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Lipoprotein metabolism in familial hypercholesterolemia: Serial assessment using a one-step ultracentrifugation method



Hayato Tada^{a,*}, Masa-aki Kawashiri^a, Atsushi Nohara^b, Akihiro Inazu^c, Hiroshi Mabuchi^b, Masakazu Yamagishi^a, Kenshi Hayashi^a

^a Division of Cardiovascular Medicine, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, 920-8641 Kanazawa, Japan

^b Department of Lipidology, Graduate School of Medical Science, Kanazawa University, Kanazawa, Japan

^c Department of Laboratory Science, Molecular Biochemistry and Molecular Biology, Graduate School of Medical Science, Kanazawa University, Kanazawa, Japan

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ABSTRACT

Objectives: It is well known that familial hypercholesterolemia (FH) is a common inherited disorder that can markedly elevate the level of plasma LDL cholesterol. However, little data exists regarding the clinical impact of the plasma triglyceride (TG)-rich lipoprotein fraction, including VLDL and IDL, in FH. Thus, we assessed the hypothesis that the mutations in the LDL receptor modulate lipoprotein metabolism other than the LDL fraction.

Design and methods: We investigated plasma lipoprotein with a one-step ultracentrifugation method for 146 controls (mean age = 61.4 ± 17.1 yr, mean LDL cholesterol = 92.7 ± 61.2 mg/dl), 213 heterozygous mutation-determined FH subjects (mean age = 46.0 ± 18.0 yr, mean LDL cholesterol = 225.1 ± 61.2 mg/dl), and 16 homozygous/compound heterozygous mutation-determined FH subjects (mean age = 26.9 ± 17.1 yr, mean LDL cholesterol = 428.6 ± 86.1 mg/dl). In addition, we evaluated cholesterol/TG ratio in each lipoprotein fraction separated by ultracentrifugation.

Results: In addition to total cholesterol and LDL cholesterol levels, VLDL cholesterol (19.5 ± 10.4 , 25.2 ± 19.3 , 29.5 ± 21.4 mg/dl, respectively) and IDL cholesterol (8.3 ± 3.7 , 16.8 ± 11.5 , 40.0 ± 37.3 mg/dl, respectively) exhibited a tri-modal distribution according to their status in LDL receptor mutation(s). Moreover, the ratios of cholesterol/TG of each lipoprotein fraction increased significantly in heterozygous FH and homozygous/compound heterozygous FH groups, compared with that of controls, suggesting that the abnormality in LDL receptor modulates the quality as well as the quantity of each lipoprotein fraction.

Conclusions: Our results indicate that cholesterol in TG-rich lipoproteins, including VLDL and IDL, are significantly higher in FH subjects, revealing a tri-modal distribution according to the number of LDL receptor mutations.

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1. Introduction

Familial hypercholesterolemia (FH) is the most common and most severe monogenic hypercholesterolemia characterized by excess deposition of cholesterol in tissues leading to tendon xanthomas and premature coronary artery disease [1–3]. Patients with FH have mutant allele(s) of either of three FH-associated genes (FH genes), namely LDL receptor, apolipoprotein B-100 and proprotein convertase subtilisin/kexin 9 (PCSK9) genes [2], among which, LDL receptor is the leading cause of this disorder. Individuals with two mutations in the LDL receptor gene (homozygous FH) display extremely severe hyper-LDL-cholesterolemia, usually over 400 mg/dl, and develop cutaneous xanthomas, coronary artery disease, and aortic valve stenosis in childhood [1,2]. If the LDL-cholesterol level is not effectively treated, homozygous FH die prematurely from an acute coronary event or heart failure [4]. The frequency of heterozygous FH in the general population has been estimated at about 1 in 500 almost all over the world. Recent advances in genetic analysis has enabled the accuracy of

Abbreviations: FH, familial hypercholesterolemia; PCSK9, proprotein convertase subtilisin/kexin 9; LDL-C, LDL cholesterol; TC, total cholesterol; TG, triglyceride; HDL-C, HDL cholesterol; VLDL-C, VLDL cholesterol; IDL-C, IDL cholesterol

* Corresponding author. Tel.: +81 76 265 2000x2251; fax: +81 76 234 4251.

