INTRODUCTION

Researchers started to explore new array-based technologies to map metabolites to provide signature characteristics for each disease state by taking snapshot of metabolism. Metabolome analysis is the systematic analysis of metabolites present in a cell which represent hundreds of diverse classes of small or organic molecules, including amino acids, nucleotides, carbohydrates, carboxylic acids, vitamins, and coenzymes [1].

Metabolomics can be used for 2 major different purposes: screening for differences between metabolic fingerprints of cohorts of populations or to understand the regulatory structure of metabolic pathways and its dynamics [2]. Although the general metabolic needs of parasites for their maintenance, growth, and reproduction are similar to those of all living forms, the specific details of pathways vary greatly [3].

Parasitic helminths have an absolute dependency on carbohydrates for their energy source, and one of the key features of carbohydrate catabolism in parasites is the excretion of a wide range of end products from carbohydrate breakdown; which are di- and tricarboxylic acids, most of which have low molecular absorptivity and thus are poorly detectable compounds by photometric detection [1]. Because of the large number and low concentration of many metabolites, metabolome studies require sensitive, selective, and high throughput separation techniques [4]. There are 2 different approaches to metabolite analysis, i.e., comprehensive and selective [5].

The major physiologic and metabolic mechanisms of snail-schistosome relationships that are crucial for survival, growth, and efficient transmission of parasites, remain partially known [6]. Recent studies on the topic have used high performance thin layer chromatography (HPTLC) to examine the pathobiocchemical effects of Schistosoma mansoni infection on the amino acid [7], neutral lipid [8], carbohydrate [9], and content of
Biomphalaria glabrata. Qualitative and quantitative changes in certain analytes were observed as a function of schistosome infection in the snail in all of the afore-mentioned studies.

Carboxylic acids, such as pyruvic, fumaric, malic, oxalic, and acetic play important roles in both aerobic and anaerobic metabolic pathways, as they are involved in the intermediary metabolism of the snail [10]. In a recent work by the authors [11], the potentiality of these acids to be used as diagnostic and therapeutic biomarkers was studied. They were found to be good discriminators between infected and uninfected snails as well as between the different stages of infection. The most suitable acids to be used as drug targets were also detected.

The present study aimed at qualitatively and quantitatively determining these acids in the hemolymph (blood) and digestive gland-gonad complex (DGG) of B. alexandrina after infection with S. mansoni in order to establish correlations between carbohydrate metabolites in different stages of infection.

MATERIALS AND METHODS

Biological materials were obtained from the Medical Malacology Laboratory, Theodor Bilharz Research Institute, Imbaba, Giza, Egypt. A total of 300 snails of an Egyptian strain, including 150 infected cases and 150 controls, were used in this study.

Snail maintenance

Snails were maintained in plastic trays, each containing 10 snails and 1 L of aerated tap water (26 ± 2˚C), replaced twice a week, and fed boiled fresh lettuce and blue green algae [12]. Aquaria were cleaned weekly for removal of feces and dead snails [13]. Laboratory reared B. alexandrina reaching 6-7 mm in shell diameter was exposed to S. mansoni miracidial infection according to Massa et al. [14]. Harvesting of the snails was done as follow: group 1 (G1) included 50 snails of 2 weeks after infection; group 2 (G2) was 50 snails at the time of patency development; and group 3 (G3) was 50 snails of 2 weeks after patency. Uninfected cohort snails of the same shell diameter were maintained in the same manner and harvested at corresponding intervals.

Sample preparation

Hemolymph samples were collected from each snail, after cleaning with a paper towel, using a Pasteur pipette inserted through a tiny hole made in the pericardial region. The samples were then centrifuged at 120 g for 5 min to separate the supernatant from hemocytes and cell debris, and then the supernatant was kept at -20˚C till the time of extraction [14]. The DGG tissue extract samples were prepared by dissection free from the snail body, and then carboxylic acid extraction was done using 50% Ringer’s solution (Carolina Biological Supply, North Carolina, USA). Centrifugation of the extract was done at 250 g for 15 min to collect the supernatants which were stored at -20˚C until use [15].

