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Original

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Image Analysis Workflow for 2-D Electrophoresis Gels Based on ImageJ

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Abstract: A number of commercial software packages are currently available to perform digital two-dimensional electrophoresis (2D-GE) gel analysis. However, both the high cost of the commercial packages and the unavailability of a standard data analysis workflow, have prompted several groups to develop freeware systems to perform certain steps of gel analysis. Unfortunately, to the best of our knowledge none of them offer a package that performs all the steps envisaged in a 2D-GE gel analysis. Here we describe an ImageJ-based procedure, able to manage all the steps of a 2D-GE gel analysis. ImageJ is a free available image processing and analysis application developed by National Institutes of Health (NIH) and widely used in different life sciences fields as medical imaging, microscopy, western blotting and PAGE. Nevertheless no one has yet developed a procedure enabled to compare spots on 2D-GE gels. We collected all used ImageJ tools in a plug-in that allows us to perform the whole 2D-GE analysis. To test it, we performed a set of 2D-GE experiments on plasma samples from 9 patients victims of acute myocardial infarction and 8 controls, and we compared the results obtained by our procedure to those obtained using a widely diffuse commercial package, finding similar performances.

Keywords: 2-D gels, bioinformatics, image analysis, myocardial infarction, spot matching

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Introduction

Two-dimensional gel electrophoresis (2D-GE) is a powerful technology to compare complex protein mixtures; it has been applied to many fields of biomedical research and is widely used in biomarker discovery. In a 2D-GE gel thousands of proteins are separated in well defined spots; these protein spots can be revealed via a variety of staining techniques (Coomassie, Silver Stain, Sypro),¹ and captured by one or more digitized computer images per gel (CCD camera, laser scanner, and optical scanner).² The image capturing phase transforms the biological information of the 2D-GE gel into a quantitative computer-readable data set. Once all the studied gels have been collected and digitized the software-based image analysis can be started. Image analysis is crucial in extracting biologically relevant information from a two-dimensional gel electrophoresis experiment.

Despite the availability of several software applications to analyze 2D-GE images, there is no general consensus on 2D-GE data analysis protocol. Moreover several authors reported that the commercial packages are time consuming, can often miss values or give false positives, and induce variance in quantitative measures.^{3–9}

The commercially available software perform the analysis workflow in two different ways. The classical package condensed the information onto spots. The spot detection is performed prior to matching and expression profile extraction. The second image analysis software group is based on the whole image information. These packages apply a warping procedure to remove running differences between gels, and the spot detection and protein expression profiles extraction occurred in a separated and independent step.⁵ The emphasis in this analysis software has been on reducing the subjectivity of the image analysis.

The fact that the alignment step is performed prior to the spot detection facilitates simultaneous spot detection on all gel images in an experiment and the resulting spot boundaries are identical on all gel images.¹⁰ In Table 1 are collected the most popular commercial software for 2D-GE gel analysis.

Several research groups have developed freeware systems to handle certain key aspects of gel analysis, including archiving (SwissProt 2D),¹¹ comparison (Flicker),¹² interactive exploration (WebGel),¹³ registration (bUnwarpJ and Sili2DGel),^{14,15} spot detection,¹⁶ spot quantification precision and differential expression (Pinnacle).¹⁷ However nobody has developed a complete package freely available and platform independent able to perform all the steps of a 2D-GE gel analysis experiment.¹⁸

Leveraging also on these experiences we have developed an image analysis workflow based on the popular public domain image analysis software package ImageJ (<http://rsb.info.nih.gov/ij/>). ImageJ and its plug-in is easy-to-use software and can be used in routine applications. Our workflow has been developed according to the whole image information procedure.¹⁹ It is based on six steps: aligning all the images, computing image fusion, creating a consensus spot pattern, propagating the consensus spot pattern to all gel images for quantification, and finally the statistic analysis.

In order to test our procedure, we performed a 2D-GE study of plasma from patients immediately after an acute myocardial event, comparing the results obtained using a widely diffused commercial package (Melanie; GeneBio, Geneva) to those obtained with our ImageJ-based procedure. We looked for biomarkers of pathology and/or treatment in acute myocardial infarction (AMI) patients treated with common anti-coagulant protocols. The authors confirm that ethical approval was obtained for this research.

Table 1. The most popular commercial software for 2D-GE gel analysis.

| Software package | Company | Type | Web link |
|---------------------------|-----------------------------------|------------|--|
| PDQuest | BioRad, Hercules, CA | Spot based | www.bio-rad.com |
| ImageMaster 2D or DeCyder | GE Healthcare | Spot based | www.gehealthcare.com |
| Dymension | Syngene, Cambridge, UK | Spot based | www.syngene.com |
| Melanie | GeneBio, Geneva, Switzerland | Spot based | www.genebio.com |
| Delta2D | Decodon, Greifswald, Germany | Warping | www.decodon.com |
| Progenesis SameSpots | Nonlinear Dynamics, Newcastle, UK | Warping | www.nonlinear.com |

**Table 2.** List of steps that describes how to perform the analysis.

| Step | Description | Web link |
|------|--|---|
| 1 | Download and install ImageJ on your computer following the installation instructions specific to your platform (Windows, Mac OS, Linux, etc.); | http://rsbweb.nih.gov/ij/download.html |
| 2 | Align all images in pairs using bUnwarpJ plugin and taking always the same image as reference, and save the warped images; | http://biocomp.cnb.uam.es/~iarganda/bUnwarpJ/ |
| 3 | Open all the warped images and save these in a stack as a sequence using the “Image>Stacks>Images To Stack command”; | |
| 4 | Sum image using “Image>Stacks>Z Project...>Sum Slices”; | |
| 5 | Perform spot detection on the fused image by the Watershed plug-in. Selected the binary output; | http://bigwww.epfl.ch/sage/soft/watershed/index.html |
| 6 | Apply the blob analyzer of ImageJ using “Analyze>Analyze Particles...”, to measure the catchment basins and save the blots as a list of ROI; | |
| 7 | Open the stack image(saved in point 4) and propagated to all gel images the list of ROI obtained in by the spot detection procedure “ROI Manager>Show All”; | |
| 8 | Measure the spots volume values using “ROI Manager>Measure” and save the Results as OpenOffice compatible (.ods) file; | http://www.openoffice.org |
| 9 | For quantitative comparison of spot intensities choose Integrated Density measure. This value is the integral of all pixel intensities within the spot boundary; | |
| 10 | Normalize the volume of each spot on a given gel image versus the total volume of all spots on that image, perform the ANOVA Test on normalized data. | |

Material and Methods

2-DE page

With this aim, we enrolled 9 patients admitted within 6 hours after the onset of chest pain symptoms, with myocardial infarction defined according to ESC/ACC criteria. All subjects signed informed consent forms prior to standard sample collection. 2D-GE was performed according to Maresca et al²⁰ and each sample was run in duplicate. For the first-dimension electrophoresis of plasma samples 200 µg (approximately 3 µl) were applied to 18-cm linear IPG strips 4–7 GE Healthcare (Uppsala, Sweden) and focused until 72000 V/hr were reached. Prior to SDS-PAGE, the IPG strips were equilibrated twice for 15 min in equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and traces of bromophenol blue) containing 1% (w/v) DTT for the first equilibration step and 2.5% (w/v) iodoacetamide for the second step. SDS-PAGE was performed on 12.5% polyacrylamide gels according to Laemmli.²¹

The run was carried out at 60 mA/gel at 16 °C and terminated when the dye front reached the lower end of the gel. Gels of plasma samples were visibly stained with Coomassie Blue, scanned using transmission mode to avoid saturation effects and saved in 16-bit TIFF format.

