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**Chronic intermittent mental stress promotes atherosclerotic plaque
vulnerability, myocardial infarction and sudden death in mice**

Lynn Roth^a, Miche Rombouts^a, Dorien M. Schrijvers^a, Katrien Lemmens^b, Gilles W. De
Keulenaer^a, Wim Martinet^a and Guido R.Y. De Meyer^a

^a Laboratory of Physiopharmacology, University of Antwerp, Antwerp, Belgium

^b Laboratory of Pharmacology, University of Antwerp, Antwerp, Belgium

Address for correspondence: Lynn Roth, University of Antwerp, Universiteitsplein 1, B-2610
Antwerp, Belgium.

E-mail: lynn.roth@uantwerpen.be

Abstract

Vulnerable atherosclerotic plaques are prone to plaque rupture leading to acute cardiovascular syndromes and death. Elucidating the risk of plaque rupture is important to define better therapeutic or preventive strategies. In the present study, we investigated the effect of chronic intermittent mental stress on atherosclerotic plaque stability and cardiovascular mortality in apolipoprotein E-deficient (ApoE^{-/-}) mice with a heterozygous mutation in the fibrillin-1 gene. This mouse model displays exacerbated atherosclerosis with spontaneous plaque ruptures, myocardial infarction and sudden death, when fed a Western-type diet (WD).

Female ApoE^{-/-}Fbn1^{C1039G+/-} mice were fed a WD for up to 25 weeks. After 10 weeks WD, mice were divided in a control (n=27) and mental stress (n=29) group. The chronic intermittent mental stress protocol consisted of 3 triggers: water avoidance, damp bedding and restraint stress, in a randomly assigned order lasting 6 hours every weekday for 15 weeks.

Chronic intermittent mental stress resulted in a significant increase in the amount of macrophages in atherosclerotic plaques of the proximal ascending aorta, whereas type I collagen and fibrous cap thickness were decreased. The coronary arteries of mental stress-treated mice showed larger plaques, more stenosis, and an increased degree of perivascular fibrosis. Moreover, myocardial infarctions occurred more frequently in the mental stress group. As compared to the control group, the survival of stressed ApoE^{-/-}Fbn1^{C1039G+/-} mice decreased from 67% to 52% at 25 weeks WD, presumably due to myocardial infarctions.

In conclusion, chronic intermittent mental stress promotes plaque instability, myocardial infarctions, and mortality of ApoE^{-/-}Fbn1^{C1039G+/-} mice.

Keywords: mental stress - atherosclerosis - myocardial infarction - coronary stenosis - perivascular fibrosis - survival

1 Introduction

2 Atherosclerosis is a progressive inflammatory disease of the large and medium-sized arteries,
3 characterized by the formation of plaques in the vessel wall. During the development of the
4 disease, the stability of the atherosclerotic plaque plays a major role. Features of plaque
5 instability are a large necrotic core, a high infiltration of inflammatory macrophages and a
6 thin fibrous cap, composed of few smooth muscle cells (SMCs) and collagen fibers. When a
7 plaque develops such an unstable phenotype, it may easily rupture, leading to thrombosis and
8 subsequent myocardial infarction, stroke or even sudden death [16, 17, 34]. Despite the
9 significant therapeutic advances in cardiology over the past decades, atherosclerotic plaque
10 rupture remains a leading cause of acute cardiovascular death. Therefore, investigating risk
11 factors of atherosclerosis is very important because it may lead to new therapeutic targets or
12 prevention methods.

13 Recent evidence suggests that mental stress is an important trigger for atherosclerosis and its
14 complications [29, 31]. For instance, grieving over the death of a loved-one, the recession of
15 the stock market but also major sporting events, can increase the risk of an acute myocardial
16 infarction [6, 15, 21]. Moreover, marital stress and job insecurity can have a negative
17 influence on coronary health [5, 26].

18 The aim of this study was to determine the impact of chronic intermittent mental stress on
19 atherosclerotic plaque stability and cardiovascular mortality. To this end, apolipoprotein E
20 deficient mice (ApoE^{-/-}) with a heterozygous mutation in the fibrillin-1 gene (Fbn1^{C1039G^{+/-}})
21 were used. Recently, we reported that this unique mouse model shows an accelerated plaque
22 progression, spontaneous plaque ruptures, myocardial infarction and sudden death [32, 33].
23 Therefore, it is an adequate model to study the effects of mental stress on plaque vulnerability
24 and the occurrence of myocardial infarctions.