E-mail address: ht240z@sa3.so-net.net (H. Tada).

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diagnosis of heterozygous FH and revealed a much higher frequency of FH in certain populations, such as the Afrikaners, Christian Lebanese, Finns, and French–Canadians due to a founder gene effect [1].

Triglyceride (TG)-rich lipoprotein, such as VLDL and IDL, are associated with coronary artery disease [5,6]. However, little data exists regarding the impact of LDL receptor mutations on the metabolism of TG-rich lipoprotein in FH, which could be an additive risk factor for the development of coronary atherosclerosis. Here, we investigated whether the mutations in the LDL receptor modulate lipoprotein metabolism other than the LDL fraction using a one-step ultracentrifugation method.

2. Materials and methods

2.1. Study subjects

We investigated plasma lipoprotein by an ultracentrifugation method for 146 controls, 213 heterozygous mutation-determined FH subjects, and 16 homozygous/compound heterozygous mutation-determined FH subjects. All of the FH subjects have (a) mutant allele (s) in LDL receptor gene. The characteristics of the study subjects are listed in Table 1.

2.2. Genetic analyses

Genomic DNA was isolated from peripheral blood white blood cells using Genomic DNA Purification Kit (Genra Systems, Minneapolis, MN) and was used for PCR. The genotypes of all the participants in this study were determined as previously described [7–10]. Genetic analyses were approved by the Ethics Committee of Kanazawa University and carried out in accordance with the Declaration of Helsinki (2008) of the World Medical Association. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consents were obtained from all subjects.

2.3. Biochemical analyses

Fasting blood samples were drawn for assays either before the lipid-lowering treatment or after discontinuation of medication for at least 4 weeks. Blood samples were stored at 4 °C immediately in the pharmaceutical refrigerators (MPR-721, Panasonic healthcare, Tokyo, Japan) until the process to obtain serum and plasma for within an hour. Serum concentrations of total cholesterol (TC), TG, and HDL cholesterol (HDL-C) were determined enzymatically (Qualigent®, Sekisui Medical, Tokyo, Japan) using automated instrumentation (LABOSPECT 008, Hitachi High-Technologies, Tokyo, Japan) based on the assays previously described [11–13]. LDL-C concentrations were derived using the Friedewald formula [14]. Plasma were processed to ultracentrifugation immediately after the acquisition.

2.4. Ultracentrifugation analyses

We used a one-step ultracentrifugation method as described in Fig. 1 [15–17]. Plasma (600 µL) was divided into three different tubes (each containing 200 µL), then NaBr solutions with different specific gravity ($d=1.006$, 1.032, and 1.120) were added. The least dense solution ($d=1.006$) was overlaid with the plasma, and the other two solutions ($d=1.032$, and 1.120) were mixed with plasma, adjusting the gravities to $d=1.019$, and 1.063, respectively. Subsequently, the same three tubes were ultracentrifuged at 42,000 rpm, for 12 h at 10 °C in a Beckman 42 Ti rotor. The top 200 µL containing VLDL ($d < 1.006$ g/ml), and the bottom 200 µL containing IDL, LDL, and HDL were extracted separately in the tube with the least dense solution. Similarly, the top 200 µL containing VLDL and IDL ($d < 1.019$ g/ml), and the bottom 200 µL containing LDL and HDL were extracted separately in the tube with the higher density solution. Finally, the top 200 µL containing VLDL, IDL, and LDL ($d < 1.063$ g/ml), and the bottom 200 µL containing HDL were extracted separately in the tube with the highest density solution. Cholesterol ester (calculated as total cholesterol minus free cholesterol), and TG were analyzed in each ultracentrifugally separated lipoprotein as described above.

Table 1
Characteristics of the study subjects.

