Fatty acid free bovine serum albumin (BSA, Roche Cat # 70335128), chloroform, methanol, butylated hydroxytoluene (BHT), thin layer chromatography (TLC) solvent: chloroform:methanol:petroleum benzene:acetic acid:boric acid (40:20:30:10:1.8; v/v/v/v/w), 2,5-bis-(5'-tert-butylbenz-oxazolyl-2')thiophene (BBOT, CAS# 7128-64-5), saline, CMS (chlorophorm:methanol:saline, 86:14:1), Malachite green phosphate detection kit from R&D Systems (Cat # DY996), 6 N KOH, perchloric acid, BCA Protein Assay Reagent A (Pierce #23223), Reagent B (4 % $CuSO_4.5H_2O$), 0.1 mg BSA standard, glacial acetic acid, anhydrous sodium sulphate, Triton X-100, SDS solution (2% sodium dodecyl sulphate, 20 mM $NaHCO_3$, 2 mM EDTA)

5.3.2. Maintenance of HepG2 cell culture

HepG2 cells (ATCC HB-8065) were cultured in 75 cm^3 flasks with culture mediums described above and sub-cultured at 80% confluency. Culture medium was replaced when necessary.

5.3.3. Preparation of fatty acids

Stocks (10 mM) of the individual FAs (*c*9*t*11, *t*10*c*12, ALA, LA) were complexed with FA-free BSA for treatment of cell cultures, according to a previously described method (Ellsworth et al., 1986). Briefly, the required FAs were measured relative to the amount needed to make up the final concentration and mixed with 1 ml pure ethanol and 1 mg phenolphthalein. A 0.1M NaOH solution was used to titrate the mixture and solvent was evaporated on a 37°C heating block under N_2 gas. The FA salt was then dissolved in DPBS,

5.4.6.4 Delta 6 desaturase substrate and product (Delta 6-S and Delta 6-P)

5.4.6.4.1. PC

***t*10*c*12 CLA:** Compared to the control, *t*10*c*12 CLA treatment significantly increased Delta 6 desaturase substrate and product at 0.5 mM. Concentration differences were seen where 0.05 mM showed a significantly higher ($P<0.05$) effect than 0.075 and 0.15 mM.

LA: Treatment with LA significantly increased ($P<0.05$) Delta 6 S/P ratio. A significant (<0.05) dose increase was seen between the highest concentration and the lowest concentration.

***c*9*t*11 CLA:** Delta 6 substrate was significantly increased by *c*9*t*11 CLA treatment, with dose difference between 0.2 mM and the two lower concentrations. Compared to control, Delta 6 product showed similar increase, which resulted in an insignificant response in the Delta 6 S/P ratio.

ALA: Compared to control, ALA treatment significantly increased the Delta 6 substrate, with dose differences seen between 0.3 mM and the two lower concentrations. Slight reduction in Delta 6 product can be seen, which is dose significant ($P<0.05$). This effect significantly increased ($P<0.05$) the Delta S/P ratio in a dose dependent manner ($P<0.05$).

5.4.6.4.2. PE

***t*10*c*12 CLA:** Compared to the control, *t*10*c*12 CLA treatment significantly decreased delta 6 desaturase substrate at 0.075 and 0.15 mM. The Delta 6 S/P ratio was significantly increased ($P<0.05$) at 0.5 mM.

LA: Treatment with LA significantly increased (P<0.05) Delta 6 S in a dose dependent manner (P<0.05). Delta 6 P was also significantly increased, but dose difference seen between the higher concentrations and the lowest concentration. The Delta 6 S/P was not significantly affected.

***c*9*t*11 CLA:** Delta 6 substrate was significantly increased (P<0.05) by *c*9*t*11 CLA treatment, and a slight increase in Delta 6 P is evident, therefore the effect on the ratio did not differ significantly from the control.

ALA: Treatment with ALA significantly increased (P<0.05) Delta 6 S. A dose increase was notable between 0.3 mM and the two lower concentrations. No effect on Delta 6 P was seen, therefore the Delta 6 S/P ratio elevated significantly (P<0.05) in a dose dependent manner (P<0.05).

5.4.6.5. AA PC/PE (arachidonic acid PC/PE ratio)

Treatment with t10c12 CLA was significantly higher than the control at 0.075 mM, which decreased as the concentration increased. LA, c9t11 CLA and ALA significantly increased (P<0.05) the AA PC/PE ratio compared to control, but no do differences between FA concentrations were seen.

Table 5.1: Fatty acid analysis (µg fatty acid/mg protein) of the phosphotidylcholine (PC) phospholipid fraction in HepG2 cells

