

Supporting information – cover page

Discovery of biomarkers for Tasmanian devil cancer (DFTD) by metabolic profiling of serum

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Table S-1. LC-MS/MS information for features in Table 2, discriminating between DFTD and healthy devils.

Table S-2. Mann-Whitney U-test comparing the means of features in Table 2 between control samples (n=35; n=24 without 10-13months old devils) and early stage DFTD (n=19), and between early and mid-late stages DFTD (n=14). Two samples with DFTD score 2 and 4 were excluded from this comparison. The order of features is identical to Tables 2 and S-1.

Figure S-1. Box plots of LC-MS intensity per sample, before and after median-fold normalisation. 19 Replicates of pooled QC sample are indicated in text. Note that this figure contains all 145 samples analysed as part of the project for characterisation of Tasmanian devil metabolome, and only 70 were selected for the presented work.

Figure S-2. Scores plot of PCA obtained for 70 serum samples and 19 replicates of a pooled QC sample, before feature selection. Data were log-transformed and Pareto scaled after replacing missing values by 20% of minimum peak area.

Figure S-3. Scores plot of PLS/DA model obtained for age-matched serum samples (training set), using 232 features (log-transformed and Pareto scaled).

Figure S-4. Classification error in random forests model obtained for age-matched serum samples (training set), using 232 features (log-transformed and Pareto scaled). The model grew 1000 trees and tested 12 variables per node.

Figure S-5. Volcano plot of features differentiating between age-matched DFTD and healthy controls (n=2x20), out of 232 features. All features have at least 1.3 fold-change between the classes. Markers above the blue line have FDR $q < 0.05$, and the red line FDR $q < 0.1$ (considered for selection).

Figure S-6. Spearman-rank correlation map between features in Table 2, after log transformation, based on age-matched samples (n=20x2)

Supp1 - Further investigation of potentially confounding factors (Age and Sex)

Supp2 - Scatter plots of cortisol levels as a function of number of total blood tests along the devil's life.

Table S-1. LC-MS/MS information for features in Table 2, discriminating between DFTD and healthy devils.

Analyte (putative or identified)	retention time (min)	m/z	base peak or adduct	MW [M+H] ⁺ ion	error (ppm)	predicted formula (M)	other adducts detected ^a	Fragments detected ^a	notes /peptide sequences
Urea ¹	2.47	121.0719	[2M+H] ⁺	61.0396	-0.31	CH4N2O	[M+ACN+H] ⁺ m/z 102.066 ^a [M+K] ⁺ m/z 98.99		
Peptide ²	7.75	432.2090	[M+H] ⁺	432.2089	-0.28	C17H29N5O8			EGGGI
Cortisol ¹	12.21	363.2168	[M+H] ⁺	363.2166	0.20	C21H30O5		327.199	
Peptide	8.19	503.2459	[M+H] ⁺	503.2387	-0.63	C20H34N6O9			AEGGGI (-log ₁₀ p = 23.23)
Peptide ²	8.1	386.7190	[M+2H] ²⁺	772.430	0.44	C32H57N11O11			IAEGGGIR
Peptide ²	3.00	230.1375	[M+2H] ²⁺	458.2601	-0.21	C18H34N8O6			GGGIR
Ser-Tyr ³	10.32	251.1027	[M+H-H2O] ⁺	268.1059	+0.27	C12H14N2O4	highly correlated [M+Na] ⁺ 273.08 ^a		
Tauro-chenodeoxycholate glucuronide	10.94	658.3257	[M+H-H2O] ⁺	658.3254	1.03	C24H47N7O14	[M+NH4] ⁺ 693.36 ^a [M+Na] ⁺ 698.32 ^a	M+H-H2O 482.29 ^a M+H-2H2O 464.28 ^a	fragments are taurodeoxycholate after loss of 176.3 (glucuronic acid)
Peptide ²	9.08	526.2824	[M+3H] ³⁺	1576.8329	-0.19	C69H113N19O23			IKEEGTFIAEGGGIR
Peptide ²	3.30	304.1505	[M+H] ⁺	304.1503	0.04	C12H21N3O6	[M+Na] ⁺ m/z 326.1320		hits: 3AA (eg. Thr Ser Pro); Acetyl-glutamyl-ornithine
Kynurenine ¹	5.17	209.0922	[M+H] ⁺	209.0921	0.63	C10H12N2O3		[M-NH3] ⁺ m/z 192.0653	
Peptide ²	8.96	488.5880	[M+3H] ³⁺	1463.7489	0.39	C63H102N18O22			KEEGTFIAEGGGIR
Peptide	9.63	710.8694	[M+2H] ²⁺	1420.7275	-0.22	C63H101N15O22			IKEEGTFIAEGGGI (-log ₁₀ p = 84.57)
Peptide ²	9.92	437.2393	[M+H] ⁺	437.2495	-0.11	C21H32N4O6			GTFI

Analyte (putative or identified)	retention time (min)	m/z	base peak or adduct	MW [M+H] ion	error (ppm)	predicted formula (M)	other adducts detected ^a	Fragments detected ^a	notes /peptide sequences
Peptide ²	2.99	201.6267	[M+2H] ²⁺	401.2387	0.46	C16H31N7O5			GGIR
Peptide	9.51	468.7555	[M+2H] ²⁺	936.4993	0.05	C43H69N9O14			IKEEGTFI (-log ₁₀ p = 52.02)
Peptide	9.54	654.3277	[M+2H] ²⁺	1307.6434	0.27	C57H90N14O21			KEEGTFIAEGGGI (-log ₁₀ p = 49.05)
Peptide	9.32	412.2135	[M+2H] ²⁺	823.4153	0.14	C37H58N8O13			IKEEGTF (-log ₁₀ p = 24.98)
Peptide ²	3.10	338.6794	[M+2H] ²⁺	676.3508	0.48	C28H49N7O12			IKEEGT
Peptide ²	8.11	355.6715	[M+2H] ²⁺	710.3355	0.25	C31H47N7O12			KEEGTF
Creatinine ¹	2.40	114.0660	[M+H] ⁺	114.0662	-0.16	C4H7N3O	[M+Na] ⁺ m/z 136.048		
Hexanoyl carnitine	10.40	204.1232	[M+H] ⁺	260.1856	0.79	C13H25NO4	260.1860 ^a [M+H] ⁺ of hexanoyl carnitine) 242.1753 [M+H-H2O] ⁺		acetyl carnitine fragment of hexanoyl carnitine
Peptide ²	9.2	510.7769	[M+2H] ²⁺	1019.5394	-0.73	C45H73N13O14			TFIAEGGGIR
Peptide ²	6.37	303.1663	[M+H] ⁺	303.1663	0.12	C12H22N4O5	[M+Na] ⁺ m/z 325.15		GGGI
Creatine ¹	2.44	132.0767	[M+H] ⁺	132.0768	-0.25	C4H9N3O2		[M-CHN3] ⁺ m/z 89.0477	
Glu-Phe ³	9.11	295.1290	[M+H] ⁺	295.1288	0.51	C14H18N2O5			
Cholic acid ¹	14.01	373.2742	[M+H-2H2O] ⁺	408.2875	1.2	C24H40O5	468.3321 M+H 409.2900 ^a	355.26 ^a	373 is usually the strongest peak

