Table 3.1
Gene expression ratio (Exp/Cont) at 4 h and the results of the Williams' test and Dunnett's test.

No.	Gene symbol	Gene expression ratio (Exp/Cont) and Williams' test									Dunnett's test	
		Genotoxic hepatocarcinogens					Non-genotoxic hepatocarcinogen		Non-genotoxic non-hepatocarcinogen		G/DEHP	G/PNT
		DEN (mg/kg bw)			DNT (mg/kg bw)		DEHP (mg/kg bw)		PNT (mg/kg bw)			
		12.5 mg	25 mg	50 mg	125 mg	250 mg	1000 mg	2000 mg	500 mg	1000 mg		
1	Aen	1.60 ± 0.25**	2.96 ± 1.05**	7.40 ± 1.47**	0.86 ± 0.12	1.22 ± 0.62	1.02 ± 0.13	0.72 ± 0.17*	0.74 ± 0.05	0.94 ± 0.40	$P<0.01$	$P<0.01$
2	Bax	4.63 ± 1.60**	4.67 ± 0.78**	5.08 ± 1.49**	0.87 ± 0.22	0.95 ± 0.22	3.37 ± 0.49**	0.95 ± 0.27	0.75 ± 0.14	0.78 ± 0.08		$P<0.01$
3	Btg2	1.78 ± 0.67*	2.72 ± 0.75**	4.72 ± 1.95**	2.81 ± 1.55**	4.40 ± 0.85**	1.78 ± 1.68	1.32 ± 0.36	1.71 ± 0.33**	3.02 ± 0.85**	$P<0.01$	
4	Ccnf	3.34 ± 0.67**	3.45 ± 0.59**	2.31 ± 0.65**	1.11 ± 0.39	1.38 ± 0.41	0.89 ± 0.29	1.41 ± 0.55	0.73 ± 0.11	0.84 ± 0.05	$P<0.01$	$P<0.01$
5	Ccng1	3.45 ± 1.10**	5.55 ± 2.26**	13.6 ± 3.12**	1.16 ± 0.22	1.75 ± 0.30**	0.90 ± 0.09	0.45 ± 0.02**	0.96 ± 0.11	1.40 ± 0.23*	$P<0.01$	$P<0.01$
6	Cdkn1a	1.90 ± 0.17**	3.38 ± 0.19**	8.20 ± 1.88**	1.69 ± 0.87	1.95 ± 0.24**	2.14 ± 0.67*	1.89 ± 0.41*	3.10 ± 0.57**	4.87 ± 0.28**		
7	Cyp21a1	1.01 ± 0.23	1.08 ± 0.10	2.28 ± 0.80**	0.91 ± 0.38	1.09 ± 0.33	0.94 ± 0.17	0.77 ± 0.29	0.70 ± 0.21	0.66 ± 0.07		
8	Cyp4a1	2.68 ± 1.13	1.44 ± 0.25	1.34 ± 0.61	1.62 ± 0.11**	1.44 ± 0.43	3.53 ± 1.25**	6.75 ± 0.30**	0.60 ± 0.04	1.66 ± 0.34		
9	Ddit4l	15.1 ± 5.10**	22.2 ± 8.85**	16.1 ± 7.37**	1.91 ± 0.55*	4.27 ± 1.82**	0.33 ± 0.09*	0.71 ± 0.55	0.55 ± 0.10*	0.62 ± 0.11	$P<0.01$	$P<0.01$
10	Egfr	1.77 ± 0.91	1.09 ± 0.55	0.87 ± 0.20	1.00 ± 0.35	1.31 ± 1.44	2.02 ± 0.88	3.71 ± 0.91**	0.78 ± 0.10	1.49 ± 0.53		
11	Ephx1	2.73 ± 0.20**	2.33 ± 0.27**	2.48 ± 0.28**	0.97 ± 0.37	1.39 ± 0.30	1.43 ± 0.36	2.12 ± 0.42**	0.81 ± 0.26	0.99 ± 0.21		$P<0.01$
12	Gadd45b	1.08 ± 0.44	1.53 ± 0.87	3.09 ± 1.12**	1.69 ± 0.51*	2.11 ± 0.56**	1.62 ± 0.92	2.50 ± 1.02*	3.97 ± 0.46**	5.41 ± 0.63**		
13	Gadd45g	0.98 ± 0.44	1.17 ± 0.76	1.21 ± 0.39	0.95 ± 0.43	0.75 ± 0.11	14.2 ± 9.08	3.30 ± 1.26**	0.84 ± 0.30	1.96 ± 0.71*	$P<0.01$	
14	Gdf15	1.69 ± 0.45	2.24 ± 0.25**	4.31 ± 1.48**	2.56 ± 0.76**	8.66 ± 1.05**	1.70 ± 0.99	2.30 ± 0.68**	0.67 ± 0.04*	0.78 ± 0.19	$P<0.05$	$P<0.01$
15	Hhex	0.82 ± 0.25	0.54 ± 0.17	1.24 ± 0.37	1.24 ± 0.12*	1.68 ± 0.40*	1.07 ± 0.50	1.20 ± 0.33	0.68 ± 0.25	0.70 ± 0.06*		
16	Hmox1	0.44 ± 0.08	0.61 ± 0.26	1.29 ± 0.35	1.33 ± 0.30	4.79 ± 2.60**	1.26 ± 0.28	0.70 ± 0.15	0.77 ± 0.10	1.33 ± 0.44		
17	Hspb1	2.50 ± 1.27**	2.48 ± 0.35**	1.98 ± 0.42*	1.30 ± 0.33	1.42 ± 0.16*	0.92 ± 0.16	0.59 ± 0.06*	0.86 ± 0.14	0.94 ± 0.19	$P<0.01$	$P<0.01$
18	Igfbp1	1.42 ± 0.84	0.44 ± 0.15	1.04 ± 0.45	2.24 ± 0.99*	2.34 ± 0.96*	0.74 ± 0.31	0.91 ± 0.21	2.59 ± 0.42**	3.94 ± 0.79**		
19	Jun	1.56 ± 0.49*	2.27 ± 0.51**	7.62 ± 3.56**	3.39 ± 0.83**	5.33 ± 1.40**	0.71 ± 0.56	0.51 ± 0.15*	1.15 ± 0.55	1.14 ± 0.20	$P<0.01$	$P<0.01$
20	Lpp	1.58 ± 0.11	1.41 ± 0.30	0.63 ± 0.36	0.89 ± 0.36	1.12 ± 0.71	2.35 ± 0.85*	1.52 ± 0.43	0.78 ± 0.18	0.95 ± 0.14	$P<0.05$	
21	Ly6al	0.98 ± 0.06	1.03 ± 0.09	1.63 ± 0.24**	0.74 ± 0.11	1.02 ± 0.41	1.65 ± 0.50	0.97 ± 0.16	0.81 ± 0.21	0.72 ± 0.04*		
22	Mdm2	0.79 ± 0.12	1.53 ± 0.98	2.05 ± 0.66*	1.25 ± 0.45	2.00 ± 0.31**	1.72 ± 0.27**	1.07 ± 0.19	1.11 ± 0.32	1.12 ± 0.25		
23	Myc	2.33 ± 0.97*	1.02 ± 0.38	10.0 ± 2.40**	5.43 ± 1.48**	12.4 ± 2.67**	1.71 ± 0.95	0.83 ± 0.18	1.12 ± 0.47	2.07 ± 0.40*	$P<0.01$	$P<0.01$
24	Net1	3.04 ± 1.30**	1.71 ± 0.39*	2.62 ± 1.30**	1.29 ± 0.16	1.46 ± 0.30*	0.22 ± 0.07**	1.08 ± 0.64	0.66 ± 0.11	0.85 ± 0.13	$P<0.01$	$P<0.01$
25	Phlda3	3.99 ± 0.64**	5.03 ± 0.81**	6.60 ± 1.68**	0.99 ± 0.31	1.93 ± 0.42*	0.95 ± 0.19	1.01 ± 0.18	0.55 ± 0.12*	0.75 ± 0.20	$P<0.01$	$P<0.01$
26	Plk2	0.57 ± 0.09	1.06 ± 0.71	2.02 ± 0.58*	1.16 ± 0.48	1.70 ± 0.21**	2.42 ± 0.55**	1.50 ± 0.27	0.54 ± 0.26*	0.63 ± 0.09**	$P<0.05$	$P<0.01$
27	Pml	2.01 ± 0.64*	1.86 ± 0.49*	1.71 ± 0.24**	1.18 ± 0.28	0.97 ± 0.25	2.98 ± 0.66**	2.08 ± 0.15**	0.66 ± 0.27	0.94 ± 0.21	$P<0.01$	$P<0.05$
28	Pmm1	2.18 ± 0.36**	2.86 ± 0.72**	2.73 ± 0.47**	0.85 ± 0.07	1.22 ± 0.30	0.77 ± 0.06	1.22 ± 0.23	1.13 ± 0.16	1.08 ± 0.13	$P<0.01$	$P<0.05$
29	Rcan1	1.14 ± 0.41	2.72 ± 0.30**	3.41 ± 0.37**	1.75 ± 0.5*	3.24 ± 0.81**	0.51 ± 0.13	1.02 ± 0.06	0.56 ± 0.34	0.50 ± 0.08	$P<0.01$	$P<0.01$
30	Tnf	0.87 ± 0.17	0.91 ± 0.14	1.44 ± 0.24	1.31 ± 0.33	1.01 ± 0.42	0.74 ± 0.24	0.74 ± 0.23	1.67 ± 0.40*	1.98 ± 0.31**	$P<0.01$	$P<0.01$
31	Tp53	1.12 ± 0.19	1.34 ± 0.34	1.33 ± 0.11	1.63 ± 0.64	1.78 ± 0.63	0.61 ± 0.19	1.43 ± 0.29	0.63 ± 0.19	1.14 ± 0.29		
32	Tubb2c	2.10 ± 0.22**	4.38 ± 1.41**	4.79 ± 1.02**	1.30 ± 0.36	1.59 ± 0.13**	0.45 ± 0.09	0.96 ± 0.16	0.77 ± 0.11	1.33 ± 0.24	$P<0.01$	$P<0.01$
33	Gapdh	0.94 ± 0.12	0.79 ± 0.10	0.52 ± 0.07	0.90 ± 0.16	0.87 ± 0.18	0.84 ± 0.08	0.75 ± 0.06	1.19 ± 0.38	1.13 ± 0.08		

Total RNA was extracted from individual livers, and cDNA was prepared. The expression of the 33 genes was quantified by qPCR, and the gene expression ratio (Exp/Cont) was calculated. The results were analyzed statistically using the Williams' test for each chemical (**significant at $P<0.01$, *significant at $P<0.05$) and the Dunnett's test to compare the genotoxic hepatocarcinogens and the non-genotoxic hepatocarcinogen (DEHP) or the non-genotoxic non-hepatocarcinogen (PNT).

experimental groups (experimental group/control group; Exp/Cont) was within the range of 0.52 to 1.58, as shown in Tables 3.1 and 3.2.