Variable	Controls (n=146)	Heterozygous FH (n=213)	Homozygous/compound heterozygous FH (n=16)
Age (yr)	61.4 ± 17.1	46.0 ± 18.0*	26.9 ± 17.1**
Men (%)	91 (62%)	87 (41%)	11 (69%)
BMI (kg/m ²)	25.8 ± 3.8	23.4 ± 2.9	25.4 ± 4.4
TC (mg/dl)	193 ± 21	321 ± 66**	701 ± 160**
VLDL-C (mg/dl)	19.5 ± 10.4	25.2 ± 19.3*	29.5 ± 21.4**
IDL-C (mg/dl)	8.3 ± 3.7	16.8 ± 11.5**	40.0 ± 37.3**
LDL-C (mg/dl)	93 ± 61	225 ± 61**	429 ± 86**
HDL-C (mg/dl)	57 ± 17	53 ± 25	39 ± 10*
TG (mg/dl)	88 ± 12	142 ± 73**	170 ± 86**
VLDL-TG (mg/dl)	42.0 ± 24.0	56.2 ± 55.3*	43.9 ± 28.6
IDL-TG (mg/dl)	7.0 ± 3.2	11.5 ± 7.2*	13.8 ± 10.4**
LDL-TG (mg/dl)	19.0 ± 6.3	35.0 ± 16.4**	68.2 ± 29.5**
HDL-TG (mg/dl)	12.1 ± 6.3	12.8 ± 5.4	12.0 ± 7.2

FH: familial hypercholesterolemia, BMI: body mass index, TC: total cholesterol, VLDL-C: VLDL cholesterol, IDL-C: IDL cholesterol, LDL-C: LDL cholesterol, HDL-C: HDL cholesterol, TG: triglyceride, VLDL-TG: VLDL triglyceride, IDL-TG: IDL triglyceride, LDL-TG: LDL triglyceride, and HDL-TG: HDL triglyceride.

* $p < 0.05$.

** $p < 0.0001$ vs controls.

2.5. Statistical analysis

Continuous variables with a normal distribution were shown as mean (\pm SD), and were compared using unpaired Student *t*-tests, and those with non-normal distribution were compared using Mann–Whitney U test. Categorical variables were expressed as percentages, and were compared using chi-square test. Analyses were conducted using R statistical software [18]. We determined data were normally distributed using Jarque–Bera test through R package (tseries). All *p* values < 0.05 were considered statistically significant.

3. Results

3.1. Baseline characteristics of study subjects

Baseline characteristics, including lipid profiles determined enzymatically in 146 controls (mean age = 61.4 ± 17.1 yr, mean LDL-C = 92.7 ± 61.2 mg/dl), 213 heterozygous mutation-determined FH subjects (mean age = 46.0 ± 18.0 yr, mean LDL-C = 225.1 ± 61.2 mg/dl), and 16 homozygous/compound heterozygous mutation-determined FH subjects (mean age = 26.9 ± 17.1 yr, mean LDL-C = 428.6 ± 86.1 mg/dl), are listed in Table 1. Tables 2 and 3 provide the genetic backgrounds of the heterozygous FH and homozygous/compound heterozygous FH subjects, respectively.

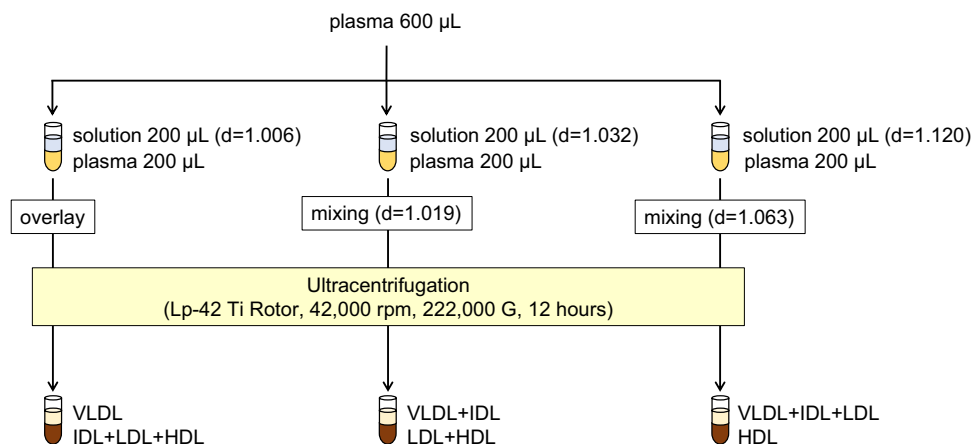


Fig. 1. Schema of one-step ultracentrifugation analysis for lipoprotein. Light blue liquid indicates solution. Orange liquid indicates plasma. Light orange indicates the top 200 μ L after ultracentrifugation. Brown indicates the bottom 200 μ L after ultracentrifugation.