Methods

Mice

Female ApoE^{-/-}Fbn1^{C1039G^{+/+}} mice were fed a Western-type diet (WD; TD88137, Harlan Teklad) starting at an age of 6 weeks. The animals were housed in a temperature-controlled room with a 12-hour light/dark cycle and had free access to water and food. Cases of sudden death were documented. At the end of the experiment (25 weeks WD), plasma samples were obtained from the retro-orbital plexus of anesthetized mice (sodium pentobarbital 75 mg/kg, i.p.). Subsequently, the mice were sacrificed with sodium pentobarbital (250 mg/kg, i.p.). Analysis of total plasma cholesterol was performed via a commercially available kit (Randox, Crumlin, UK). The animal procedures were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and all experiments were approved by the ethics committee of the University of Antwerp.

Mental stress protocol

ApoE^{-/-}Fbn1^{C1039G^{+/+}} mice were divided in a control (n=27) and mental stress (n=29) group. At 10 weeks WD, a mental stress protocol adapted from Marcondes et al. [18], was initiated and consisted of 3 different triggers: water avoidance, damp bedding and restraint stress. To induce water avoidance stress, mice were placed in an empty cage filled with 0.3 cm of water. A platform (diameter of 3 cm) was placed in the center of the cage as a dry environment. During restraint stress, mice were placed in Plexiglas restrainers that prevented them from inverting their position. Damp bedding was created by adding sufficient water to wet the bedding of the cage. These 3 triggers were randomly assigned over time, to avoid habituation. The control group remained in the home cage during the experiment, while the mental stress group was subjected to daily stress triggers that lasted 6 hours for 5 consecutive days,

1 followed by 2 days of rest. The protocol was executed for 15 weeks and, at the end, all mice
2 had received the same number of each stress trigger. To document the efficacy of the
3 protocol, body weight was measured before and after the 5-days mental stress period.
4 Moreover, plasma corticosterone and aldosterone were measured using ELISA (EIA kit, Enzo
5 Life Sciences, Farmingdale, NY).

6 7 Echocardiography

8 Transthoracic echocardiograms were performed at the start of the stress protocol (10 weeks
9 WD), at 17 weeks WD and at the end of the experiment (n=10-11 per group). The procedure
10 was performed on anesthetized mice (sevoflurane; 8% for induction and 4.5% for
11 maintenance, SevoFlo[®], Penlon vaporizer) using a Toshiba diagnostic ultrasound system
12 (SSA-700A), equipped with a 15 MHz transducer. End-diastolic diameter (EDD) and end-
13 systolic diameter (ESD) were measured and fractional shortening (FS) was calculated.

14 15 Histology

16 After sudden death or sacrifice of ApoE^{-/-}Fbn1^{C1039G+/-} mice, the proximal ascending aorta and
17 the heart were collected. Tissues were fixed in 4% formaldehyde (pH 7.4) for 24 hours,
18 dehydrated overnight in 60% isopropanol and embedded in paraffin. Serial cross sections (5
19 µm) of the proximal ascending aorta and heart were prepared for histological analysis.
20 Atherosclerotic plaque size, necrotic core and the occurrence and size of plaque calcifications
21 of the proximal ascending aorta were analyzed on haematoxylin-eosin (H-E) stained sections.
22 Immunohistochemical staining with a primary antibody against Mac-3 (Pharmingen, San
23 Diego, CA) was used to determine the percentage of macrophages in the plaques. Collagen
24 type I content was measured in Sirius red stained sections under polarized light. Fibrous cap
25 thickness was determined as the median value of 10 measurements per atherosclerotic plaque

on α -SMC actin (Sigma, St Louis, MO) stained sections. The occurrence of myocardial infarctions and perivascular fibrosis, measured as the perivascular collagen area divided by the luminal area (PVCA/LA) of 10 coronary arteries per mouse, was analyzed on Masson's trichrome stained sections (cut from the middle of the heart to the apex). Septal wall thickness (median value of 3 measurements per heart) was determined on H-E stained sections. If plaques were present in the coronary arteries, plaque size and percentage stenosis were measured on Masson's trichrome stained sections.