Analyte (putative or identified)	retention time (min)	m/z	base peak or adduct	MW [M+H] ⁺ ion	error (ppm)	predicted formula (M)	other adducts detected ^a	Fragments detected ^a	notes /peptide sequences
							M-H ₂ O+H 391.2847 M+NH ₃ 426.32 ^a M+Na 431.28 ^a		
unknown	10.56	233.0922	[M+ACN+H] ⁺	192.0655	+0.75	C ₁₀ H ₉ NO ₃	[M+H] ⁺ m/z 192.0656	130.064 ^a	m/z 130.064 corresponds to an indole group
Serine ¹	2.43	106.0497	[M+H] ⁺	106.0499	-1.6	C ₃ H ₇ NO ₃	[M+2Na-H] ⁺ m/z 150.01	[M+H-H ₂ O] ⁺ m/z 88.0390	
N-carbamoyl sarcosine	2.89	155.0427	[M+Na] ⁺	133.0608	0.01-0.6	C ₄ H ₈ N ₂ O ₃	[M+H] ⁺ m/z 133.0608		
C5 carnitine ?	9.23	246.1701	[M+H] ⁺	246.1700	0.47	C ₁₂ H ₂₃ NO ₄			
Tyrosine ¹	3.2	182.0811	[M+H] ⁺	182.0812	0.17	C ₉ H ₁₁ NO ₃			
Phenylacetyl glycine ³	10.27	194.0813	[M+H] ⁺	194.0812	0.67	C ₁₀ H ₁₁ NO ₃	239.14/10.3? same box plots	[M+H-H ₂ O] ⁺ m/z 176.0707 m/z 91.0540 (C ₇ H ₇)	
Inosine ¹	3.38	269.0881	[M+H] ⁺	269.0880	0.20	C ₁₀ H ₁₂ N ₄ O ₅	[M+Na] ⁺ m/z 291.07 ^a [M+K] ⁺ m/z 307.04 400.18/3.4?	137.045 ^a (C ₅ H ₅ N ₄ O)	
Kynurenic acid ¹	9.4	190.0500	[M+H] ⁺	190.0499	0.69	C ₁₀ H ₇ NO ₃	[M-H] ⁻ m/z 188.036		
Heptanedioylcarnitine	8.5	304.1754	[M+H] ⁺	304.1755	-0.34	C ₁₄ H ₂₆ NO ₆			
Peptide ²	3.40	330.1773	[M+2Na] ²⁺	659.3644	1.5	C ₂₇ H ₅₀ N ₈ O ₈			IAEGGGI
trihydroxy-β-cholestanoyl- taurine	13.4	558.3463	[M+H] ⁺	558.3457	-0.38	C ₂₉ H ₅₁ NO ₇ S	[M+NH ₃] ⁺ m/z 575.3722	[M-C ₅ H ₉ SO ₂] ⁺ m/z 424.3056	
Citric acid ¹	3.08	215.0163	[M+Na] ⁺	193.0343	1.3	C ₆ H ₈ O ₇	[M+2Na-H] ⁺ m/z 236.99		

Analyte (putative or identified)	retention time (min)	m/z	base peak or adduct	MW [M+H] ⁺ ion	error (ppm)	predicted formula (M)	other adducts detected ^a	Fragments detected ^a	notes /peptide sequences
							[M+NH3] ⁺ ^a		
OH-PheAceGly	8.07	210.0762	[M+H] ⁺	210.0761	-0.31	C ₁₀ H ₁₁ NO ₄			
trimethyllysine	2.2	189.1600	[M+H] ⁺	189.1598	1.09	C ₉ H ₂₀ N ₂ O ₂			
glucose ⁴	2.4	203.0528	[M+Na] ⁺	181.0707	0.84	C ₆ H ₁₂ O ₆	[2M+Na] ⁺ [M+ACN+Na] ⁺		

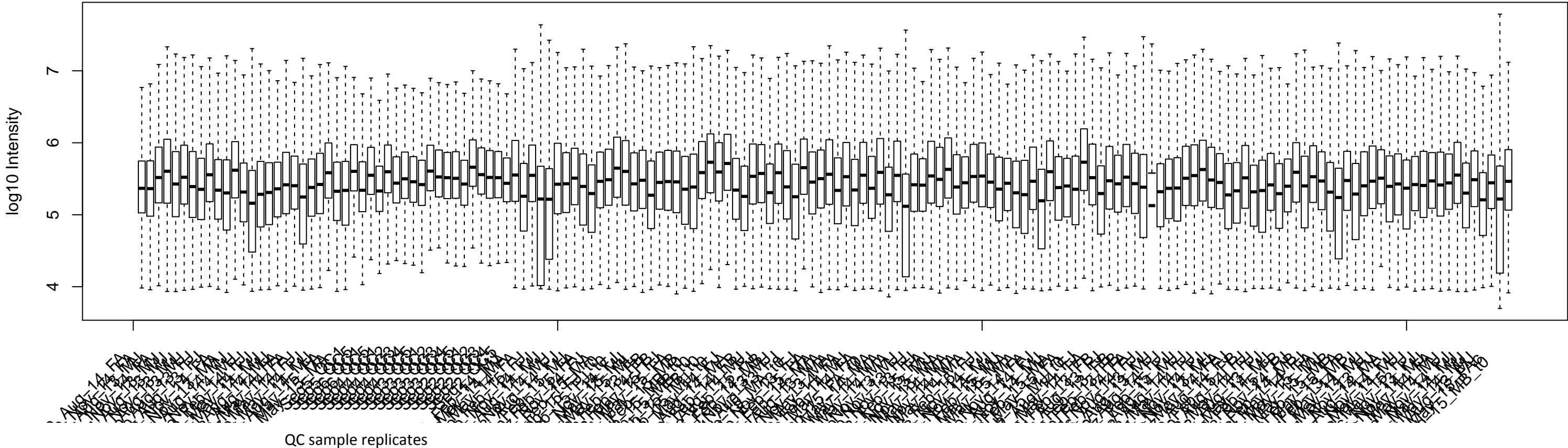
¹ Validated using authentic standard; ² peptide sequence deduced by matching calculated and observed masses (+/- 2 ppm); ³ elemental composition or by searching m/z values against chemical databases; Multiple options with same formula; ⁴ confirmed by ¹H NMR(data not shown); ^a Detected adducts or fragments showed significance between DFTD and Healthy devils in the age-matched dataset

Table S-2. Mann-Whitney U-test comparing the means of features in Table 2 between control samples (n=35; n=24 without 10-13months old devils) and early stage DFTD (n=19), and between early and mid-late stages DFTD (n=14). Two samples with DFTD score 2 and 4 were excluded from this comparison. The order of features is identical to Tables 2 and S-1.

