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# Glycerol derived process contaminants in refined coconut oil induce cholesterol synthesis in HepG2 cells

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ABSTRACT

Despite its 50-year history, the conventional diet-heart hypothesis holding that dietary saturated fats raise serum cholesterol, and with it, cardiovascular risk, remains controversial. Harsh chemical and physical treatment generates process contaminants, and refined oils raise serum and tissue cholesterol *in vivo* independent of saturated fat content. We developed an *in vitro* bioassay for rapidly assessing the influence of oils on cholesterol metabolism in the human liver HepG2 cell line, and tested it using coconut oil (CO) of various stages of refinement. CO was dissolved with dipalmitoyl phosphatidylcholine (DPPC) surfactant, solvent evaporated, and emulsified into fat-free cell culture media. After 24 h treatment cellular cholesterol and triacylglycerol increased; *HMG-CoA* Reductase (HMGCR) increased and *CYP7A1* (cholesterol 7α-hydroxylase) decreased with sequential processing steps, deacidification, bleaching, deodorization, while fatty acid profiles were not affected. Glycerol-derived process contaminants glycidyl esters and monochloropropandiol (MCPD) increased with processing. Addition of glycidyl or MCPD to virgin CO (VCO) had similar effects to processing, while addition of phenolic antioxidants to fully refined CO reduced HMGCR and increased *CYP7A1*. We conclude that harsh processing creates contaminants that raise cholesterol levels *in vitro*, consistent with a role as a contributing atherosclerotic factor.

## 1. Introduction

The traditional diet-heart hypothesis posits that the atherogenicity of food fats is related to their saturated fat character through raising of serum cholesterol and/or lipoprotein sub-fractions, specifically LDL cholesterol (Bier, 2016). Saturated animal fats, tallow, dairy fat (e.g. butter), and lard among others, removed and replaced by polyunsaturated vegetable fats, corn oil and later soy oil, reliably lowered serum cholesterol (Anderson et al., 1957), which was assumed to reduce accumulation of cholesteryl ester rich fatty streaks in the coronary arteries (Hegsted et al., 1965). Later, saturated plant fats, chiefly coconut oil and palm kernel oils originating from equatorial regions (hence “tropical oils”) were shown also to raise serum cholesterol (Edem, 2002; Elson, 1992). Dozens of studies in rats and rabbits produced concordant data, showing that both these tropical plant fat sources dramatically increase cholesterol levels in the liver and serum so reliably that they were routinely used as positive controls in comparative animal feeding studies (Tyburczy et al., 2009). As recently as the 1990s, activist groups in the US successfully campaigned for the removal of coconut oil from common foods such as popcorn toppings (Grimes, 1994). Similarly, partially hydrogenated vegetable oils containing trans fats, among the most harshly processed oils, were removed from the US food supply as the 2006 requirement mandates that *trans* fat content be listed on Nutrition Facts labels and unless specifically authorized must no longer be a component of foods as of June 2018 (US Food and Drug Administration, 2017). The American Heart Association recently issued a Presidential Advisory against consumption of saturated fat for cardiovascular health, including specific advice against

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coconut oil as among the most saturated of food fats (Sacks et al., 2017).

Since about 2000, virgin coconut oil (VCO) has come to be a common commodity in US natural and healthy food stores as an alternative to saturated animal fats. VCO is produced by a wet low temperature process applied to fresh coconut intended to retain maximal constituents (Marina et al., 2009a, 2009b) whereas refined-bleached-deodorized (RBD) coconut oil, produced from coconut kernel dried for several days, pulverized, solvent extracted, deacidified, bleached, and deodorized, many of the latter steps at high temperature for extended periods. Such steps alter the oil chemically by generating or introducing process contaminants, including trans fatty acids. As a general matter, processing is required to extract oils and fats from their native state, and some chemical processing is required to make many oils edible and/or acceptable to the consumer. Recent advances in analysis have led to careful evaluation of process contaminants induced by harsh chemical processing, as well as process contaminants entering the oils from external sources (Cassiday, 2016). Health effects of process contaminants are not well understood, though prudence suggests that chemical compounds appearing in foods incidental to processing, detectable by modern methods or not, should be minimized because oxidation products and contaminants may have potent effects.

Human cholesterol biosynthesis and metabolism is highly regulated via complex mechanisms. Among the many steps in cholesterol biosynthesis from 2 to 4 carbon units is the target of statin therapy, 3-hydroxy-3-methylglutaryl-CoA (HMGCoA) reductase (*HMGCR*), widely recognized as the rate limiting step. The first committed step in cholesterol degradation is mediated by the P450 gene *CYP7A1* coding for cholesterol 7α-hydroxylase, leading to the classic bile salt synthesis pathway (Pikuleva, 2006).