	*t*10*c*12 CLA			LA			*c*9*t*11 CLA			ALA			Control
Dose (mM)	**0.5**	**0.15**	**0.075**	**0.6**	**0.3**	**0.15**	**0.2**	**0.1**	**0.05**	**0.3**	**0.15**	**0.075**	**0**
SATS													
C14:0	0.25±0.11	0.39±0.26	0.60±0.23	0.44±0.05*b*	0.37±0.07*b*	1.14±0.12**a*	1.47±0.34**b*	0.94±0.26	0.63±0.30*a*	0.40±0.16	0.43±0.18	0.37±0.13	0.45±0.15
C16:0	3.81±1.12*b*	5.31±0.66*b*	7.46±0.*86***a*	7.40±0.97**b*	5.79±0.43*b*	11.4±1.48**a*	19.5±3.26**c*	15.4±2.14**b*	11.3±0.75**a*	5.10±1.31	5.24±1.72	4.97±0.35	4.53±0.99
C18:0	0.80±0.14*b*	1.48±0.*18***a*	1.55±0.59**a*	0.91±0.12**b*	1.01±0.37**b*	1.62±0.07*a*	2.71±0.14**c*	2.14±0.17**b*	1.61±0.18**a*	0.39±0.09	0.40±0.14	0.45±0.12	0.46±0.19
Total SATS	4.57±1.51*b*	7.18±0.74	9.80±1.*39a*	8.75±1.13c	7.17±0.79*b*	14.2±1.578*a*	24.6±5.12**c*	18.5±2.52**b*	13.5±0.92**a*	6.18±1.60	6.36±2.08	6.02 ±0.55	6.73±2.71
MUFA													
C16:1n-7	0.15±0.05*	0.41±0.11*	0.56±0.37*	0.44±0.04**b*	0.45±0.03**b*	1.22±0.15*a*	4.25±0.69*	4.37±0.68*	3.24±0.52*	0.55±0.15*	0.73±0.24*	0.87±0.11*	1.73±0.54
C18:1n-9	0.81±0.18**b*	1.66±0.14*	2.78±1.02**a*	0.91±0.11**b*	1.07±0.21**b*	2.44±0.*12***a*	7.66±1.33*	8.94±0.08*	8.03±0.86*	0.84±0.21*	1.19±0.35*	1.64±0.09*	3.29±0.36
C18:1n-7	0.46±0.12*	0.81±0.08*	1.32±0.64*	0.70±0.09**b*	0.87±0.09**b*	2.27±0.30*a*	3.76±0.46**b*	4.92±0.13**a*	4.60±0.58*	0.63±0.15*	0.84±0.26*	1.16±0.10*	2.34±0.57
C20:1n-9	0.33±0.12*	0.20±0.03	0.19±0.07	0.07±0.*00***b*	0.10±0.02*b*	0.22±0.04*a*	1.33±0.07**b*	1.11±0.22**b*	0.82±0.10**a*	0.08±0.03	0.08±0.03	0.08±0.02	0.18±0.08
Total MUFA	1.69±0.45*	3.08±0.22*	4.85±1.71*	2.15±0.20**b*	2.48±0.32*	6.15±0.55*a*	17.0±2.38*	18.4±2.73*	16.7±1.43*	2.10±0.54*	2.84±0.86*	3.76±0.27*	8.42±2.76
N6 PUFA													
C18:2	0.45±0.14	0.57±0.03	0.57±0.19	8.86±0.58**c*	6.54±0.73**b*	4.98±0.*64***a*	2.95±0.52**b*	2.27±0.*17***a*	1.85±0.*23***a*	1.67±0.15**c*	1.39±0.64**b*	0.88±0.*17***a*	0.60±0.22
C18:3	ND	ND	ND	2.24±0.17**c*	2.71±0.34**b*	0.10±0.01*a*	ND	ND	ND	ND	ND	ND	ND
C20:2	ND	ND	ND	0.67±0.09**b*	0.39±0.04**b*	0.45±0.*04***a*	ND	ND	ND	ND	ND	ND	0.21±0.00
C20:3	0.01±0.01*	0.04±0.03	0.04±0.04	0.34±0.04**b*	0.36±0.08**b*	0.80±0.*10***a*	0.20±0.04*	0.22±0.03*	0.21±0.04*	0.04±0.01*	0.05±0.01*	0.06±0.00	0.09±0.03
C20:4	0.13±0.03*b*	0.24±0.04*a*	0.33±0.04**a*	1.51±0.15**b*	1.46±0.13**b*	2.95±0.*45***a*	0.92±0.10**b*	0.80±0.11	0.68±0.09*a*	0.15±0.03	0.14±0.04	0.16±0.01	0.17±0.07
C22:5	ND	ND	ND	0.06±0.01**b*	0.07±0.01**b*	0.16±0.02**a*	ND	ND	ND	ND	ND	ND	ND
Total N6	0.73±0.26	0.85±0.05	0.94±0.15	13.6±0.77**c*	11.5±0.81**b*	9.62±1.*09***a*	4.07±0.52*b*	3.29±0.*31***a*	2.74±0.*35***a*	1.86±0.68	1.29±0.82	1.30±0.45	0.92±0.35
N3 PUFA													
C18:3	ND	ND	ND	ND	ND	ND	ND	ND	ND	3.79±0.88**c*	1.83±0.57**b*	0.85±0.11**a*	ND
C20:3	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.58±0.12**b*	0.40±0.13**a*	0.27±0.03**a*	ND
C20:5	ND	ND	ND	ND	ND	ND	0.55±0.09*	0.51±0.10*	0.43±0.04*	0.81±0.17*	0.80±0.24*	0.77±0.11*	ND
C22:5	ND	ND	ND	0.03±0.*01***a*	0.02±0.00**b*	0.03±0.*00***a*	0.04±0.00*	0.04±0.01*	0.04±0.00*	0.06±0.02*	0.06±0.02*	0.07±0.01*	ND
C22:6	0.03±0.*03***b*	0.11±0.02	0.14±0.03*a*	0.25±0.05**b*	0.18±0.02**b*	0.39±0.*03***a*	0.50±0.06*	0.51±0.05*	0.48±0.08*	0.09±0.02*b*	0.11±0.03	0.18±0.03*a*	0.14±0.06
Total N3	0.03±0.03**b*	0.11±0.02	0.14±0.03*a*	0.28±0.06**b*	0.20±0.02*b*	0.42±0.*03***a*	1.09±0.15*	1.06±0.15*	0.95±0.11*	5.32±1.20**b*	3.21±0.98**a*	2.14±0.28**a*	0.15±0.07
PUFA	0.79±0.33	0.96±0.07	1.13±0.13	13.9±0.*77***c*	11.7±0.82**b*	10.0±1.11**a*	5.17±0.65**b*	4.35±0.45*	3.69±0.*47***a*	7.18±1.88**c*	4.50±1.48**b*	3.44±0.48**a*	1.07±0.37
LC PUFA	0.16±0.06**b*	0.39±0.04*a*	0.56±0.13*a*	2.84±0.*06***b*	2.47±0.*26***b*	4.96±0.*71***a*	2.21±0.15*	2.08±0.29*	1.84±0.23*	1.72±0.36*	1.57±0.46*	1.50±0.18*	0.46±0.11
LC /PUFA	0.18±0.03*b	0.40±0.08a	0.49±0.07a	2.84±0.06**b*	2.47±0.26**b*	4.96±0.71**a*	2.21±0.15*	2.08±0.29*	1.84±0.23*	1.72 ± 0.36*	1.57±0.46*	1.50±0.18*	0.46±0.11
CLA													
***c*9*t*11**	0.65±0.10**c*	0.46±0.07**b*	0.22±0.90**a*	ND	ND	ND	11.1±0.*32***c*	5.89±1.*06***b*	2.77±0.*24***a*	ND	ND	ND	ND
***t*10*c*12**	4.85±0.77**c*	3.76±0.57**b*	2.60±0.29**a*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Phospholipids	46.12±12.41	73.02±25.03	69.65±27.00	43.14±6.92	46.45±5.11	42.15±4.42	64.57±3.87	55.97±5.72	61.99±3.03	30.33±3.03*	32.12±2.94*	25.28±2.85*	65.32±23.98

Values are expressed as mean (µg fatty acid/mg protein) ± SD of 4 replications. * denotes significant (P<0.05) difference compared to control. The letters (*a,b and c)* denote a significant (P<0.05) difference between treatment according to ANOVA (analysis of variance) analyses, where *a*, *b* and *c* are all different. FA = fatty acid, mM = treatment concentration in mM, SATS = saturated fatty acids, MUFA = monounsaturated fatty acids, N6 PUFA = omega 6 polyunsaturated fatty acids, N3 PUFA = omega 3 polyunsaturated fatty acids, PUFA = polyunsaturated fatty acids, FA containing 2 or more double bonds, LCPUFA = long chain polyunsaturated fatty acids greater than 20 carbons in length, LC/PUFA = the ration between long chain FA and PUFA, CLA = conjugated linoleic acid, C9T11 = *cis*9-*trans*11 CLA, T10C12 = *trans*10-*cis*12 CLA, ND = not detected.

Table 5.2: Fatty acid analysis (µg fatty acid/mg protein) of the phosphotidylethanolamine (PE) phospholipid fraction in HepG2 cells