2.3. Statistical analysis

For statistical analysis, we performed a logarithmic ($\log_2$) transformation of the data to stabilize the variance, and the gene expression profiles were normalized to the median gene expression level for the entire sample set.

The significance of dose-dependent increases or decreases in the individual qPCR data was statistically determined using the Williams' test at 4 and 48 h. The experimental groups were compared to a control group. The statistical significance for each gene between the genotoxic hepatocarcinogens, the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen was assessed with the Dunnett's test at 4 and 48 h. The statistical significance between the control water group and olive oil group was assessed using Welch's *t*-test.

Differentiation of the gene expression profiles associated with genotoxic hepatocarcinogens from those associated with the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen was achieved through statistical analysis using PCA. PCA involves a mathematical procedure that transforms a number of potentially correlated variables into a smaller number of uncorrelated variables referred to as "principal components". The first principal component (PC1) accounts for as much of the variability in the data as possible, and each subsequent component accounts for as much of the remaining variability as possible. PCA was performed using the PCA programs in GeneSpringGX11.0.1 (Agilent Technologies, Santa Clara, CA, USA). Initially, PCA was applied to all 32 logarithmically ($\log_2$) transformed ratios (Exp/Cont), with the exception of *Gapdh* and was subsequently tested with various candidate gene sets until the optimal discrimination was achieved. The optimal candidate genes were primarily selected based on the results of Dunnett's test at 4 h and 48 h. The results are presented in two-dimensional (PC1 and PC2) and three-dimensional figures (PC1, PC2 and PC3).

2.4. Gene ontology, pathways and network analysis

Gene ontology analysis was performed using the Gene Ontology Database (http://geneontology.org/) and Ingenuity Pathways Analysis 7.0 (IPA) (http://www.Ingenuity.com). The results were confirmed using the references available in PubMed (http://www.ncbi.nlm.nih.gov/pubmed). The gene pathways and networks were generated with GeneSpringGX11.0.1 and IPA, which enable the visualization and analysis of biologically relevant networks to allow for discovery, visualization, and exploration of therapeutically relevant networks, as previously described [9,10].

2.5. Immunohistochemistry

Immunohistochemical staining was performed using monoclonal antibodies against Cdkn1a/p21 [(p21 (F-5): sc-6246), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA)] and Hmox1 [(Anti-HO-1), Stressgen Bioreagents (Brussels, Belgium)], as described in the manufacture's protocol, on the livers of 4 rats in each group. The TUNEL method was applied using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Flowgen Bioscience Ltd., Nottingham, UK).

3. Results

3.1. Changes in gene expression determined by qPCR and analyzed with the Williams' test and the Dunnett's test

The individual qPCR gene expression results (Exp/Cont) were calculated for each group (4 rats in triplicate assays), the mean ± SD was determined, and statistical significance was assessed using the Williams' test. All 32 genes, with the exception of *Gapdh*, exhibited statistically significant changes in gene expression at least once, at 4 h and/or 48 h, as calculated using the Williams' test (Tables 3.1 and 3.2). The changes in gene expression were generally greater at 4 h than at 48 h. Furthermore, at 4 h, statistical significance was observed in the Dunnett's test between the genotoxic hepatocarcinogens (DEN and DNT) and the non-genotoxic hepatocarcinogen (DEHP) for 19 genes (*Aen*, *Btg2*, *Ccnf*, *Ccng1*, *Ddit4l*, *Gadd45g*, *Gdf15*, *Hspb1*, *Jun*, *Lpp*, *Myc*, *Net1*, *Phlda3*, *Plk2*, *Pml*, *Pmm1*, *Rcan1*, *Tnf* and *Tubb2c*) and between genotoxic hepatocarcinogens and the non-genotoxic non-hepatocarcinogen (PNT) for 18 genes (*Aen*, *Bax*, *Ccnf*, *Ccng1*, *Ddit4l*, *Ephx1*, *Gdf15*, *Hspb1*, *Jun*, *Myc*, *Net1*, *Phlda3*, *Plk2*, *Pml*, *Pmm1*, *Rcan1*, *Tnf* and *Tubb2c*), as shown in Table 3.1. At 48 h, statistical significance was observed between genotoxic hepatocarcinogens (DEN and DNT) and the non-genotoxic hepatocarcinogen (DEHP) for 14 genes (*Aen*, *Ccng1*, *Cdkn1a*, *Cyp21a1*, *Cyp4a1*, *Hhex*, *Igfbp1*, *Ly6al*, *Mdm2*, *Myc*, *Phlda3*, *Pml*, *Pmm1* and *Tubb2c*) and between the genotoxic hepatocarcinogens and the non-genotoxic non-hepatocarcinogen (PNT) for 8 genes (*Ccng1*, *Cdkn1a*, *Cyp4a1*, *Gdf15*, *Igfbp1*, *Mdm2*, *Phlda3 and Plk2*) using the Dunnett's test, as shown in Table 3.2. The results for the housekeeping gene *Gapdh* are also shown in Tables 3.1 and 3.2. This gene was used to normalize the gene expression ratio, as it did not show any changes in expression.

The changes in gene expression detected for 10 major genes (*Aen*, *Btg2*, *Ccng1*, *Cdkn1a*, *Ddit4l*, *Gdf15*, *Jun*, *Phlda3*, *Rcan1* and *Tubb2c*) are shown in Fig. 1. At 4 h, DEN and DNT produced a dose-dependent increase in all of these 10 genes, with the exception of *Aen* under DNT treatment. At 48 h, DEN and DNT produced dose-dependent increases in *Ccng1*, *Cdkn1a* and *Phlda3*. However, DEHP and PNT did not cause dose-dependent increases in these 10 genes at 4 or 48 h. Furthermore, statistical significance (using the Dunnett's test) was observed between the genotoxic hepatocarcinogens and one non-genotoxic hepatocarcinogen (DEHP) and/or the non-genotoxic non-hepatocarcinogen (PNT) for 9 of the genes, with the exception of *Cdkn1a*, at 4 h and for *Aen*, *Ccng1*, *Cdkn1a*, *Gdf15*, *Phlda3* and *Tubb2c* at 48 h. No single gene completely discriminated genotoxic hepatocarcinogens (DEN and DNT) from the non-genotoxic hepatocarcinogen (DEHP) and/or the non-genotoxic non-hepatocarcinogen (PNT).

3.2. Differentiation of the gene expression profiles of the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen by statistical analysis using PCA

Differentiation of the gene expression profile obtained from the genotoxic hepatocarcinogens and from the non-genotoxic hepatocarcinogen and/or from the non-genotoxic non-hepatocarcinogen was achieved *via* statistical analysis using PCA. PCA of all 32 genes was able to differentiate genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogen and/or the non-genotoxic non-hepatocarcinogen at 4 and 48 h (data not shown). Furthermore, we selected specific genes to obtain optimal separation between the genotoxic hepatocarcinogens and the non-genotoxic hepatocarcinogen and/or the non-genotoxic non-hepatocarcinogen using PCA. PCA of 16 genes (*Ccnf*, *Ccng1*, *Cyp4a1*, *Ddit4l*, *Egfr*, *Gadd45g*, *Gdf15*, *Hspb1*, *Ighbp1*, *Jun*, *Myc*, *Net1*, *Phlda3*, *Pml*, *Rcan1* and *Tubb2c*) at 4 h and of 10 genes (*Aen*, *Ccng1*, *Cdkn1a*, *Cyp21a1*, *Cyp4a1*, *Gdf15*, *Igfbp1*, *Mdm2*, *Phlda3* and *Pmm1*) at 48 h optimally differentiated the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogen as well as the non-genotoxic non-hepatocarcinogen, with principal component 1 (PC1) (Fig. 2A-1 at 4 h and Fig. 2B-1 at 48 h). At 4 h, the genotoxic hepatocarcinogens exhibited a PC1 of less than −0.24, while the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen exhibited a PC1 of greater than 2.4 (Fig. 2A-1). At 48 h, the genotoxic hepatocarcinogens presented a PC1 less than 0.06, whereas the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen presented a PC1 greater than 1.8 (Fig. 2B-1). The hepatocarcinogens (in the green circle) were distinguished from the non-hepatocarcinogen (PNT, in the blue circle) with PC1, PC2 and PC3 in 3 dimensions at 4 and 48 h (Fig. 2A-2 and B-2).

3.3. Gene ontology and biologically relevant gene networks

We analyzed the gene ontology of the examined genes using the Gene Ontology Database (in *Rattus norvegicus*) to clarify which categories of genes contributed to the differentiation between the genotoxic hepatocarcinogens and the non-genotoxic hepatocarcinogen and/or the non-genotoxic non-hepatocarcinogen; the

Table 3.2
Gene expression ratio (Exp/Cont) at 48 h and the results of the Williams' test and Dunnett's test.

