different locations in the heart and the ranges of contents per gram wet weight skeletal tissue are shown. Units: troponin I (Ac) , troponin I (Ax) and troponin T: µg/g wet weight tissue; myoglobin: mg/g wet weight tissue; HBD: U/g wet weight tissue. Abbreviations:RV: right ventricle, LV: left ventricle; RA: right atrium; LA: left atrium; SM: skeletal muscle. Troponin I (Ac): troponin I^{Access} ,Troponin I (Ax): troponin I^{AxSYM}. Statistically significant differences: for all markers the contents of the right ventricle and left ventricle are higher than those of the right atrium as well as the left atrium; for the markers troponin I^{Access}, troponin T, myoglobin and HBD the contents of the left ventricle are higher than those of the right ventricle.

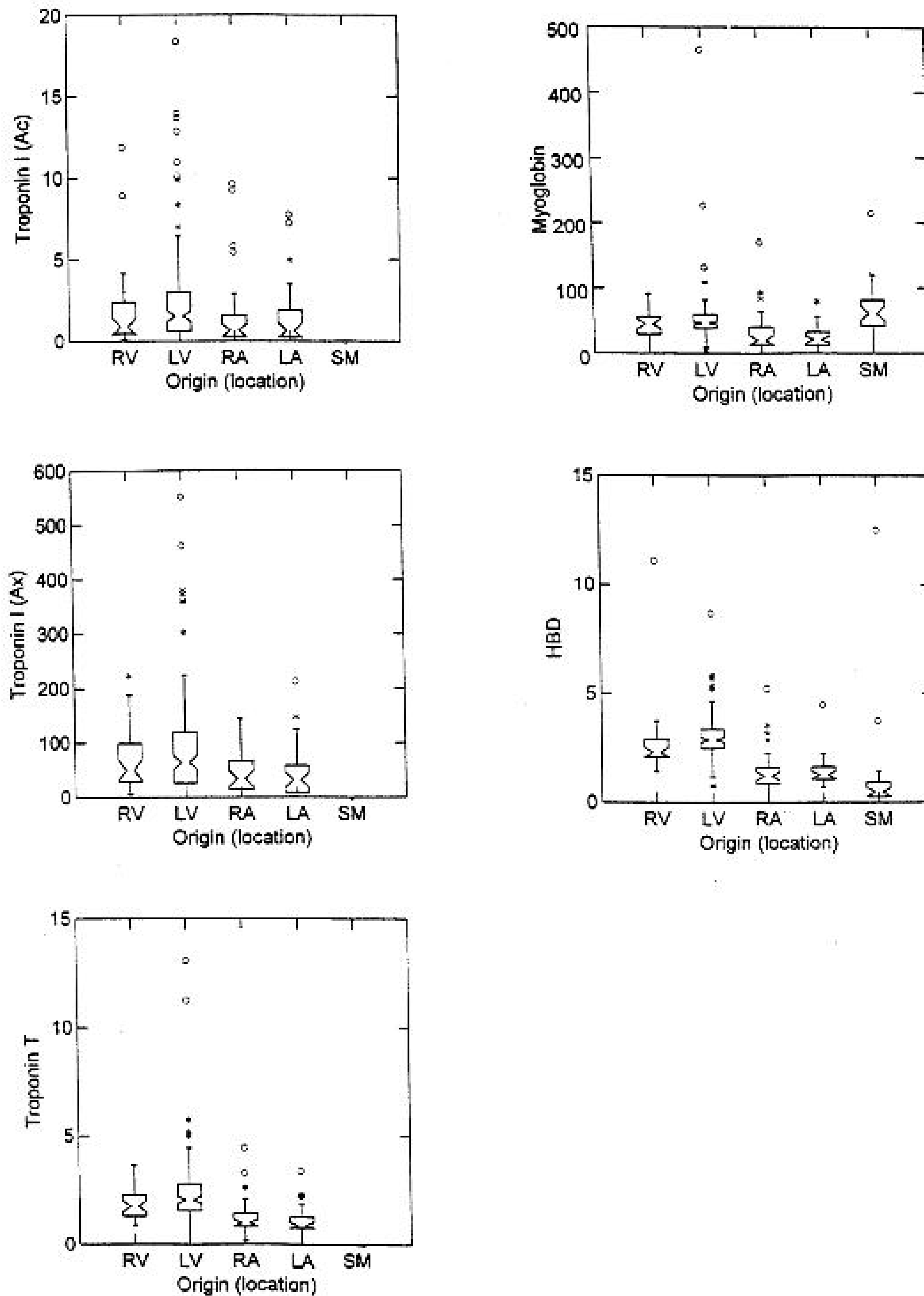

Figure 2. The ranges of contents expressed per gram protein for several cardiac markers sampled at different locations in the heart and the ranges of contents per gram protein in skeletal tissue are shown. Units: troponin I (Ac), troponin I (Ax) and troponin T: µg /g protein; myoglobin: mg/g protein; HBD: U/g protein. For abbreviations see text figure 1. Statistically significant differences: for all markers the contents of the right ventricle and left ventricle are higher than those of the right atrium as well as the left atrium; there is no difference between the right and left ventricle.

Table 2. Median (range) content of various biochemical markers per gram wet weight myocardial tissue sampled at different places in the left ventricle (LV) of the human heart.

Biochemical marker (Unit)	LV posterior wall	LV lateral wall	LV anterior wall	LV septum	LV overall
Troponin I^{Access} (µg)	76.7 (.18 - 543)	69 (.04 - 620)	68.4 (.1 - 459)	66.6 (.1- 491)	71.3 (.04-620)