FA	*t*10*c*12 CLA			LA			*c*9*t*11 CLA			ALA			Control
Dose (mM)	0.5	0.15	0.075	0.6	0.3	0.15	0.2	0.1	0.05	0.3	0.15	0.075	0
SATS													
C14:0	0.06±0.03	0.04±0.01	0.05±0.02	0.13±0.02**b*	0.11±0.02*	0.08±0.01*a*	0.16±0.03**b*	0.10±0.02*a*	0.07±0.02*a*	0.03±0.03	0.06±0.03	0.05±0.01	0.06±0.03
C16:0	0.90±0.17	1.00±0.09	1.63±0.71	2.24±0.41*	1.62±0.09*b*	2.51±0.54**a*	3.10±0.47**b*	2.78±0.28**b*	1.88±0.21**a*	0.98±0.39	1.13±0.26	1.02±0.05	1.11±0.37
C18:0	0.84±0.17*b*	1.23±0.07	1.40±0.16*a*	2.16±0.32**b*	2.08±0.31**b*	3.08±0.34**a*	2.97±0.48**b*	2.87±0.12*	2.21±0.38**a*	1.35±0.25	1.29±0.30	1.03±0.03	1.06±0.32
Total SATS	1.81±0.35	2.28±0.17	4.04±1.67	4.53±0.22*	3.81±0.38**b*	5.68±0.77**a*	6.24±0.93**b*	5.76±0.41**b*	4.16±0.29**a*	2.36±0.66	2.53±0.59	2.11±0.04	2.11±0.04
MUFA													
C16:1n-7	0.11±0.04**b*	0.19±0.03*	0.39±0.18**a*	0.24±0.02**b*	0.23±0.02**b*	0.45±0.06*a*	1.37±0.22*	1.32±0.13*	1.16±0.21*	0.17±0.08*	0.27±0.07	0.28±0.02	0.43±0.13
C18:1n-9	0.98±0.21*	1.41±0.11	1.72±0.04	1.34±0.18**b*	1.73±0.15*b*	3.02±0.28*a*	5.33±0.71*	5.70±0.24**b*	4.33±0.45**a*	1.09±0.25*	1.38±0.33	1.39±0.09	2.27±0.76
C18:1n-7	0.18±0.08*	0.28±0.02*	0.58±0.22*	0.78±0.12*b*	0.91±0.09*b*	2.37±0.91**a*	1.65±0.23	1.97±0.04*	1.70±0.24*	0.55±0.11*	0.65±0.17	0.66±0.02	1.15±0.40
C20:1n-9	0.17±0.02	0.11±0.01	0.18±0.04	0.08±0.01*b*	0.12±0.02*b*	0.28±0.06**a*	1.16±0.15**b*	0.88±0.08**a*	0.69±0.10**a*	0.09±0.04	0.10±0.02	0.09±0.02	0.13±0.05
Total MUFA	1.40±0.43*	2.00±0.13*	2.84±0.41	2.43±0.31*b*	2.99±0.23*b*	6.13±1.17**a*	9.51±1.27*	9.77±0.57*	7.87±0.90*	1.90±0.48	2.39±0.57	2.43±0.10	3.98±1.29
N6 PUFA													
C18:2	0.24±0.18	0.17±0.01	0.23±0.06	5.95±0.36**c*	3.59±0.97**b*	2.00±0.56**a*	1.52±0.21**b*	1.03±0.31**a*	1.06±0.25*	1.85±0.65*	1.38±0.62*	1.47±0.44*	0.26±0.09
C18:3	ND	ND	ND	2.54±0.39**b*	2.85±0.17**b*	0.04±0.01**a*	ND	ND	ND	ND	ND	ND	ND
C20:2	ND	ND	ND	0.75±0.15**b*	0.36±0.03**a*	0.33±0.05**a*	ND	ND	ND	ND	ND	ND	ND
C20:3	0.02±0.02*b*	0.05±0.01	0.06±0.03*a*	0.36±0.07**b*	0.31±0.03**b*	0.53±0.05**a*	0.52±0.17**b*	0.21±0.01*a*	0.18±0.03*a*	0.05±0.01	0.05±0.01	0.05±0.00	0.10±0.05
C20:4	0.53±0.15	0.70±0.06	0.54±0.2	2.03±0.46**b*	2.42±0.20**b*	4.48±0.36**a*	1.42±0.23*	1.62±0.03*	1.23±0.25*	0.42±0.07*	0.44±0.10*	0.43±0.02*	0.76±0.21
C22:4	ND	ND	ND	0.28±0.07**b*	0.20±0.02**a*	0.20±0.03**a*	ND	ND	ND	ND	ND	ND	ND
C22:5	ND	ND	ND	0.32±0.09**b*	0.40±0.05**b*	0.67±0.08**a*	ND	ND	ND	ND	ND	ND	ND
Total N6	0.83±0.56	0.91±0.27	0.83±0.14*	12.2±1.23**b*	10.1±1.23**a*	8.28±0.79**a*	3.46±0.56*	2.86±0.29*	2.48±0.52	2.32±0.74	1.87±0.64	2.01±0.37	1.70±0.65
N3 PUFA													
C18:3	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.32±0.53**c*	1.03±0.23**b*	0.47±0.09**a*	ND
C20:3	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.49±0.08**c*	0.25±0.06**b*	0.12±0.01**a*	ND
C20:5	0.10±0.02	0.04±0.01*	0.03±0.02*	ND	ND	ND	0.65±0.04**b*	0.59±0.03**b*	0.39±0.09**a*	0.98±0.19*	1.09±0.26*	0.99±0.07*	0.09±0.05
C22:5	ND	ND	ND	0.08±0.02	0.09±0.01	0.11±0.02	0.13±0.02	0.15±0.00**b*	0.11±0.01*a*	0.25±0.05*	0.23±0.06*	0.17±0.01	0.11±0.02
C22:6	0.14±0.06*	0.23±0.03*	0.21±0.08*	0.54±0.05**b*	0.61±0.04**b*	0.88±0.09**a*	0.87±0.10	1.02±0.07**b*	0.79±0.05*a*	0.42±0.08*	0.49±0.13*	0.56±0.04	0.70±0.08
Total N3	0.13±0.08*	0.27±0.03*	0.26±0.07*	0.62±0.07	0.70±0.04	0.99±0.11	1.80±0.42*	1.76±0.09*	1.39±0.30	4.47±0.93**b*	3.09±0.73**a*	2.31±0.16**a*	0.85±0.42
PUFA	0.96±0.64*	1.19±0.10*	1.09±0.20*	12.8±1.29**b*	10.7±1.26**a*	9.26±0.87**a*	5.27±0.87*	4.62±0.29*	3.87±0.83	6.76 ±1.67**b*	4.96±1.11*	4.26±0.59*a*	2.72±0.68
LC PUFA	0.57±0.32*	1.02±0.09*	0.86±0.25*	4.37±0.90**b*	4.30±0.37**b*	7.22±0.62**a*	3.74±0.74*	3.59±0.11*	2.80±0.58*	2.62±0.49*	2.55±0.62	2.31±0.13	1.76±0.44
LC /PUFA	0.18±0.03*b	0.18±0.03*b	0.40±0.08a	0.49±0.07a	2.84±0.16	2.47±0.24	4.96±0.22**b*	2.21±0.13*a*	2.08±0.07*a*	1.84±0.52	1.72±0.49	1.57±0.46	1.50±0.18
CLA													
***c*9*t*11**	0.43±0.10**b*	0.28±0.03*	0.26±0.13**a*	ND	ND	ND	9.98±0.99**c*	4.89±0.40**b*	2.02±0.31**a*	ND	ND	ND	ND
***t*10*c*12**	1.76±0.56*	1.26±0.15*	1.25±0.64*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Phospholipids	22.16±7.42**b*	36.63±8.0.3	43.08±12.13*a*	29.70±4.15*	21.82±4.64*	28.11±3.30*	38.62±12.93	33.23±7.93	33.22±8.48	23.12±5.45*	28.76±7.71*	23.27±2.04*	43.03±7.46

Values are expressed as mean (µg fatty acid/mg protein) ± SD of 4 replications. * denotes significant (P<0.05) difference compared to control. The letters (*a,b and c)* denote a significant (P<0.05) difference between treatment according to ANOVA (analysis of variance) analyses, where *a*, *b* and *c* are all different. FA = fatty acid, mM = treatment concentration in mM, SATS = saturated fatty acids, MUFA = monounsaturated fatty acids, N6 PUFA = omega 6 polyunsaturated fatty acids, N3 PUFA = omega 3 polyunsaturated fatty acids, PUFA = polyunsaturated fatty acids, FA containing 2 or more double bonds, LCPUFA = long chain polyunsaturated fatty acids greater than 20 carbons in length, LC/PUFA = the ration between long chain FA and PUFA, CLA = conjugated linoleic acid, C9T11 = *cis*9-*trans*11 CLA, T10C12 = *trans*10-*cis*12 CLA, ND = not detected.