Animal studies have compared VCO with RBD CO and found low serum and liver cholesterol with VCO and high serum cholesterol with RBD CO (Arunima and Rajamohan, 2014; Brenna and Kothapalli, 2014). For instance, a recent rat study found that serum and liver cholesterol were 29% and 104% greater, respectively, in the RBD group compared to the VCO group (Arunima and Rajamohan, 2014). A study of purified elaidic acid (trans-9-octadecenoic acid), the most prominent trans fatty acid in hydrogenated soy oil and many other hydrogenated oils, was found to lower serum cholesterol and raise *LDL* receptor levels in Syrian golden hamsters compared to rapeseed oil controls, opposite expectations and in stark contrast to the hydrogenated soy oil positive control (Tyburczy et al., 2009). Similarly, vaccenic acid (trans-11-octadecenoic acid) did not alter plasma cholesterol compared to the rapeseed control (Tyburczy et al., 2009). In human studies, the scant epidemiology around high traditional consumers of coconut fat supports low, not high, levels of cardiovascular disease, for instance in populations on Pacific islands, The Philippines, Indonesia, India, and Sri Lanka (Brenna and Kothapalli, 2014). Careful evaluation/re-evaluation of data from early studies on saturated fat show no evidence of benefit and some evidence of harm from the substitution of high PUFA oils for butter (Ramsden et al., 2016), though this interpretation is controversial (Sacks et al., 2017).

The totality of evidence leads us to hypothesize that process contaminants created, as well as native compounds lost, contributes to atherogenicity of fats and oils. We tested this hypothesis considering cholesterol synthesis and degradation using a novel method of introducing whole oil, rather than fatty acids, into human HepG2 cells.

## 2. Materials and methods

### 2.1. Chemicals

Solvents for lipid extraction were HPLC grade from Sigma-Aldrich (St. Louis, MO) and from Burdick & Jackson (Muskegon, MI). Cell culture and fatty acid supplementation media, PBS, FBS and reagents for cell culture work were obtained from Life Technologies (NY), Corning (MA) and Thermo Fisher Scientific (MA). The 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine (DPPC) was purchased from Avanti Polar Lipids (Alabaster, AL). Tocopherols (α, β, γ and δ), β-sitosterol, campesterol and stigmasterol standards were purchased from Sigma Chemical Co. (St Louis, MO, USA). 3-MCPDE (rac-1,2-distearoyl-3-chloropropanediol 3-MCPD-SS, purity > 98%) and glycidyl stearate GE-S (purity > 98%) were obtained from Toronto Research Chemicals (Toronto, Ontario, Canada). Total phenolics were extracted from virgin coconut oil according to previous methods (Gouvinhas et al., 2014).

### 2.2. Coconut oil

Two sets of virgin/processed CO samples were evaluated. A set of four coconut oils starting with J1 VCO (kind gift of Coconut Research Institute of Chinese Academy of Tropical Agricultural Sciences) was generated by processing at our Jiangnan University laboratories by J2) deacidification, J3) bleaching, and J4) deodorization according to existing protocols (Canapi et al., 2005). Briefly, for deacidification a 12% NaOH solution was added dropwise to stirred oil held at 90 °C and stirred for 30 min, centrifuged for 20 min, and washed with hot water to remove soap and later dried under vacuum at 90 °C. Deacidified oil was bleached at 110 °C, under vacuum with constant stirring for 30 min using 2% activated palygorskite clay (a magnesium aluminum silicate) and 0.5% activated carbon (% of oil weight) and filtered. Finally, deodorization was carried out by molecular distillation at 185 ± 2 °C under $9.7 \times 10^{-4}$ mbar, collecting the remaining heavy fraction as deodorized CO.

A second pair of oils was obtained commercially, U1) VCO (SimplyNature, distributed by ALDI Inc. Batavia, IL 60510) and U2) refined CO (Nutiva, 213 W. Cutting Blvd. Richmond, CA 94804).

### 2.3. Cell culture

HepG2 cells were grown in MEM-α with 10% FBS, in a humidified environment at 37 °C with 5% $CO_2$. Cells ($1 \times 10^6$) were treated with oils for 24 h, washed twice with 1*X* PBS and harvested using trypsin for analysis.

### 2.4. Delivery of oil to cells

Our intent was to deliver oil retaining all components to the cells rather than, for instance, only fatty acids absent nonsaponifiables. The coconut oil (average molecular weight was calculated as trilaurin) was solubilized in tetrahydrofuran (THF) and ethanol (1:1, *v:v*), and DPPC dissolved in chloroform were evaporated under nitrogen and resuspended in THF and ethanol to a final concentration of 0.5 M oil in solvent. The oil-in-water emulsion was produced by adding the lipid mix into a flask containing MEM-α and sonicating for 5 min then rapid vortex mixing for final concentration of 500 μM DPPC and 50 μM coconut oil. The final concentrations of THF and ethanol were about 0.1% each in the incubation media.

### 2.5. Fatty acid extraction and analysis

Harvested cell pellets were used for fatty acid extraction and analysis. Fatty acid methyl esters (FAME) were prepared using a modified one step method (Garces and Mancha, 1993). Samples were analyzed by gas chromatography with flame ionization detection (GC-FID) as described in detail elsewhere (Brenna, 2016). Response factors from an equal weight mixture were used to adjust responses and data are reported as percent by weight (%, w/w).