Analyte (identified or putative)	Feature (m/z POS /retention time)	DFTD/ control	Mann-Whitney <i>U</i> test		
			control (n=35) vs. early DFTD	control (n=24) vs. early DFTD	early DFTD vs. mid-late DFTD
urea	121.07/2.5	Down	0.003	4.7E-04	0.08
peptide?	432.21/7.7	Up	0.002	8.1E-04	0.04
cortisol	363.22/12.2	Down	1.5E-04	< 0.0001	5.8E-04
peptide	503.25/8.2	Up	6.7E-04	2.3E-04	7.0E-04
peptide	386.72/8.1	Up	< 0.0001	< 0.0001	3.6E-04
peptide	230.14/3.0	Up	< 0.0001	< 0.0001	0.016
Ser-Tyr?	251.1/10.3	Down	4.3E-04	0.004	0.66
cholate glucuronide	658.33/10.9	Down	0.24	0.07	< 0.0001
peptide	526.28/9.1	Up	8.1E-04	0.001	0.008
peptide	304.15/3.3	Up	0.003	4.6E-04	0.04
kynurenine	209.09/5.1	Down	0.001	0.013	0.18
peptide	488.59/9.0	Up	5.7E-04	0.001	0.01
peptide	710.87/9.7	Up	2.7E-04	4.3E-04	0.005
peptide	437.24/9.9	Up	0.031	0.018	0.05
peptide	201.63/3.0	Up	0.006	0.002	0.13
peptide	468.76/9.5	Up	0.002	0.001	0.02
peptide	654.33/9.6	Up	6.6E-04	0.001	0.004
peptide	412.21/9.3	Up	0.003	0.002	0.07
peptide	338.68/3.1	Up	0.003	0.001	0.14
peptide	355.67/8.1	Up	0.003	0.001	0.16
creatinine	114.07/2.4	Down	0.29	0.050	0.02
hexanoyl carnitine	204.12/10.4	Down	0.025	0.033	0.02
peptide	510.78/9.2	Up	0.008	0.015	8.9E-04
peptide	303.17/6.3	Up	0.004	0.004	0.10
creatine	132.08/2.4	Down	0.012	0.005	0.26
Glu-Phe	295.13/9.1	Down	< 0.0001	2.4E-04	0.88
cholic acid	373.27/14.0	Up	0.054	0.015	0.28
?	233.09/10.6	Down	0.014	0.074	0.15
serine	106.05/2.4	Up	0.021	0.048	0.66
N-carbamoyl sarcosine	155.04/2.9	Down	< 0.0001	0.002	0.44
C ₅ carnitine	246.17/9.2	Down	0.038	0.12	0.049
Tyrosine ¹	182.08/3.2	Down	0.01	0.042	0.19
PheAceGly	194.08/10.3	Down	0.066	0.078	0.05
inosine	291.07/3.4	Up	0.009	0.003	0.74
kynurenic acid	190.05/9.4	Up	0.014	0.014	0.27
Heptanedioylcarnitine	304.18/8.5	Up	0.40	0.32	0.02
peptide?	330.18/3.4	Up	0.31	0.28	0.003
triOH-β-chole- taurine	558.35/13.4	Up	0.23	0.02	0.13
citric acid	215.02/3.1	Down	6.0E-04	0.01	0.97
OH-PheAceGly	210.08/8.1	Down	0.009	0.056	0.42
trimethyllysine	189.16/2.2	Down	0.40	>0.5	0.01
glucose	203.05/2.4	Up	>0.5	>0.5	0.04

Figure S-1. Box plots of LC-MS intensity per sample, before and after median-fold normalisation. 19 Replicates of pooled QC sample are indicated in text. Note that this figure contains all 145 samples analysed as part of the project for characterisation of Tasmanian devil metabolome, and only 70 were selected for the presented work.

Un Normalized Intensity Values



Normalized Intensity Values

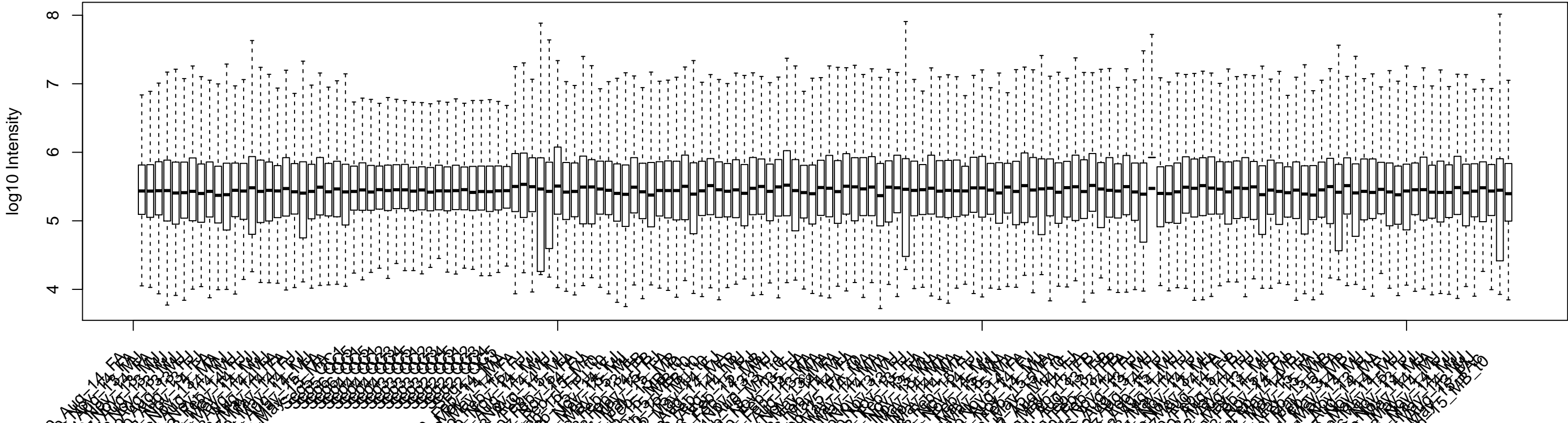


Figure S-2. Scores plot of PCA obtained for 70 serum samples and 19 replicates of a pooled QC sample, before feature selection. Data were log-transformed and Pareto scaled after replacing missing values by 20% of minimum peak area.