Image analysis

Once all gels in the study had been collected and digitalized, they were analyzed using the ImageJ and some of its plugins or the commercial software Melanie.

We now go through the ImageJ-based procedure, and then we will compare the results obtained to the Melanie output. In Table 2 is collected the list of steps that describes how to perform the analysis using ImageJ and its plug-in.

First, all images were warped by bUnwarpJ,¹⁴ an algorithm for elastic and consistent image registration developed as an ImageJ plug-in. It performs a simultaneous registration of two images, allowing us to solve

the problem of spatial distortions due to run-time differences and dye-front deformations.

We used the software to align all images in pairs, taking always the same image as reference and producing the corresponding warped images of the others. bUnwarpJ can be freely downloaded from <http://biocomp.cnb.uam.es/~iarganda/bUnwarpJ/>.

The reference image and warped images were subsequently displayed in a single stack image and summed to generate a fused image. We followed an image sum approach to retain as much information as possible from the original images.

Spot detection was performed on the fused image by the watershed plug-in written by Daniel Sage and freely downloaded from <http://bigwww.epfl.ch/sage/soft/watershed/index.html>. This plug-in is able to segment an image using the watershed algorithm by flooding directly on graylevel image. Of the several kind of outputs provided, we selected the binary output that allows us to apply the blob analyzer of ImageJ, so as to measure the catchment basins and save the blots, one for each protein spot, as a list of regions of interest (ROI). Each ROI corresponds exactly to a spot in the fused image. The list of ROI obtained by the spot detection procedure was our consensus spot pattern that is valid for the whole gel set of the experiment.

In other words, the list of ROI obtained is equivalent to the grid used in gene chip analysis, this grid was imposed on each of the aligned gel images so that a defined number of areas were quantified on every gel image of the experiment. The spot volume values extracted from each image were listed in a ImageJ “Results table”. The resulting table of “the whole image information procedure” did not have empty cells, while some commercial software, such as Melanie, are not able to eliminate all bias due to missing spot values.²² All the data were analyzed by Calc (OpenOffice), a open source spreadsheet program downloadable from the web site <http://www.openoffice.org/>. For the normalization the volume of each spot on a given gel image was divided by the total volume of all spots on that image.²³ The resulting table of our method did not have empty cells, while some commercial software, such as Melanie, are not able to eliminate all bias due to missing spot values.²²

Results

The warping step has produced a good alignment of all 2D-GE images. In Figure 1 is shown an example

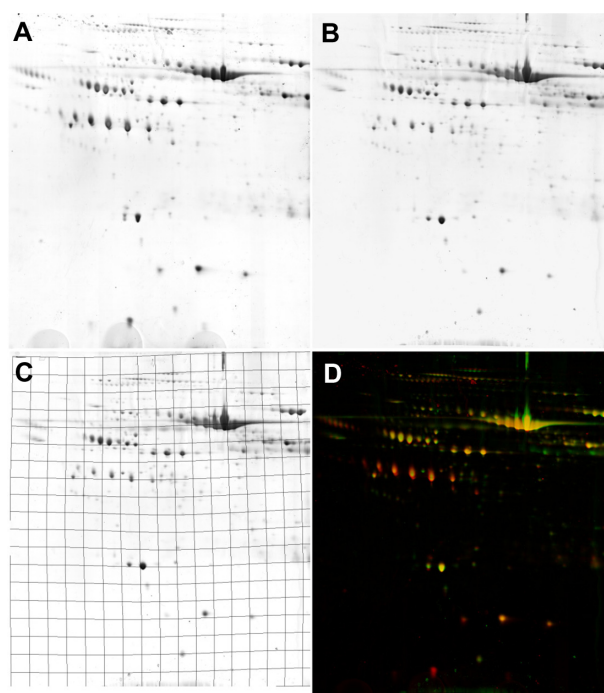


Figure 1. (A and B) shown two different 2D-GE images of two control subjects. In (C) is shown the elastic registration obtained during using bUnwarpJ plug-in. In Figure 1D is shown the overlap of the two gels after the warping step, in the red channel is shown the reference gel (Fig. 1A) and in the green channel is shown a warped gel.

of warping step results. Figure 1A and B show two different 2D-GE images, in Figure 1C is shown the elastic registration obtained during the warping, and in Figure 1D the overlap of the two gels after the warping step. For the image fusion process all the warped images were used and the fused images do not show multiple spots thanks to the strength of the elastic alignment.

Using the ImageJ procedure we were able to study 232 conserved spots, while with Melanie we analyzed

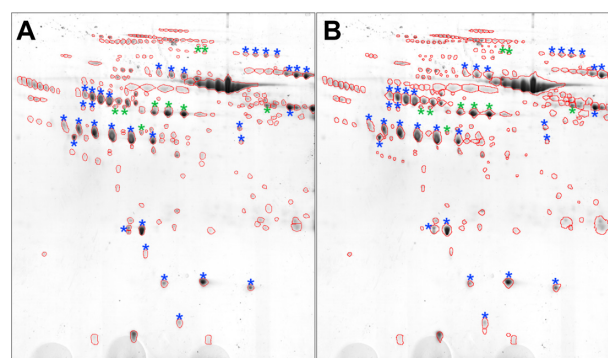


Figure 2. 2D-GE from the plasma of a control individual. (A) Spots detected and matched using Melanie. (B) Spots detected using the ImageJ procedure.

Note: The stars show the spots used for comparison of quantification methods (unchanged spots in blue, differential spots in green).

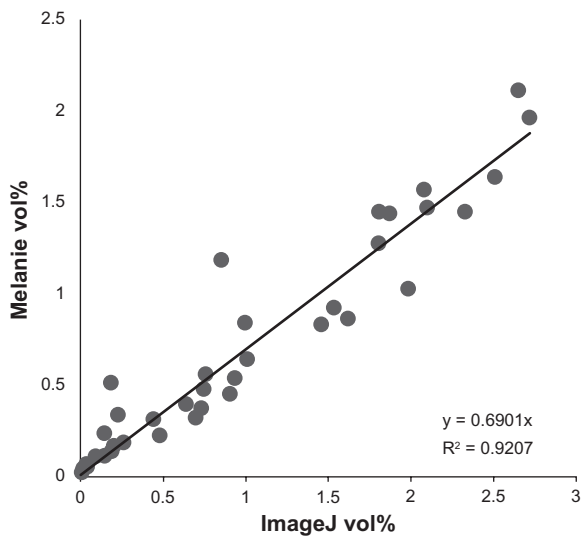


Figure 3. Scatter plot of spot mean volumes as evaluated by Melanie and ImageJ.