### 2.6. Isolation of total genomic RNA

RNeasy Mini kit was used to isolate total RNA from HepG2 cells (Qiagen, MD). RNA concentration and quality were determined by 260/

**Table 1**
PCR primer sequences.

| Genes | Sequence(5′-3′) |
|---|---|
| HMG-CR | F: AGCTTGTGTGTCCTTGGTATT<br>R:CTGAGTTACAGGATTCGGCTTAT |
| CYP7A1 | F: CCAGCGACTTTCTGGAGTTTAT<br>R: GATTGCCTTCCAAGCTGACT |
| SREBP-2 | F: GGTCTGGAGACCATGGAGAC<br>R: GGGAACTCTCCCACTTGATTAC |
| SCARB1 | F: CATCAAGCAGCAGGTCCTTA<br>R:CGTCAAAGAAGTAGACGGAGAG |
| CYP51A1 | F: AACGCAGACAGTCTCAAGAAA<br>R: AAGCATCCCTGCTACTTCATC |
| β-ACTIN | F: ATTGCCGACAGGATGCAGAA<br>R: AAGCATTTGCGGTGGACGAT |
| GAPDH | F: AACGGATTTGGTCGTATTGGGC<br>R: TTGACGGTGCCATGGAATTTGC |

F: Forward Primer.
R: Reverse Primer.

280 nm ratio using a UV–Vis spectrophotometer (NanoDrop, 2000, Thermo Scientific). Total RNA (800-ng) was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, NY) as per manufacturer's instructions. The resulting cDNA was used as the template for semi-quantitative real time PCR reactions.

### 2.7. cDNA synthesis and RT-PCR

Gene specific primers were designed using PrimerQuest software from Integrated DNA Technologies (Coralville, IA). All the gene specific primer sequences are presented in Table 1. Semiquantitative RT-PCR amplification reactions were run on a gradient thermal cycler (Eppendorf, NY) using EmeraldAmp GT PCR Master Mix (Clontech, CA). Expression levels of transcripts were normalized to expression values of the control GAPDH and/or Beta-Actin genes.

### 2.8. Oxidation products and phytochemicals

The total 3-chloropropane-1,2-diol (3-MCPD) esters and glycidyl esters were determined using AOCS method Cd 29c-13. Total phytosterol content was determined according to the method described by Shi (Shi et al., 2015). The extraction and determination of total phenolic content (TPC) of coconut oil samples was carried out according to Appaiah and Sunil (Appaiah et al., 2014). AOCS methods were used for determination of peroxide value (PV, Cd 8-53) and Acid value (AV, Cd 3d-63).

*Total cholesterol (T-CHO) and Triacylglycerol (TG) analysis*. T-CHO content and TG content of packed cell pellets was measured using the Total Cholesterol Assay Kit and Triacylglycerol Assay Kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer protocols.

*Tests of minor components on the key genes of cholesterol metabolism.* We tested whether isolated minor process contaminants and depleted phytochemicals influence HMGCR and/or CYP7A1 gene expression.

Genuine 3-MCPDE or GEs were added to J1 (virgin coconut oil) at levels measured in J4 (fully refined) to yield J1 + 3-MCPDE and J1 + GEs coconut oils. Similarly, total phytosterols and total phenolics measured in J1 (virgin coconut oil), total phytosterols standard and total phenolics extracts were respectively added back to J4 (deodorization) in order to get J4 + Phytosterols and J4 + Phenolics.

### 2.9. Statistical analysis

For analysis of virgin and refined oils, data are presented as means ± SD of two independent biological replicates. Statistical

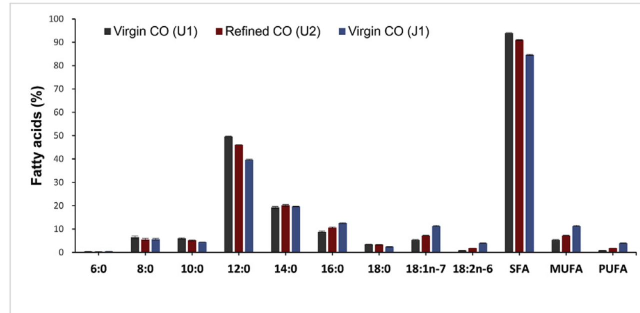


**Fig. 1.** Fatty acid composition of virgin CO (U1), refined CO (U2) and virgin CO (J1). No significant differences were found between the oils, though refined oil tended to be lower in saturates and higher in PUFA.

analysis was performed by one-way analysis of variance and when significant was followed by two sided pairwise *t*-test comparison using Microsoft Excel 2013 for the in-house refined samples, and simple pairwise *t*-test for the commercial samples. $p < 0.05$ was considered significant; $p < 0.1$ marginally significant.

For analysis of virgin and fully refined oils with added components, results are presented as the mean ± SD for at least three replicates. Statistical analyses were performed using the SPSS program, version 20.0 (SPSS Inc., IBM Corp, Armonk, NY). Asterisks indicate significant ($p < 0.05$) and highly significant ($p < 0.01$).