Table 5.3: Fatty acid ratios (µg fatty acid/mg protein) of the PC and PE phospholipid fractions in HepG2 cells following treatment for 48 hours

FA		*t10c12* CLA			LA			*c9t11* CLA			ALA		Control
Dose (mM)	0.5	0.15	0.075	0.6	0.3	0.15	0.2	0.1	0.05	0.3	0.15	0.075	0
Phospholipid ratio PC/PE	2.22±0.65	1.96±0.29	1.75±0.99	1.48±0.35	1.98±0.40	1.51±0.19	1.85±0.58	1.98±0.80	1.69±0.38	1.34±0.18	1.15±0.18	1.09±0.07	1.63±0.41
PC FA ratios													
N6/N3	13.3±6.85**b*	7.90±1.08	5.90±2.88*a*	51.5±12.3**b*	58.7±6.01**b*	22.8±1.97**a*	3.74±0.31**b*	3.13±0.24*	2.89±0.06**a*	0.34±0.05*	0.42±0.30*	0.62±0.22*	5.76±0.45
P/S	0.15±0.03	0.13±0.01	0.15±0.05	1.61±0.20**b*	1.65±0.13**b*	0.71±0.05**a*	0.21±0.02	0.24±0.01*	0.27±0.04*	1.16±0.04**b*	0.72±0.15**a*	0.57±0.07**a*	0.15±0.06
AA/EPA	NA	NA	NA	NA	NA	NA	1.70±0.37*	1.60±0.22*	1.56±0.11*	0.18±0.01	0.18±0.02	0.20±0.03	NA
16:1/16:0	0.04±0.00	0.08±0.01	0.12±0.00	0.06±0.01*b*	0.08±0.01*a*	0.11±0.00*a*	0.22±0.01	0.28±0.01	0.29±0.03	0.11±0.00	0.14±0.00	0.17±0.01	0.29±0.02
18:1/18:0	1.01±0.07*	1.13±0.11*	1.32±0.18*	1.00±00.01*	1.11±0.20*	1.51±0.14*	2.59±0.06	3.91±0.30	4.99±0.30	2.13±0.02	0.±0.81	5.10±0.77	3.39±0.81
18:1/16:0	0.12±0.02	0.15±0.02	0.20±0.05	0.09±0.00*	0.15±0.01*	0.20±0.02*	0.19±0.01	0.29±0.02	0.41±0.06	0.12±0.00	0.16±0.01	0.23±0.02	0.53±0.11
Desaturation index	0.49±0.22*	0.43±0.03*	0.57±0.09*	0.25±0.01*	0.35±0.01*	0.44±0.04*	0.70±0.04**b*	0.99±0.03*	1.23±0.11**a*	0.34±0.00**c*	0.45±0.02**b*	0.62±0.03**a*	1.30±0.22
Delta 6 S	0.45±0.14	0.57±0.03	0.57±0.19	8.89±0.57**c*	6.56±0.73**b*	5.01±0.64**a*	2.99±0.53**b*	2.31±0.18**a*	1.89±0.24**a*	5.52±1.53**b*	2.99±1.12**a*	2.01±0.45*a*	0.61±0.30
Delta 6 P	0.05±0.02**b*	0.11±0.02*a*	0.19±0.10*a*	2.54±0.17**c*	2.93±0.36**b*	0.65±0.06**a*	0.50±0.06*	0.51±0.05*	0.48±0.08*	0.09±0.0**c*	0.11±0.01*b*	0.18±0.03*a*	0.15±0.07
Delta 6 S/P	11.17±4.59**b*	5.29±0.82*a*	3.68±0.62*a*	3.50±0.19*b*	2.25±0.44*b*	7.77±1.17**a*	5.90±0.41*b*	4.56±0.14	3.99±0.17*a*	63.9±4.81**c*	26.55±7.81*b*	11.57±2.85*a*	4.97±3.48
PE FA ratios													
N6/N3	7.12±2.36**b*	3.36±0.17*a*	3.29±0.82*a*	19.6±0.26**c*	16.9±1.*48***b*	8.40±0.78**a*	1.96±0.35	1.63±0.19	1.79±0.03	0.51±0.06*	0.63±0.26*	0.84±0.14*	2.22±1.01
P/S	0.46±0.31*	0.53±0.03*	0.31±0.16*	2.84±0.29**b*	2.82±0.30**b*	1.64±0.10**a*	0.85±0.11	0.80±0.03	0.96±0.12	2.89±0.16**b*	1.99±0.35**a*	1.93±0.28**a*	1.01±0.30
AA/EPA	23.4±6.7**b*	17.9±2.06*	14.3±5.55*a*	NA	NA	NA	1.90±0.55*	2.7±0.13*	3.2±0.16*	0.43±0.01*	0.40±0.01*	0.43±0.02*	10.78±2.5
16:1/16:0	0.12±0.03*	0.19±0.03*	0.17±0.05*	0.11±0.02*	0.14±0.01*	0.18±0.02*	0.44±0.02*b*	0.47±0.01	0.62±0.14**a*	0.17±0.02	0.28±0.01	0.28±0.01	0.40±0.09
18:1/18:0	1.16±0.14*	1.15±0.05*	1.25±0.15*	0.62±0.01**b*	0.84±0.11**a*	0.98±0.05**a*	1.80±0.08	1.98±0.02	1.98±0.15	0.80±0.04	1.34±0.06	1.34±0.06	1.98±0.35
18:1/16:0	0.20±0.06*	0.28±0.01*	0.28±0.08*	0.36±0.10**b*	0.56±0.05*	0.93±0.15*a*	0.54±0.05*	0.72±0.08*	0.91±0.14	0.60±0.13	0.71±0.12	0.71±0.12	1.08±0.29
Desaturation index	0.65±0.23*	0.88±0.02*	0.84±0.07*	0.54±0.07**b*	0.79±0.04*	1.07±0.10**a*	1.53±0.03	1.70±0.03	1.89±0.07	0.81±0.02	0.94±0.04	1.19±0.07	1.68±0.32
Delta 6 S	0.42±0.39	0.17±0.01*	0.23±0.06*	6.31±0.35**c*	3.87±0.98**b*	2.32±0.65**a*	1.65±0.23*	1.28±0.28*	1.18±0.28*	4.43±1.23**b*	2.64±0.71**a*	2.20±0.46**a*	0.71±0.28
Delta 6 P	0.14±0.6*	0.23±0.03*	0.21±0.08*	3.41±0.52**b*	3.77±0.22**b*	1.60±0.17**a*	0.87±0.10	1.02±0.07	0.88±0.18	0.42±0.08	0.49±0.13	0.56±0.04	0.64±0.31
Delta 6 S/P	2.60±1.61*	0.73±0.07	1.34±0.85	1.88±0.28	1.02±0.22	1.46±0.36	1.91±0.26	1.17±0.32	1.35±0.06	10.3±0.87**c*	5.58±1.52**b*	3.76±0.82**a*	1.44±0.98
AA PC/PE	0.25±0.04*b*	0.35±0.03*b*	0.57±0.27**a*	0.76±0.13*	0.60±0.04*	0.66±0.10*	0.66±0.13*	0.50±0.07*	0.55±0.06*	0.35±0.01*	0.33±0.06*	0.31±0.15*	0.20±0.06

Values are expressed as mean (µg fatty acid/mg protein) ± SD of 4 replications. * denotes significant (P<0.05) difference compared to control. The letters (*a,b and c)* denote a significant (P<0.05) difference between treatment according to ANOVA (analysis of variance) analyses, where *a*, *b* and *c* are all different. FA = fatty acid, mM = treatment concentration in mM, N6 PUFA = omega 6 polyunsaturated fatty acids, N3 PUFA = omega 3 polyunsaturated fatty acids, PUFA = polyunsaturated fatty acids, FA containing 2 or more double bonds, CLA = conjugated linoleic acid, C9T11 = *cis*9-*trans*11 CLA, T10C12 = *trans*10-*cis*12 CLA, N6/N3 = omega 6/omega 3 ratio, P/S = polyunsaturated./saturated fatty acid ratio, AA/EPA = arachidonic acid/eisocapentanoic acid ratio, C20:4n6/C20:5n3, Delta 6 S = substrate for Δ-6 desaturase, Delta 6 P = product of Δ-6 desaturase, Delta 6 S/P = ratio of Δ-6 desaturase substrate/product, NA = not available, EPA is undetected.

5.5. Discussion

This study explores the effect of *c*9*t*11 CLA, *t*10*c*12 CLA, LA and ALA on fatty acid incorporation, phospholipids and cholesterol in the membranes of HepG2 cells. The cellular membrane consists of a phospholipid bilayer. Each phospholipid contains two FA chains and it is well documented that the types of FA they contain affect membrane fluidity, the transfer of nutrients across the membrane and intercellular communication (Spector and Yorek, 1985). The FA composition of the cell membrane can easily be modified by dietary intake of fatty acids.