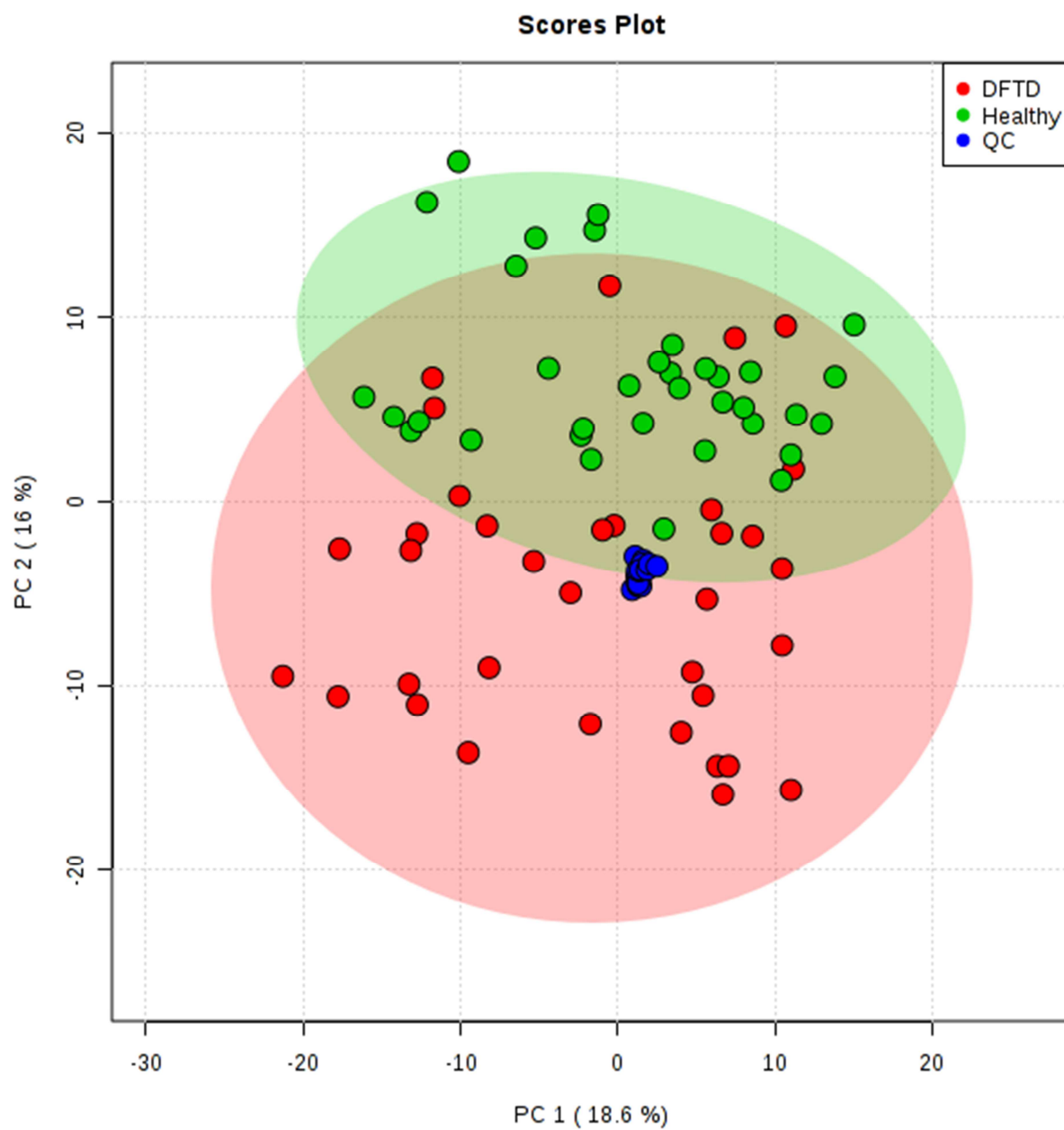


Figure S-3. Scores plot of PLS/DA model obtained for age-matched serum samples (training set), using 232 features (log-transformed and Pareto scaled).