Troponin I^{AxSYM} (µg)	2072 (15.9 -17520)	2338 (8.4 - 22374)	3182 (8.6 - 15756)	2787 (6.5 - 10678)	2453 (6.5-22374)
Troponin T (µg)	84.5 (17 - 234)	117 (18 - 213)	94 (24 - 244)	107 (13.5 - 230)	95 (13.5-244)
Myoglobin (mg)	2.40 (.78- 4.05)	2.50 (.67 - 5.21)	2.20 (.38 - 11.7)	2.40 (.25 - 3.69)	2.40 (.25-11.7)
HBD (U)	143 (38 - 247)	151 (12.7 - 270)	138 (62 - 196)	135 (5 - 183)	140 (5-270)

No statistically significant differences for each examined cardiac marker could be detected between the various left ventricle locations.

Table 3. Median content per gram wet weight myocardial tissue and per gram protein of various biochemical markers sampled at different places in the human heart and of skeletal muscle tissue.

Biochemical marker (Unit)	Right Ventricle		Left Ventricle		Right Atrium		Left Atrium		Skeletal Muscle	
	w.w[a]	protein[b]	w.w[a]	protein[b]	w.w[a]	protein[b]	w.w[a]	protein[b]	w.w[a]	protein[b]
Troponin I^{Access} (µg)	41.1	0.88	71.3	1.54	28.1	0.66	26	0.65	.08	0.004
Troponin I^{AxSYM} (µg)	65	50.0	2453	64.1	1291	33.9	1264	31.4	2.25	0.04
Troponin T (µg)	75	1.77	95	2.06	39.0	0.99	39	0.88	0.04	0.001
Myoglobin (mg)	1.90	45.1	2.40	46.1	1.00	20.1	0.80	20.9	3.85	60.8
HBD (U)	116	2.30	140	2.87	50	1.21	52	1.26	38	0.49

[a] content expressed per gram wet weight tissue;
[b] content expressed per gram protein.
left atrium) and of the skeletal muscle tissue for both cardiac troponin I methodologies, cardiac troponin T, myoglobin and HBD.

We observe no heart-weight dependency of contents of the examined biochemical markers for all sampled locations between normal (n=25) and hypertrophic (n=10) hearts (all p-values were > 0.05). Evenso, we observe no differences in biochemical contents between the hearts of the male and the female patients (all p-values > 0.05).