HPLC analysis

Synthetic standards of acetic, fumaric, oxalic, malic, and pyruvic acids (Sigma-Aldrich, St. Louis, Missouri, USA) in the highest purity grade available were determined by the C18 ion-suppression reversed-phase HPLC according to Lian et al. [16]. Adjusted HPLC graded water as the mobile phase, isocratic elution at a flow rate of 1 ml/min, and the ultraviolet diode array detector at 210 nm with a sensitivity of 0.02 absorbance units were used to obtain chromatograms of the carboxylic acid standards and to determine the retention time of each acid. Calibration curves of each standard were done separately by plotting the peak area of each standard against its concentration, and then pooling of studied carboxylic acid standards was done to obtain calibration curves of studied acid mixtures.

Identification of carboxylic acids in studied samples was made by matching the peak area retention time between standards and studied sample chromatograms. Validation was made by comparing the standards and sample ultraviolet spectra collected by the detector during the separation. Quantitative analyses of the identified acids were done by generating graphs that relate the standard concentrations of the acids to their peak areas. Results were achieved by interpolation within the calibration curve.

Pooling each of 10 snails was used throughout the study, and the mean of 10 trails for each HPLC analysis was done for each sample. The conversions to part per million concentrations (ppm) were done according to Fishel and Mossier [17]. The concentration (ppm) of each acid in snail DGGs was calculated by multiplying the sample solution concentration in parts per million by the original sample volume (V) (ml) and division of the product by the mass of the snail DGG (M) (g) and for hemolymph. Carboxylic acid concentration (µg/dl) was calculated by multiplying I times V times 100, and division of the product by the hemolymph volume (HV) (ml) according to Massa et al. [14]. Statistical analysis of the results was done with Microsoft Excel.
software using the Pearson correlation analysis.

RESULTS

Descriptive statistics of the studied acid concentrations are shown in Tables 1 and 2, and the correlations between the studied acids in hemolymph (H) and tissue samples (T) in different groups (control group, 2-week post exposure group, shedding group, and 2-week post shedding group) are shown in Tables 3-7.

**Table 1.** Concentrations (mean± SD) of 5 acids in the 4 groups of hemolymph samples

|                | Oxalic acid hemolymph | Malic acid hemolymph | Acetic acid hemolymph | Pyruvic acid hemolymph | Fumaric acid hemolymph |
|----------------|-----------------------|----------------------|-----------------------|------------------------|------------------------|
| Control        | 335.62± 229.87        | 10.15± 6.38          | 0.04± 0.05            | 0.13± 0.22             | 0.051± 0.12            |
| G1             | 17.92± 7.02           | 0.92± 0.49           | 0.003± 0.001          | 0.014± 0.005           | 0.007± 0.011           |
| G2             | 61.47± 48.87          | 5.99± 2.52           | 0.019± 0.004          | 0.09± 0.058            | 0.0002± 0.0001         |
| G3             | 214.3± 103.24         | 33.85± 8.65          | 0.090± 0.062          | 0.39± 0.19             | 0.013± 0.005           |

**Table 2.** Concentrations (mean± SD) of 5 acids in the 4 groups of tissue samples

|                | Oxalic acid in tissue | Malic acid in tissue | Acetic acid in tissue | Pyruvic acid in tissue | Fumaric acid in tissue |
|----------------|-----------------------|----------------------|-----------------------|------------------------|------------------------|
| Control        | 208.71± 190.65        | 13.96± 7.06          | 0.02± 0.03            | 2.16± 1.52             | 0.04± 0.02             |
| G1             | 57.26± 24.14          | 2.63± 1.79           | 0.03± 0.01            | 0.32± 0.33             | 0.003± 0.006           |
| G2             | 232.37± 123.69        | 17.08± 6.07          | 0.17± 0.12            | 0.72± 0.63             | 0.005± 0.004           |
| G3             | 566.92± 180.87        | 17.516± 11.89        | 0.29± 0.13            | 0.71± 0.18             | 0.06± 0.01             |