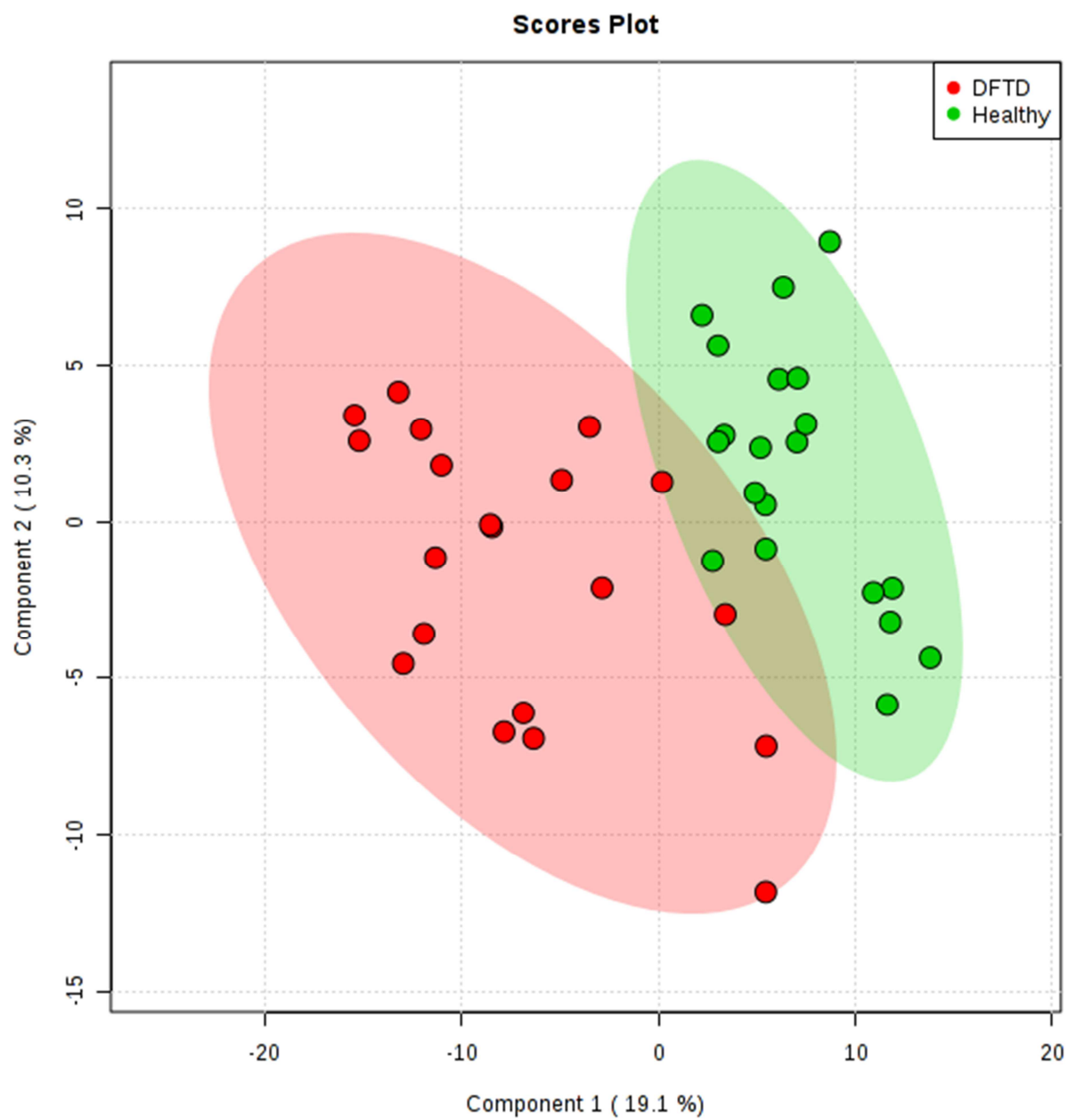


Figure S-4. Classification error in random forests model obtained for age-matched serum samples (training set), using 232 features (log-transformed and Pareto scaled). The model grew 1000 trees and tested 12 variables per node.

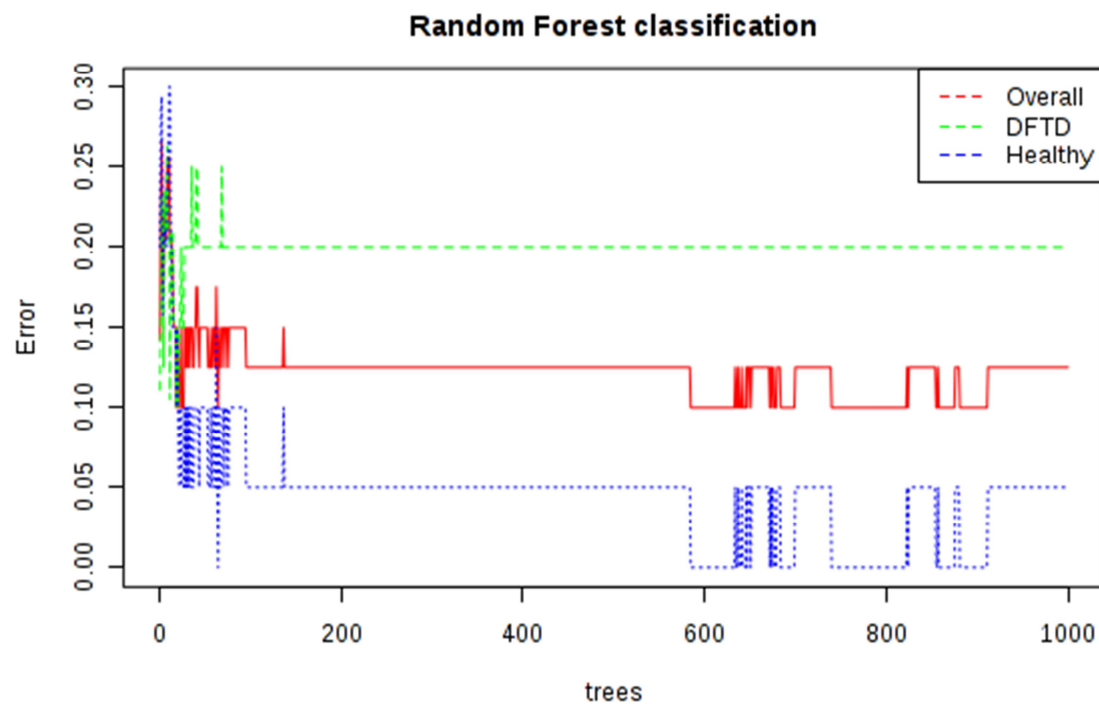


Figure S-5. Volcano plot of features differentiating between age-matched DFTD and healthy controls (n=2x20), out of 232 features. All features have at least 1.3 fold-change between the classes. Markers above the blue line have FDR $q < 0.05$, and the red line FDR $q < 0.1$ (considered for selection).