Discussion

In this study we have investigated the contents per gram wet weight tissue as well as per gram protein and the distributions of cardiac troponin I, cardiac troponin T, HBD and myoglobin in the human heart. In summary, the person to person variety of the contents is high (the contents differ for some persons more than five times). Furthermore, the distribution of the markers over the heart is not homogeneous. The biochemical contents in the right and in the left atrium are statistically significant lower than those in the right and in the left ventricle, whereas the contents in the right and in the left atrium are not statistically significant different for all examined markers. Furthermore, the contents expressed per gram wet weight tissue in the right ventricle are, in contrast to the contents expressed per gram protein, statistically significant lower than those in the left ventricle for troponin I^{Access}, troponin T, myoglobin, and HBD, whereas the contents within the left ventricle show no differences for all examined biochemical markers regarding the locations posterior-, lateral-, anterior wall and interventricular septum. We have also looked for a gender and a heart-weight dependency. However, we did not observe statistically significant differences between the contents in female and male hearts, although the mean heart-weight of the female patients (365 gram) was lower than that of the male patients (441 gram). Furthermore, we did also not observe statistically significant differences between the contents of 'normal' and hypertrophic hearts.

In clinical practice it is of common use to estimate myocardial tissue damage from levels of cardiac markers in serum. But the afore reported non-homogeneous distribution of cardiac markers in the heart implicates, that it is necessary to know the location of the damage (right or left ventricle?), if the tissue damage is expressed per gram tissue. It is not necessary to differentiate between the locations right and left atrium. However, these locations should be considered different from both locations right and left ventricle. A consequence of the (high) person to person variety in contents is that it is not reliable to state that an individual patient with somewhat higher levels of cardiac markers in serum after AMI experiences more myocardial damage than an other individual patient who has somewhat lower levels of cardiac markers in serum after AMI. In contrast, it is allowed to use the estimations of myocardial damage with levels of cardiac markers in serum to compare groups of patients, if these groups have an equal composition, so that the person to person variety is comparable in both groups, and, if the size of these groups is big enough (to be determined by a power calculation).

The difference between the results of the troponin I^{Access} and the troponin I^{AxSYM} methodologies is caused by the lack of standardization of the troponin I analysis. On the one hand, this is due to the lack of consensus concerning the use of the antibody, which is used to detect troponin I. On the other hand, there is no consensus about the composition of a troponin standard, which may be used by each troponin manufacturer as the 'gold' standard for calibration. The results of the various troponin I methodologies will become more comparable, if such a 'gold' standard will be accepted and introduced by the different troponin manufacturers. However, the composition of a 'gold' troponin standard is complicated, because troponin is in various forms present in circulation. These