**Table 3.** Correlations between the studied acids in hemolymph (H) and tissue samples (T) in control group

|                | Oxalic H | Oxalic T | Malic H | Malic T | Acetic H | Acetic T | Pyruvic H | Pyruvic T | Fumaric H |
|----------------|----------|----------|---------|---------|----------|----------|-----------|-----------|-----------|
| Oxalic T       | R 0.637  |          |         |         |          |          |           |           |           |
|                | P 0.005  |          |         |         |          |          |           |           |           |
| Malic H        | R 0.27   | 0.056    |         |         |          |          |           |           |           |
|                | P 0.165  | 0.421    |         |         |          |          |           |           |           |
| Malic T        | R -0.016 | 0.031    | -0.168  |         |          |          |           |           |           |
|                | P 0.477  | 0.457    | 0.274   |         |          |          |           |           |           |
| Acetic H       | R -0.041 | -0.03    | 0.096   | 0.175   |          |          |           |           |           |
|                | P 0.442  | 0.132    | 0.367   | 0.267   |          |          |           |           |           |
| Acetic T       | R 0.22   | 0.189    | -0.532  | 0.325   | -0.186   |          |           |           |           |
|                | P 0.215  | 0.25     | 0.021   | 0.119   | 0.253    |          |           |           |           |
| Pyruvic H      | R -0.017 | -0.181   | 0.385   | 0.118   | 0.617    | -0.315   |           |           |           |
|                | P 0.262  | 0.26     | 0.078   | 0.338   | 0.007    | 0.127    |           |           |           |
| Pyruvic T      | R -0.011 | 0.451    | -0.389  | 0.655   | -0.11    | 0.572    | -0.105    |           |           |
|                | P 0.484  | 0.048    | 0.076   | 0.348   | 0.013    | 0.355    |           |           |           |
| Fumaric H      | R 0.462  | 0.192    | 0.198   | -0.101  | -0.065   | -0.118   | -0.117    | -0.256    |           |
|                | P 0.042  | 0.247    | 0.239   | 0.361   | 0.409    | 0.339    | 0.339     | 0.179     |           |
| Fumaric T      | R -0.003 | -0.4     | -0.444  | -0.236  | 0.271    | 0.24     | -0.359    | -0.194    | 0.179     |
|                | P 0.495  | 0.05     | 0.049   | 0.198   | 0.164    | 0.195    | 0.094     | 0.244     | 0.261     |

R, correlation coefficient; P, P-value.

*Significant; **Highly significant.

**Control group**

In hemolymph samples, a significant positive correlation was found between oxalic and fumaric acids, and a highly significant positive correlation was found between acetic and pyruvic acids. In tissue samples, a significant positive correlation was found between oxalic and pyruvic acids and between acetic and pyruvic acids, a highly significant positive correlation was found between malic and pyruvic acids, and a significant negative correlation was found between fumaric and oxalic acids. Other significant negative correlations were also detected between the concentrations of malic acid in hemolymph and acetic acid in tissue ($r = -0.532$) and between the concentrations...
Table 4. Correlations between the studied acids in hemolymph (H) and tissue samples (T) in the 2 weeks post exposure group

|          | Oxalic H | Oxalic T | Malic H | Malic T | Acetic H | Acetic T | Pyruvic H | Pyruvic T | Fumaric H | Fumaric T |
|----------|----------|----------|---------|---------|----------|----------|-----------|-----------|-----------|-----------|
| Oxalic T | R        | 0.41     | P       | 0.247   |          |          |           |           |           |           |
| Malic H  | R        | 0.874    | P       | 0.026*  | 0.441    |          |           |           |           |           |
| Malic T  | R        | 0.057    | P       | 0.464   | 0.215    | 0.36     |           |           |           |           |
| Acetic H | R        | 0.041    | P       | 0.252   | 0.023*   | 0.382    | 0.5       |           |           |           |
| Acetic T | R        | 0.578    | P       | 0.154   | 0.269    | 0.401    | 0.199     | 0.407     |           |           |
| Pyruvic H| R        | 0.394    | P       | 0.256   | 0.136    | 0.144    | 0.104     | 0.277     | 0.477     |           |
| Pyruvic T| R        | 0.099    | P       | 0.437   | 0.256    | 0.384    | 0.05      | 0.45      | 0.164     | 0.149     |
| Fumaric H| R        | -0.7     | P       | 0.094   | 0.161    | 0.327    | 0.304     | 0.218     | 0.01*     | 0.47      | 0.281     |
| Fumaric T| R        | -0.657   | P       | 0.114   | 0.172    | 0.353    | 0.362     | 0.202     | 0.018*    | 0.453     | 0.339     |

R, correlation coefficient; P, P-value.
*Significant; **Highly significant.

of malic acid in hemolymph and fumaric acid in tissue (r = -0.444) (Table 3).