## 3. Results

### 3.1. Fatty acid profiles of test oils

The fatty acid composition of test oils is presented in Fig. 1. No significant trends are found in the in-house refined oil so only the profile of the VCO is presented. The commercially obtained pair oils differed slightly in fatty acid profile, with the refined CO having *more* PUFA than the VCO.

### 3.2. HepG2 cell uptake of coconut oil

A convenient indicator of test oil uptake was needed to ensure that, in this case, CO, was taken up by cells. CO is rich in 12:0 but has negligible 16:1. To verify uptake of CO by HepG2 cells, the ratio of 12:0 to 16:1 was determined in the cells post-treatment. A ratio of 10 to 1 of DPPC to CO was used, based on previous reports (Fujisawa et al., 2004). The ratio of (DPPC/VCO) dependent on uptake of liposomes shows that the 12:0 to 16:1 ratio increased 15 fold post-treatment (Fig. 2), verifying that the lipid was taken up by the cells.

### 3.3. Transcriptional regulation of cholesterol homeostasis genes

Initially, five genes with metabolic relationships to cholesterol levels were probed for transcriptional changes. Of these, two were found responsive to oil treatment, *HMGCR* and *CYP7A1*. The mean HMGCR significantly increased with deacidification and further with bleaching, and was marginally significantly increased with deodorization, compared to VCO. In contrast, *CYP7A1* followed an opposite trend, decreasing after de-acidifcation and bleaching, and leveling off after deodorization (Fig. 3). No trends were found for the other genes, *SREBP-2, SCARB1,* and *CYP51A1*. The mean increase in HMGCR for fully processed (deodorized) oil was 57%, while the mean decrease for *CYP7A1* was 35%. Compared to VCO (J1) group total cholesterol and TG levels are significantly higher in the J2, J3 and J4 groups (Fig. 3, Lower panel). The results for the commercially obtained VCO and RBD CO were concordant with the in-house processed samples. *HMGCR* increased significantly *VCO* to RBD CO, while CYP7A1 decreased (Fig. 4)

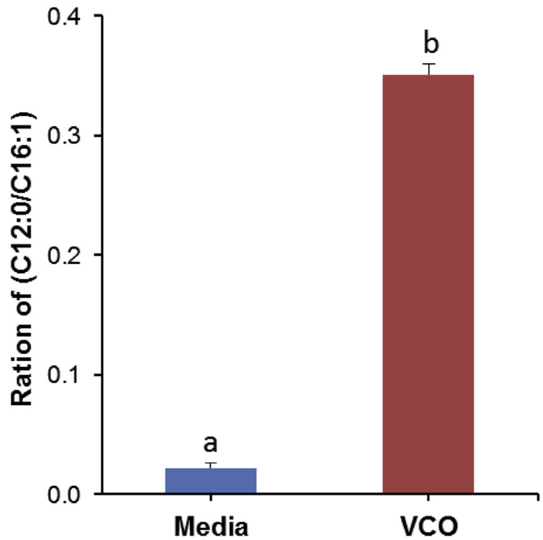


**Fig. 2.** Uptake of oil in cells as measured by the ratio of 12:0 to 16:1 incorporation in cells for VCO liposome or blank (media only) incubations. VCO increased in the cells by 15-fold ($p < 0.05$) compared to blank.

by +79% and −35%, respectively.

### 3.4. Oxidation products and phytochemicals of CO

Indices of processing effects on the oils indicated increases in processing contaminants and a decrease in phytochemical content (Table 2). 3-MCPD was undetectable in either sample of VCO. In the in-house processed samples, it was detectable after deacidification, doubled with bleaching, and then increased by a factor of 10 in the deodorization step. The commercial RBD oil had detectable 3-MCPD but with substantially lower absolute concentration than our in-house fully processed (deodorized) oil. Total glycidyl esters were not detectable in either commercial CO.

Phytosterols and polyphenols both decreased with each step for the in-house processed oils and the commercial oils. In both sets of oils, the phytosterol content dropped to about half its VCO value in the full processed oils. Similarly, the polyphenol content dropped to about half its VCO value for in-house processing, and the commercial RBD oil was about two thirds the value of the commercial VCO. We also measured the acid value as a routine measure of oil status. The in-house processed oil decreased as expected for deacidification, then increased in the bleaching step presumably due to heating, and finally settled out to a value between the deacidified and VCO. Both commercial samples were lower than any of the in-house oils. Peroxides were undetectable in VCOs but were detectable in the processed oils.

#### 3.4.1. Process contaminants-phytochemicals and HMGCR expression

Fig. 5 shows HMGCR expression levels in VCO (J1) with process contaminants added to reach J4 levels, and in fully processed (J4) oils with phytochemicals added to simulate J1 levels. HMGCR expression increased significantly with addition of GEs or 3-MCPD, and was not different between those two groups. For the fully processed CO, phenolics addition significantly reduced HMGCR expression, whereas addition of phytosterols to J4 had no effect.