Notes: The ImageJ values are plotted on the X-axis, the Malenie values on the Y-axis. Spots were normalized based on total spot volume.

a pattern of 205 matched spots. The spot detection and the matching were manually checked in both the procedures; Figure 2 shows the spots detected by the two procedure on one of the control subject gels (the reference gel used for the Melanie analysis).

The scatter plot in Figure 3 shows that there is a linear relationship between the spot volumes evaluated by Melanie and the corresponding values obtained by the ImageJ procedure. In particular 42 spots, 33 more abundant spots (blue stars in Fig. 2) and the 9 differentially expressed spots (green stars in Fig. 2, see the next paragraph for the identification procedure) were considered for the comparison; the fact that the straight line in Figure 3 has a slope <1 means that volume values calculated for the same spot are on average larger by using the ImageJ procedure, which can be related to the slightly larger area segmented for each spot by ImageJ due to the fact that spots were segmented on the fused image (and not on every single gel, as Melanie does). Similar results were obtained for the spot list of every other gel as well as for the average volumes (data not shown).

The 9 differential spots (shown in Figure 4), whose mean normalized volume was significantly decreased in the myocardial infarction versus the control group, were all identified by *t* test (P -value <0.001); the test was run independently on the list of the spots, quantified by our procedure or by Melanie. 7 out

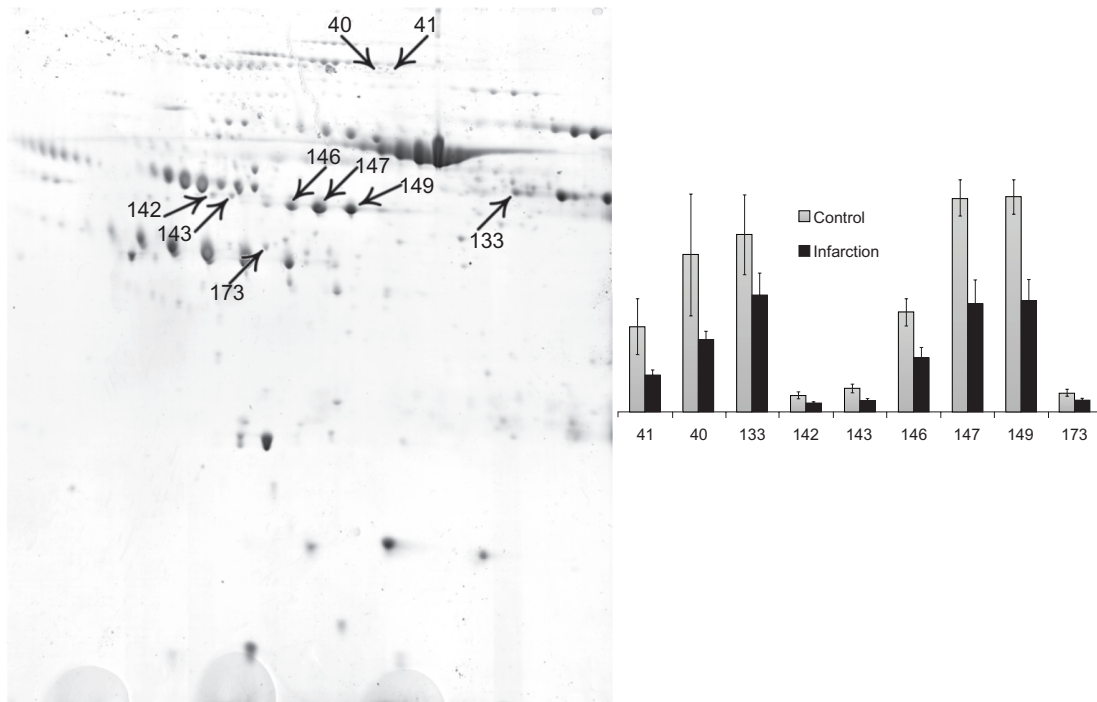


Figure 4. Profile of proteins differentially expressed in the plasma of myocardial infarction (MI) patients vs. control subjects. 7 spots were found significantly decreased in the plasma of the myocardial infarction patients by both methods (P -value <0.001 , ANOVA test). Spots 133 and 173 were found to be differentially expressed by one method only (see text for explanation). Spots 142, 143, 146, 147 and 149 were identified as fibrinogen gamma chain fragments. In supplementary materials is shown the table with all raw data of the spots.



of the 9 spots were well above the selected P -value threshold for both Melanie and the ImageJ-based procedure, while each of the 2 methods identified an additional spot which was missed by the other (with reference to Figure 4, spots 133 and 173 where identified only by Melanie and ImageJ respectively). These 2 spots have a P -value slightly higher than the threshold and anyway with a significance under 0.05. All data of the spots are shown in table in supplementary material.

By using the procedure described by Lemkin et al,²⁴ ie, by matching the spots of a gel with those of a reference map of human plasma (<http://expasy.org/swiss-2dpage/viewer>), we were able to tentatively identify 5 of the 7 significantly different spots as fibrinogen gamma chain fragments (with reference to Figure 4, spots 142, 143, 146, 147, and 148).

Discussion and Conclusions

Previous proteomic studies reported protein expression differences in plasma from patients during an acute coronary syndrome and from patients with moderate hypercholesterolemia,²⁵ and proteomic differences in the plasma of coronary ischemic patients resistant to aspirin as compared to aspirin-sensitive patients.²⁶ Interestingly, 3 of the very same gamma fibrinogen spots were reported as increased in untreated myocardial infarction,²⁵ but thrombolytic (fibrinolytic) therapy reduces the level of all fibrinogen chains (see for example²⁷). Since all the enrolled patients received an anticoagulant therapy, this decrease is possibly connected to the therapy, and thus may reflect modifications of the fibrin/fibrinogen balance in the patient blood.

Further experimental work in a larger patient cohort will be needed to confirm these data, and the study of the effects of anticoagulant therapy on hospitalized patients goes beyond the purpose of this work.

In conclusion, we have developed a free and easy alternative to a common commercial package for the segmentation and quantification of 2D gel spots; the procedure proved to be so effective, to confirm the results obtained by an established commercial solution.

We hope that the provided solution can help proteomic laboratories to quickly and inexpensively evaluate 2D-gel experimental results, without losing the required accuracy and providing a common reference for future analyses.