G1 (2 weeks post exposure group)

In hemolymph samples, a significant positive correlation was found between oxalic and malic acids. In tissue samples, a highly significant positive correlation was found between malic and pyruvic acids, and a significant negative correlation was found between acetic and fumaric acids. Other significant correlations were also detected between the followings: a significant positive correlation between the concentrations of acetic acid in hemolymph and oxalic acid in tissue (r = 0.885) and a
Significant negative correlation between the concentrations of fumaric acid in hemolymph and acetic acid in tissue ($r = -0.934$) (Table 4).

**G2 (the shedding group)**

In hemolymph samples, a significant positive correlation was found between oxalic and fumaric acids. In tissue samples, a highly significant positive correlation was found between acetic and pyruvic acids. Other significant correlations were also detected between the followings: a significant positive correlation between the concentrations of oxalic acid in hemolymph and acetic acid in tissue ($r = 0.925$), between the concentrations of fumaric acid in hemolymph and acetic acid in tissue ($r = 0.915$), and between the concentrations of fumaric acid in hemolymph and pyruvic acid in tissue ($r = 0.874$). A highly significant positive correlation was found between the concentrations of oxalic acid in hemolymph and pyruvic acid in tissue ($r = 0.94$) (Table 5).

**G3 (2 weeks post-shedding group)**

In hemolymph samples, a significant positive correlation was found between oxalic and malic acids. In tissue samples, a significant positive correlation was found between fumaric and oxalic acids, and a highly significant negative correlation was found between malic and pyruvic acids. Other significant negative correlations were also detected between the concentrations of fumaric acid in hemolymph and pyruvic acid in tissue ($r = -0.845$) (Table 6).

Finally, highly significant positive correlations were detected between oxalic acid concentration in both hemolymph and tissue samples in the control group and between fumaric acid concentration in both hemolymph and tissue samples in 2...
weeks post-exposure group (Table 7).

DISCUSSION

Despite the difficulties in assessing the causal origin of a specific correlation in metabolomics data, the observed pattern provides information about the metabolic system that allows greater understanding of the underlying process. The observed correlations are properties of the whole system, not of any particular metabolite, enzyme, or reaction [18]. When measuring a population of biological replicates, intrinsic fluctuations may arise due to at least 2 different mechanisms; first, organisms are never actually identical even under identical experimental conditions, there are always inevitable small differences in enzyme concentrations [19], and second, the effects of environmental factors on cellular metabolism [18].

Pooling of each 10 snails was used throughout the study and the mean of 10 trails for each HPLC analysis was done for each sample. Van Saun [20] encouraged the use of pooled samples and stated that, though some variation may be masked, pooled samples may provide an economic alternative to traditional metabolic profiling, as most of the important measures of metabolic status showed minimal differences between pooled and individual samples and found them to be statistically equivalent. He stated that the real challenge of using pooled samples is interpretation. Empirically one can interpret pooled samples by determining how far they deviate from the midpoint of the reference range for the control [19].

Comparing correlations obtained with each single state is more relevant than combining samples of different states, this is particularly important when, in one state there is a high correlation, but not in the other, or when the correlations are both high but of opposite sign [19]. In the present study, some correlations were found to be high within the control group but not in other groups; between oxalic acid concentrations in hemolymph and tissue samples, oxalic and pyruvic acids in tissue samples, pyruvic and acetic acids in hemolymph samples, malic acid concentration in hemolymph, and both acetic and fumaric acid concentrations in tissue samples.

The more striking finding was the existence of reversed correlations, i.e., a situation in which the correlation between 2 metabolites changes its sign [21]. In the present work, although no significant correlation was found between malic and pyruvic acids in all groups in hemolymph samples, a highly significant positive correlation was found between malic and pyruvic acids in the control group and group 1, reversed to a highly significant negative correlation in group 3 in tissue samples. Also, the significant negative correlation that was found between fumaric and oxalic acids in tissue samples in the control group was reversed to a significant positive correlation in group 3. Moreover, the significant negative correlation found between the concentrations of fumaric acid in hemolymph and acetic acid in tissue samples in group 1 was found to be reversed to a positive correlation in group 2, which was then reversed again to a negative correlation in group 3. This points to a marked change in the underlying regulation of the system and possibly reflects the existence of multiple steady states. Indeed, the phenomenon of reversed correlations is also observed in the numerical models of cellular metabolism, involving multi-stationary and switching between different states [22]. However, other causes of reversed correlations are also conceivable and a more detailed evaluation is still needed.