## 4. Discussion

Our results show that harsh processing increases cholesterol synthesis as well as TAG accumulation in human cells. We further show that processing-induced creation of glycidol and 3-MCPD recapitulated this activity in VCO. The magnitude of increase in HMGCR upon addition of glycidyl esters or 3-MCPD to VCO was similar to that in fully

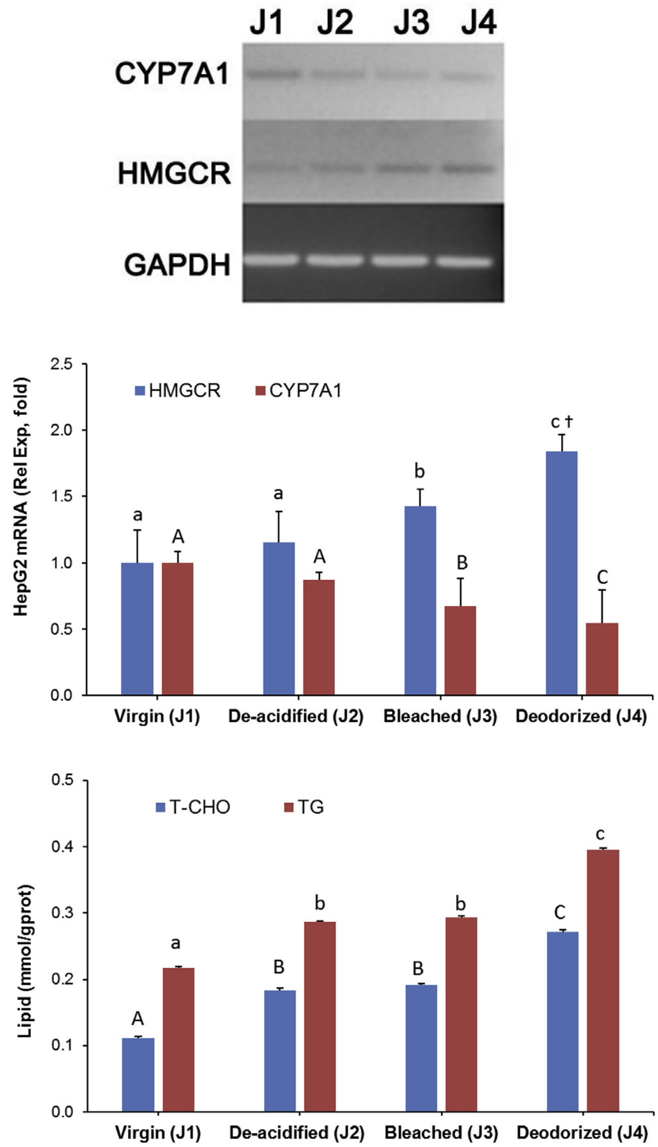


**Fig. 3.** mRNA expression levels of HMGCR and CYP7A1 genes in HepG2 cells (n = 2 independent biological replicates), J1) VCO and processed in our Jiangnan University laboratories J2) deacidification, J3) bleaching, and J4) deodorization. One-way analysis of variance was significant for both genes, pairwise *t*-test significantly different for bars with different letters. †p = 0.082 vs Virgin. Lower panel. Cholesterol and TG in cells. Bars with different superscripts are significantly different ($p < 0.05$).

processed oil, suggesting that these process contaminants are major drivers of HMGCR gene expression within the chemically complex oil matrix. We also show that reintroduction of phenolics generally considered to be antioxidants reduces this activity in RBD CO.

Our method is novel in that it enables study of the effect of the whole oil *in vitro*, rather than the isolated fatty acids. In this respect the method represents a rapid bioassay for a key property of edible oils, their effects on cholesterol synthesis and degradation, long recognized as key to health indicators. Net functional activity, for instance by metabolite concentrations, may be regulated at any level from the abundance and activation of transcription factors, splicing and splice variants, translation, post-translational modifications, and substrate activation. As a result the correlation between gene expression and, for instance, protein abundance, is weak on average across the genome (Maier et al., 2009). In the present cases, HMGCR is regulated at multiple levels including at transcription (Vock et al., 2008), while CYP7A1