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Disclosures

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Supplementary Materials

The table shows the data obtained for ImageJ based analysis. The spots in green have a P -value < 0.001 (these spots are shown in Fig. 3). In yellow is shown the spot 173, this spot has a P -value < 0.05 in ImageJ analysis and P -value < 0.001 in Melanie analysis (this spot is shown in Fig. 3).

| | Control mean | Control St_Dev | Control median | Infarction mean | Infarction St_Dev | Infarction median | T test P-value | Wilcoxon P-value |
|-----|--------------|----------------|----------------|-----------------|-------------------|-------------------|----------------|------------------|
| 149 | 1.401 | 0.114 | 1.421 | 0.721 | 0.146 | 0.705 | 0.0000001 | 0 |
| 147 | 1.394 | 0.117 | 1.355 | 0.702 | 0.155 | 0.656 | 0.0000001 | 0 |
| 146 | 0.653 | 0.092 | 0.656 | 0.351 | 0.068 | 0.329 | 0.0000179 | 0 |
| 143 | 0.154 | 0.028 | 0.148 | 0.071 | 0.016 | 0.066 | 0.0000692 | 0 |
| 41 | 0.016 | 0.005 | 0.013 | 0.007 | 0.001 | 0.007 | 0.0001684 | 0 |
| 142 | 0.107 | 0.022 | 0.096 | 0.055 | 0.010 | 0.051 | 0.0004514 | 0 |
| 133 | 0.124 | 0.023 | 0.118 | 0.076 | 0.015 | 0.067 | 0.0006462 | 0 |
| 40 | 0.029 | 0.011 | 0.024 | 0.013 | 0.002 | 0.013 | 0.0009521 | 0 |
| 79 | 0.052 | 0.014 | 0.054 | 0.025 | 0.009 | 0.022 | 0.0020000 | 0.0020000 |
| 154 | 0.196 | 0.018 | 0.191 | 0.150 | 0.031 | 0.158 | 0.0020000 | 0.0020000 |
| 135 | 1.156 | 0.258 | 1.180 | 0.759 | 0.144 | 0.771 | 0.0050000 | 0.0040000 |
| 173 | 0.119 | 0.040 | 0.137 | 0.059 | 0.024 | 0.051 | 0.0060000 | 0.0120000 |
| 144 | 0.018 | 0.004 | 0.017 | 0.013 | 0.002 | 0.012 | 0.0070000 | 0.0000000 |
| 157 | 0.006 | 0.001 | 0.006 | 0.005 | 0.001 | 0.005 | 0.0070000 | 0.0060000 |
| 25 | 0.151 | 0.059 | 0.164 | 0.218 | 0.031 | 0.219 | 0.0250000 | 0.0120000 |
| 124 | 0.352 | 0.043 | 0.365 | 0.297 | 0.046 | 0.300 | 0.0260000 | 0.0320000 |
| 199 | 0.019 | 0.006 | 0.016 | 0.027 | 0.005 | 0.027 | 0.0260000 | 0.0320000 |
| 4 | 0.093 | 0.034 | 0.075 | 0.130 | 0.025 | 0.131 | 0.0340000 | 0.0420000 |
| 150 | 0.386 | 0.106 | 0.359 | 0.277 | 0.057 | 0.266 | 0.0390000 | 0.0080000 |
| 182 | 0.197 | 0.018 | 0.199 | 0.221 | 0.024 | 0.211 | 0.0430000 | 0.0900000 |
| 224 | 0.175 | 0.031 | 0.165 | 0.141 | 0.030 | 0.153 | 0.0450000 | 0.0420000 |
| 22 | 0.191 | 0.085 | 0.206 | 0.273 | 0.049 | 0.255 | 0.0490000 | 0.0540000 |
| 1 | 0.036 | 0.011 | 0.032 | 0.054 | 0.021 | 0.050 | 0.0550000 | 0.0720000 |
| 177 | 0.461 | 0.096 | 0.463 | 0.626 | 0.212 | 0.676 | 0.0610000 | 0.1420000 |
| 129 | 0.135 | 0.051 | 0.114 | 0.189 | 0.058 | 0.185 | 0.0660000 | 0.0720000 |
| 5 | 0.065 | 0.024 | 0.052 | 0.088 | 0.024 | 0.090 | 0.0780000 | 0.0900000 |
| 183 | 0.202 | 0.011 | 0.203 | 0.226 | 0.034 | 0.224 | 0.0800000 | 0.2100000 |
| 46 | 0.034 | 0.011 | 0.034 | 0.024 | 0.010 | 0.021 | 0.0810000 | 0.0420000 |
| 21 | 0.227 | 0.104 | 0.251 | 0.320 | 0.088 | 0.334 | 0.0820000 | 0.1740000 |
| 2 | 0.087 | 0.030 | 0.073 | 0.115 | 0.029 | 0.113 | 0.0830000 | 0.0900000 |
| 223 | 0.025 | 0.005 | 0.024 | 0.042 | 0.026 | 0.029 | 0.0860000 | 0.0220000 |
| 103 | 0.439 | 0.036 | 0.452 | 0.402 | 0.046 | 0.400 | 0.0950000 | 0.1420000 |
| 73 | 0.469 | 0.133 | 0.484 | 0.606 | 0.179 | 0.555 | 0.1030000 | 0.0900000 |
| 165 | 0.128 | 0.047 | 0.105 | 0.205 | 0.119 | 0.140 | 0.1040000 | 0.1140000 |
| 3 | 0.094 | 0.038 | 0.072 | 0.123 | 0.024 | 0.128 | 0.1090000 | 0.1740000 |
| 139 | 0.686 | 0.091 | 0.685 | 0.552 | 0.209 | 0.496 | 0.1110000 | 0.0900000 |
| 136 | 0.146 | 0.028 | 0.138 | 0.121 | 0.030 | 0.130 | 0.1140000 | 0.1740000 |
| 118 | 0.416 | 0.035 | 0.408 | 0.379 | 0.056 | 0.383 | 0.1220000 | 0.3000000 |
| 110 | 0.087 | 0.009 | 0.084 | 0.079 | 0.010 | 0.079 | 0.1280000 | 0.1420000 |
| 43 | 0.006 | 0.005 | 0.003 | 0.002 | 0.000 | 0.002 | 0.1300000 | 0.0120000 |

(Continued)



(Continued)