Table 2
Genetic background of the study subjects with heterozygous FH.

Nucleotide change	Mutation type	Effect on protein	Number of patients
c.68-?_313+?del	Large deletion	Truncated protein	15
c.191-?_940+?dup	Large duplication	Truncated protein	6
c.344G > A	Missense	Arg-His	1
c.413C > G	Nonsense	Ser-stop	3
c.539G > A	Nonsense	Trp-stop	1
c.662_665dupACTG	Duplication	Frameshift/stop	1
c.682G > A	Missense	Glu-Lys	2
c.797A > G	Missense	Asp-Gly	1
c.901G > T	Missense	Asp-Tyr	1
c.1012T > A	Missense	Cys-Ser	2
c.1285G > A	Missense	Val-Met	1
c.1297G > C	Missense	Asp-His	2
c.137G > A	Missense	Cys-Tyr	1
c.1432G > A	Missense	Gly-Ala	1
c.1474G > A	Missense	Asp-Asn	1
c.1567G > A	Missense	Val-Met	1
c.1689dupC	Duplication	Frameshift/stop	1
c.1702C > G	Missense	Leu-Val	1
c.1778dupG	Duplication	Frameshift/stop	1
c.1845+2T > C	Splicing	exon13 skip	1
c.1871_1873delTCA	Deletion	603 Ile deletion	5
c.2054C > T	Missense	Pro-Leu	32
c.2140+1G > T	Splicing	exon15 skip	3
c.2141-?_2311+?del	Large deletion	Truncated protein	7
c.2312-3C > A	Splicing	exon16 skip	25
c.2431A > T	Nonsense	Lys-stop	97

Table 3
Genetic background of the study subjects with homozygous/compound heterozygous FH.

Nucleotide changes	Mutation types	Effect on proteins	Number of patients
c.901G > T/c.901G > T	Missense/missense	Asp-Tyr/Asp-Tyr	1
c.2054C > T/c.2431A > T	Missense/nonsense	Pro-Leu/Lys-stop	2
c.68-?_313+?del/c.68-?_313+?del	Large deletion/large deletion	Truncated protein/Truncated protein	1
c.1246C > T/c.1246C > T	Missense/missense	Arg-Trp/Arg-Trp	1
c.796G > A/c.796G > A	Missense/missense	Asp-Asn/Asp-Asn	1
c.1285G > A/c.418G > A	Missense/missense	Val-Met/Glu-Lys	1
c.1502C > T/c.241dupC	Missense/missense	Ala-Val/Arg-Cys	1
c.1845+2T > C/c.1845+2T > C	Splicing/splicing	exon13 skip/exon13 skip	2
c.2201_2202delCA/c.2201_2202delCA	Nonsense/nonsense	Gln-stop/Gln-stop	1
c.661G > A/c.1845+2T > C	Missense/splicing	Asp-Asn/exon13 skip	1
c.1012T > A/c.68-?_313+?del	Missense/large deletion	Cys-Ser/Truncated protein	2
c.796G > A/c.361T > G	Missense/missense	Asp-Asn/Cys-Gly	1
c.1567G > A/c.1567G > A	Missense/missense	Val-Met/Val-Met	1