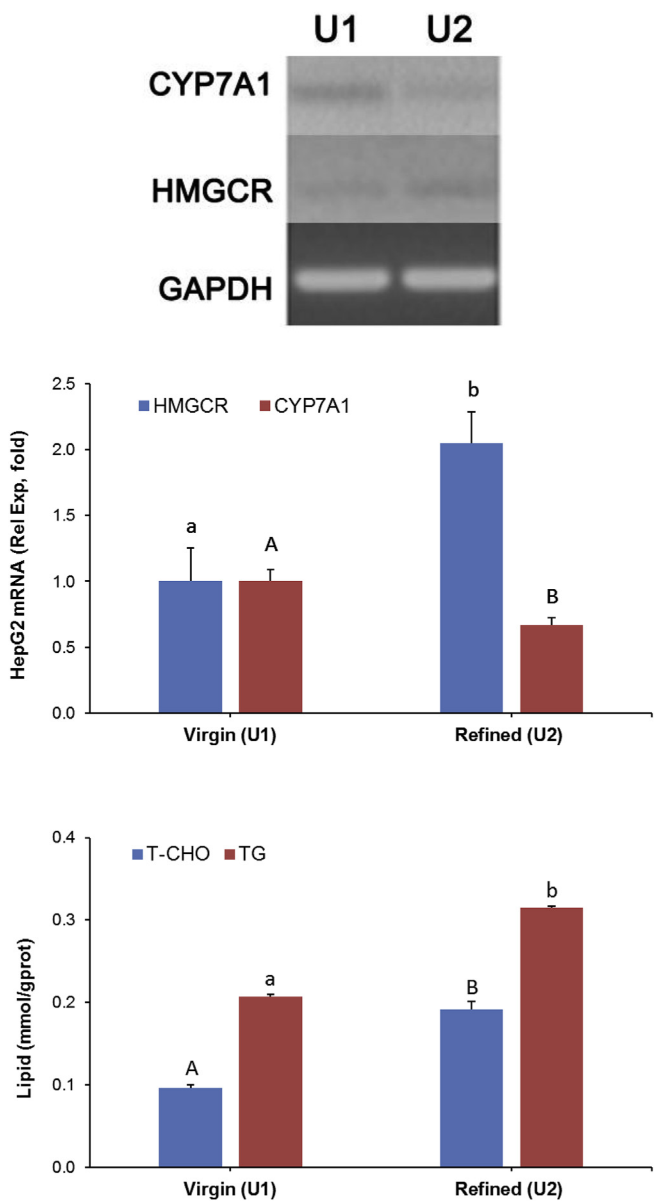


**Fig. 4.** mRNA expression levels of HMGCR and CYP7A1 genes in HepG2 cells. U1) VCO and U2) refined CO, were obtained in the USA. Refined oil significantly altered both genes (pairwise *t*-test, $p < 0.05$). Lower panel. Cholesterol and TG in cells. Bars with different superscripts are significantly different ($p < 0.05$).

is primarily regulated at transcription (Gupta et al., 2001), and our results show that cellular cholesterol did change in the direction expected from gene expression.

Glycidol and MCPD have long been known as carcinogens, however to our knowledge the present data are the first to indicate that they induce hepatic cholesterol synthesis. When these results are confirmed *in vivo*, they can be considered an identifiable chemical risk for cardiovascular disease. In rats, glycidyl esters are hydrolyzed to fatty acids and glycidol in the intestinal lumen and glycidol is absorbed, distributed widely, metabolized, and excreted. In humans, consumption of glycidyl ester containing oil cause glycidol urinary metabolites to rise. Glycidol is a recognized non-mutagenic carcinogen, however its activity as a stimulator of cholesterol synthesis has not yet previously been shown in any biological system, to our knowledge (EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2016).

Saturated fat content cannot be the source of the changes in cholesterol metabolism because the fatty acid profiles of the in-house processed oils did not differ with stage of processing, while the commercial VCO had more saturated fat than the commercial RBD oil. HMGCR is the rate-limiting step for cholesterol biosynthesis and the target of statin therapy, which lowers serum cholesterol through inhibition of HMGCR and the "gold standard" drug class which has clearly been shown to lower risk for cardiovascular disease events (Jacobson et al., 2014; Stone et al., 2014). Thus, intake of oils that oppose the statin mechanism are likely to have the opposite effect. CYP7A1 is the principal rate-limiting step in the degradation of cholesterol to bile salts. Humans with a genetic polymorphism that renders the product of the CYP7A1 gene inoperative are hypercholesterolemic, and CYP7A1 polymorphorisms are associated with cardiovascular events, (Pullinger et al., 2002). Moreover, CYP7A1 is transcriptionally regulated *in vivo*, (Gilardi et al., 2007). Modifying cholesterol levels in humans by modulating this mechanism is not a focus of current therapy except secondarily via bile acid sequestrants (Jacobson et al., 2014; Wada et al., 2008). Harshly processed fats may induce CYP7A1 indirectly either via inducing liver X receptor (LXR) or farnesoid X receptor (FXR) (Chiang et al., 2000). The net effect of an increase in synthesis and a decrease in degradation is expected to be an increase in the overall cholesterol level, though our *in vitro* data cannot be used to estimate its magnitude without further studies.

Because fatty acid profile cannot be the cause of changes in gene expression, we looked at other parameters known to change with processing. Process contaminant 3-MCPD increased with each step of in-house processing. Total glycidyl esters increased at the last step of in house processing but were below the detection limits of our method for other steps. 3-MCPD or other process contaminants for which it serves as a proxy could be the causative agent in altering transcript levels.

Total phytosterols and polyphenolics decreased with processing steps. Others have suggested that it is their reduction that leads to less healthfulness of processed oils (Marina et al., 2009b). Our results are consistent with this hypothsis for polyphenoics, and to our knowledge ours are the first data to demonstrate *in vitro* reduction in an atherogenic risk factor directly by phenolics in oils.