No.	Gene symbol	Gene expression ratio (Exp/Cont) and Williams' test									Dunnett's test	
		Genotoxic hepatocarcinogens					Non-genotoxic hepatocarcinogen		Non-genotoxic non-hepatocarcinogen		G/DEHP	G/PNT
		DEN (mg/kg bw)			DNT (mg/kg bw)		DEHP (mg/kg bw)		PNT (mg/kg bw)			
		12.5 mg	25 mg	50 mg	125 mg	250 mg	1000 mg	2000 mg	500 mg	1000 mg		
1	Aen	1.90 ± 0.40	0.88 ± 0.13	1.43 ± 0.21	1.22 ± 0.52	1.42 ± 1.11	0.51 ± 0.10**	0.60 ± 0.06**	0.87 ± 0.27	1.10 ± 0.21	$P<0.01$	
2	Bax	0.61 ± 0.10**	0.71 ± 0.15*	0.53 ± 0.22*	1.34 ± 0.57	1.41 ± 0.59	0.77 ± 0.12	0.62 ± 0.11**	0.81 ± 0.23	0.82 ± 0.21		
3	Btg2	0.61 ± 0.37	0.54 ± 0.09	1.10 ± 0.13	1.11 ± 0.31	2.07 ± 0.94	0.97 ± 0.15	1.06 ± 0.34	0.93 ± 0.15	1.13 ± 0.16		
4	Ccnf	0.67 ± 0.09*	0.60 ± 0.18*	0.52 ± 0.11**	1.63 ± 0.65	2.30 ± 1.19*	0.97 ± 0.20	0.69 ± 0.32	0.80 ± 0.31	1.10 ± 0.31		
5	Ccng1	1.90 ± 1.01	2.04 ± 0.54**	4.22 ± 0.45**	1.30 ± 0.78	2.22 ± 2.34	0.49 ± 0.04**	0.70 ± 0.16*	0.72 ± 0.26	0.73 ± 0.17	$P<0.01$	$P<0.01$
6	Cdkn1a	3.12 ± 0.42**	5.88 ± 0.93**	7.79 ± 1.51**	2.26 ± 0.79*	3.31 ± 2.04*	1.63 ± 0.27**	1.53 ± 0.29*	1.03 ± 0.14	1.16 ± 0.21	$P<0.01$	$P<0.01$
7	Cyp21a1	1.32 ± 0.44	1.14 ± 0.42	1.18 ± 0.37	0.91 ± 0.08	1.32 ± 0.26	2.04 ± 0.61**	2.68 ± 0.66**	0.93 ± 0.20	1.73 ± 0.56*	$P<0.01$	
8	Cyp4a1	0.56 ± 0.11**	0.50 ± 0.14**	0.29 ± 0.09**	0.72 ± 0.20	0.70 ± 0.36	5.43 ± 2.30**	9.66 ± 3.13**	1.04 ± 0.42	0.91 ± 0.35	$P<0.01$	$P<0.01$
9	Ddit4l	0.59 ± 0.21	0.80 ± 0.22	1.05 ± 0.26	2.04 ± 1.41	1.93 ± 1.01	0.92 ± 0.13	0.52 ± 0.04**	1.48 ± 0.21**	1.25 ± 0.25		
10	Egfr	0.65 ± 0.18*	0.73 ± 0.15*	0.73 ± 0.24	1.10 ± 0.41	1.02 ± 0.36	1.03 ± 0.18	0.66 ± 0.12*	0.97 ± 0.34	1.01 ± 0.44		
11	Ephx1	0.85 ± 0.13	1.09 ± 0.16	2.05 ± 0.20**	1.80 ± 0.75	1.32 ± 0.16	1.15 ± 0.32	0.91 ± 0.12	0.98 ± 0.15	1.12 ± 0.32		
12	Gadd45b	0.58 ± 0.16	1.18 ± 0.07	0.62 ± 0.24	0.60 ± 0.08	1.00 ± 0.57	1.07 ± 0.16	0.68 ± 0.31	0.72 ± 0.19	0.89 ± 0.17		
13	Gadd45g	1.05 ± 0.05	1.69 ± 0.35*	2.14 ± 0.53**	1.62 ± 0.49	1.42 ± 0.29	0.63 ± 0.17	3.03 ± 4.49	0.83 ± 0.19	2.46 ± 1.77		
14	Gdf15	1.42 ± 0.48	1.49 ± 0.36*	2.29 ± 0.51**	0.70 ± 0.19	1.38 ± 0.79	1.31 ± 0.33	1.41 ± 0.26*	0.81 ± 0.19	0.73 ± 0.16		$P<0.01$
15	Hhex	0.31 ± 0.06**	0.38 ± 0.11**	0.35 ± 0.07**	0.85 ± 0.11	1.02 ± 0.25	1.20 ± 0.42	1.30 ± 0.43	0.57 ± 0.15*	0.84 ± 0.41	$P<0.01$	
16	Hmox1	0.77 ± 0.19	0.58 ± 0.10	1.29 ± 0.28	1.16 ± 0.38	1.63 ± 0.98	0.74 ± 0.11	1.05 ± 0.07	0.95 ± 0.04	1.02 ± 0.09		
17	Hspb1	0.55 ± 0.22**	0.53 ± 0.12**	0.55 ± 0.15**	1.56 ± 0.37*	1.54 ± 0.69	1.33 ± 0.25	0.93 ± 0.25	0.91 ± 0.19	0.97 ± 0.19		
18	Igfbp1	0.27 ± 0.03**	0.35 ± 0.16**	0.44 ± 0.17**	0.59 ± 0.24	0.45 ± 0.23*	0.73 ± 0.16	0.77 ± 0.25	0.62 ± 0.30	0.97 ± 0.18	$P<0.01$	$P<0.01$
19	Jun	0.54 ± 0.02**	0.45 ± 0.07**	0.60 ± 0.20	0.43 ± 0.15	1.10 ± 0.90	1.06 ± 0.20	0.73 ± 0.11	0.52 ± 0.15*	0.46 ± 0.09**		
20	Lpp	0.51 ± 0.09**	0.47 ± 0.22*	0.38 ± 0.11**	1.23 ± 0.16	1.42 ± 0.35	0.59 ± 0.15*	0.37 ± 0.04**	0.54 ± 0.16	0.66 ± 0.14		
21	Ly6al	1.33 ± 0.34	1.17 ± 0.44	1.24 ± 0.23	0.67 ± 0.40	0.92 ± 0.18	1.51 ± 0.26	1.87 ± 0.52*	0.66 ± 0.28	1.45 ± 0.24	$P<0.01$	
22	Mdm2	1.99 ± 0.63*	2.29 ± 0.35**	4.40 ± 0.99**	1.77 ± 0.82	2.33 ± 2.11	1.16 ± 0.19	1.75 ± 0.22**	1.10 ± 0.29	1.05 ± 0.30	$P<0.05$	$P<0.01$
23	Myc	0.17 ± 0.05**	0.37 ± 0.09**	0.48 ± 0.15	1.04 ± 0.61	1.02 ± 0.34	1.18 ± 0.48	0.85 ± 0.52	0.66 ± 0.26	0.73 ± 0.56	$P<0.05$	
24	Net1	0.69 ± 0.10	0.79 ± 0.25	0.40 ± 0.10**	1.15 ± 0.29	1.15 ± 0.36	0.81 ± 0.11	0.65 ± 0.09*	0.90 ± 0.26	1.84 ± 0.94		
25	Phlda3	3.10 ± 1.21**	3.86 ± 1.00**	2.80 ± 0.32**	1.11 ± 0.61	1.76 ± 1.74	0.90 ± 0.28	0.83 ± 0.19	0.67 ± 0.22	0.69 ± 0.17	$P<0.01$	$P<0.01$
26	Plk2	2.16 ± 1.04**	2.91 ± 0.40**	3.14 ± 0.65**	1.01 ± 0.23	1.07 ± 0.38	1.09 ± 0.30	1.75 ± 0.80*	0.86 ± 0.16	0.81 ± 0.18		$P<0.01$
27	Pml	1.02 ± 0.42	1.01 ± 0.28	1.61 ± 0.47	1.01 ± 0.30	1.14 ± 0.60	0.92 ± 0.29	0.57 ± 0.08**	0.76 ± 0.21	1.20 ± 0.27	$P<0.05$	
28	Pmm1	0.90 ± 0.13	0.98 ± 0.03	3.06 ± 0.63**	1.50 ± 0.82	2.98 ± 2.32	1.22 ± 0.16	1.12 ± 0.15	0.76 ± 0.17	1.11 ± 0.26	$P<0.05$	
29	Rcan1	0.84 ± 0.24	1.03 ± 0.17	0.59 ± 0.06*	0.51 ± 0.26*	0.66 ± 0.25	0.85 ± 0.20	0.67 ± 0.07**	0.56 ± 0.13**	0.52 ± 0.13**		
30	Tnf	0.99 ± 0.42	0.59 ± 0.12	1.15 ± 0.19	0.57 ± 0.28	1.01 ± 0.57	1.19 ± 0.16	1.21 ± 0.07	0.57 ± 0.07	1.07 ± 0.57		
31	Tp53	1.29 ± 0.39	1.18 ± 0.16	1.60 ± 0.19**	0.99 ± 0.38	0.90 ± 0.48	1.14 ± 0.21	0.92 ± 0.16	1.02 ± 0.44	0.68 ± 0.36		
32	Tubb2c	0.46 ± 0.09**	0.52 ± 0.07**	0.26 ± 0.07**	0.99 ± 0.19	1.39 ± 0.70	1.18 ± 0.08	1.37 ± 0.29	0.75 ± 0.16	1.01 ± 0.21	$P<0.01$	
33	Gapdh	1.03 ± 0.12	1.00 ± 0.16	1.08 ± 0.15	1.58 ± 0.15	1.55 ± 0.05	0.73 ± 0.14	0.76 ± 0.08	1.08 ± 0.15	1.07 ± 0.20		

Total RNA was extracted from individual livers, and cDNA was prepared. The expression of the 33 genes was quantified by qPCR, and the gene expression ratio (Exp/Cont) was calculated. The results were analyzed statistically using the Williams' test for each chemical (**significant at $P<0.01$, *significant at $P<0.05$) and the Dunnett's test to compare the genotoxic hepatocarcinogens and the non-genotoxic hepatocarcinogen (DEHP) or the non-genotoxic non-hepatocarcinogen (PNT).