For the purpose of this study, cells were exposed to FA conjugated to BSA in the culture medium. This clearly affected the FA profile of the cell membrane after 48 hour incubation. All supplemented FA incorporated well into the cell membrane of HepG2 cells. The treatment concentrations were based on the ATP IC_{50}, BrdU IC_{50} results and a concentration positioned between the two IC_{50}s as defined in Chapter 4 - Table 4.1, were chosen. The FAs were incorporated into the PC and PE membrane fractions in a dose dependent manner. Thus, as the treatment concentration increased, the amount of FA detected in the sample increased proportionally and vice versa. The fatty acids were however incorporated better into the PC fraction than the PE phospholipid fraction of the cell membrane. Based on supplementation concentration, LA and *c*9*t*11 CLA were the best incorporated fatty acids and *t*10*c*12 was the least best incorporated fatty acid. CLA isomers did not affect the phospholipid concentration of the PC fraction, which was reduced dose dependently in the PC phospholipid fraction of HepG2 cell membranes. Regarding the effect on membrane composition, the *t*10*c*12 CLA seems to have a lesser effect than *c*9*t*11 CLA in that the *t*10*c*12 CLA mostly has effect on MUFA while *c*9*t*11 CLA seems to affect all FA groups, i.e. SATS, MUFA, N6, N3 and this

pattern seems consistent for PC and PE. As expected, ALA increased N3 FA and LA increased the composition of N6 FA, which is consistent in both PC and PE.

FA supplementation did not significantly affect the cholesterol content in the cell membranes of HepG2 cells. This finding was consistent with a previous study that looked at the effect of CLA isomers and LA on the distribution of cholesterol in lipid rafts and non-raft lipids (Subbaiah et al., 2011).

CLA alters the desaturation index and Δ9-desaturase activity in HepG2 cells and *t*10*c*12 CLA has the greater effect than *c*9*t*11 CLA (Subbaiah et al., 2011). CLA reduces the expression and activity of Δ9 desaturase, the enzyme involved in converting SATS to MUFA by catalysing the formation of a *cis*-double bond at carbon-9 position of the SATS (Zhang et al., 2010). The desaturation ratios that can be identified in the present study are 16:1n7/16:0, 18:1n9/18:0 and 18:1n7/16:0. All desaturation ratios have been greatly reduced following FA supplementation by all FA. The most outspoken effect in the desaturation of SATS to MUFA was observed in samples treated with *t*10*c*12 CLA and LA. The *t*10*c*12 CLA also showed low Δ6 (desaturation of 18:2n6 to 18:3n6) and Δ5 (desaturation of 18:3n6 to 18:4n6) desaturase activity. These findings are consistent with previous studies indicative of the FA metabolism altering effect of *t*10*c*12 CLA, and increased prostaglandin formation (Eder et al., 2002).

N6 FA cannot be converted to N3 FA in humans because of the absence of the Δ15-desaurase enzyme. Cancer cells are known to produce FA to maintain cell integrity through *de novo* synthesis (Berquin et al., 2011). The process involves the conversion of Acetyl-CoA leading to the formation of C18:1n9 and C20:4n3. This could explain the result of treatment with *c*9*t*11 CLA in Table 5.1 and 5.2. C18:1n9 is significantly higher than the control and the long chain N3 FA also increased significantly, indicating a possible response of *de novo* FA synthesis.

C20:4n6 (Arachidonic acid, AA) and C20:5n3 (Eicosapentaenoic acid, EPA) are important precursors of lipid mediator molecules involved in inflammation, proliferation, apoptosis and angiogenesis. These FA are substrates for a number of enzymes belonging to COX and LOX families in the production of prostaglandins, thromboxanes and leukotrienes (Berquin et al., 2011). EPA and DHA are precursors for anti-inflammatory lipid mediators while AA is a precursor for pro-inflammatory lipid mediators (Azrad et al., 2013). The results show that *c*9*t*11 CLA increase AA, EPA and DHA in both PC and PE cellular membrane fractions, suggesting an antagonistic effect with regard to pro- and anti-inflammatory lipid mediators. The *t*10*c*12 CLA isomer showed insignificant suppression of AA, EPA and DHA, suggesting possible suppression in eicosanoid production, consistent with previous findings (Eder et al., 2002, Eder et al., 2003).

The results show that treatment with FA alters the lipid profile and FA metabolism of HepG2 cells. It is clear that the two CLA isomers, *c*9*t*11 CLA and *t*10*c*12 CLA, produced different outcomes with regard to the proliferation altering mechanism of each isomer. The *c*9*t*11 CLA increased desaturation of PUFA and lowered the N6/N3 ratio, whereas *t*10*c*12 suppressed desaturation of SATS and increase the N6/N3 ratio. AA and EPA were increased by *c*9*t*11 and suppressed by *t*10*c*12, suggesting altering effects on lipid mediator production. Indicating that the anti-carcinogenic mechanism of *c*9*t*11 CLA involves inflammation, apoptosis and cell proliferation, however the exact mechanism of *t*10*c*12CLA is yet to be established. Therefore further studies in cycle events and gene expression are required to fully uncover the anticancer effect of CLA.

5.6. References

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CHAPTER 6

6. General discussion and conclusion

6.1. Introduction

Cancer cells possess a number of characteristics that are vital to their immortality. These phenotypic characteristics contrast to what is considered normal characteristics of normal cells, i.e. altered pathways in growth signals, evasion of apoptosis and protection from cellular stress signals (Santos and Schulze, 2012). Cellular stress signals resulting from changes in cancer cell metabolism or unfavourable tumour microenvironment, such as hypoxia and glucose deprivation, initiate the cellular stress response to ensure the cell's survival (Kim et al., 2014). The cellular stress response includes: evasion of apoptosis, unfolded protein response and autophagy, which may contribute to carcinogenesis, tumour progression, and also treatment resistance, since most current anticancer treatments act by stimulating cell death pathways, like apoptosis in cancer cells. (Fulda, 2010).

Fatty acids are essential building blocks of cell membrane and play an important role in cellular energy and signalling molecules. The fatty acid composition of cell membranes has a significant effect on many cellular processes. Research suggests that altering the fatty acid profile of cellular membranes may play a role in human health (Bondia-Pons et al., 2007). Modifications to the lipid profile of cellular membranes alter membrane fluidity as well as many cellular functions, including transport of extracellular molecules via carrier-mediated transport, prostaglandin production and cell growth (Spector and Yorek, 1985).

The present study explored three different areas that could possibly affect the tumour development process. Firstly, the antioxidant activity of CLA isomers was studied. Antioxidants are chemicals that block the activity of free radicals and reactive oxygen species, which are highly reactive and have the potential to cause damage to cellular

components that may lead to cancer. Secondly, this study explored the cytotoxicity of CLA on HepG2 cells and addressed their effect on cell viability, apoptosis and cell proliferation. Finally, the effect of CLA on cell membrane fatty acid composition was evaluated and proposed possible effects that contribute to the anti-tumour properties of CLA.

6.2. Antioxidant activity (Chapter 3)

The antioxidant activity of *c*9*t*11 CLA and *t*10*c*12 CLA was evaluated by measuring the protection against iron-induced lipid peroxidation in rat liver microsomes by the TBARS method. When the microsomes were treated with varying concentrations of C18 FA's, there was a clear difference in the level of iron-induced lipid peroxidation. Each fatty acid showed varying degrees of protection, but certain groupings were evident. Mono-unsaturated FA displayed the greatest protection, which can be attributed to the availability of a single hydrogen bond for free radical attack thereby producing fewer lipid peroxides. Polyunsaturated fatty acids were clustered together, with the highest and lowest effect at each concentration level spanning less than 20%.