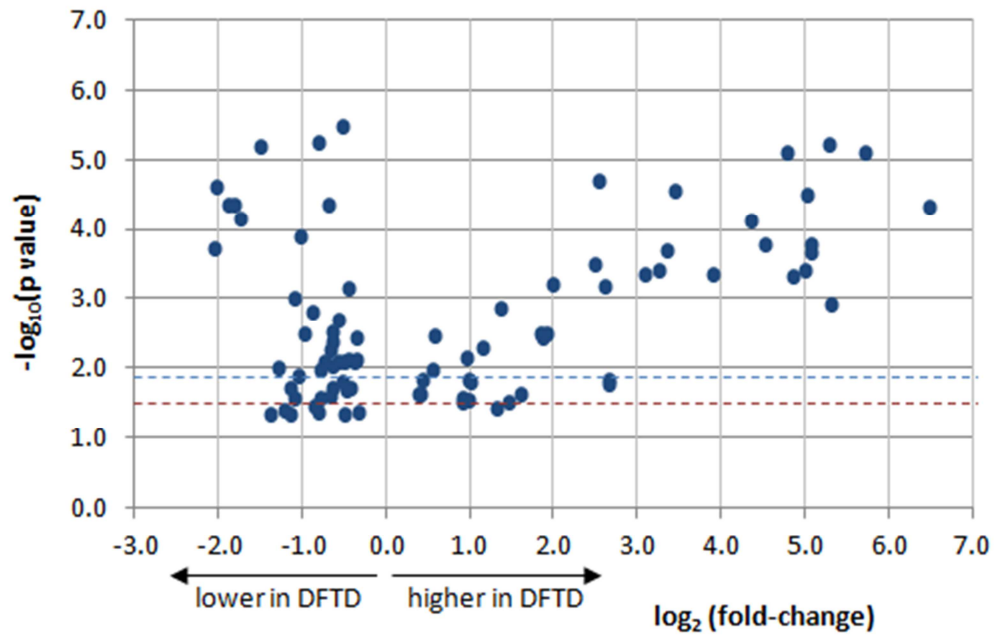
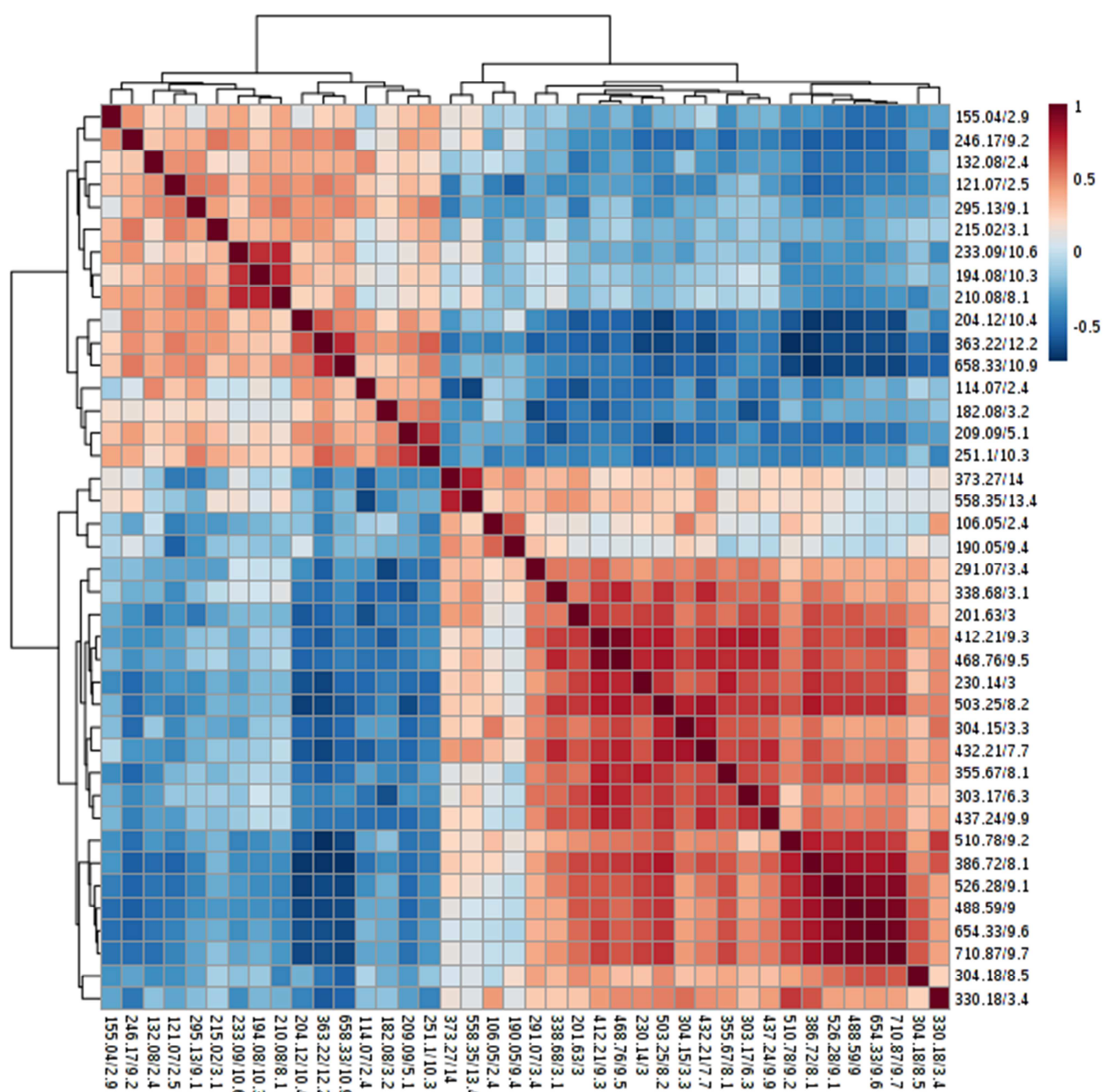


Figure S-6. Spearman-rank correlation map between features in Table 2, after log transformation, based on age-matched samples (n=20x2)