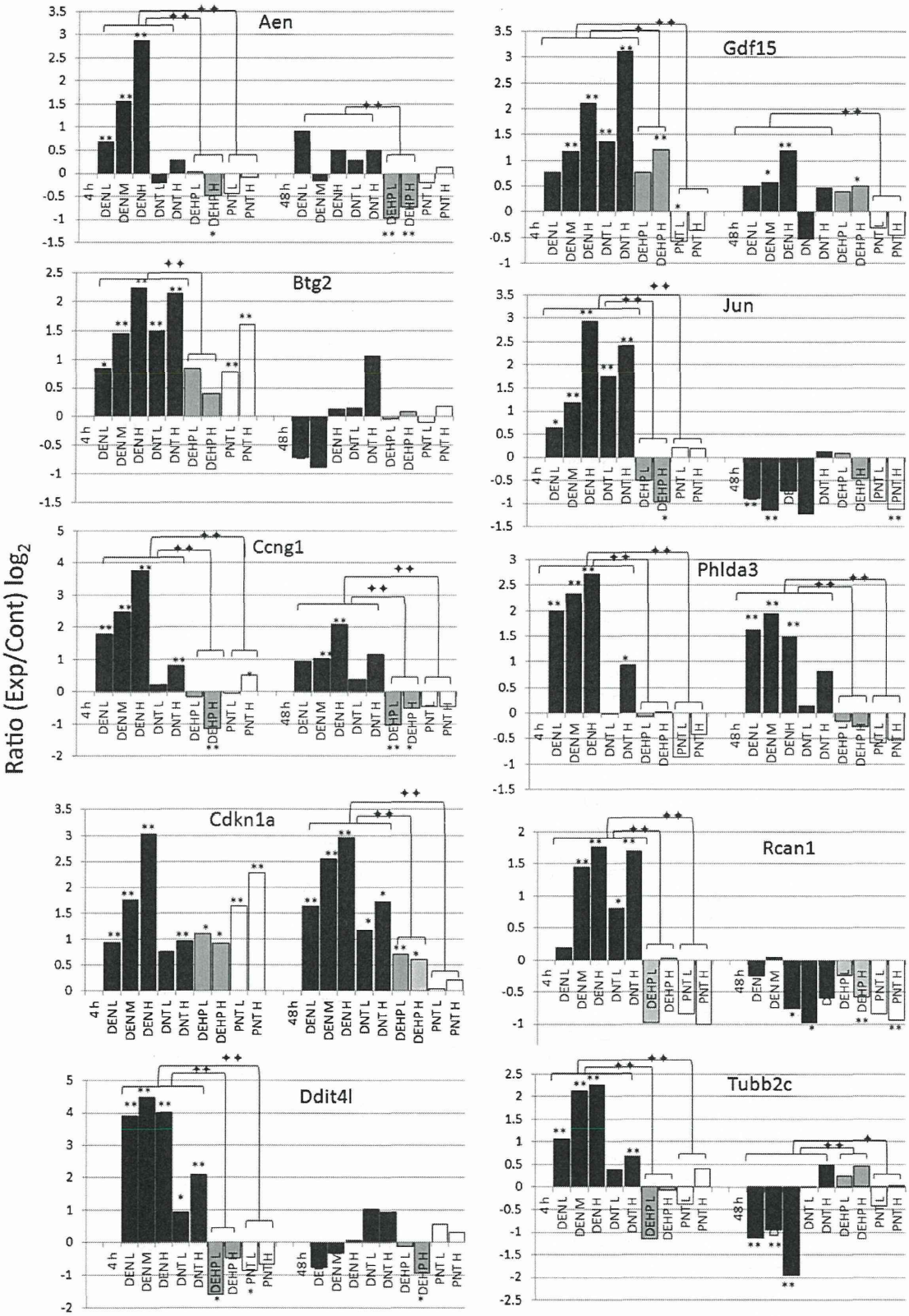

Fig. 1. Changes in the gene expression of 10 genes (*Aen*, *Btg2*, *Ccng1*, *Cdkn1a*, *Ddit4l*, *Gdf15*, *Jun*, *Phlda3*, *Rcan1* and *Tubb2c*) as quantified by qPCR at 4 h and 48 h. DEN L: DEN low dose, DEN M: DEN middle dose, DEN H: DEN high dose, DNT L: DNT low dose, DNT H: DNT high dose, DEHP L: DEHP low dose, DEHP H: DEHP high dose, PNT L: PNT low dose and PNT H: PNT high dose. The statistical significance for each chemical was analyzed using the Williams' test. $^{*}P < 0.05$; $^{**}P < 0.01$. The statistical significance between genotoxic hepatocarcinogens and non-genotoxic hepatocarcinogens or the non-genotoxic non-hepatocarcinogen was analyzed using the Dunnett's test, ♦$P < 0.05$, and ♦♦$P < 0.01$ outside the framework. ■: Genotoxic hepatocarcinogen, ■ : non-genotoxic hepatocarcinogen, □: non-genotoxic non-hepatocarcinogen. Total RNA was extracted from individual livers (4 rats/group) and reverse-transcribed into cDNA. Changes in gene expression were determined in triplicate by qPCR.

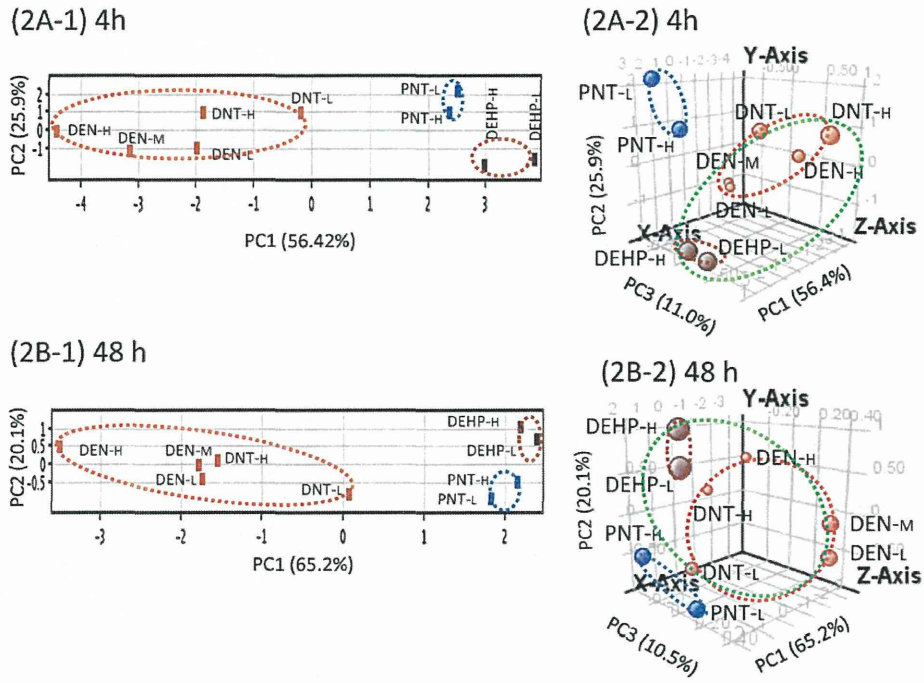

Fig. 2. Principal component analysis (PCA) of the gene expression levels under treatment with 3 types of carcinogens as quantified by qPCR. Genotoxic hepatocarcinogens (red-colored, DEN-L: DEN low dose, DEN-M: DEN middle dose, DEN-H: DEN high dose, DNT-L: DNT low dose and DNT-H: DNT high dose), a non-genotoxic hepatocarcinogen (brown-colored, DEHP-L: DEHP low dose and DEHP-H: DEHP high dose) and a non-genotoxic non-hepatocarcinogen (blue-colored, PNT-L: PNT low dose and PNT-H: PNT high dose). The mean values of triplicate qPCR assays for each sample were analyzed statistically using the PCA program in GeneSpringGX11.0.1. The results of the PCA are shown as the two- or three-dimensional contribution scores for component numbers 1, 2 and 3 (PC1, PC2 and PC3). The contribution scores were produced by conversion from each eigenvector value. A: 4 h, with 16 genes (*Ccnf*, *Ccng1*, *Cyp4a1*, *Ddit4l*, *Egfr*, *Gadd45g*, *Gdf15*, *Hspb1*, *Ighbp1*, *Jun*, *Myc*, *Net1*, *Phlda3*, *Pml*, *Rcan1* and *Tubb2c*), B: 48 h, with 10 genes (*Aen*, *Ccng1*, *Cdkn1a*, *Cyp21a1*, *Cyp4a1*, *Gdf15*, *Igfbp1*, *Mdm2*, *Phlda3* and *Pmm1*). PCA successfully differentiated the genotoxic hepatocarcinogen (red circle) from the non-genotoxic hepatocarcinogen (brown circle) and non-genotoxic non-hepatocarcinogen (blue circle) with principal component 1 at 4 and 48 h (A-1 and B-1). The hepatocarcinogens (green circle) were distinguished from the non-hepatocarcinogen (blue circle) with PC1, PC2 and PC3 at 4 and 48 h (A-2 and B-2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