Each PUFA displayed a slight difference in effect as indicated by the different TBARs IC_{50} levels depicting protection against lipid peroxidation. In order of most to least protection, LA has the lowest IC_{50}, followed by *c*9*t*11, *t*10*c*12 and finally ALA with the highest IC_{50}. This drove the conclusion, that the number and position of the double bond played an effectual role in the susceptibility to iron-induced lipid peroxidation. MUFA showed the highest protection and the lowest IC_{50}, PUFAs with 2 double bonds (LA and CLA) were in the middle, while PUFAs with three double bonds showed least protection and therefore the highest IC_{50} concentration. Linoleic acid and the 2 CLA isomers each have 2 double bonds

and differ only in their position and arrangement. The same can be said with ALA and GLA, with 3 double bonds differing in their position. These two groups did not produce the same effect, but effectual differences could be seen possibly linked to the distance of the double bond closest to the methyl end of the fatty acid chain. Linoleic acid (C18:2n6) has its first double bond at carbon position 6 from the methyl end, *c*9*t*11 CLA at position 9 and *t*10*c*12 at position 10. Similarly, the effect can be seen with ALA (C18:3n3), with its first double bond methyl end at carbon position 3, and GLA (C18:3n6), which has its first double bond at carbon position 6 from the methyl end. This led to the summation that the positions of the double bond also play a role in iron-induced lipid peroxidation.

Polyunsaturated fatty acids are highly susceptible to lipid peroxidation when exposed to free radicals. Free radicals attack the double bonds closest to the tail of the FA chain, thereby forming lipid peroxides. Lipid peroxidation plays an important role in cell membrane damage and increase in membrane permeability due to the presence of oxidized lipids (Wong-Ekkabut et al., 2007). Not only are the cell membranes affected, intracellular oxidative stress as well as irreversible oxidative damage also occurs, ultimately leading to cell death (Repetto et al., 2012). In this study we saw that *c*9*t*11 and *t*10*c*12 CLA isomers did not significantly affect iron-induced lipid peroxidation differently compared to supplementation with LA or ALA. If the outcome was to reduce lipid peroxidation, supplementation with MUFA would be the best option, but in the scope of this study, this was not the main objective. The present study showed that introducing CLA isomers to the cell membranes did not significantly differ from what was observed when introducing LA or ALA. This indicates that if CLA was introduced via the diet for example, has a similar effect to LA with regard to lipid peroxidation and the protection thereof.

6.3. Growth parameters (Chapter 4)

FAs possess multifaceted effects on the survival of cancer cells. They have been implicated in altering cancer cell proliferation and growth signals (Stephenson et al., 2013), and affecting apoptosis (Corsetto et al., 2011). In ideal situations, an effectual anticancer agent would be cytotoxic to only the cancerous cells and not healthy cells and tissue. The agent should decrease cell viability, increase apoptosis and retard the proliferation of these cells.

The present study showed the effect of *c*9*t*11 CLA and *t*10*c*12 CLA on cytotoxicity, cell viability, apoptosis and cell proliferation in HepG2 cells was determined and compared to effects elicited by LA and ALA. The results have shown that each FA that was used affected each parameter differently, but the outcomes were generally the same. These results were increased cytotoxicity, decreased cell viability, increased apoptosis and a decreased cancer cell proliferation at different levels for each FA. Between the 2 CLA isomers, *c*9*t*11 CLA showed greater cytotoxicity than *t*10*c*12 CLA, which is also reflected in cell viability. *c*9*t*11 CLA reduced cell viability to a greater extent than *t*10*c*12 CLA. All supplemented FAs reduced cell proliferation, however slight differences were evident with least effect observed with LA where no IC_{50} could be calculated. The *c*9*t*11 CLA and ALA effect was the greatest and almost overlapped (Chapter 4; Figure 4.3). With regards to apoptosis, caspases are effector molecules in all the apoptotic pathways leading to DNA fragmentation, membrane blebbing and formation of apoptotic bodies (Tawa et al., 2004). Here again *c*9*t*11 CLA showed a much greater effect on apoptosis fold increase than the *t*10*c*12 CLA isomer. These findings suggest that different mechanisms of action exist between *c*9*t*11 and *t*10*c*12 CLA in reducing cancer cell growth parameters.

With regard to cytotoxicity, cell viability and cell proliferation, the effect between the 2 CLA isomers were not the same but showed similar trends. However, the effect on apoptosis was

substantially different. This could mean that *t*10*c*12 CLA may possess anticancer properties that to a lesser extent involve apoptosis and more actively involve effects on cell growth involving cell cycle arrest and senescence. Of interest is that, *t*10*c*12 CLA was shown to be a greater inhibitor of the genes regulating cell cycle and growth than *c*9*t*11 CLA (Kelley et al., 2007).

6.4. Lipid profile (Chapter 5)

Fatty acids play a major role in the structure and function of cellular membranes. Certain FAs are important for maintaining membrane structure and integrity and are involved in signalling and regulating the cell's response to its environment. Modifications to the lipid profile of the cell membrane have been shown to alter a number of cellular processes such as cell proliferation and prostaglandin production (Berquin et al., 2011).

HepG2 cells were supplemented with *c*9*t*11 CLA, *t*10*c*12 CLA, LA and ALA to determine effects on the membrane lipid profile. A distinct change in the FA composition of the cellular membrane was observed compared to unsupplemented control samples. In general, LA increased omega 6 (N6) fatty acids and ALA increased omega 3 (N3) FA, as predicted.

Supplementation with *t*10*c*12 CLA reduced the MUFA of both PC and PE fractions of the cell membrane. LA, ALA and *t*10*c*12 CLA also decreased the desaturation index in both PC and PE phospholipid fractions. This finding suggests that these FA may moderate the activity of Δ9-desaturase, which is the enzyme involved in converting SATS to MUFA. Tumour cells obtain most fatty acids, to maintain high levels of proliferation, by *de novo* synthesis. Therefore, increased expressions of biosynthetic enzymes that are required to produce the large amounts of FA that are essential for maintaining the cancer phenotype

(Mohammadzadeh et al., 2014). The Δ9-desaturase enzyme is an important regulatory enzyme in cellular *de novo* fatty acid synthesis and has been implicated in the proliferation of cancer cells (Luyimbazi et al., 2010).

When looking at the outcome of this study (Chapter 5), it is evident that supplementation with *c*9*t*11 CLA significantly increased C20:4n6 (Arachidonic acid, AA) and C20:5n3 (Eicosapentaenoic acid, EPA) in both PC and PE phospholipid fractions of the cell membrane. AA and EPA are important precursors of lipid mediator molecules involved in inflammation, proliferation, apoptosis and angiogenesis. EPA is a precursors for anti-inflammatory lipid mediators while AA is a precursor for pro-inflammatory lipid mediators (Azrad et al., 2013).

6.5. Conclusion

Cell proliferation, death and senescence control in normal mammalian cells and cancer cells are related to the regulation of metabolism, in particular the synthesis and remodelling of lipid modulators and structures (Igal, 2010). Elevated rate of aerobic glycolysis and an accelerated biosynthesis of macromolecules, including DNA, proteins and lipids, are characteristic in the metabolism in cancer cells (DeBerardinis et al., 2008). An essential aspect of the remodelled metabolism of transformed cancer cells is an increased rate of lipid biosynthesis (Baenke et al., 2013). Lipid metabolism modifications can influence many cellular processes, including cell growth, proliferation, differentiation and motility (Santos and Schulze, 2012).

It is shown in the present study that *c*9*t*11 and *t*10*c*12 CLA are incorporated into the cell membrane of HepG2 cells. Their incorporation has a consequential inhibiting effect on cell

proliferation of cancer cells that may be associated with the altered lipid profile of the cellular membrane.

The *c*9*t*11 CLA isomer markedly increased apoptosis and the overall FA content of the cellular membrane. This increased a number of factors that contribute to the effect of programmed death seen on cancer cells. These include increased MUFA concentrations, which reduces overall susceptibility to lipid peroxidation, thereby preventing cellular stress and allowing the cells to follow their normal cycle towards cell death. Moreover, increasing the total FA content of the cell membrane suggest increase *de novo* synthesis of FA, that are required for the formation of new cells, but could also be implicated in the formation of apoptotic bodies, which are membranous bodies containing cell fragments that are formed at the end of the apoptosis process.