| | Control mean | Control St_Dev | Control median | Infarction mean | Infarction St_Dev | Infarction median | T test P-value | Wilcoxon P-value |
|-----|---------------------|-----------------------|-----------------------|------------------------|--------------------------|--------------------------|-----------------------|-------------------------|
| 74 | 1.095 | 0.279 | 1.187 | 1.341 | 0.332 | 1.273 | 0.1300000 | 0.2520000 |
| 122 | 0.018 | 0.003 | 0.017 | 0.015 | 0.003 | 0.015 | 0.1300000 | 0.1140000 |
| 170 | 0.028 | 0.002 | 0.028 | 0.041 | 0.023 | 0.033 | 0.1340000 | 0.1420000 |
| 54 | 0.010 | 0.002 | 0.009 | 0.012 | 0.003 | 0.012 | 0.1360000 | 0.1140000 |
| 60 | 0.052 | 0.010 | 0.052 | 0.060 | 0.011 | 0.062 | 0.1360000 | 0.2100000 |
| 163 | 0.155 | 0.014 | 0.157 | 0.142 | 0.020 | 0.142 | 0.1420000 | 0.2100000 |
| 44 | 0.007 | 0.006 | 0.003 | 0.003 | 0.000 | 0.003 | 0.1480000 | 0.1140000 |
| 78 | 0.077 | 0.023 | 0.082 | 0.110 | 0.058 | 0.128 | 0.1480000 | 0.2100000 |
| 86 | 0.007 | 0.001 | 0.007 | 0.008 | 0.002 | 0.008 | 0.1540000 | 0.2100000 |
| 141 | 0.078 | 0.072 | 0.042 | 0.033 | 0.012 | 0.028 | 0.1540000 | 0.3520000 |
| 42 | 0.009 | 0.008 | 0.004 | 0.004 | 0.001 | 0.004 | 0.1570000 | 0.2520000 |
| 219 | 0.244 | 0.031 | 0.233 | 0.201 | 0.077 | 0.164 | 0.1570000 | 0.0720000 |
| 6 | 0.023 | 0.008 | 0.020 | 0.029 | 0.008 | 0.030 | 0.1590000 | 0.1740000 |
| 30 | 0.064 | 0.025 | 0.066 | 0.087 | 0.038 | 0.079 | 0.1590000 | 0.2100000 |
| 29 | 0.114 | 0.041 | 0.127 | 0.144 | 0.037 | 0.153 | 0.1610000 | 0.1740000 |
| 168 | 0.036 | 0.013 | 0.035 | 0.054 | 0.034 | 0.044 | 0.1660000 | 0.1420000 |
| 39 | 0.016 | 0.004 | 0.014 | 0.014 | 0.003 | 0.014 | 0.1730000 | 0.3520000 |
| 47 | 0.088 | 0.027 | 0.077 | 0.111 | 0.038 | 0.100 | 0.1740000 | 0.3000000 |
| 178 | 0.025 | 0.006 | 0.027 | 0.029 | 0.006 | 0.029 | 0.1780000 | 0.1740000 |
| 121 | 0.347 | 0.112 | 0.336 | 0.421 | 0.102 | 0.420 | 0.1970000 | 0.1740000 |
| 115 | 1.627 | 0.167 | 1.669 | 1.733 | 0.147 | 1.691 | 0.2090000 | 0.3000000 |
| 231 | 0.043 | 0.012 | 0.040 | 0.091 | 0.104 | 0.041 | 0.2120000 | 0.5360000 |
| 58 | 0.078 | 0.024 | 0.079 | 0.061 | 0.027 | 0.057 | 0.2130000 | 0.1740000 |
| 137 | 0.050 | 0.041 | 0.033 | 0.028 | 0.013 | 0.021 | 0.2140000 | 0.2520000 |
| 15 | 0.029 | 0.026 | 0.018 | 0.046 | 0.028 | 0.035 | 0.2170000 | 0.1740000 |
| 215 | 0.614 | 0.039 | 0.597 | 0.579 | 0.069 | 0.587 | 0.2200000 | 0.2520000 |
| 111 | 0.164 | 0.030 | 0.152 | 0.141 | 0.041 | 0.132 | 0.2280000 | 0.1740000 |
| 123 | 0.208 | 0.028 | 0.217 | 0.232 | 0.048 | 0.211 | 0.2340000 | 0.7580000 |
| 172 | 0.359 | 0.133 | 0.391 | 0.286 | 0.091 | 0.300 | 0.2370000 | 0.2520000 |
| 229 | 0.183 | 0.138 | 0.095 | 0.106 | 0.096 | 0.075 | 0.2380000 | 0.6060000 |
| 159 | 0.037 | 0.008 | 0.036 | 0.044 | 0.013 | 0.043 | 0.2410000 | 0.3000000 |
| 200 | 0.100 | 0.055 | 0.110 | 0.073 | 0.025 | 0.063 | 0.2490000 | 0.5360000 |
| 228 | 0.528 | 0.225 | 0.587 | 0.659 | 0.203 | 0.681 | 0.2500000 | 0.2100000 |
| 48 | 0.192 | 0.056 | 0.174 | 0.222 | 0.041 | 0.217 | 0.2580000 | 0.1740000 |
| 195 | 0.017 | 0.002 | 0.017 | 0.023 | 0.016 | 0.017 | 0.2590000 | 0.3000000 |
| 107 | 0.049 | 0.011 | 0.045 | 0.044 | 0.003 | 0.044 | 0.2650000 | 0.6800000 |
| 131 | 0.595 | 0.075 | 0.577 | 0.652 | 0.123 | 0.673 | 0.2700000 | 0.1140000 |
| 102 | 0.172 | 0.026 | 0.181 | 0.160 | 0.014 | 0.160 | 0.2710000 | 0.2520000 |
| 20 | 0.289 | 0.133 | 0.270 | 0.360 | 0.109 | 0.362 | 0.2720000 | 0.4080000 |
| 19 | 0.217 | 0.116 | 0.206 | 0.276 | 0.074 | 0.269 | 0.2740000 | 0.3520000 |
| 76 | 0.028 | 0.009 | 0.028 | 0.032 | 0.007 | 0.033 | 0.2780000 | 0.6060000 |
| 98 | 0.453 | 0.088 | 0.456 | 0.498 | 0.064 | 0.515 | 0.2790000 | 0.3000000 |
| 184 | 0.058 | 0.023 | 0.053 | 0.046 | 0.015 | 0.049 | 0.2890000 | 0.2520000 |
| 132 | 0.275 | 0.063 | 0.291 | 0.246 | 0.031 | 0.258 | 0.2900000 | 0.3000000 |
| 160 | 0.013 | 0.002 | 0.013 | 0.015 | 0.004 | 0.016 | 0.2990000 | 0.3520000 |
| 210 | 0.016 | 0.001 | 0.016 | 0.019 | 0.007 | 0.016 | 0.3020000 | 0.6800000 |
| 188 | 0.200 | 0.035 | 0.205 | 0.179 | 0.044 | 0.200 | 0.3130000 | 0.4080000 |

(Continued)



(Continued)