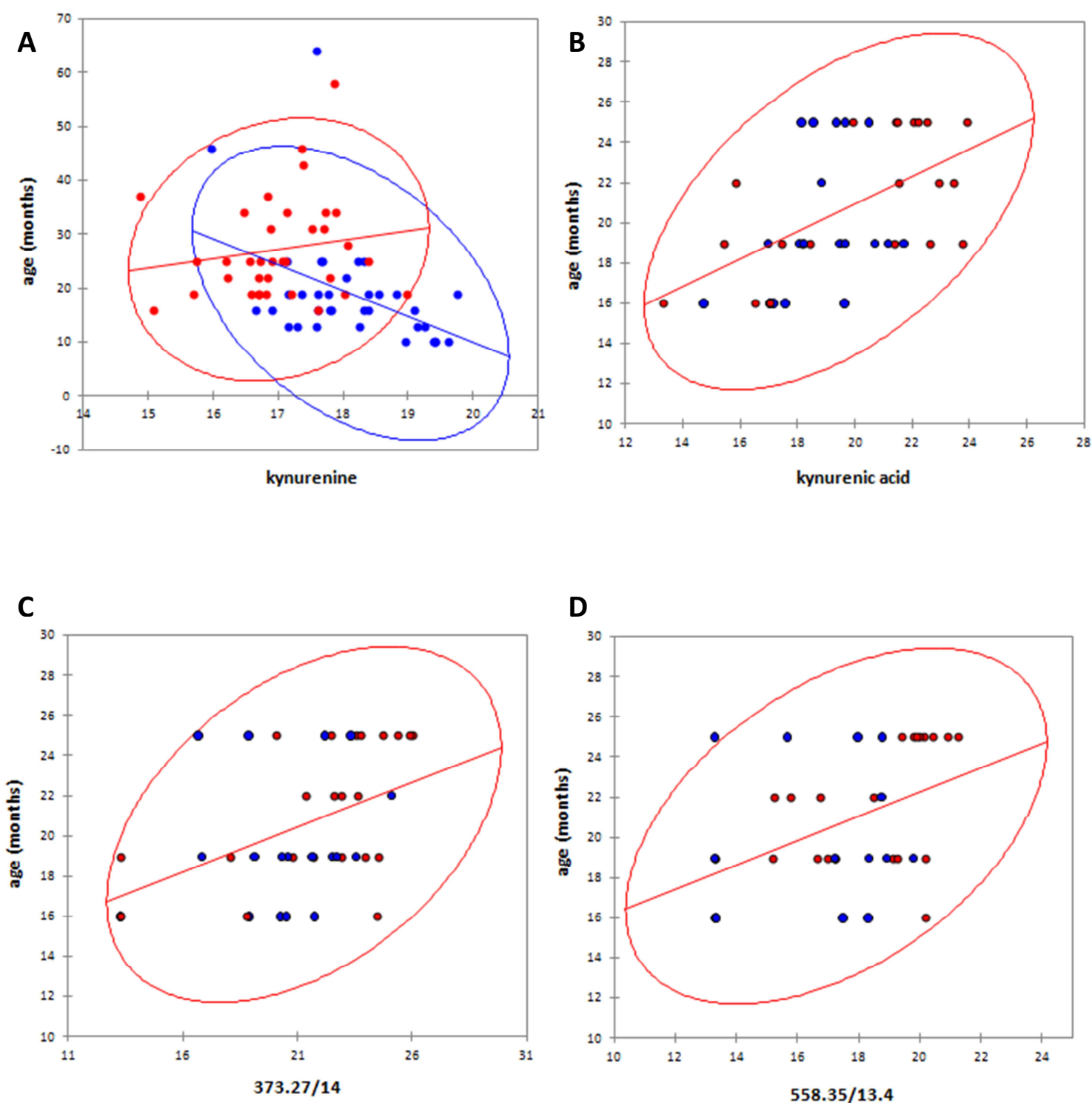


Supp1 - Further investigation of potentially confounding factors

1) Age

The correlation between age and features was examined, using either overall correlation in age-matched subsample or within-class correlation for the total cohort. The only feature significantly associated with age was kynurenine within the control class (Spearman $R=-0.429$, $p=0.011$, FDR $q>0.1$) but not DFTD ($R=0.205$, $p=0.236$), as shown in Figure 1 below **(A)**. For the age-matched subsample, significant correlations were found for kynurenic acid ($R=0.556$; $p=0.0002$) and the putative bile acids m/z 373 ($R=0.434$; $p=0.006$) and m/z 558 ($R=0.465$; $p=0.003$). These are depicted in figures **B-D** below, and the distribution of class-coded markers reveals no apparent scrutiny of the DFTD-related differentiating ability of the features. In addition, 2-way ANOVA could not find interactions between DFTD class and sex or age, and a marginal interaction with season was found for m/z 295 ($p=0.04$), however, similarly to other factors, it should be further examined in a larger cohort.

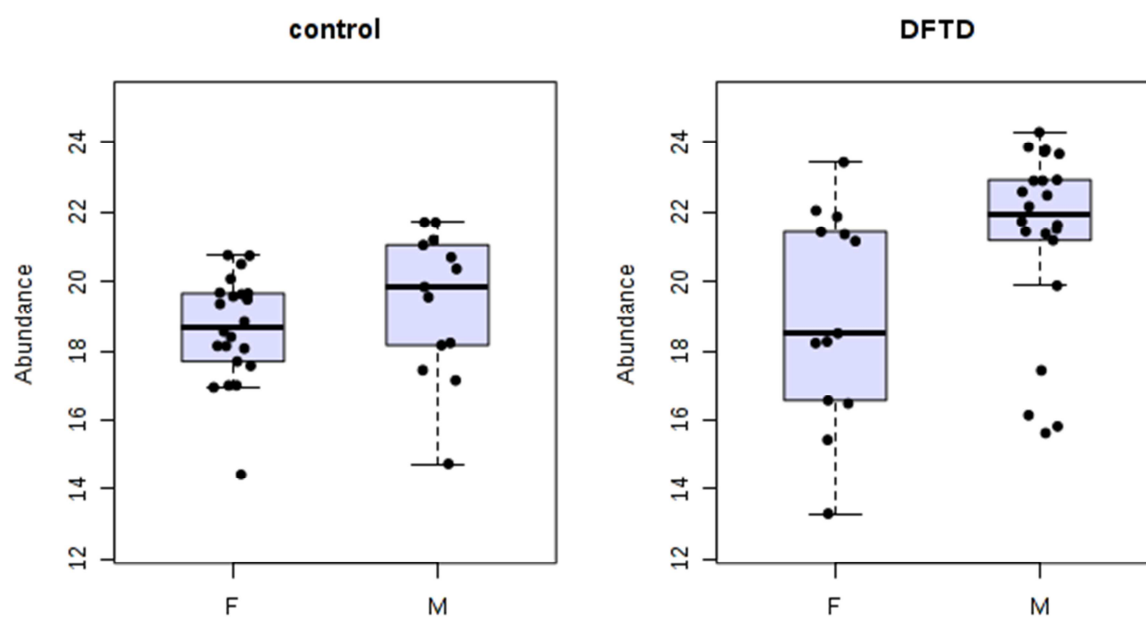
Figure 1. Scatter plots for age (in months) vs. \log_2 values of metabolites in control samples (blue markers) and tumour samples (red markers), with 95% confidence interval ellipses. Significant Spearman correlation: A) kynurenine, within-class correlation, $R=-0.429$, $p=0.011$ in the control class only ($n=35$). Plots B-D shows overall correlation in age-matched samples ($n=20 \times 2$): B) kynurenic acid, $R=0.556$; $p=0.0002$; C) putative cholic acid m/z 373, $R=0.434$; $p=0.006$; putative cholate derivative m/z 558, $R=0.465$; $p=0.003$.