The transition to a different state may not only involve changes in the average levels of the measured metabolites, but also involve changes in their pair-wise correlations. Likewise, a metabolite which shows no significant change in the average level between 2 different experimental conditions may still show an alteration of its pair-wise correlations with other metabolites. This observation leads to an interpretation of the resulting pattern of correlation as a global fingerprint of the state [18].

In our study, the majority of metabolite pairs did not show significant correlations. This was explained by different reports [18,19,21], which stated that metabolite correlations do not necessarily correspond to proximity in the biochemical network as it is noted that neighboring metabolites and directly interacting metabolites in the metabolic network may have little or no correlation. It is not because they are not related, but because the variance in the enzymes that control them also affects them in equal amounts and different directions. This is what happens to most of the metabolite pairs and is the consequence of the systemic nature of metabolic control.

Strong correlations between 2 metabolites are likely explained by the chemical equilibrium between them. Also an interesting prediction, still to be confirmed, is that metabolites sharing conserved moieties should have high correlations and at least one of them being negatively correlated with the others. However, most high correlations may be due to either stronger mutual control by a single enzyme or variation of a single enzyme level much above others. In both cases, it is impossible to identify the responsible enzyme from these data alone, thou-
Although hints can be obtained from the set of metabolites forming correlation clique [22]. Ultimately, further data are required for resolving the responsible regulatory events with protein profiles being the most promising for this effect [18,19].

When 2 metabolites are moderately correlated, it may be due to a large concentration response coefficient towards a common enzyme, or an enzyme that has carries unusually high variance. However, to identify this enzyme requires further data [23].

Metabolites in chemical equilibrium will have nearly perfect positive correlation. As a consequence, metabolites with negative correlation are not in equilibrium. In this case, the correlation does not originate from the enzyme that catalyzes the equilibrium reaction, as the metabolites have very small response towards it [19].

That is, differences or distance in terms of observed correlations should reflect and correspond to differences or distance in states, tissue types, and experimental conditions. The observed correlations should be robust with respect to minor changes in the underlying system, while at the same time they should be susceptible for marked changes in the underlying biochemical system. While the preliminary studies seem to support this view [21,22], large scale comparisons of metabolic correlations are still sparsely reported. Thus, to distinguish the specific mechanisms responsible for an observed correlation does require additional knowledge, and a concluding evaluation of the validity and applicability of large-scale metabolomic correlation analysis requires further experimental verification [18,19].

Consistent differences in both the hemolymph and tissue carbohydrate metabolite correlation profiles of the studied groups represent a promising additional source of information about the state of a metabolic system. However, their interpretation in terms of the underlying biochemical pathways is not straightforward and largely defies an intuitive analysis. These findings highlight the potential of metabolomics as a novel approach for fundamental investigations of host-pathogen interactions as well as for disease surveillance and control.

Identifying enzymes involved in specific metabolic pathway detected only in S. mansoni-infected snails may allow the use of certain enzyme inhibitors as target chemotherapeutic control agents. Alteration in the metabolism or metabolic status may also be used as a control measure without killing snails to keep balanced ecology.

REFERENCES

1. Kaddurah-Daouk R, Kristal B, Bogdhanov M, Matson W, Beal F. Metabolomics: A new approach towards identifying biomarkers and therapeutic targets in CNS disorder. In: Vaidyanathan S, Harrigan G, Goodacre R eds, Metabolome Analyses: Strategies for Systems Biology. New York, USA. Springer. 2005, p 45-62.

2. Nicholson JK, Holmes E, Lindon JC. Metabonomics and metabolomics techniques and their applications in mammalian systems. In: Lindon JC, Nicholson JK, Holmes E eds, Handbook of Metabonomics and Metabolomics. Oxford, UK. Elsevier. 2007, p 1-33.

3. Bush AO, Fernandez JC, Esch GW, Seed BJ. Parasitism: The diversity and ecology of animal parasites. 1st ed. London, UK. Cambridge University Press. 2001.

4. Villas-Bôas SG, Hojer-Pedersen J, Akesson M, Smedsgaard J, Nielsen J. Global metabolite analysis of yeast: evaluation of sample preparation methods. Yeast 2005; 22: 1155-1169.