Brief comments on the putative atherogenicity of saturated fatty acids per se will be useful. Through the last quarter of the 20th century coconut oil was widely assumed to be inalterably atherogenic because of its highly reproducible influence on serum cholesterol in experimental animal models. Much but not all of the published work used

**Table 2**
Minor components and properties of sample coconut oils (CO; mean ± SD).

| | *Virgin CO (J1)* | *De-acidified CO (J2)* | *Bleached CO (J3)* | *Deodorized CO (J4)* | *Virgin CO (U1)* | *Refined CO (U2)* |
|---|---|---|---|---|---|---|
| ***(mg/kg)*** | *n.d.* | *0.12 ± 0.017[a] n.d.* | *0.23 ± 0.012[b] n.d.* | *2.37 ± 0.01[c]* | *n.d.* | *0.44 ± 0.03 n.d.* |
| ***Total 3-MCPD*** | *n.d.* | | | *1.48 ± 0.04* | *n.d.* | |
| ***Total Glycidyl esters*** | | | | | | |
| ***(mg/kg)*** | *82.31 ± 0.00[a]* | *71.24 ± 1.58[b]* | *66.56 ± 0.70[c]* | *43.13 ± 0.85[d]* | *97.2 ± 0.10[A]* | *59.8 ± 1.45[B]* |
| ***Total Phytosterols*** | *8.10 ± 0.11* | *6.25 ± 0.90* | *5.11 ± 1.30* | *4.23 ± 0.95* | *6.75 ± 0.15[A]* | *4.65 ± 0.34[B]* |
| ***Total Phenolics*** | | | | | | |
| ***AV (mg KOH/g)*** | *0.37 ± 0.01* | *0.16 ± 0.01* | *0.73 ± 0.01* | *0.23 ± 0.00* | *0.08 ± 0.01* | *0.12 ± 0.02* |
| ***PV(mmol/KG)*** | *0* | *0.15 ± 0.02* | *0.53 ± 0.02* | *0.11 ± 0.01* | *0* | *0.32 ± 0.01* |

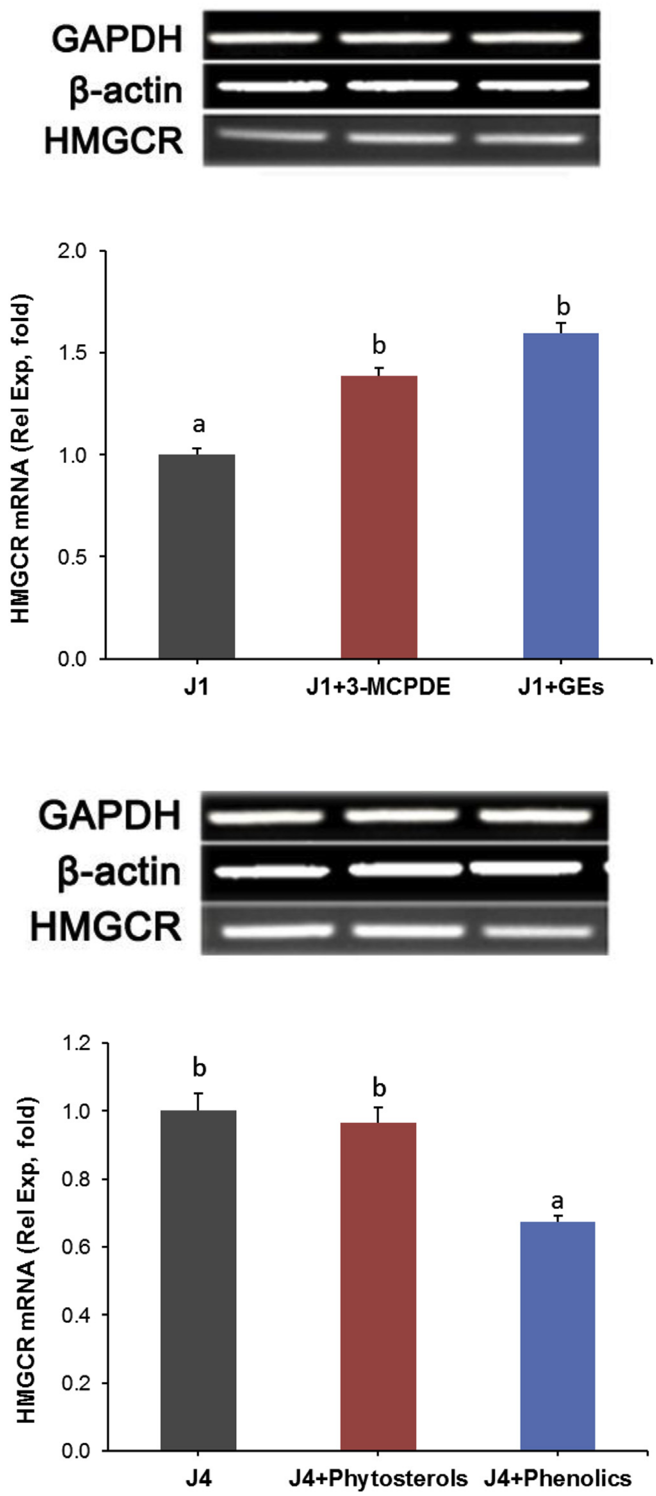


**Fig. 5.** Process contaminant-phytochemical recapitulation of activity. Top. VCO (J1) with GE2 increased HMGCR mRNA significantly (+59%) while the increase in 3-MCPDE was less and did not reach significance. Bottom. Fully processed CO (J4) plus phenolics significantly attenuated the rise in HMGCR (33% decrease) while phytosterols had no effect. Process contaminants contents (mg/kg): J1+3-MCPDE (2.33 ± 0.21); J1+GEs (1.45 ± 0.18); J4+Phytosterols (82.30 ± 1.05); J4+Phenolics (8.00 ± 0.92).