Statistical analysis

All data are expressed as mean $\pm$ SEM. Statistical analyses were performed using SPSS software (version 20, SPSS Inc., Chicago, IL). Statistical tests are specified in the figure legends. Histological data of the proximal ascending aorta only include mice that survived the mental stress protocol (25 weeks WD). Data on the heart include all mice. Differences were considered significant at $p < 0.05$.

Results

Plasma corticosterone and aldosterone, body weight and total plasma cholesterol

Both plasma corticosterone and aldosterone levels were significantly higher (2 to 3 times) in mice subjected to chronic intermittent mental stress as compared to controls (Table 1).

Control and stress ApoE^{-/-}Fbn1^{C1039G+/-} mice were weighed at the beginning and end of each 5-day stress period. Control mice did not show significant fluctuations, but stressed mice revealed weight loss after each mental stress period of 5 days. At the start of the following stress period (i.e. after 2 recovery days), the weight of stressed mice was (almost) returned to

baseline levels (Supplemental Figure 1A). The weight loss per week was significantly higher in stressed mice as compared to controls (Supplemental Figure 1B).

Total plasma cholesterol levels were not different between control and stressed ApoE^{-/-} Fbn1^{C1039G+/-} mice (Table 1).

Plaque size and composition in the proximal ascending aorta

Chronic intermittent mental stress did not affect atherosclerotic plaque size in the proximal ascending aorta of ApoE^{-/-} Fbn1^{C1039G+/-} mice (Table 1). The size of the necrotic core was also not different between the control and stress group (Table 1). However, the amount of macrophages was significantly higher in stressed mice ($3.0 \pm 0.6\%$) versus controls ($1.5 \pm 0.3\%$, Figure 1A). Moreover, type I collagen in the plaque was decreased from $4.3 \pm 0.4\%$ in the control group to $2.7 \pm 0.5\%$ after mental stress treatment (Figure 1B). Furthermore, the thickness of the fibrous cap was $12.8 \pm 1.6 \mu\text{m}$ in the controls and $6.5 \pm 1.0 \mu\text{m}$ in the stressed mice (Figure 1C). The occurrence of plaque calcifications (control: 28%, stress: 33%) and the calcified plaque area (control: $2.6 \pm 1.1\%$, stress: $1.3 \pm 0.4\%$) was not influenced by mental stress.

Coronary plaque, stenosis and perivascular fibrosis

Although atherosclerotic plaques were present in the coronary arteries of both treatment groups (Figure 2A), the occurrence was higher in mice subjected to mental stress (Table 1).

Plaque size in the coronary arteries was also significantly larger in stressed mice (Figure 2B).

Accordingly, coronary stenosis was more pronounced after mental stress (Figure 2C).

Perivascular fibrosis of the coronary arteries was measured by dividing the perivascular collagen area by the luminal area of the blood vessel. This fibrosis was significantly more pronounced in the mental stress group ($47 \pm 6\%$ versus $28 \pm 4\%$ in control mice; Figure 2A and

D). Moreover, perivascular fibrosis correlated significantly with corticosterone (Pearson $r^2=0.58$, $p=0.001$) and aldosterone levels (Pearson $r^2=0.34$, $p=0.028$).

Echocardiography

At the beginning of the mental stress protocol (10 weeks WD), both treatment groups did not differ in EDD, ESD or FS (Figure 3A, B and C). After 7 weeks of intermittent mental stress, ESD was significantly larger as compared to control mice (Figure 3B). Accordingly, fractional shortening was markedly reduced by mental stress (Figure 3C). At the end of the experiment (25 weeks WD) no differences were measured between the treatment groups (Figure 3A, B and C).

Myocardial infarction and septal wall thickness

The number of animals with myocardial infarctions (Figure 4A) was higher in the mental stress group (Table 1). The infarcts were located in the septum wall, the left and right ventricle, with no specific preference and no difference between control and stress-treated mice (data not shown). Moreover, the thickness of the septal wall was significantly less in the mental stress group ($1310 \pm 31 \mu\text{m}$ versus $1455 \pm 47 \mu\text{m}$ in control mice, Figure 4B).