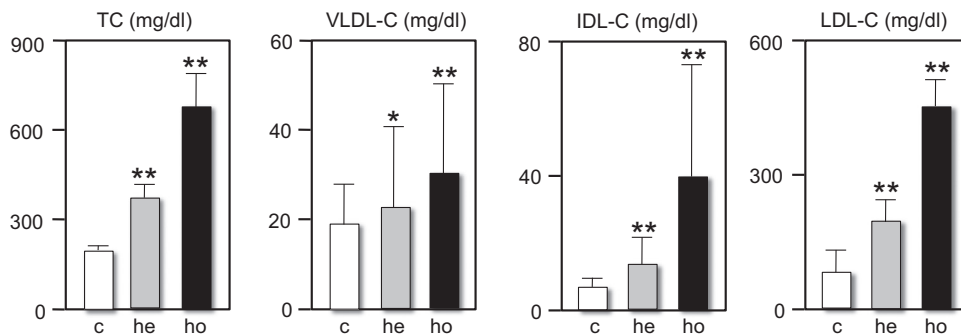


Fig. 2. Lipoprotein subfractions determined by ultracentrifugation. White bars indicate controls. Gray bars indicate heterozygous FH. Black bars indicate homozygous FH. TC: total cholesterol, VLDL: very low-density lipoprotein, IDL: intermediate-density lipoprotein, LDL: low-density lipoprotein, c: controls; he: heterozygous FH; ho: homozygous or compound heterozygous FH. * $p < 0.05$, ** $p < 0.0001$ vs controls.

3.2. Lipoprotein subfractions separated by ultracentrifugation and ratios of cholesterol/triglyceride in each lipoprotein fractions

In addition to TC and LDL-C levels, cholesterol levels in TG-rich lipoprotein fractions separated by ultracentrifugation also exhibited tri-modal distributions according to the mutation status in the LDL receptor (VLDL-C levels were 19.5 ± 10.4 , 25.2 ± 19.3 , and 29.5 ± 21.4 mg/dl, respectively; IDL-C levels were 8.3 ± 3.7 , 16.8 ± 11.5 , and 40.0 ± 37.3 mg/dl, respectively; Fig. 2).

Moreover, the ratios of cholesterol/TG in each lipoprotein fraction also exhibited similar tri-modal distributions, suggesting that the status of mutation(s) in the LDL receptor modulates not only metabolism in the LDL fraction, but also in VLDL and IDL fractions (Fig. 3). We also investigated the correlations between lipoprotein fractions separated by ultracentrifugation and enzymatically determined lipid profiles (Supplemental Tables 1–3, and Supplemental Figs. 1–12). As expected, enzymatically determined TG levels exhibited good correlations with levels of VLDL-TG and IDL-TG.

4. Discussion

Using a FH cohort of 213 heterozygous and 16 homozygous/compound heterozygous mutation-determined subjects, we sought to evaluate if cholesterol in TG-rich lipoprotein levels separated by ultracentrifugation are higher in FH subjects. We found that cholesterol in those TG-rich lipoproteins, including VLDL and IDL, were significantly higher in FH subjects, revealing a tri-modal distribution according to the number of mutations. In addition to the total amount, the ratio of cholesterol/TG in each lipoprotein fraction was also significantly higher in FH subjects, showing a tri-modal distribution.

Patients with heterozygous FH, which is estimated to be at least 20 million worldwide exhibit premature coronary atherosclerosis, thus their risk of future coronary events needs to be assessed [19,20]. There are a number of biomarkers for the prediction of coronary events, such as age, sex, LDL-C level and cholesterol in TG-rich lipoprotein levels [21–23]. In this study, we clearly demonstrated that cholesterol in TG-rich lipoprotein levels separated by ultracentrifugation were significantly and quantitatively higher in FH subjects. In addition, our study has a strength in the determination of mutation status in LDL receptor, providing a comparison of TG-rich lipoproteins between patients with FH exhibiting single LDL receptor mutation and those with double mutations.

Possible mechanisms of those increased cholesterol in TG-rich lipoproteins could be (1) increased production of VLDL, as demonstrated in several kinetic studies in vivo [24], or (2) disturbed clearance of TG-rich lipoproteins via a dysfunctional LDL receptor [25]. In accordance with these hypotheses, we have previously demonstrated that the clearance of post-prandial remnant lipoproteins in FH was also disturbed [26]. In addition, lipoprotein lipase and hepatic lipase which are expected to remove TG and therefore enrich cholesterol content in TG-rich lipoproteins have been shown to be elevated in FH subjects and in Watanabe heritable hyperlipidaemic rabbits [27,28]. Such elevation of lipase should contribute to the modulation of cholesterol content in FH observed in this study. Furthermore, the clearance of TG-rich lipoproteins seem to be preserved in autosomal recessive hypercholesterolemia which is caused by loss of function mutation in