| | Control mean | Control St_Dev | Control median | Infarction mean | Infarction St_Dev | Infarction median | T test P-value | Wilcoxon P-value |
|-----|--------------|----------------|----------------|-----------------|-------------------|-------------------|----------------|------------------|
| 7 | 0.039 | 0.016 | 0.042 | 0.046 | 0.014 | 0.049 | 0.3260000 | 0.2520000 |
| 31 | 0.023 | 0.009 | 0.026 | 0.027 | 0.007 | 0.029 | 0.3380000 | 0.2520000 |
| 70 | 0.207 | 0.073 | 0.195 | 0.241 | 0.062 | 0.229 | 0.3380000 | 0.3000000 |
| 203 | 0.025 | 0.004 | 0.025 | 0.034 | 0.027 | 0.026 | 0.3400000 | 1.0000000 |
| 230 | 0.278 | 0.257 | 0.192 | 0.421 | 0.326 | 0.229 | 0.3440000 | 0.1740000 |
| 45 | 0.224 | 0.237 | 0.136 | 0.132 | 0.031 | 0.121 | 0.3470000 | 0.6060000 |
| 66 | 0.405 | 0.052 | 0.394 | 0.377 | 0.063 | 0.396 | 0.3480000 | 0.5360000 |
| 212 | 0.009 | 0.001 | 0.009 | 0.010 | 0.003 | 0.009 | 0.3490000 | 0.5360000 |
| 10 | 0.114 | 0.057 | 0.122 | 0.137 | 0.025 | 0.141 | 0.3510000 | 0.4080000 |
| 67 | 1.791 | 0.326 | 1.845 | 1.975 | 0.436 | 1.851 | 0.3510000 | 0.4080000 |
| 57 | 0.016 | 0.007 | 0.015 | 0.013 | 0.004 | 0.013 | 0.3520000 | 0.4080000 |
| 130 | 0.090 | 0.012 | 0.090 | 0.083 | 0.015 | 0.089 | 0.3530000 | 0.6060000 |
| 164 | 0.043 | 0.004 | 0.046 | 0.045 | 0.007 | 0.045 | 0.3560000 | 0.4700000 |
| 11 | 0.101 | 0.050 | 0.107 | 0.121 | 0.026 | 0.118 | 0.3580000 | 0.4700000 |
| 82 | 0.019 | 0.018 | 0.013 | 0.012 | 0.002 | 0.012 | 0.3620000 | 0.5360000 |
| 27 | 0.005 | 0.001 | 0.005 | 0.005 | 0.001 | 0.005 | 0.3650000 | 0.3000000 |
| 117 | 0.238 | 0.063 | 0.243 | 0.280 | 0.115 | 0.259 | 0.3660000 | 0.6800000 |
| 65 | 0.239 | 0.076 | 0.249 | 0.268 | 0.031 | 0.264 | 0.3700000 | 0.4700000 |
| 204 | 0.062 | 0.014 | 0.068 | 0.055 | 0.020 | 0.056 | 0.3700000 | 0.4700000 |
| 64 | 0.105 | 0.029 | 0.097 | 0.116 | 0.012 | 0.115 | 0.3760000 | 0.3520000 |
| 96 | 0.230 | 0.042 | 0.223 | 0.246 | 0.027 | 0.245 | 0.3880000 | 0.2520000 |
| 8 | 0.055 | 0.025 | 0.061 | 0.066 | 0.019 | 0.069 | 0.3930000 | 0.4080000 |
| 55 | 37.791 | 2.655 | 38.727 | 39.108 | 3.319 | 39.207 | 0.3930000 | 0.6800000 |
| 9 | 0.091 | 0.045 | 0.097 | 0.108 | 0.025 | 0.120 | 0.3940000 | 0.3520000 |
| 214 | 1.888 | 0.160 | 1.946 | 1.793 | 0.271 | 1.846 | 0.3960000 | 0.3520000 |
| 33 | 0.068 | 0.026 | 0.075 | 0.078 | 0.014 | 0.077 | 0.3970000 | 0.6060000 |
| 201 | 0.020 | 0.006 | 0.019 | 0.026 | 0.019 | 0.020 | 0.3980000 | 0.7580000 |
| 53 | 0.015 | 0.005 | 0.012 | 0.017 | 0.005 | 0.017 | 0.4050000 | 0.5360000 |
| 221 | 0.153 | 0.019 | 0.149 | 0.143 | 0.030 | 0.135 | 0.4090000 | 0.1140000 |
| 13 | 0.036 | 0.031 | 0.024 | 0.049 | 0.032 | 0.036 | 0.4110000 | 0.4080000 |
| 56 | 0.023 | 0.009 | 0.024 | 0.019 | 0.007 | 0.018 | 0.4110000 | 0.4080000 |
| 227 | 0.457 | 0.236 | 0.447 | 0.364 | 0.191 | 0.370 | 0.4110000 | 0.6060000 |
| 80 | 0.177 | 0.048 | 0.165 | 0.160 | 0.021 | 0.161 | 0.4120000 | 0.5360000 |
| 104 | 0.043 | 0.007 | 0.044 | 0.040 | 0.007 | 0.041 | 0.4130000 | 0.3520000 |
| 151 | 0.300 | 0.162 | 0.246 | 0.244 | 0.082 | 0.226 | 0.4270000 | 0.5360000 |
| 12 | 0.087 | 0.042 | 0.084 | 0.101 | 0.021 | 0.101 | 0.4320000 | 0.3520000 |
| 89 | 0.066 | 0.031 | 0.053 | 0.056 | 0.017 | 0.050 | 0.4360000 | 0.3520000 |
| 209 | 0.018 | 0.001 | 0.018 | 0.020 | 0.006 | 0.018 | 0.4390000 | 1.0000000 |
| 232 | 0.043 | 0.056 | 0.021 | 0.026 | 0.011 | 0.022 | 0.4510000 | 0.6800000 |
| 94 | 0.667 | 0.141 | 0.668 | 0.716 | 0.102 | 0.767 | 0.4520000 | 0.4700000 |
| 51 | 0.128 | 0.057 | 0.126 | 0.109 | 0.039 | 0.112 | 0.4540000 | 0.5360000 |
| 114 | 1.760 | 0.348 | 1.904 | 1.873 | 0.238 | 1.887 | 0.4780000 | 0.6800000 |
| 26 | 0.049 | 0.019 | 0.043 | 0.043 | 0.012 | 0.043 | 0.4800000 | 0.7580000 |
| 208 | 0.211 | 0.034 | 0.206 | 0.223 | 0.032 | 0.227 | 0.4820000 | 0.6060000 |
| 72 | 0.085 | 0.032 | 0.083 | 0.095 | 0.016 | 0.091 | 0.4850000 | 0.2520000 |
| 38 | 0.005 | 0.002 | 0.005 | 0.004 | 0.001 | 0.004 | 0.4890000 | 0.9180000 |
| 108 | 1.147 | 0.336 | 1.278 | 1.253 | 0.226 | 1.254 | 0.4890000 | 0.7580000 |

(Continued)