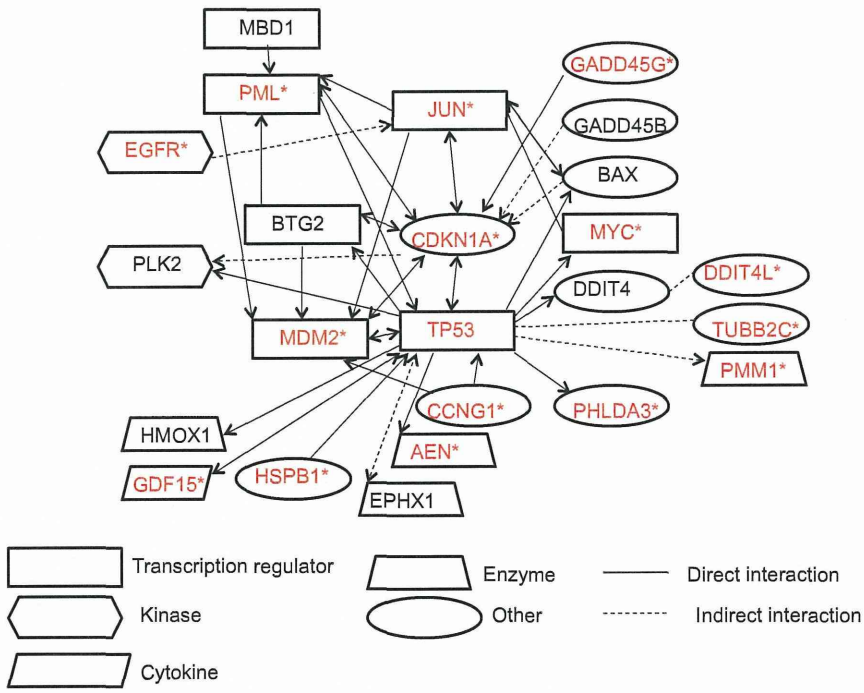

Fig. 3. The gene networks and pathways of 24 genes as determined by qPCR. The network was constructed from the results of Ingenuity Pathways Analysis, GeneSpring software and references from PubMed. The 15 red-colored genes indicated with an asterisk are genes that significantly contributed to the discrimination of the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen by PCA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

results are shown in Table 4. Eight major biological processes were extracted from this gene ontology analysis. The first process, which included 18 genes, was associated with apoptosis; the second was associated with the cell cycle and included 14 genes; the third was associated with cell proliferation and included 11 genes; the fourth process, which included 10 genes, was associated with DNA damage; the fifth was associated with DNA repair and included 1 gene; the sixth was associated with oxidative stress and included 3 genes; the seventh was oncogenes and included 2 genes; and the eighth process was tumor suppressors and included 1 gene. A considerable number of genes classified in the apoptosis, cell cycle, cell proliferation and DNA damage categories exhibited differential gene expression between the genotoxic hepatocarcinogens and the non-genotoxic hepatocarcinogen as well as the non-genotoxic non-hepatocarcinogen. The DNA damage response, which functions *via* signal transduction through a p53 class mediator and results in the induction of apoptosis, was characteristically suggested as an associated biological process. Sixteen genes (*Aen*, *Bax*, *Btg2*, *Ccng1*, *Cdkn1a*, *Ephx1*, *Gdf15*, *Hmox1*, *Hspb1*, *Mdm2*, *Myc*, *Phlda3*, *Plk2*, *Pmm1*, *Pml* and *Tbb2c*) from the present study were reported to be associated with *Tp53*. Among these, 9 genes (*Aen*, *Ccng1*, *Cdkn1a*, *Gdf15*, *Hspb1*, *Mdm2*, *Myc*, *Pml* and *Phlda3*) contributed to the differentiation of the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogen and/or the non-genotoxic non-hepatocarcinogen in the PCA. The summarized gene networks are shown in Fig. 3. The major gene pathway suggested by the network was the *Tp53*-mediated DNA damage response.

3.4. The expression of Cdkn1a and Hmox1 proteins, the level of apoptosis and histological changes

Changes in the expression of Cdkn1a and Hmox1 proteins, the level of apoptosis measured by the TUNEL assay and histology were observed in the genotoxic hepatocarcinogen-treated rats at 48 h (Table 5) but were nearly undetectable at 4 h (results not shown) in all groups. Cdkn1a-positive cells and TUNEL-positive cells were observed in 2 of 4 and all 4 DEN-treated rats at the highest doses, respectively. Cdkn1a-positive cells, Hmox1-positive cells and TUNEL-positive cells were observed in all 8, 6 of 8 and 4 of 8 DNT-treated rats, respectively. An increase in the number of mitotic cells was observed in all 4 DEN-treated rats at the highest dose and 2 of the 4 DNT-treated rats at the highest dose, as determined by HE staining.

3.5. Relative gene expression ratio between the control olive oil and water groups

In the present study, DEN was dissolved in sterile water, while the other chemicals were dissolved or suspended in olive oil. Although olive oil is often used as a non-toxic solvent in animal studies, its effect on gene expression has rarely been examined. Table 6 shows the relative gene expression in the liver in the control olive oil and water groups at 4 and 48 h. Although statistically significant differences were observed in 18 genes based on Welch's *t*-test, the differences in 9 genes did not exceed 2-fold, which could be considered within normal variations, while only 2 genes (*Myc* and *Pml*) showed a 3-fold difference at 48 h. These differences did not appear to affect the results regarding the gene expression ratio (Exp/Cont) (Tables 3.1 and 3.2).

4. Discussion

In the present study, we applied our selected candidate marker genes, which were previously demonstrated to discriminate genotoxic hepatocarcinogens from non-genotoxic hepatocarcinogens in the mouse liver [8–10], to rat hepatocarcinogens in the young rat liver. Consequently, we suggest that the selected genes are also useful for differentiating genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen examined (in the present study) in the young rat liver; these differences were determined by qPCR and PCA at 4 and 48 h after a single administration of these chemicals. Although we did not examine nitroaromatic compounds in our previous experimental method in the mouse, our selected candidate marker genes were also useful for discriminating DNT from the non-genotoxic hepatocarcinogen in the young rat liver. Present results were also congruent with the results of micronucleus assay in young rats [11,12].

In the present study, 32 genes, with the exception of *Gapdh*, exhibited statistically significant changes in gene expression (Exp/Cont) at least once, at 4 and/or 48 h, as detected using the Williams' test (Tables 3.1 and 3.2). The changes in gene expression were generally greater at 4 h than at 48 h. Furthermore, statistical significance was observed, using the Dunnett's test, between the genotoxic hepatocarcinogens and the non-genotoxic hepatocarcinogen (DEHP) and/or the non-genotoxic non-hepatocarcinogen (PNT) for 29 genes (with the exceptions being *Egfr*, *Hmox1*, *Tp53* and *Gapdh*) at 4 and/or 48 h (Tables 3.1 and 3.2). In PCA, the optimal differential gene expression was detected for 16 genes (*Ccnf*, *Ccng1*, *Cyp4a1*, *Ddit4l*, *Egfr*, *Gadd45g*, *Gdf15*, *Hspb1*, *Ighbp1*, *Jun*, *Myc*, *Net1*, *Phlda3*, *Pml*, *Rcan1* and *Tubb2c*) at 4 h and 10 genes (*Aen*, *Ccng1*, *Cdkn1a*, *Cyp21a1*, *Cyp4a1*, *Gdf15*, *Igfbp1*, *Mdm2*, *Phlda3* and *Pmm1*) at 48 h. Seven of these candidate genes (*Aen*, *Ccng1*, *Cdkn1a*, *Mdm2*, *Myc*, *Phlda3* and *Pml*) were classified as DNA damage-associated genes in the Gene Ontology analysis (Table 4), while 11 genes (*Aen*, *Ccng1*, *Cdkn1a*, *Gadd45g*, *Hspb1*, *Jun*, *Mdm2*, *Myc*, *Net1*, *Phlda3* and *Pml*) were classified as apoptosis-associated genes. Fifteen genes (*Aen*, *Ccng1*, *Cdkn1a*, *Ddit4l*, *Egfr*, *Gadd45g*, *Gdf15*, *Hspb1*, *Jun*, *Mdm2*, *Myc*, *Phlda3*, *Pml*, *Pmm1* and *Tubb2c*) were associated with a *Tp53*-mediated signaling pathway (Fig. 3). These genes were characteristically suggested to be induced in the DNA damage response.

Table 4
Gene ontology analysis of the rat genes examined in the present study.

Biological process	Genes
Apoptosis	**Aen***, **Bax***, **Btg2***, **Ccng1***, **Cdkn1a***, Egfr, **Gadd45g***, Hmox1, **Hspb1***, **Jun***, **Mdm2***, **Myc***, **Net1***, **Phlda3***, **Plk2***, **Pml***, **Tnf***, Tp53
Cell cycle	**Bax***, **Ccnf***, **Ccng1***, **Cdkn1a***, Egfr, Gadd45b, **Gadd45g***, **Hhex*** **Jun***, **Mdm2***, **Myc***, **Plk2***, **Pml***, Tp53
Cell proliferation	**Bax***, **Ccng1***, **Cdkn1a***, Egfr, **Hhex***, Hmox1, **Jun***, **Myc***, **Pml***, **Tnf***
DNA damage	**Aen***, **Bax***, **Btg2***, **Ccng1***, **Cdkn1a***, Hmox1, **Mdm2***, **Myc***, **Phlda3***, **Pml***
DNA repair	Egfr
Oxidative stress	Egfr, Hmox1, **Pml***
Oncogene	**Jun***, **Myc***
Tumor suppressor	Tp53

Gene ontology analysis of the examined genes, based on Gene Ontology annotation (http://www.geneontology.org/) and references. Boldface with an asterisk (*) indicates differential gene expression between the genotoxic hepatocarcinogens and the non-genotoxic hepatocarcinogen and/or the non-genotoxic non-hepatocarcinogen at 4 and/or 48 h that was statistically significant based on the Dunnett's test.

Table 5
Immunohistochemistry and histopathological findings in the liver 48 h after treatment with the test chemicals.