hydrogenated coconut oil and with few documenting the sources of those oils (Kritchevsky et al., 1976, 1980, 1982, 2003). In mid-2017, the American Heart Association issued a Presidential Advisory against consumption of satuated fats based on a new meta-analysis of four cornerstone studies said to compare high saturated with high polyunsaturated fat intake (Sacks et al., 2017). Careful assessment of these studies reveals that three of the four studies were conducted in Europe in the 1960 and 70s with soybean oil as a test fat to replace habitual oil intake which served as the control. Habitual fat and oil intake at that time was rich in partially hydrogenated fish oil; the Oslo study specifically estimated that daily intake of partially hydrogenated fish oil was 40–50 g per day (Leren, 1970). The widespread use of partially hydrogenated fish oil as a feed stock for European margarines nearly assures that comparator habitual diets in the studies in Finland (Leren, 1970) and the UK(Haigh et al., 1968) were similar. These diets therefore compared soybean oil as a parttial substitute for a mixture of highly processed hydrogenated fish oil rather than as asserted in the paper, oil with no trans fatty acids. The sole US study in the meta-analysis specifically tested replacement of the saturated animal fats and hydrogenated shortenings of the conventional diet by equal quantities of unsaturated fat in the form of vegetable oils. The study took place in a domicilied participants and a designed control fat was used with non-hydrogenated margarine (Hiscock et al., 1962). No significant reduction in coronary disease was found in this study (Sacks et al., 2017).

The carcinogenicity of glycidyl esters and 3-MCPD were not identified until decades after the conclusion of these studies (B.S. et al., 1993), and there were no standard checks on process contaminants in oils of the day. The AHA Advisory acknowledges the atherogenicity of "trans unsaturated fat", though we interpret this statement as recognition of the atherogenicity of partially hydrogenated vegetable oil. We also note that others have found no evidence for the atherogenicity of saturated fat (Ramsden et al., 2016) nor evidence that full fat dairy should be avoided (Mozaffarian, 2016). Integrating these data with animal and the present *in vitro* data, the totality of evidence suggest that the AHA meta-analysis considered mostly studies which were tests of harshness of processing rather than the saturated fatty acids per se, and that saturated fats are not inherently athergenic compared to mildly processed vegetable oils.

The widespread availability of VCO in the U.S. has precipitated a renaissance in use of these oils in home cooking and prepared products which, in the absence of specific studies, remains controversial (Sacks et al., 2017). A recent systematic review (Eyres et al., 2016) of eight controlled human trials of coconut oils versus cis unsaturated vegetable oils found no evidence that coconut oil is different in its effect on blood lipid parameters compared to cis unsaturated oils (Sacks et al., 2017). However, only one of these studies reported the processing method for the coconut oil tested (Ng et al., 1991). Of the others, only two reported the commericial source of the oils (Reiser et al., 1985; Voon et al., 2011), and the others did not report the source or the processing (Cox et al., 1995, 1998; Fisher et al., 1983; Mendis and Kumarasunderam, 1990; Mendis et al., 2001; Ng et al., 1991). None specifically tested RBD versus verified VCO. The widespread availability of RBD coconut oils as well as the relatively recent and rare availabiity of virgin coconut oils suggest that the majority of the oils in these studies were RBD coconut oil. A plausible explanation for the net results obtained in these studies is an overemphasis on the fatty acid profile without considering the differences in minor and trace chemical constituents of oils induced by differences in processing that may have definitive biological effects, particularly over the time scales of years relevant to chronic disease development. Head-to-head *in vivo* comparison of VCO and RBD-CO in animals resulting in different outcomes for oils of the same fatty acid profile are definitive in showing, at a minimum, that more than a fatty acid profile is an issue for the healthfulness of oils. (Arunima and Rajamohan, 2014; Nevin and Rajamohan, 2004, 2009).

Our method is not inherently restricted to coconut oil but applies to all oils and, in modified form, probably all foods. As a practical matter, rapid methods for assessing the effects of novel foods on chronic disease are needed to improve the commercial food chain on which a large fraction of the human population depends. Ours and similar biology-based strategies have the potential to move nutritional science to a more nuanced view of food processing.

## Author contributions

RL and JTB conceived and designed the research. RL, MC, KSDK, ZW, EM, HGP executed the research. All authors contributed writing and approved the final version of the manuscript.

## Notes

The authors declare no competing financial interest.

## Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.03.005.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.03.005.

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