Survival

At 25 weeks WD, 33% of the control ApoE^{-/-}Fbn1^{C1039G+/-} mice had suddenly died. Mental stress increased sudden death to 48% (Figure 4C).

Discussion

Mental stress is considered a strong risk factor for cardiovascular disease [3, 12, 23] and our data provide direct evidence that it is associated with plaque vulnerability, myocardial infarction and death.

Mental stress can exert its effects by activating two main pathways. The first pathway is the sympathetic nervous system (SNS), which results in an increased heart rate, blood pressure, myocardial oxygen consumption and thrombogenicity [2, 12]. Moreover, the release of noradrenaline accelerates hematopoiesis via the β_3 -adrenergic receptor. This leads to an increase in circulating neutrophils and monocytes, eventually resulting in plaque progression and vulnerability [10]. The SNS is also able to activate the renin-angiotensin-aldosterone system (RAAS), resulting in higher levels of angiotensin II and aldosterone [1, 2, 12, 14]. Aldosterone is known to activate the mineralocorticoid receptor (MR), which can influence the process of atherosclerosis by increasing vascular inflammation [14, 19]. The second pathway activated by mental stress is the hypothalamic-pituitary-adrenal axis (HPA), which leads to the release of glucocorticoids in the circulation [1, 3, 8, 9, 12]. In humans the main endogenous glucocorticoid is cortisol, while in mice it is corticosterone [20]. Glucocorticoids have immunosuppressive properties under normal circumstances, but may contribute to inflammation under pathological conditions. The intensity and duration of the stress response will determine the outcome. Therefore, it is conceivable that the early phase of intermittent mental stress results in an inflammatory reaction in mice, while later phases might induce immunosuppression [3, 9, 27].

In the present study, we observed a significant increase in plasma levels of aldosterone and corticosterone after mental stress, which confirms the activation of RAAS and HPA. The presence of higher corticosterone levels in combination with the weekly weight fluctuations, confirmed that the stress protocol was effective. Both aldosterone and corticosterone can

1 contribute to atherogenesis in several ways. Aldosterone upregulates genes involved in
2 inflammation and enhances monocyte adhesion to the vascular wall. Moreover, when the MR
3 is blocked in animal models, the atherosclerotic burden is diminished [14, 19]. Cortisol (in
4 humans) or corticosterone (in mice) can induce a redistribution of monocytes from the
5 peripheral blood to areas of inflammation, such as the atherosclerotic plaque [3].

6
7 Atherosclerotic plaques in the proximal ascending aorta of mentally stressed ApoE^{-/-}
8 Fbn1^{C1039G+/-} mice were more inflammatory, as indicated by their higher amount of
9 macrophages. This observation can be explained by the inflammatory properties of
10 aldosterone and corticosterone as described above, but can also be the result of accelerated
11 hematopoiesis resulting in increased levels of inflammatory monocytes [10]. Other key
12 characteristics of unstable plaques, such as a thin fibrous cap and a low amount of type I
13 collagen, were seen in stressed ApoE^{-/-}Fbn1^{C1039G+/-} mice, confirming previous reports in
14 ApoE^{-/-} mice [10, 24, 35]. In the present study, we also examined the effect of intermittent
15 mental stress on plaque formation in the coronary arteries, the occurrence of myocardial
16 infarctions and on heart function. This was possible because, in contrast to ApoE^{-/-} mice,
17 ApoE^{-/-}Fbn1^{C1039G+/-} mice develop significant atherosclerotic plaques in the coronary arteries
18 within 25 weeks WD. We observed a significant increase in coronary plaque size and degree
19 of stenosis after chronic intermittent mental stress, which presumably contributed to the
20 increased occurrence of myocardial infarctions. Also Cynomolgus monkeys exposed to
21 mental stress develop more severe coronary artery atherosclerosis [13] and job insecurity in
22 humans is known to negatively affect coronary heart disease [5], confirming the validity of
23 the ApoE^{-/-}Fbn1^{C1039G+/-} mouse model.