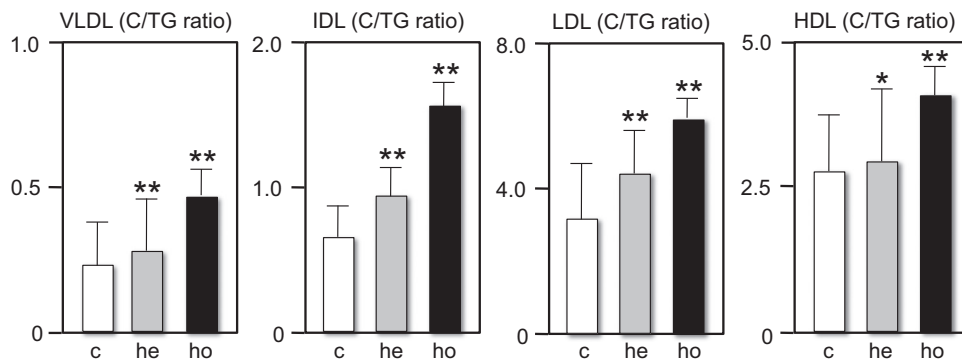


Fig. 3. Cholesterol/triglyceride ratio in each lipoprotein subfractions. White bars indicate controls. Gray bars indicate heterozygous FH. Black bars indicate homozygous FH. VLDL: very low-density lipoprotein, IDL: intermediate-density lipoprotein, LDL: low-density lipoprotein, HDL: high-density lipoprotein, C: cholesterol; TG: triglyceride, c: controls; he: heterozygous FH; ho: homozygous or compound heterozygous FH. * $p < 0.05$, ** $p < 0.0001$ vs controls.

LDL receptor adapter protein 1 gene, contributing to milder phenotype than FH with LDL receptor mutation [29]. This finding suggests that TG-rich lipoprotein metabolism is ligand-sensitive. In addition, LDL receptor can transfer such TG-rich lipoproteins to an additional receptor for uptake by the liver when its internalization is impaired. These pathways are not always via LDL receptor, LDL receptor related protein and heparan sulfate proteoglycan [30]. Recent Mendelian randomization trials suggested that TG level was the cause of coronary artery disease, independent of LDL cholesterol [31–34]. Thus, activating such pathway(s) could be one of the desirable therapies for patients with FH.

Our study has several limitations. This study was an analysis of data from a single center with a relatively small sample size, thus our results need to be validated through multi-center studies. There were significant differences between the subjects' ages in the three groups (controls, heterozygous FH, and homozygous/compound heterozygous FH), which could affect the results. The controls groups exhibiting high TC level without mutations in LDL receptor could provide the evidence to support our results were specific to FH.

In conclusion, our results indicate that cholesterol in TG-rich lipoproteins, including VLDL and IDL, are significantly higher in FH subjects, revealing a tri-modal distribution according to the number of LDL receptor mutations.

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Conflict of interest

Hayato Tada has received research grants from the Banyu Life Science Foundation International, SENSHIN Medical Research Foundation, and The Uehara Memorial Foundation. Masa-aki Kawashiri has received payments for lectures from Shionogi & Co., Ltd., Daiichi-Sankyo Co., Ltd., Astellas Pharma Inc., AstraZeneca K.K., Kissei Pharmaceutical Co., Ltd., Bayer Yakuhin, Ltd., Kyowa Hakko Kirin, Co., Ltd. Atsushi Nohara and Horoshi Mabuchi have received research grants from MSD K.K., Sanofi K.K., Shionogi & Co., Ltd., Kowa Co., Ltd., Astellas Pharma Inc., AstraZeneca K.K., Keiai-Kai Medical Corp., and Biopharm of Japan Co. Akihiro Inazu has no financial or other relations that could lead to a conflict of interest. Masakazu Yamagishi has received research grants from MSD K.K., Astellas Pharma Inc., Daiichi-Sankyo Co., Ltd., Otsuka Pharmaceutical Co., Ltd., and he has received payments for lectures from Astellas Pharma Inc., Daiichi-Sankyo Co., Ltd., Shionogi & Co., Ltd., Kowa Co., Ltd.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.plabm.2015.03.001>.

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