The *t*10*c*12 CLA isomer reduced cell proliferation comparable to *c*9*t*11 CLA (Chapter 4; Table 4.1); however *c*9*t*11 was more effective in increasing apoptosis and decreasing cell viability, similar to ALA. This suggests that the cell proliferation reducing effect of *t*10*c*12 CLA may be the result of a different mechanism. The *t*10*c*12 CLA isomer showed to reduce delta-9 desaturase activity, which is an essential enzyme in *de novo* synthesis of FA, indicated by reduction in MUFA composition of the cell membrane of HepG2 cells. Limiting *de novo* FA synthesis effectively reduces the FA availability required for proliferation of new cells. This could explain the effect seen in the cell proliferation assay. EPAs are precursors to anti-inflammatory modulatory molecules that have been implicated in prostaglandin production, which could suggest another mechanism of action of CLA.

When looking at Table 4.1 in Chapter 4, *c*9*t*11 seems to mimic the effectiveness of ALA on of viability, cell proliferation and apoptosis in HepG2 cells. *c*9*t*11 also increased N6 and N3 PUFA, potentially increasing cell susceptibility to decreased survival and growth despite the

increased SATS and MUFA. The antioxidative potential of *c*9*t*11 CLA was greater than *t*10*c*12 CLA; this in addition to *c*9*t*11's greater effectiveness on cell viability and apoptosis, and the difference between the 2 CLA isomers could make *c*9*t*11 a more effective anticancer agent.

The evidence exists in literature that reduction of cell proliferation exhibited by CLA may be attributed to the aggregation of cells in the S phase of the cell cycle and apoptosis via mechanisms involving the mitochondrial pathway (Miglietta et al., 2006). CLA also elicited cell cycle arrest in G1 phase and induced tumour suppressor protein accumulation (Kemp et al., 2003). Isomer specifically, t10c12 indicated that t10c12-induced p21 (CIP1/WAF1) binds to cyclin dependent kinases (CDK), thereby inhibiting the enzyme activity, which contributed to the decrease in the G1-S progression potentially. (Kim et al., 2006). There are yet to be fully explained; therefore further studies in cycle events and gene expression are required to fully uncover the anticancer mechanisms of both *c*9*t*11 and *t*10*c*12 CLA isomers.

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12 April 2000

Dr WCA Gelderblom
PROMEC
PO Box 19070
TYGERBERG
7505

Dear Dr Gelderblom

ECRA MEETING : 28 FEBRUARY 2000

Thank you for your letter of 21 February 2000. We discussed the issues you raised and should like to comment as follows:

- Provision will be made to discuss unclear issues or questions with relevant applicants when necessary.

- Animals used for "harvesting tissue" for supporting standard biochemical techniques are not classified as animal experiments and therefore need no ethical approval. ECRA, however, requests that a register be kept for this specific purpose containing the following information:
  - Date
  - Animal used
  - Number of animals
  - Tissue harvested
  - Purpose of procedure
  - Method of termination
  - Researcher's signature

- Changes to protocol while experiment is in progress: Any changes in procedures must be reported, in writing, to the Chairperson who will use his discretion as to whether the request needs to be discussed at committee level. This will especially apply when radical changes are introduced.

Yours sincerely

DR AJS BENADÉ
CONVENOR : ECRA

BCA (Pierce) Protein Determination Assay

Solutions required

- 2% SDS Solution
- BCA Protein Reagent A
- BCA Protein Reagent B (4% $CuSO_4.5H_2O$)
- BSA Standard (1mg/ml)
- Distilled H_2O

2% SDS Solution	Reagent A	Reagent B	BSA
Make your own	Purchase from Seperations	Make your own	
2Mm EDTA		4% $CuSO_4.5H_2O$	
20Mm $NaHCO_3$			
2% Sodium dodecyl sulphate			

Working Reagent preparation

Mix Solution A and Solution B in a 50:1 ratio

Standard preparation

	BSA(µl)	2% SDS(µl)	Working Reagent(µl)
Blank	0	100	200
S1	5	95	200
S2	10	90	200
S3	20	80	200
S4	30	70	200
S5	40	60	200
S6	50	50	200

Unknown samples

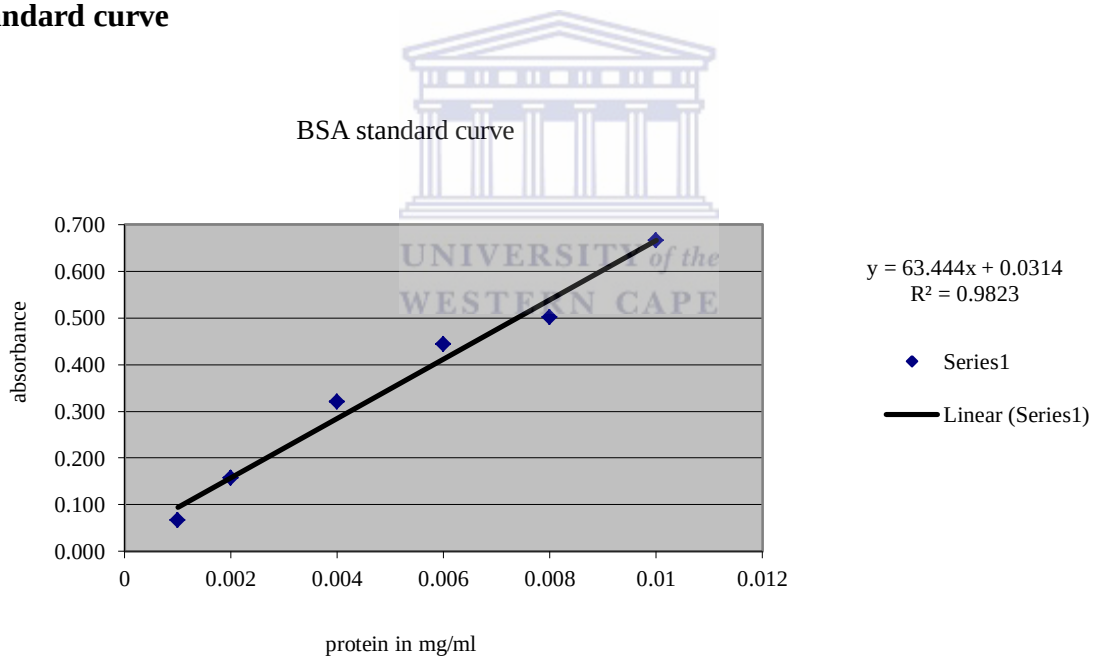
Sample(µl)	2% SDS(µl)	Working Reagent(µl)
25	75	200

*Smaller volume of sample can be used – just ensure that sample and SDS does not exceed 100µl.

Procedure

1. Pipette each standard and sample into a microplate well.
2. Add the working reagent.
3. Cover plate and incubate at 37°C for 30 minutes.
4. Cool plate to room temperature.
5. Measure the absorbance at 562nm on plate reader.

Standard curve



Equation: $y = mx + c$

y = Absorbance reading (nm)

m = slope

x = protein concentration (mg/ml)

c = y-intercept

Esterbauer and Cheeseman (1990), Methods Enzymol. 186: 407-421

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ASSESSMENT OF LIPID PEROXIDATION:

MDA (Malondialdehyde) or TBARS ASSAY

REAGENTS:

- TCA Reagent (10 % TCA, BHT [see below] & 1mM EDTA) – Make up fresh
- 0.67% TBA solution in distilled water – Make up fresh:Weigh out amount of TBA powder needed and mix with less than needed amount of water, heat at approx. 45°C-50°C until dissolved, cool to room temp. Then make up to desired volume in a volumetric flask.
- 1.15% KCl containing 0.01 M potassium phosphate buffer (pH 7.4)
- $FeSO_4$ solution (2.5mM ferrous sulphate; 69.5mg/100ml dist. water) – Make up fresh
- BHT (stock = 8g/100ml ethanol, kept in walk in fridge), add 0.125ml/100ml TCA-EDTA (BHT does not dissolve easily in an aqueous solution, will flocculate)

NB: For all steps work on ice

Only make up the required amount of reagents (TCA, TBA, as required per assay)

Homogenate preparation

- Homogenise tissue samples on ice in 19 volumes of 1.15% KCl in phosphate buffer using glass hand Dounce (dounce approx. 10 x) and store at -80°C.
- Microsomes (stored at -80°C) for determination of anti-oxidative activity should be dounced on ice before use (10x, loose dounce)
- Determine protein concentration

  (NB: Dilute samples for protein assay with 2% SDS solution approx. 50-60x, microsomes 50x).