2) Sex

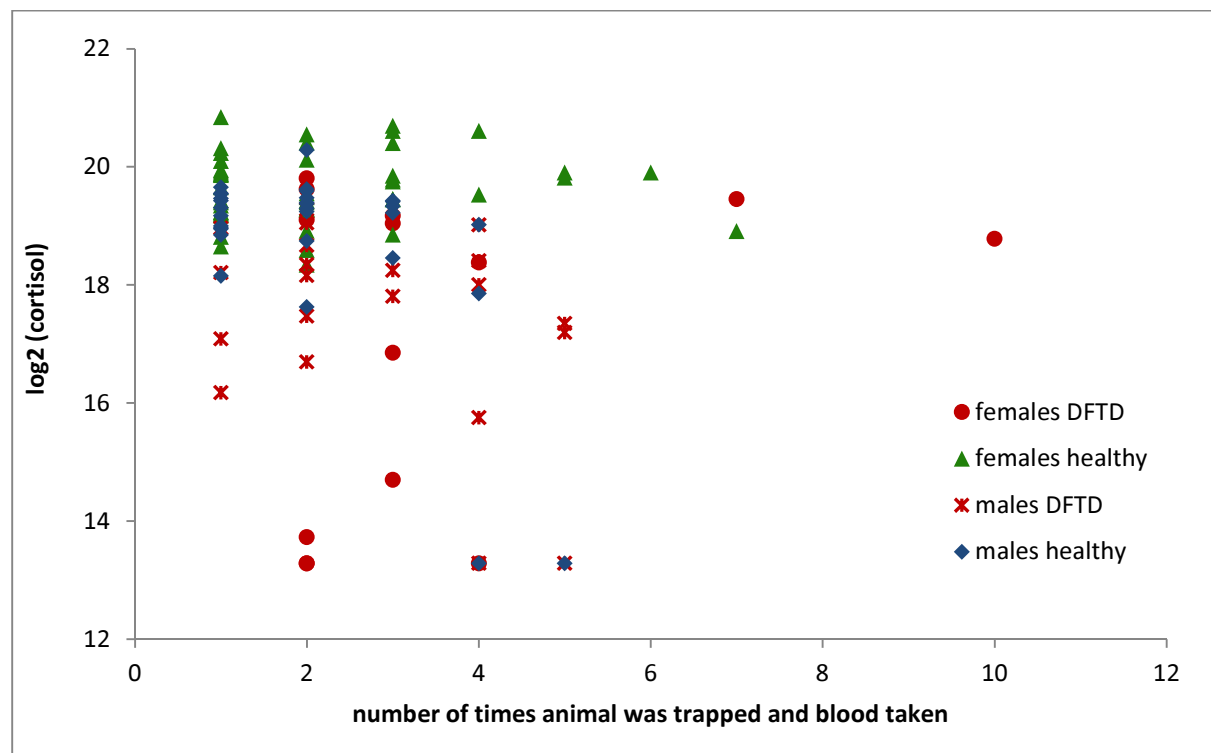
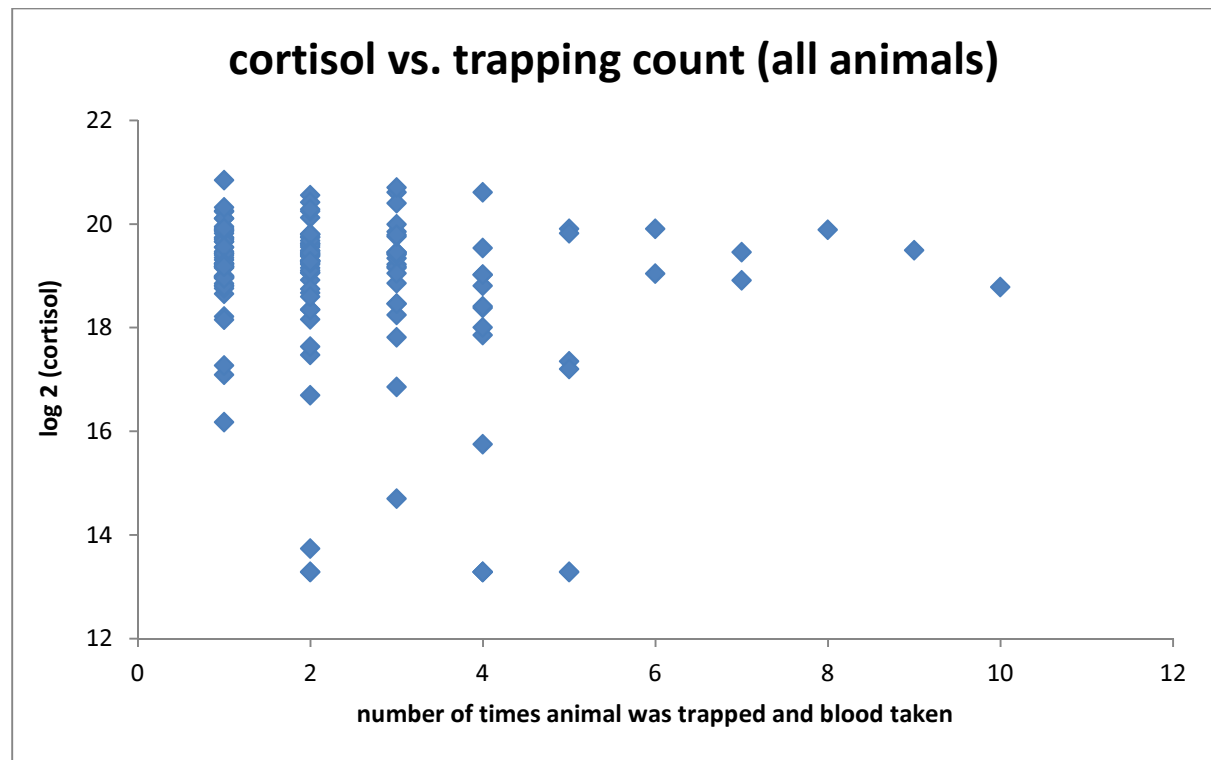
The potentially confounding factor of sex suffers from unbalanced representation in the training set. In the full cohort, PLS/DA analysis resulted in poor prediction of sex class ($Q^2 < 0.2$), and insignificant model (permutation tests $p > 0.5$). PLS/DA of sex, either within DFTD or control class, resulted in negative Q^2 values, and $p > 0.6$ in permutation tests. Logistic regression between sex and each metabolite gave only a few models which seemed significant (for kynurenic acid, C₅carnitine and cholic acid). Box plots of kynurenic acid values in the stratified classes indicate some inconsistency (Fig.2 below), and this should be further investigated in a larger cohort, and with targeted analysis. For the latter two, despite AUROC of ~ 0.7 , these were rather weak predictors of sex (average accuracy below 0.6 after 100 cross validations) and the permutation tests indicated a random model ($p > 0.2$).

Figure 2. Box plots of kynurenic acid levels in DFTD and controls (full cohort), according to sex (F/M).



Supp2 - Cortisol levels vs. number of trips an animal had been trapped:

Note that an animal may be trapped more than once during each 10-day trip, but a blood sample is taken only in the first trapping in each trip. Therefore, the number of times included in the following analysis does not indicate the total times of handling.



Cortisol levels (total) in animals with blood tests from more than one trip. Each set of dots represents one devil, and the name is compiled of: sample name_ male/female_ health status in consecutive samples. For example, female #412 was asymptomatic (“healthy”) in the first sample, possibly latent in the second sample, and DFTD in the 3rd and 4th; #452 was asymptomatic in all trips.