(Continued)

| | Control mean | Control St_Dev | Control median | Infarction mean | Infarction St_Dev | Infarction median | T test P-value | Wilcoxon P-value |
|-----|---------------------|-----------------------|-----------------------|------------------------|--------------------------|--------------------------|-----------------------|-------------------------|
| 77 | 0.897 | 0.118 | 0.914 | 0.951 | 0.195 | 0.937 | 0.5030000 | 0.6800000 |
| 197 | 0.014 | 0.004 | 0.015 | 0.013 | 0.002 | 0.013 | 0.5060000 | 0.4700000 |
| 207 | 0.019 | 0.004 | 0.019 | 0.018 | 0.005 | 0.017 | 0.5080000 | 0.5360000 |
| 34 | 0.050 | 0.020 | 0.062 | 0.056 | 0.013 | 0.058 | 0.5180000 | 0.6800000 |
| 125 | 0.044 | 0.006 | 0.047 | 0.046 | 0.006 | 0.045 | 0.5210000 | 0.8380000 |
| 156 | 0.026 | 0.005 | 0.025 | 0.032 | 0.026 | 0.022 | 0.5220000 | 0.5360000 |
| 88 | 0.296 | 0.091 | 0.265 | 0.273 | 0.033 | 0.272 | 0.5290000 | 1.0000000 |
| 218 | 1.225 | 0.365 | 1.215 | 1.125 | 0.192 | 1.101 | 0.5290000 | 0.6060000 |
| 18 | 0.132 | 0.065 | 0.133 | 0.150 | 0.035 | 0.150 | 0.5370000 | 0.7580000 |
| 153 | 0.018 | 0.004 | 0.016 | 0.016 | 0.003 | 0.017 | 0.5370000 | 0.4700000 |
| 206 | 0.207 | 0.056 | 0.196 | 0.189 | 0.052 | 0.193 | 0.5370000 | 0.6060000 |
| 158 | 0.205 | 0.044 | 0.186 | 0.222 | 0.061 | 0.234 | 0.5380000 | 0.3000000 |
| 155 | 0.107 | 0.025 | 0.098 | 0.099 | 0.024 | 0.102 | 0.5470000 | 0.7580000 |
| 93 | 0.954 | 0.218 | 0.873 | 1.014 | 0.153 | 1.101 | 0.5520000 | 0.5360000 |
| 190 | 0.015 | 0.005 | 0.015 | 0.016 | 0.005 | 0.015 | 0.5550000 | 0.7580000 |
| 92 | 0.195 | 0.084 | 0.164 | 0.175 | 0.024 | 0.169 | 0.5580000 | 0.8380000 |
| 32 | 0.046 | 0.016 | 0.051 | 0.050 | 0.013 | 0.056 | 0.5610000 | 0.4080000 |
| 116 | 0.074 | 0.008 | 0.074 | 0.078 | 0.020 | 0.081 | 0.5640000 | 0.4080000 |
| 225 | 1.178 | 0.652 | 1.517 | 0.993 | 0.659 | 1.110 | 0.5840000 | 0.7580000 |
| 52 | 0.010 | 0.004 | 0.008 | 0.012 | 0.005 | 0.011 | 0.5910000 | 0.6060000 |
| 24 | 0.025 | 0.009 | 0.023 | 0.022 | 0.005 | 0.023 | 0.5920000 | 0.7580000 |
| 87 | 0.215 | 0.070 | 0.204 | 0.199 | 0.040 | 0.190 | 0.5960000 | 0.6800000 |
| 176 | 0.220 | 0.029 | 0.227 | 0.231 | 0.051 | 0.216 | 0.5980000 | 0.8380000 |
| 120 | 0.164 | 0.039 | 0.160 | 0.154 | 0.031 | 0.139 | 0.6020000 | 0.5360000 |
| 14 | 0.057 | 0.026 | 0.055 | 0.062 | 0.011 | 0.062 | 0.6030000 | 0.6060000 |
| 71 | 0.699 | 0.071 | 0.686 | 0.675 | 0.110 | 0.684 | 0.6070000 | 0.6800000 |
| 23 | 0.081 | 0.025 | 0.084 | 0.087 | 0.014 | 0.085 | 0.6180000 | 0.5360000 |
| 191 | 0.011 | 0.003 | 0.011 | 0.012 | 0.003 | 0.012 | 0.6200000 | 0.6800000 |
| 140 | 0.075 | 0.051 | 0.046 | 0.063 | 0.040 | 0.038 | 0.6240000 | 0.9180000 |
| 187 | 0.131 | 0.037 | 0.127 | 0.141 | 0.035 | 0.150 | 0.6280000 | 0.5360000 |
| 75 | 1.820 | 0.202 | 1.802 | 1.741 | 0.450 | 1.728 | 0.6470000 | 0.9180000 |
| 16 | 0.044 | 0.018 | 0.048 | 0.047 | 0.008 | 0.046 | 0.6480000 | 1.0000000 |
| 37 | 0.053 | 0.013 | 0.054 | 0.051 | 0.009 | 0.049 | 0.6480000 | 0.4700000 |
| 167 | 0.076 | 0.020 | 0.079 | 0.081 | 0.019 | 0.082 | 0.6480000 | 0.4700000 |
| 222 | 0.059 | 0.018 | 0.054 | 0.063 | 0.011 | 0.058 | 0.6480000 | 0.3000000 |
| 68 | 0.230 | 0.072 | 0.238 | 0.244 | 0.034 | 0.240 | 0.6540000 | 0.8380000 |
| 194 | 0.041 | 0.011 | 0.041 | 0.044 | 0.010 | 0.043 | 0.6570000 | 0.6060000 |
| 217 | 2.982 | 0.719 | 3.044 | 2.837 | 0.501 | 2.737 | 0.6580000 | 0.6060000 |
| 91 | 0.142 | 0.068 | 0.115 | 0.130 | 0.023 | 0.129 | 0.6600000 | 0.6060000 |
| 181 | 0.392 | 0.109 | 0.425 | 0.368 | 0.100 | 0.343 | 0.6600000 | 0.6800000 |
| 192 | 0.025 | 0.007 | 0.023 | 0.026 | 0.006 | 0.026 | 0.6630000 | 0.5360000 |
| 126 | 1.043 | 0.241 | 1.040 | 0.996 | 0.177 | 1.068 | 0.6700000 | 1.0000000 |
| 84 | 0.024 | 0.005 | 0.024 | 0.025 | 0.005 | 0.023 | 0.6720000 | 1.0000000 |
| 106 | 0.403 | 0.071 | 0.395 | 0.388 | 0.064 | 0.412 | 0.6720000 | 0.7580000 |
| 97 | 1.123 | 0.287 | 1.052 | 1.175 | 0.179 | 1.200 | 0.6840000 | 0.5360000 |
| 83 | 0.012 | 0.002 | 0.012 | 0.011 | 0.002 | 0.010 | 0.6860000 | 0.9180000 |
| 152 | 0.430 | 0.111 | 0.394 | 0.411 | 0.066 | 0.391 | 0.6920000 | 0.9180000 |

(Continued)