METHOD

- Use 1mg protein per unknown sample/microsomes

Note: *Use 1mg protein per test tube, therefore 0.5ml sample must contain 2mg protein. This will equate to 1mg protein per 1ml total test tube reaction volume.*

- Run 2x blanks through the procedure containing sample buffer only (1.15% KCl in phosphate buffer)
- Dilution of tea samples may need to be pre-determined, a range of dilutions should be used to determine the IC_{50} value

Treatment with iron (Table 1)

- Vortex and incubate at 37°C for 1 hr in a shaking waterbath.
- Thereafter, place tubes on ice and follow the assay procedure.

Table 1 pipetting scheme for testing antioxidant potential of tea/oil etc. samples using microsomes

	Antioxidant	***KCl-buffer***	***$FeSO_4$***	***Microsomes (2mg/ml)***	***Total volume***
Reagent blank	---------	0.8ml	0.2ml	---------	1ml
Microsomes blank	---------	0.5ml	---------	0.5ml	1ml
Positive control	---------	0.3ml	0.2ml	0.5ml	1ml
Samples (teas, oils, etc.)	0.1ml	0.2ml	0.2ml	0.5ml	1ml

Table 2 pipetting scheme for determination of TBARS in tissue homogenates

	protein	***KCl-buffer***	***$FeSO_4$***	***Homogenate***	***Total volume***
Reagent blank	---------	0.8ml	0.2ml	---------	1ml
Samples as is (liver homogenates)	±10mg/ml	0.7ml	0.2ml	0.1ml	1ml

Assay procedure:

- add 2ml TCA reagent (10% TCA with BHT & EDTA) to each tube (Addition of EDTA and BHT to the TCA reagent prevents further oxidative damage during the assay procedure)
- Vortex and centrifuged at 2000 rpm for 15 min.
- Take off 2ml of the supernatant, add to a new test tube and add 2 ml 0.67% TBA solution
- Vortex and heat in capped tubes at 90ºC for 20 min in a water-bath.
- Allow mixture to cool (ice bath)
- Measure absorbency at 532 nm on spectrophotometer, use distilled water as blank and read sample blanks as well as unknown samples. **Use glass 2ml or 4ml cuvettes.** Plastic cuvettes get bubbles forming.

Lipid peroxidation is expressed as nmol MDA equivalents per mg protein, using the molar extinction coefficient of 1.56 x 10^5 M^{-1} cm^{-1} at 532 nm for MDA or the standard curve (Esterbauer and Cheeseman, 1990).*Esterbauer and Cheeseman (1990), Methods Enzymol. 186: 407-421*

Addendum 4

Product	size	Cat.#
CellTiter-Glo®Luminescent Cell Viability Assay	10 ml	G7570

Substrate is sufficient for 100 assays at 100µl/assay in 96-well plates or 400 assays at 25µl/assay in 384-well plates. Includes:

• 1 × 10ml CellTiter-Glo® Buffer

• 1 vial CellTiter-Glo® Substrate (lyophilized)

Mix together before use.

Protocol:

1. Prepare opaque-walled multiwell plates with mammalian cells in culture medium 100µl per well for 96-well plates.

2. Prepare control wells containing medium without cells to obtain a value for background luminescence.

3. Add the test compound to experimental wells, and incubate according to culture protocol.

4. Equilibrate the plate and its contents at room temperature for approximately 30 minutes.

5. Add a volume of CellTiter-Glo® Reagent equal to the volume of cell culture medium present in each well (e.g., add 100µl of reagent to 100µl of medium containing cells for a 96-well plate, or add 25µl of reagent to 25µl of medium containing cells for a 384-well plate).

6. Mix contents for 2 minutes on an orbital shaker to induce cell lysis.

7. Allow the plate to incubate at room temperature for 10 minutes to stabilize luminescent signal.

8. Record luminescence.

Note: Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline.

Addendum 5

Product	size	Cat.#
Caspase-Glo®3/7 Assay	100ml	ZZG8092

Each system contains sufficient reagents for 1,000 assays at 100µl per assay in a 96-well plate or 4,000 assays of 25µl per assay in a 384-well plate. Includes:

• 1 × 100ml Caspase-Glo® 3/7 Buffer

• 1 bottle Caspase-Glo® 3/7 Substrate (lyophilized)

Mix together before use.

Standard Protocol for Cells Cultured in a 96-Well Plate

1. Before starting the assay, prepare the Caspase-Glo®3/7 Reagent. Allow the reagent to equilibrate to room temperature. Mix well.

2. Remove 96-well plates containing cells from the incubator and allow plates to equilibrate to room temperature.

3. Add 100µl of Caspase-Glo®3/7 Reagent to each well of a white-walled 96-well plate containing 100µl of blank, negative control cells or treated cells in culture medium.

4. Gently mix contents of wells using a plate shaker at 300–500rpm for 30 seconds. Incubate at room temperature for 30 minutes to 3 hours, depending upon the cell culture system.

5. Measure the luminescence of each sample in a plate-reading Luminometer as directed by the luminometer manufacturer.

CHOLESTEROL DETERMINATION

Adapted from – Richmond (1973). Clin. Chem., 19: 1350-1356.

– Smuts *et al.* (1994). Cor. Art. Dis. 5: 331-338

Reagents:

(1) Cholesterol Colour Reagent:

To one liter volumetric flask add the following:

KH_2PO_4	22,1827 g
$K_2HPO_4.3H_2O$	8,4445 g
KI	19,9212 g
NaN_3	9,7515 g
Alkylbenzyldimethylammonium chloride	100 mg
Ammonium molbydate	12,4 mg
Triton X-100 (polyethylene glycol monether; peroxide free)	2 g

(2) Cholesterol Reagent B:

Cholesterol esterase	83.3 µl
Cholesterol oxidase	125 µl
3M NaCl	1792 µl

Method:

(1) Use 20 µl of the lipid sample from CMS extraction used for TLC spotting.

(2) Add 50 µl CM, vortex.

(3) Add 300 µl 1% Tritin-X100 (peroxide free), vortex.

(4) Evaporate emulsion under N_2, vortex until clear.

(5) Add 1.7 ml cholesterol reagent.

(6) Standards: - 10 µl from 4 different cholesterol standard concentrations
- 300 µl 1% Triton-X100
- Add 1.7 ml cholesterol reagent

(7) Carry 1 ml over from (5) and (6) into small sample tubes and add 20 µl cholesterol Reagent B.

(8) Add 20 µl 3 M NaCl to original tubes from (5) and (6).

(9) Incubate at room temperature.

(10) Read at 365 nm with both VIS and UV bulbs on. Read each sample together with its respective blank.

Cholesterol content calculation:

Colon polyps/mucosa

$$C_{cholesterol} = \frac{f_{standard} \times f_{aliquot} \times (^{100}/_{CLW}) \times absorbance_{sample}}{m_{colon\_protein}\ [mg]}$$

RBC membranes

$$C_{cholesterol} = \frac{f_{standard} \times f_{aliquot} \times absorbance_{sample}}{C_{protein}}$$

$f_{standard}$	average of n values: $m_{standard}$ [µg]/ $absorbance_{standard}$
$f_{aliquot}$	$V_{total\ CMS\ of\ sample}$ [µl]/ $V_{aliquot\ of\ CMS\ extract\ spotted\ for\ TLC}$ [µl]
CLW	colon lipid weight (mg)
$m_{colon\_protein}$ [mg]	mg sample protein per 100 mg colon protein dry weight
$C_{protein}$	RBC protein concentration (mg per ml)
unit:	µg cholesterol per mg protein

This letter serves to inform the reader that necessary corrections have been made to the MSc thesis titled: *The Modulating Effect of Conjugated Linoleic Acid CLA on Cancer Cell Survival in vitro.*

Lyle Arendse

15 February 2015