Furthermore, the coronary arteries of stressed ApoE^{-/-}Fbn1^{C1039G+/-} mice showed a marked increase in perivascular fibrosis, in which the renin-angiotensin-aldosterone system is a known key player [25]. Moreover, it has been reported that perivascular fibrosis is correlated with an impaired coronary blood flow [4]. Indeed, the higher degree of stenosis in the coronary arteries of mentally stressed ApoE^{-/-}Fbn1^{C1039G+/-} mice as compared to controls might have blunted blood flow and we confirmed the activation of the renin-angiotensin-aldosterone system, as indicated by the increased plasma levels of aldosterone. Thus both factors might contribute to the augmented perivascular fibrosis.

Importantly, the cardiac function of ApoE^{-/-}Fbn1^{C1039G+/-} mice deteriorated as a result of chronic intermittent mental stress. A significant increase in ESD and a decrease in FS were observed at 17 weeks WD. At 25 weeks WD, this effect on heart function was not observed anymore, which is probably due to a higher number of stressed mice that had suddenly died before the end of the study.

In humans, mental stress contributes to the development of myocardial infarctions [6, 21, 29]. Also in the present study, the number of mice showing myocardial infarctions was significantly higher in the mental stress group. Importantly, our study is the first one that utilizes mental stress as a trigger to induce myocardial infarctions in a mouse model. Furthermore, the septal wall was thinned, which might be indicative of left ventricular dilatation, but it may also be the result of myocardial cell death [30]. Although we do not know the exact cause of increased mortality in stressed ApoE^{-/-}Fbn1^{C1039G+/-} mice, this study strongly suggests that a heart-related problem was an important contributor. The increased occurrence of myocardial infarctions in the stressed mice was presumably the main reason for

the increased mortality, but also subsequent ventricular remodeling and heart failure or arrhythmias cannot be excluded.

In future experiments, it might be interesting to investigate the role of accelerated hematopoiesis, as a result of increased noradrenaline levels, in the occurrence of MI in stressed ApoE^{-/-}Fbn1^{C1039G+/-} mice. Heidt et al., reported that treatment of stressed ApoE^{-/-} mice with a β_3 -adrenergic receptor blocker resulted in a reduced number of neutrophils and macrophages in plaques [10]. It would therefore be fascinating to investigate the effect of a β_3 -adrenergic receptor antagonist in stressed ApoE^{-/-}Fbn1^{C1039G+/-} mice. Furthermore, to determine the direct role of aldosterone in plaque vulnerability, MI and sudden death in the current study, an aldosterone receptor antagonist might be used. It has been reported that treatment with aldosterone receptor antagonists, such as spironolactone or eplerenone, reduces inflammation, plaque progression, cardiac fibrosis and mortality [7, 11, 22, 28]. Thus, treating stressed ApoE^{-/-}Fbn1^{C1039G+/-} mice with an aldosterone receptor antagonist, might prove the significance of this steroid hormone in stress-induced cardiovascular disease.

In conclusion, we report that chronic intermittent mental stress increased plaque vulnerability in the proximal ascending aorta of ApoE^{-/-}Fbn1^{C1039G+/-} mice. The coronary arteries showed larger and more stenotic plaques, which culminated in an increased occurrence of myocardial infarctions and more cases of sudden death.

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Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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1 **Figure legends**

2 **Figure 1: Plaque composition of control and stress ApoE^{-/-}Fbn1^{C1039G/+} mice.** (A) Mac-3
 3 staining of atherosclerotic plaques showed an increase in the number of macrophages in the
 4 stress group (n=9) when compared to control mice (n=12, Independent samples t test;
 5 *p<0.05). (B) Analysis of Sirius red staining under polarized light revealed a significant
 6 decrease of type I collagen in stress-treated mice (control n=11, stress n=9, Independent
 7 samples t test; *p<0.05). (C) α SMC actin staining showed the number of smooth muscle cells
 8 present in the fibrous cap. Stress resulted in a decrease in fibrous cap thickness when
 9 compared to control conditions (control n=12, stress n=7, Independent samples t test;
 10 *p<0.05). Scale bar = 50 μ m

12 **Figure 2: Coronary plaque size, stenosis and fibrosis.** (A) Trichrome Masson staining
 13 showed the presence of plaques and perivascular fibrosis in coronary arteries of control and
 14 stress-treated mice. Scale bar = 50 μ m, L= lumen, P= plaque. (B) Plaque size in the coronary
 15 arteries of stress-treated (n=11) mice was more than doubled when compared to controls (n=7,
 16 Independent samples t test; *p<0.05). (C) The percentage of stenosis was significantly higher
 17 in stressed mice (control n=7, stress n=13, Independent samples t test; *p<0.05). (D) A
 18 significant increase of perivascular fibrosis (perivascular collagen area divided by luminal
 19 area = PVCA/LA) in the stress group was observed (control n=27, stress n=29, Independent
 20 samples t test; *p<0.05).