5. Nicholson JK, Holmes E, Lindon JC, Wilson ID. The challenges of modeling mammalian biocomplexity. Nat Biotechnol 2004; 22: 1268-1274.

6. Khayath N, Vicogne J, Ahier A, BenYounes A, Konrad C, Trolet J, Viscochliosi E, Brehm K, Dissous C. Diversification of the insulin receptor family in the helminth parasite Schistosoma mansoni. FEBS J 2007; 274: 659-676.

7. Pachuski J, Fried B, Sherna J. HPTLC analysis of amino acids in Biomphalaria glabrata infected with Schistosoma mansoni. J Liq Chromatogr Relat Technol 2002; 25: 2345-2349.

8. Muller EE, Fried B, Sherna J. HPTLC analysis of neutral lipids in Biomphalaria glabrata snails infected with Schistosoma mansoni (Trematoda). J Planar Chromatogr Mod TLC 2000; 13: 228-231.

9. Janusiewicz JA, Sherna J, Fried B. Thin layer chromatographic analysis of glucose and maltose in estivated Biomphalaria glabrata snails and those infected with Schistosoma mansoni. Comp Biochem Physiol B Biochem Mol Biol 2006; 145: 346-349.

10. Thompson SN. Physiology and biochemistry of snail-larval trematode relationships. In: Fried B, Graczyl TK eds, Advances in Trematode Biology. Florida, USA. CRC Press. 1997, p 149-195.

11. Abou Elseoud SM, Abdel Fattah NS, Ezz El Din HM, Abdel Al H, Mossalem H, Elleboudy N. Carboxylic acids as biomarkers of Biomphalaria alexandrina snails infected with Schistosoma mansoni. Korean J Parasitol 2010; 48: 127-132.

12. Becker W, Lamprecht I. Microcalorimetric investigations of the host-parasite relationship between Biomphalaria glabrata and Schistosoma mansoni (author’s transl.). Z Parasitenkd 1977; 53: 297-305.

13. Schneck JL, Fried B. Growth of Biomphalaria glabrata (NMRI strain) and Helisoma trivolvis (Colorado strain) under laboratory conditions. Am Malacol Bull 2005; 20: 71-73.

14. Massa DR, Chejilva MJ, Fried B, Sherna J. High performance column liquid chromatographic analysis of selected carboxylic acids in Biomphalaria glabrata patently infected with Schistosoma
1. Mansoni. Parasitol Res 2007; 101: 925-928.

15. Massa DR, Chejlava MJ, Fried B, Sherma J. Thin layer and high performance column liquid chromatographic analysis of selected carboxylic acids in standards and from Helisoma trivolvis (Colorado Strain) snails. J Liq Chromatogr Relat Technol 2007; 30: 2221-2229.

16. Lian HZ, Mao L, Ye XL, Miao J. Simultaneous determination of oxalic, fumaric, maleic and succinic acids in tartaric and malic acids for pharmaceutical use by ion-suppression reversed-phase high performance liquid chromatography. J Pharm Biomed Anal 1999; 19: 621-625.

17. Fishel F, Mossler M. What is the significance of part per million? 2005. [cited 2011 October 10] Available from: http://edis.ifas.ufl.edu/pi116

18. Steuer R. Review: On the analysis and interpretation of correlations in metabolomic data. Brief Bioinform 2006; 7: 151-158.

19. Camacho D, de la Fuente A, Mendes P. The origin of correlations in metabolomics data. Metabolomics 2005; 1: 53-63.

20. Van Saun RI. Application of a pooled sample metabolic profile for use as a herd screening tool. In: Proceedings Danske Krvægf- dyrlægers Årsmøde (Danish bovine practitioner seminar) Middelfart, Denmark. 2007, p 24-25.

21. Martins AM, Camacho D, Shuman J, Sha W, Mendes P, Shulaev V. A systems biology study of two distinct growth phases of Saccharomyces cerevisiae cultures. Curr Genomics 2004; 5: 649-663.

22. Morgenthal K, Weckwerth W, Steuer R. Metabolomic networks in plants: Transitions from pattern recognition to biological interpretation. Biosystems 2006; 83: 108-117.

23. Steuer R, Kurths J, Daub CO, Weise J, Selbig J. The mutual information: Detecting and evaluating dependencies between variables. Bioinformatics 2002; 18: S231-S240.