Chemical	Vehicle	DEN			DNT		DEHP		PNT	
Dose (mg/kg bw)	0	12.5	25	50	125	250	1000	2000	500	1000
Animal no.	1234	1234	1234	1234	1234	1234	1234	1234	1234	1234
Description of immunohistochemistry										
Anti-Cdkn1a	----	----	----	--11	2111	3111	----	----	----	----
Anti-Hmox1	----	----	----	----	111-	211-	----	----	----	----
TUNEL	--1-	----	----	2222	---1	1-11	----	----	----	----
Test chemical-related histopathological finding										
Cell infiltration, inflammatory, Glisson's sheath	----	----	----	----	11--	2---	----	----	----	----
Hypertrophy, hepatocyte, diffuse	----	----	----	----	1---	2---	----	----	----	----
Single-cell necrosis	----	----	----	----	----	1---	----	----	----	----
Increase, mitosis	----	----	----	2222	----	--11	----	----	----	----

The liver was dissected and examined immunohistochemically. Vehicle: olive oil or sterile water. Observations were graded from 0 (–) to 3 semiquantitatively. –: no findings, 1: minimal, 2: mild, 3: severe.

When we analyzed the expression of *Tp53* itself, we identified a statistically significant but less than 2-fold increase only at 48 h post-injection of DEN (50 mg/kg bw) (Table 3.2), although the basal expression of *Tp53* in the control animals may already have been sufficient for DNA damage to occur under the experimental conditions.

In this paragraph, we compare the dose-dependent alterations in the gene expression induced by 4 h of DEN treatment in the 9-week-old male mouse livers using intraperitoneal injection [9] and the 4-week-old rat livers with oral administration. We observed generally similar changes between mice and rats. Specifically, 18 of the examined genes (*Aen*, *Bax*, *Btg2*, *Ccng1*, *Cdkn1a*, *Cyp21a1*, *Gadd45b*, *Gdf15*, *Hspb1*, *Jun*, *Mbd1*, *Mdm2*, *Myc*, *Net1*, *Plk2*, *Pmm1*, *Rcan1* and *Tubb2c*) showed similar dose-dependent alterations or positive alterations in gene expression in the rat liver at 4 h after DEN administration in the present study (Table 3.1). Among these genes, 7 (*Ccng1*, *Gdf15*, *Hspb1*, *Jun*, *Myc*, *Rcan1* and *Tubb2c*) contributed to the PCA in distinguishing the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen.

Table 6
Relative gene expression ratio between the control olive oil and water groups at 4 h and 48 h and the results of Welch's *t*-test.

No.	Gene symbol	Ratio (olive/water)	
		4 h	48 h
1	Aen	0.81 ± 0.13	1.24 ± 0.14
2	Bax	0.75 ± 0.11	**1.48 ± 0.27***
3	Btg2	1.01 ± 0.39	1.34 ± 0.21
4	Ccnf	1.10 ± 0.16	1.24 ± 0.46
5	Ccng1	0.98 ± 0.19	**1.89 ± 0.17****
6	Cdkn1a	0.58 ± 0.38	0.80 ± 0.19
7	Cyp21a1	0.89 ± 0.23	1.23 ± 0.41
8	Cyp4a1	**1.40 ± 0.25***	1.23 ± 0.56
9	Ddit4l	1.08 ± 0.21	0.79 ± 0.07
10	Egfr	1.26 ± 0.64	**1.81 ± 0.46****
11	Ephx1	1.52 ± 0.71	1.27 ± 0.33
12	Gadd45b	1.41 ± 0.59	0.93 ± 0.12
13	Gadd45g	1.10 ± 1.52	0.50 ± 0.17
14	Gdf15	0.78 ± 0.16	**0.46 ± 0.06***
15	Hhex	1.35 ± 0.51	**0.41 ± 0.11***
16	Hmox1	**0.48 ± 0.09****	**2.06 ± 0.16****
17	Hspb1	0.99 ± 0.14	0.76 ± 0.23
18	Igfbp1	**2.47 ± 1.09***	0.85 ± 0.27
19	Jun	1.11 ± 0.41	0.66 ± 0.29
20	Lpp	0.86 ± 0.17	**1.84 ± 0.18****
21	Ly6al	1.19 ± 0.11	1.60 ± 0.52
22	Mdm2	**0.63 ± 0.07***	**1.64 ± 0.14****
23	Myc	1.09 ± 1.26	**0.32 ± 0.03****
24	Net1	1.10 ± 0.26	0.84 ± 0.22
25	Phlda3	1.05 ± 0.38	**2.82 ± 0.81****
26	Plk2	1.16 ± 0.13	**1.30 ± 0.04***
27	Pml	**0.55 ± 0.25***	**3.48 ± 0.56****
28	Pmm1	**2.86 ± 0.31****	**1.58 ± 0.13****
29	Rcan1	1.04 ± 0.37	0.87 ± 0.22
30	Tnf	**2.58 ± 0.91***	1.23 ± 0.30
31	Tp53	0.90 ± 0.44	**1.35 ± 0.19***
32	Tubb2c	**0.60 ± 0.17***	1.02 ± 0.30
33	Gapdh	1.05 ± 0.14	1.20 ± 0.22

Total RNA was extracted from individual livers, and cDNA was prepared. The expression of the 33 genes was quantified by qPCR and the gene expression ratio (olive/water) was calculated. The results were analyzed statistically using Welch's *t*-test (boldface with **significant at $P < 0.01$, boldface with *significant at $P < 0.05$).

In this paragraph, we compare the gene expression changes induced by 2000 mg/kg bw DEHP at 4 and 48 h after administration between the 9-week-old male mouse livers [10] and the 4-week-old rat livers examined in the present study. The gene expression changes induced by DEHP were rather different between the mouse liver and the rat liver under the present experimental conditions. Specifically, we observed statistically significant changes in the gene expression induced by DEHP in the mouse liver in only 2 genes (*Ddit4 and Hist1h1c*) at 4 h and in 3 genes (*Bhlhe40*, *Hspb1* and *Ly6a*) at 48 h; however, we observed changes in gene expression in a greater number of genes in the rat liver induced by DEHP treatment at a dose of 2000 mg/kg bw in the present study. Statistically significant changes in gene expression were induced in the rat liver by treatment with 2000 mg/kg bw DEHP in 12 genes at 4 h and 16 genes at 48 h: however, only 3 genes (*Cyp41a*, *Egfr* and *Gadd45g*) at 4 h and only 1 gene (*Cyp41a*) at 48 h exhibited changes of greater than 3-fold in response to DEHP; these genes were not associated with DNA damage, and other genes presented rather minor changes.

We examined both the levels of protein expressions by immunohistochemistry using commercially available antibodies (anti-Cdkn1a and anti-Hmox1) and the levels of apoptosis by the TUNEL assay. Slight changes in the protein expression of Cdkn1a and Hmox1 and in the number of TUNEL-positive cells were only observed in the DEN- and DNT-treated rats at 48 h (Table 6), but not in the DEHP- and PNT-treated rats. However, dose-dependent alterations in the expression of proteins or in the level of apoptosis were not observed with DEN and DNT treatment. Although 1 of 4 rats in the vehicle control group showed positive results in the TUNEL assay, it was at a minimal grade, and it has been reported that the TUNEL assay is not necessarily completely negative in the rat liver of vehicle control groups [25]. The present results suggested the moderate induction of apoptosis in DEN-treated rats at a dose of 50 mg/kg bw and weak induction of apoptosis in DNT-treated rats at a dose of 250 mg/kg bw. The immunohistochemical results generally agreed with the results of the gene expression analyses for these proteins and with the apoptotic gene expression.

Few time-course-based differential gene expression profiles of genotoxic and non-genotoxic hepatocarcinogens in rodents have been published based on DNA microarray and real-time PCR analyses. Ellinger-Ziegelbauer et al. used the Affymetrix RG_U34 microarray system to examine the differential gene expression produced by 4 genotoxic (dimethylnitrosamine, 2-nitrofluorene, aflatoxin B1 and 4-(methylnitrosamino)1-(3-pyridyl)-1-butanone) and 4 non-genotoxic hepatocarcinogens (methapyrilene, diethylstilbestrol, Wy-14643 and piperonylbutoxide) in the livers of rats that had been administered doses of the chemicals for 1, 3, 7 and 14 days [26]. They reported the detection of 477 deregulated genes in 23 categories. A total of 9 out of our 33 genes agreed with their candidates, specifically, 5 of these genes were involved in the DNA damage response (*Bax*, *Btg2*, *Ccng1*, *Cdkn1a* and *Mdm2*), 2 genes were involved in the oxidative stress response category (*Ephx1* and *Hmox1*) and 2 genes were involved in cell survival/proliferation (*Gdf15* and *Igfbp1*). These authors proposed that there was a prominent induction of the p53 target genes (*Cdkn1a*, *Bax*, *Btg2*, *Ccng1* and *Mdm2*) by genotoxic carcinogens and of genes involved in cell cycle progression, oxidative protein damage and a regression response by the non-genotoxic carcinogens. We extracted a network associated with the *Tp53*-mediated signaling pathway, which includes these 5 p53 target genes (Fig. 3); however, our network is much more extensive.

In summary, based on our analysis of the 21 genes selected from our mouse DNA microarray and qPCR studies, we suggest that qPCR and PCA are effective methods for distinguishing genotoxic hepatocarcinogens from a non-genotoxic hepatocarcinogen and a non-genotoxic non-hepatocarcinogen in the 4-week-old male F344 rat liver at the early time points of 4 and 48 h after a single administration. The changes in gene expression were greater at 4 h than at 48 h for genotoxic hepatocarcinogens. We recommend the 4 h time point for the first experiment. We analyzed a nitroso compound (DEN) and a nitroaromatic compound (DNT) as genotoxic hepatocarcinogens, a peroxisome proliferator (DEHP) as a non-genotoxic hepatocarcinogen, and an aromatic amide (PNT) as a non-genotoxic non-hepatocarcinogen. Further analysis using a greater number of rat hepatocarcinogens with different chemical properties are required for a final selection of marker genes for discrimination of genotoxic hepatocarcinogens from non-genotoxic hepatocarcinogens as well as non-genotoxic non-hepatocarcinogens in the young rat liver.