22 **Figure 3: Cardiac function of control and stress ApoE^{-/-}Fbn1^{C1039G/+} mice.** (A) End-
 23 diastolic diameter did not show differences between the treatment groups in time. (B) End-
 24 systolic diameter was significantly increased at week 17 of WD (Independent samples t test;
 25 *p<0.05). At the end of the experiment no significant differences could be detected. (C)

Fractional shortening showed a significant decrease at 17 weeks WD (Independent samples t test; $*p<0.05$). At 25 weeks WD no differences were observed. (week 10: control n=9, stress n=11; week 17: control n=7, stress n=9; week 25: control n=6, stress n=6)

Figure 4: Myocardial infarction, septal wall thickness and survival. (A) Trichrome Masson staining of the heart of a $\text{ApoE}^{-/-}\text{Fbn1}^{\text{C1039G}/-}$ mice revealed the presence of a large fibrotic area in the septal wall, indicative of a myocardial infarction. Scale bar = 500 μm . **(B)** Measurements of septal wall thickness showed a significant decrease in the stress treatment group when compared to control mice (control n=26, stress n=29, Independent samples t test; $*p<0.05$). **(C)** At 25 weeks WD 71% of the control mice (n=27) remained alive. Stress resulted in a decreased survival, since only 50% of the stress mice (n=29) survived the experiment (Kaplan Meier: not significant).

1 **Table 1: Plasma, atherosclerotic plaque and heart characteristics**

	Control	Stress
Plasma		
Corticosterone (ng/ml)^a	47 ± 9	116 ± 21*
Aldosterone (pg/ml)^b	216 ± 33	652 ± 139*
Total cholesterol (mg/dl)^c	442 ± 31	544 ± 45
Atherosclerotic plaque: proximal ascending aorta		
Plaque size (x10³ μm²)^d	988 ± 94	1082 ± 125
Necrotic core (%)^e	13 ± 2	13 ± 2
Heart and coronary arteries		
Myocardial infarctions^f	8/27 (30%)	16/29 (55%)
Coronary plaques^g	7/27 (26%)	13/29 (45%)

2 ^{a,b} Mean ± SEM, control n=6 and stress n=8, Independent samples t test; *p<0.05

3 ^c Mean ± SEM, control n=16 and stress n=12

4 ^{d,e} Mean ± SEM, control n=18 and stress n=15

5 ^f Number of mice showing myocardial infarctions, Pearson's chi-squared test; p=0.05

6 ^g Number of mice showing coronary plaques, Pearson's chi-squared test; p=0.14

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Highlights

- Mental stress enhanced plaque vulnerability in ApoE^{-/-}Fbn1^{C1039G^{+/}-} mice.
- Coronary plaques were larger and more stenotic after mental stress treatment.
- Perivascular fibrosis of coronary arteries was increased.
- The occurrence of myocardial infarctions was higher as a result of stress.
- Survival in stressed ApoE^{-/-}Fbn1^{C1039G^{+/}-} mice was decreased.

Figure 1

Figure 2

Figure 3

Figure 4

Supplemental Figure legends

Supplemental Figure 1: Body weight of control and stress ApoE^{-/-}Fbn1^{C1039G^{+/-}} mice. (A)

The weight of the control mice (n=27) remained stable over the entire experiment. The stress group (n=29) clearly had a fluctuating pattern, showing weight loss at the end of every 5-day stress period, which was restored after 2 days of rest. **(B)** The weight loss per week was significantly higher in stress-treated (n=26) as compared to control mice (n=26, Independent samples t test; ***p<0.001).

Supplemental Figure 1