(Continued)

| | Control mean | Control St_Dev | Control median | Infarction mean | Infarction St_Dev | Infarction median | T test P-value | Wilcoxon P-value |
|-----|-------------------------|---------------------------|---------------------------|----------------------------|------------------------------|------------------------------|---------------------------|-----------------------------|
| 161 | 0.090 | 0.032 | 0.071 | 0.096 | 0.031 | 0.099 | 0.6970000 | 0.7580000 |
| 119 | 0.822 | 0.194 | 0.750 | 0.854 | 0.093 | 0.856 | 0.7060000 | 0.2100000 |
| 105 | 0.541 | 0.229 | 0.496 | 0.578 | 0.134 | 0.574 | 0.7090000 | 0.5360000 |
| 185 | 0.042 | 0.007 | 0.041 | 0.043 | 0.010 | 0.042 | 0.7090000 | 0.6800000 |
| 61 | 0.120 | 0.020 | 0.123 | 0.116 | 0.022 | 0.120 | 0.7140000 | 0.9180000 |
| 220 | 0.112 | 0.022 | 0.116 | 0.118 | 0.040 | 0.127 | 0.7140000 | 0.8380000 |
| 134 | 0.142 | 0.117 | 0.074 | 0.121 | 0.116 | 0.085 | 0.7300000 | 0.7580000 |
| 202 | 0.111 | 0.039 | 0.104 | 0.117 | 0.021 | 0.113 | 0.7310000 | 0.8380000 |
| 99 | 1.134 | 0.315 | 1.046 | 1.182 | 0.207 | 1.137 | 0.7350000 | 0.3520000 |
| 50 | 0.022 | 0.007 | 0.022 | 0.021 | 0.007 | 0.021 | 0.7400000 | 0.6060000 |
| 162 | 0.589 | 0.144 | 0.523 | 0.620 | 0.233 | 0.657 | 0.7430000 | 0.9180000 |
| 85 | 0.038 | 0.012 | 0.035 | 0.037 | 0.008 | 0.033 | 0.7500000 | 0.9180000 |
| 101 | 0.014 | 0.004 | 0.014 | 0.014 | 0.002 | 0.014 | 0.7530000 | 0.9180000 |
| 28 | 0.035 | 0.012 | 0.037 | 0.033 | 0.011 | 0.030 | 0.7660000 | 1.0000000 |
| 100 | 0.032 | 0.016 | 0.029 | 0.034 | 0.008 | 0.033 | 0.7840000 | 0.1740000 |
| 186 | 0.022 | 0.008 | 0.022 | 0.023 | 0.007 | 0.024 | 0.7850000 | 0.6060000 |
| 211 | 0.016 | 0.001 | 0.016 | 0.017 | 0.002 | 0.016 | 0.7850000 | 0.9180000 |
| 90 | 0.283 | 0.092 | 0.245 | 0.273 | 0.042 | 0.269 | 0.7940000 | 0.8380000 |
| 128 | 0.807 | 0.130 | 0.812 | 0.825 | 0.142 | 0.866 | 0.7960000 | 0.6800000 |
| 213 | 0.059 | 0.018 | 0.060 | 0.057 | 0.012 | 0.057 | 0.7980000 | 0.9180000 |
| 145 | 0.030 | 0.017 | 0.022 | 0.032 | 0.013 | 0.027 | 0.8040000 | 0.4700000 |
| 17 | 0.064 | 0.029 | 0.070 | 0.067 | 0.014 | 0.062 | 0.8120000 | 0.9180000 |
| 95 | 0.154 | 0.059 | 0.134 | 0.160 | 0.033 | 0.154 | 0.8180000 | 0.3520000 |
| 196 | 0.030 | 0.009 | 0.028 | 0.029 | 0.009 | 0.033 | 0.8190000 | 0.6800000 |
| 169 | 1.648 | 0.335 | 1.560 | 1.601 | 0.500 | 1.717 | 0.8260000 | 1.0000000 |
| 148 | 0.010 | 0.004 | 0.009 | 0.010 | 0.004 | 0.010 | 0.8560000 | 0.7580000 |
| 205 | 0.025 | 0.009 | 0.024 | 0.024 | 0.004 | 0.024 | 0.8710000 | 0.6800000 |
| 59 | 0.416 | 0.120 | 0.431 | 0.424 | 0.064 | 0.406 | 0.8750000 | 0.6060000 |
| 63 | 0.005 | 0.001 | 0.005 | 0.005 | 0.001 | 0.005 | 0.8780000 | 1.0000000 |
| 62 | 0.087 | 0.017 | 0.084 | 0.086 | 0.011 | 0.088 | 0.8870000 | 0.9180000 |
| 109 | 0.012 | 0.002 | 0.011 | 0.012 | 0.002 | 0.012 | 0.9250000 | 1.0000000 |
| 179 | 0.133 | 0.020 | 0.137 | 0.131 | 0.030 | 0.133 | 0.9270000 | 0.9180000 |
| 127 | 0.715 | 0.125 | 0.745 | 0.719 | 0.111 | 0.689 | 0.9410000 | 1.0000000 |
| 216 | 0.015 | 0.006 | 0.013 | 0.015 | 0.004 | 0.014 | 0.9420000 | 0.8380000 |
| 174 | 0.372 | 0.024 | 0.375 | 0.373 | 0.048 | 0.359 | 0.9480000 | 1.0000000 |
| 69 | 0.036 | 0.014 | 0.039 | 0.036 | 0.007 | 0.033 | 0.9580000 | 0.8380000 |
| 112 | 0.158 | 0.021 | 0.153 | 0.157 | 0.044 | 0.159 | 0.9610000 | 0.7580000 |
| 193 | 0.040 | 0.010 | 0.042 | 0.040 | 0.009 | 0.041 | 0.9610000 | 0.9180000 |
| 171 | 1.646 | 0.348 | 1.625 | 1.655 | 0.481 | 1.882 | 0.9660000 | 0.5360000 |
| 189 | 0.160 | 0.065 | 0.154 | 0.158 | 0.037 | 0.170 | 0.9660000 | 0.6060000 |
| 226 | 0.229 | 0.112 | 0.242 | 0.226 | 0.197 | 0.139 | 0.9660000 | 0.7580000 |
| 166 | 1.161 | 0.242 | 1.068 | 1.155 | 0.439 | 1.272 | 0.9730000 | 0.8380000 |
| 198 | 0.035 | 0.009 | 0.035 | 0.035 | 0.008 | 0.032 | 0.9760000 | 1.0000000 |
| 49 | 0.216 | 0.067 | 0.231 | 0.215 | 0.035 | 0.221 | 0.9800000 | 0.7580000 |
| 81 | 0.014 | 0.003 | 0.014 | 0.014 | 0.003 | 0.014 | 0.9810000 | 1.0000000 |
| 113 | 0.094 | 0.022 | 0.084 | 0.094 | 0.020 | 0.096 | 0.9820000 | 0.6800000 |
| 175 | 1.092 | 0.274 | 1.065 | 1.089 | 0.311 | 1.055 | 0.9830000 | 1.0000000 |

(Continued)



(Continued)

| | Control mean | Control St_Dev | Control median | Infarction mean | Infarction St_Dev | Infarction median | T test P-value | Wilcoxon P-value |
|-----|---------------------|-----------------------|-----------------------|------------------------|--------------------------|--------------------------|-----------------------|-------------------------|
| 180 | 0.077 | 0.011 | 0.080 | 0.077 | 0.012 | 0.080 | 0.9830000 | 0.9180000 |
| 36 | 0.036 | 0.011 | 0.036 | 0.036 | 0.008 | 0.035 | 0.9950000 | 1.0000000 |
| 138 | 0.489 | 0.104 | 0.431 | 0.489 | 0.109 | 0.447 | 0.9970000 | 0.7580000 |
| 35 | 0.020 | 0.009 | 0.015 | 0.020 | 0.007 | 0.018 | 0.9980000 | 0.8380000 |

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