Conflict of interest

We do not have any conflicts of interest, including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent application/registrations or grants or other sources of funding.

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Plasma and Serum from Nonfasting Men and Women Differ in Their Lipidomic Profiles

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Biomarkers will play important roles in disease diagnosis, drug development, and the proper use of drugs. Blood is considered the best biofluid for biomarker research because it is easy to access and a wealth of data are available. However, previous studies revealed that several ionic metabolites showed different levels (including presence or absence) in plasma and serum. Thus, attention should be paid to selecting the best biofluid for biomarker exploration. Many lipid molecules have biological significance and thus would be candidate biomarkers. However, no comprehensive study revealing differences in lipid metabolite levels between plasma and serum has been undertaken. Furthermore, gender differences have not been reported. To clarify the difference in the levels of lipid metabolites between human plasma and serum from both genders, we performed lipid metabolomic analysis using liquid chromatography-mass spectrometry-based systems for phospholipids (PLs), lysoPLs, sphingomyelins, ceramides and oxidative fatty acids. Our results revealed that most of the lipid metabolites were present at similar levels in plasma and serum and in males and females. However, several oxidative fatty acid metabolites showed differences. Of the metabolites related to clotting processes, three showed higher levels in serum than in plasma, and three were detected only in serum. Furthermore, four metabolites were present at different levels between males and females, and two were detected only in males. Thus, attention should be paid to the selection of plasma or serum when utilizing these lipid metabolites as biomarkers.

Key words lipid metabolite; plasma; serum; gender; level difference; biomarker

Biomarkers are expected to play important roles in disease diagnosis, drug development, and the proper use of drugs. The information will guide decisions regarding the selection of patient subpopulations and optimal dose, benefit–risk assessment, and regulatory approvals, as surrogate markers for clinical end points.[1] Many useful genomic biomarkers have already been found and used to predict drug responses by stratifying patient populations, such as *UGT1A1* variations for irinotecan therapy and K-ras mutations for anti-epidermal growth factor receptor (EGFR) antibody therapy.[2,3] However, differences in drug responses cannot be satisfactorily predicted only by genomic biomarkers. Metabolomics, analyzing the comprehensive profile of small molecule metabolites found in biological specimens, is expected to lead to novel diagnostic markers for disease status and drug–responses, including adverse reactions.[4]

Blood is an appropriate biofluid for biomarker research because it is easy to access and there is a wealth of background data. Serum and plasma are two distinct biofluids separated from blood after phlebotomy. Recently, several studies focused on the differences in metabolite concentrations between plasma and serum. Thus, choosing between plasma and serum for biomarker exploration must be done carefully. For example, glucose concentrations were reported to be lower in plasma than in serum, possibly as a result of fluid shift from erythrocytes to plasma caused by anticoagulants.[5] Liu *et al.* reported that 36 metabolites, mainly bearing ionic features, differed between plasma (ethylenediaminetetraacetic acid (EDTA) was the anticoagulant) and serum obtained from healthy fasting volunteers: 29 showed higher levels in serum and seven higher in plasma.[6] Most of them were amino acids and glucose derivatives involved in energy production and the urea cycle. Untargeted metabolomics revealed that 19 (mainly ionic) metabolites were detected in either plasma (heparin was the anticoagulant) or serum from small-cell lung cancer patients.[7] Thus, plasma and serum have different metabolite profiles. When seeking biomarkers with applications to clinical research, choosing between plasma and serum should be determined by the nature of the molecules to be measured.

Lipid metabolites such as lysophospholipids (lysoPLs), ceramides (Cers) and eicosanoids are important extracellular signaling molecules through specific receptor interactions or unknown mechanisms. Therefore, they were expected to become candidate biomarkers for early diagnosis of disease, drug therapy, and pathology of various diseases. For instance, several lysophospholipids (especially lysophosphatidic acid) and sphingosine-1-phosphate in plasma were reported to be potential diagnostic and prognostic biomarkers of ovarian cancers.[8] In murine plasma, it was suggested that 20-hydroxyeicosatetraenoic acid (20-HETE), an arachidonic acid-metabolite, was a mediator of rofecoxib-induced facilitation of platelet aggregation, and thus offers a possible explanation for the adverse cardiovascular events associated with its administration.[9]

Therefore, lipid molecules in blood are promising targets for the discovery of candidate biomarkers. However, previous studies have primarily focused on the levels of ionic metabolites in plasma and serum. In contrast, comprehensive studies of lipid metabolite levels have not been conducted. Furthermore, gender differences in lipid metabolite levels have not been reported in plasma and serum. To clarify possible difference in the levels of lipid metabolites between human plasma and serum from both genders and to utilize this information for future biomarker discovery studies, we conducted lipid

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metabolomic analysis using liquid chromatography-mass spectrometry (MS)-based systems. We focused on phospholipids (PLs), lysoPLs, sphingomyelins (SMs), ceramides (Cers), and oxidative fatty acids (oxFA) as target molecules.

MATERIALS AND METHODS

Subjects Venous blood was collected from 10 nonfasting Caucasian volunteers after antecubital venipuncture into 5 mL Vacutainer Serum Separator Tubes with clot activator (Becton Dickinson) and 4.5 mL Vacutainer Plasma Separator Tubes containing K_2EDTA for plasma separation (Becton Dickinson). Participants consisted of 5 males, aged 27 to 33 years old (median 32 years old) and 5 females, aged 26 to 33 years old (median 32 years old). Blood collections from all of the donors were performed in the morning, prior to noon. We could not obtain further personal information on participants such as body weight, height, the individual dietary constituents, and postprandial period except for their gender and ages. According to manufacturer's instructions the samples were centrifuged, and serum and plasma were separated within 2 h of blood collections, immediately frozen, and stored at −80°C for up to one month. The samples were prepared by PromedDX (Norton, MA, U.S.A.) after obtaining informed consent from all participants, and shipped on dry ice. This study was approved by the ethics committee of the National Institute of Health Sciences.

Lipid Metabolite Extraction Serum or plasma (100 μL) samples diluted with methanol were transferred into glass tubes, and a mixture of internal standards (ISs) was added. Internal standards consisted of the following: 1,2-dipalmitoyl D6-3-*sn* glycerophosphatydylcholine (20 nmol/100 μL serum or plasma, Larodan, Malmo, Sweden), deuterated prostaglandin E2 (PGE2-d4, five ng, Cayman Chemical, Ann Arbor, MI, U.S.A.) and deuterated leukotriene B4 (LTB4-d4, 5 ng, Cayman Chemical) were used. Then, chloroform, methanol and 20 mM potassium phosphate (Kp_i) buffer were added to achieve a volume ratio of buffer–methanol–chloroform= 0.8:2:1, and it was mixed vigorously for 5 min. Phase separation was achieved by adding 1 mL of both chloroform and 20 mM Kp_i buffer. After vortexing, the mixture was centrifuged at 1000×***g*** for 10 min. The upper aqueous layer was collected, and the lower organic layer was re-extracted by adding an equal volume of aqueous solution consisting of 100 mM potassium chloride (KCl)–methanol–chloroform=48:47:3. The organic layer was then collected, dried under a gentle stream of nitrogen, dissolved in 1 mL chloroform–methanol (1:1), and stored at −90°C until use (BD sample). To distinguish alkenylacyl and alkyl phospholipid species with the same exact mass, a small aliquot of each BD sample was acid-hydrolyzed using 0.5 N HCl as described previously (BD-HCl sample).[10] BD samples and BD-HCl samples, which contained PLs and sphingolipids, were measured using reverse-phase liquid chromatography/electrospray ionization-mass spectrometry (RPLC/TOF MS). The detailed analytical methods and data processing are described in the supplemental information.

Samples of the aqueous layer were subjected to solid extraction to obtain oxidative fatty acids (oxFAs). Briefly, samples were diluted 10-fold using water adjusted to pH 3.0 with 1 N HCl, and then applied to preconditioned Oasis SPE cartridges (60 mg, Waters, Milford, MA, U.S.A.). After washing the column with 3 mL of Milli-Q water followed by 3 mL of hexane, the samples were then eluted with 3 mL methyl formate (MF). MF fractions were dried under nitrogen, dissolved in 1 mL chloroform–methanol (1:1), and stored at −90°C until use (MF sample). MF samples were measured using RPLC-triple quadrupole mass spectrometric multiple reaction monitoring. Their detailed analytical methods and the data processing are described in the supplementary information.

Statistical Analysis Data were analyzed statistically using the Wilcoxon (matched-pairs) signed-rank test for the comparison of each metabolite level between serum and plasma (from the same subjects), and the Mann-Whitney *U*-test for the levels between males and females. The statistical analysis was performed with Prism ver. 5.0 (GraphPad Software, Inc., La Jolla, CA, U.S.A.). p values less than 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

Glycerophospholipids and Sphingolipids PLs, lysoPLs, SMs and Cers were measured using RPLC/TOF-MS. A total of 72 metabolites were identified consisting of 27 phosphatidylcholines (PCs), 6 ether-type PCs, 7 lysoPCs, 5 phosphatidylethanolamines (PE), 3 plasmalogen PEs, 2 lysoPEs, 6 phosphatidylinositol (PI), 14 SMs, and 2 Cers. Supplementary Table 1 summarizes relatively quantified data of IS-normalized peak heights of each metabolite.

There were no significant differences in the 72 lipid levels between plasma and serum in either gender. On the other hand, female serum contained significantly higher levels of 4 metabolites than did male serum: 36:2 PE (fold-change of median level in females relative to that in males was 1.8-fold, p=0.032), 36:2 SM (1.3-fold, p=0.032), 40:2 SM (1.5-fold, p=0.016) and 42:1 Cer (1.6-fold, p=0.008). These results suggested that glycerophospholipid and sphingolipid levels are generally similar between the plasma and serum and between males and females, although several metabolites showed significant but less than 2-fold differences between genders.