forms include free as well as several forms of complex bound troponin such as troponin I-troponin C, troponin I-troponin C-troponin T and troponin I-troponin T (16). Nevertheless an AACC-standardisation subcommittee is working on this subject (17).
Although for a long time increased concentrations in blood of cardiac markers are used to estimate the extent of myocardial necrosis, only a few publications adress to the distribution and content of biochemical parameters in the human heart. Van der Laarse et al. (9) reported no difference between the enzyme content expressed per gram wet weight tissue in the right and in the left ventricle of myocardial tissues obtained by biopsies during open heart surgery. They reported a mean content of 120 U/g wet weight myocadium for HBD. In contrast to our experiments, were these HBD measurements performed at 25 °C. Furthermore, these results were based on left ventricle tissue obtained by biopsies from 6 patients and right ventricle tissue obtained by biopsies from 9 patients. It is unclear whether these tissues were derived from (partly) the same patients. In contrast to their results, we observe statistically significant differences between the contents in the right and in the left ventricle. Besides the already mentioned discrepancy in number of patients for who tissue was derived from right and left ventricles (in our study n=35 versus Van derLaarse n=6 (LV) and n=9 (RV)), also the procedure of collection was different (autopsy versus biopsy). Moreover, we use the Friedman and Wilcoxon Signed Ranks tests to compare the results of the contents in the right and in the left ventricle, whereas Van der Laarse used a *t*-test to test the significance of differences between means of two groups and a Fisher's *F*-test to test the significance of differences between variances of two groups.
Kragten et al. (10) reported the content per gram wet weight tissue of HBD and of troponin T from only the left ventricle in myocardial tissue derived from 17 patients at autopsy. They reported a mean HBD content of 156 U/g wet weight myocardial tissue, which is in agreement with our findings. The mean troponin T content was reported to be 234 µg/g wet weight myocardial tissue, which is more than a factor two higher than we observe. However, in contrast to our measurements, they used the second generation troponin T reagent, whereas we used the third generation. As has recently been reported (7), this third generation troponin T assay (a) is more accurate as well as (b) demonstrates an improved linearity compared with the second generation troponin T and (c) shows lower results above values of 0.2 µg/l than the second generation troponin T assay. Between 0 and 0.2 µg/l there is no difference in results between the second and the third generation assays. The difference in results starts at a level of 0.2 µg/l. At a level of 10 µg/l the results of the third generation are 50% of those determined by the second generation. At a level of 25 µg/l the results of the third generation are 40% of those of the second generation (7). All our measurements in the tissue-homogenates were performed in diluted samples, because the undiluted tissue-homogenate solutions would be out of the analyser range. We diluted the homogenates until a concentration between 10 and 15 µg/l. As already stated, the difference between the second and third generation troponin T assays is at this level a factor between two and two-and-a-half. This might be a reasonable explanation for the difference in troponin T contents between our findings and those reported by Kragten et al.
Katus et al. (11) reported only free troponin T contents in the left ventricles of hearts from three patients undergoing heart transplantation. These patients are different from our population, because most of the patients in our study have no history of heart disease and moreover, none of the patients underwent a heart transplantation.
Bleier et al. (12) reported the content per gram wet weight tissue of myoglobin, troponin I and troponin T in the right atrium from 11 patients undergoing heart surgery. They reported a mean

myoglobin content of 0.97 mg/g wet weight, which is in good agreement with our findings of the right atrium. The troponin I and troponin T contents are different from our results. This might be explained by the already mentioned lack of standardisation of the troponin analysis, as they used other troponin I and troponin T methodologies than we did.
Voss et al. (7) reported the content per gram protein of cardiac troponin T and of myoglobin in the left as well as in the right ventricle in hearts of three healthy and of three diseased humans. The cardiac troponin T measurements were not carried out with the third generation assay, so that these results can not be compared with our results. The mean content of myoglobin of the left ventricle in healthy persons is 18.4 mg/g and in diseased persons 49.8 mg/g (overall mean of these persons is 34.1 mg/g). For the right ventricle these contents were reported to be 28.8 and 66.4 mg/g respectively (overall mean of these persons is 47.6 mg/g). We report median values of 46.1 mg/g for the left ventricle and 45.1 for the right ventricle.
From this study we conclude that there are statistically significant differences between the contents per gram wet weight tissue in the right and in the left ventricle for the biochemical markers troponin I^{Access}, troponin T, myoglobin and HBD. The biochemical contents per gram wet weight tissue as well as per gram protein in the right and in the left atrium are statistically significant lower for all examined biochemical markers than those in the right and in the left ventricle, whereas there are no statistically significant differences between the biochemical contents in the right and in the left atrium. Furthermore, within the left ventricle there is no statistically significant difference between the locations posterior-, lateral-, anterior wall and interventricular septum. For clinical practice these findings implicate that for estimation of myocardial damage with levels of cardiac markers in serum it is useful to know what the location of damage is in the heart. Furthermore, it is not reliable to compare myocardial damage between individual patients with levels of cardiac markers in serum. In contrast, this is allowed for the comparison between groups of patients, if the composition of these groups is equal (e.g. the same person to person variety), and, if the sizes of these groups are big enough.

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