One study showed that lysophosphatidylinositols (LPIs) were more abundant in plasma than in serum, possibly because their consumption was prevented in plasma by inhibition of the blood clotting cascade that activates thrombin and

Table 1. Oxidative Fatty Acid Metabolites with Different Levels in Plasmas and Sera or in Genders

Metabolites with higher levels in serum than in plasma (more than 10-fold depending on the individual)		
Thromboxane B2	12-HHT	12-HETE
Metabolites detected only in serum (plasma levels were below the detection limit)		
12-HEPE	14-HDoHE	20-Hydroxy leukotriene B4
Metabolites with different levels between males and females (Fig. 1)		
11,12-diHETrE	14,15-diHETrE	17,18-diHETE
20-hydroxy leukotriene B4		
Metabolites detected only in males (females levels were below the detection limit)		
16-HETE	18-HETE	

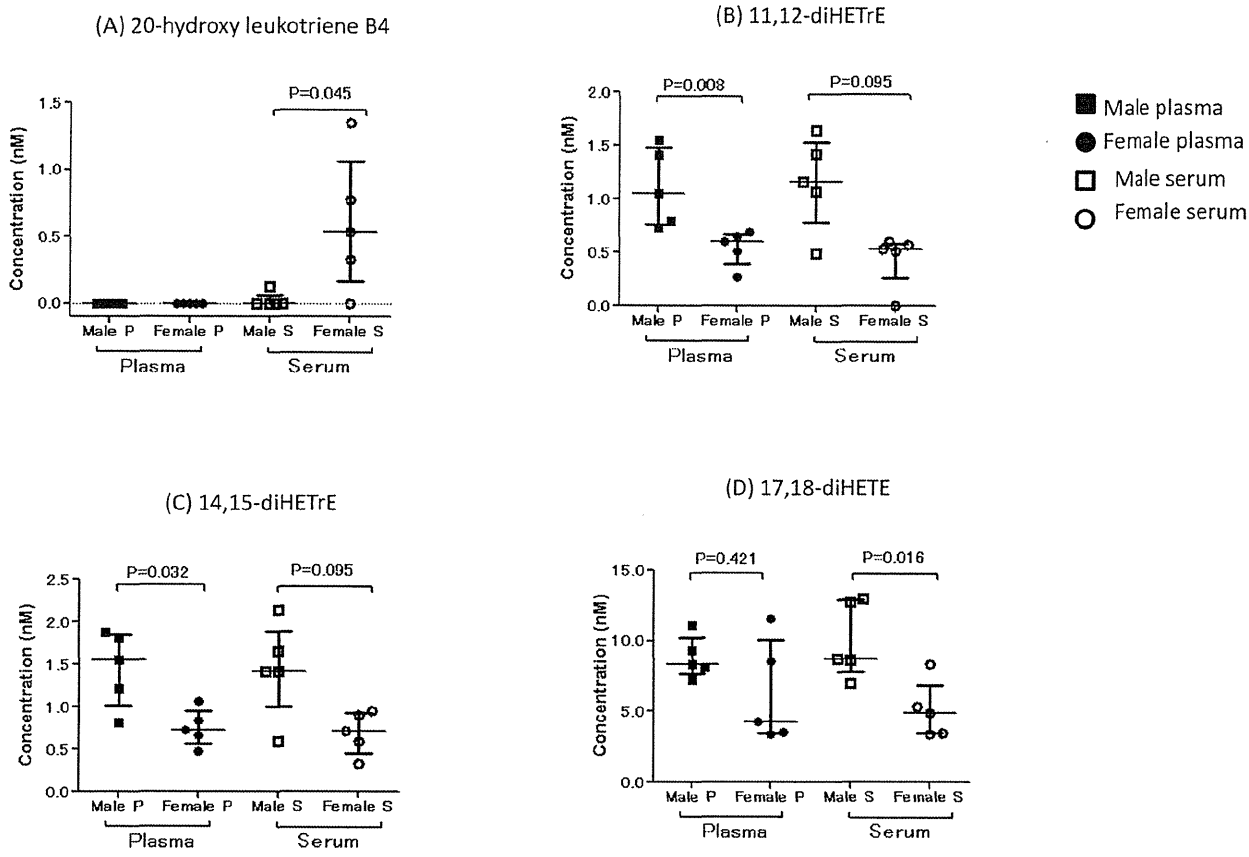

Fig. 1. Oxidative Fatty Acid Metabolites with Levels Significantly Different between Males and Females

Oxidative fatty acid metabolites were quantified in plasmas (n=5) and sera (n=5) of both genders, and compared between males and females for all matrices by Mann–Whitney U-test. The graph shows the median with interquartile range. Symbols: closed square, male plasma; closed circle, female plasma; open square, male serum; open circle, female serum.

other proteases.[11] However, we could not detect LPI because they were too hydrophilic to retain in our RPLC system.

Oxidative Fatty Acids Free polyunsaturated fatty acids and their oxidized metabolites were quantified using standard chemicals, and 30 detected species are listed in supplemental Table 2. Statistical analysis was performed for the levels in plasmas and sera, and we found that no metabolite was significantly different. However, some serum samples contained much higher (more than 10-fold) levels of three metabolites than did the corresponding plasma derived from same individuals: thromboxane B2 (TXB2) (maximally 156-fold), 12-hydroxyheptadecatrienoic acid (12-HHT, maximally 149-fold) and 12-HETE (5 to 208-fold). These variations resulted in wide inter-individual differences between serum and plasma concentration ratios. In addition, several oxFAs were detected only in serum. Two metabolites 12-hydroxyeicosapentaenoic acid (12-HEPE), and 14-hydroxydocosahexaenoic acid (14-HDoHE) were detected only in serum from both genders, while in plasma, they were below the quantification limits shown in supplemental Table 3. Moreover, 20-hydroxy LTB4 was mostly detected only in female serum (Fig. 1).

The essential difference between plasma and serum is that serum is collected after clotting, whereas plasma is collected without clotting in the presence of an anticoagulant such as ethylenediamine tetraacetic acid (EDTA) or heparin. During blood coagulation, arachidonic acid is released primarily from membrane phospholipids in platelets by phospholipase A2. The oxidative fatty acids that were increased (or only detected in serum) compared with plasma from the same blood donor were likely involved in the clotting process. The 12-HHT was co-generated in the process of TXA2 (a potent activator of platelet aggregation) generation by thromboxane synthase.[12] TXB2 is the stable metabolite of TXA2. Thrombin-activated platelets produce proinflammatory factor LTB4 *via* the 5-lipoxigenase (5-LOX) pathway and is further metabolized into the 20-hydroxy form,[13] Activation of 12-LOX in platelets could produce 12-HETE from arachidonic acid,[14] and 12-HEPE from eicosapentaenoic acid,[15] and 14-HDoHE from docosahexaenoic acid.[16] The 12-LOX products of PUFA such as 12-HETE, 12-HEPE and 14-HDoHE function as inhibitors of platelet aggregation.[17] Thus, a higher abundance of these oxFAs in serum than in plasma is likely due to the clotting process.

Next we compared the oxFAs levels between males and females. As described in the above section, 20-hydroxy LTB4 levels were significantly higher in female serum than in male serum (p=0.045). Furthermore, the levels of 11,12-dihydroxyeicosatrienoic acid (11,12-diHETrE, 0.6-fold in plasma, p=0.008), 14,15-diHETrE (0.5-fold in plasma, p=0.032) and 17,18-dihydroxyeicosatrienoic acid (17,18-diHETE, 0.6-fold in serum, p=0.016) were higher in males than in females in both plasma and serum, though the statistical significance was dependent on the blood matrices (Fig. 1). The metabolites 16-HETE and 18-HETE were detected only in some males,

while their levels in females were below the detection limits irrespective of blood matrices. The reason for these differences is currently unknown. Further studies are needed to provide a mechanistic explanation for gender differences in these metabolites.

In conclusion, current lipidomic study has revealed that most of the lipid metabolites are present at similar levels in human plasma and serum and between males and females, although several oxidative fatty acid metabolites showed different levels between both of them. The levels of 3 metabolites (TXB2, 12-HHT, 12-HETE) were more than 10-fold higher in some sera than their corresponding plasmas from the same individuals although statistical significance was not obtained due to wide inter-individual differences. Three metabolites (12-HEPE, 14-HDoHE and 20-hydroxy LTB4) were detected only in serum compared with plasma. Above 6 metabolites (TXB2, 12-HHT, 12-HETE, 12-HEPE, 14-HDoHE and 20-hydroxy LTB4) were reported to be related to the clotting process (platelet aggregation and its inhibition). Furthermore, 4 metabolites (11,12-diHETrE, 14,15-diHETrE, 17,18-diHETE, 20-hydroxy LTB4) were present at significantly different levels between males and females, and 2 (16-HETE, and 18-HETE) were detected only in males. The limitation of this study is that small numbers of subjects in each gender category might decrease the power for the detection of metabolites with wide inter-individual difference in their levels. Furthermore, the present study did not consider the individual dietary variations which may affect lipid metabolite levels in blood.[18]

Biomarkers should reflect normal biologic processes in a body (health or disease state or drug responses). Therefore, the metabolites that involved in platelet aggregation such as TXB2, 12-HHT, 12-HETE need to be measured using plasma because the clotting process caused large increases in these metabolite levels, resulting in no reflection to their real levels in the blood. As for hydrophilic metabolites such as amino acids and glucose derivatives, however, serum was recommended as an appropriate biofluid for biomarker exploration because these compounds were biochemically metabolized *in vitro* more quickly in plasma (maybe due to active metabolism in the blood cells) than in sera after blood collection.[6] Thus, the choice of which biofluids as a sample in clinical situation depends on the nature of the molecules to be measured. The present study showed that attention should be paid in selecting plasma or serum and for utilizing lipid metabolites as biomarkers. Although further studies clearly needed using larger sample sizes (such as thousands of subjects), our results provide basal information useful for future exploration and selection of biomarkers for disease diagnosis and therapeutic intervention.

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