STUDY REPORT
IN VIVO EVALUATION OF THE EFFECTS OF THS2.2 ON THE RESPIRATORY AND CARDIOVASCULAR SYSTEMS OF APO LIPOPROTEIN E-DEFICIENT MICE
PART 2: METABOLIC ENDPOINTS - INSULIN RESISTANCE
(STUDY NUMBER 15015)

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1 Review and Approval

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Fong, Kevin	Formatting and editorial revisions reviewed by QA	31 Aug 2016

Note, this version of study report part 2 report has been modified from the original study report in order to address minor formatting changes required to align with the 'Global Submit Publishing Standards'. There are no changes to the study report contents. This version will be filed with the original study report in the archive.

1.1 Study Director's Statement of Compliance

The study director acknowledges responsibility for the validity of the data.

Name	Justification	Date / Signature
Phillips, Blaine	Signed as Study Director	31 Aug 16

2 Abstract

This study is a research arm linked to the 15015 ApoE-THS2.2 switching study, was performed to assess the impact of mainstream cigarette smoke (CS)/aerosol exposure on development of insulin resistance (IR) in apolipoprotein deficient mice ($ApoE^{-/-}$) fed with regular and high fat diet (HFD). The $ApoE^{-/-}$ mice were exposed to diluted mainstream CS/aerosol from 3R4F /THS2.2. We used the HFD-fed $ApoE^{-/-}$ mouse model as it has been used as a diabetic atherosclerosis model to establish the development of IR. Furthermore, IR-related metabolic changes were also examined in the non-diabetic atherosclerosis (chow-fed) $ApoE^{-/-}$ mice.

During the development of IR condition, following increase of food intake (fuel loading), the pancreas releases more insulin to compensate for the increased blood glucose until the beta-cells can no longer secrete sufficient levels of insulin, this situation eventually leads to type 2 diabetes. Therefore, to monitor the development of IR, we measured the circulating levels of fasting glucose and insulin every 4weeks. We hypothesized that the HFD-fed $ApoE^{-/-}$ mice, which exhibit IR, through progressively increased fasting glucose and insulin levels, would exacerbate the development of IR when exposed to 3R4F CS over a period of 11weeks; whereas THS2.2 being a reduced risk product (RRP), exposure of THS2.2 aerosol would exhibit reduced risk of IR as compared to 3R4F exposure (in both chow and HFD-fed animals).

Our results showed that the body weights were increased in HFD-fed mice as compared to the chow fed mice. The HFD-3R4F group had a rapid unexpected initial gain in body weight relative to HFD-sham and HFD-THS2.2 groups. However, these three HFD-groups had no difference in weights at the end of the study (11 weeks). Lower overall body weight gain was observed in the 3R4F-exposed animals relative to either the sham or THS2.2-exposed groups (only in chow-fed mice).

Four repeated measurements of fasting blood glucose and insulin were performed every 4 weeks intervals (1 baseline and 3 measurements during the CS and aerosol exposure for 11 weeks). The HFD-sham group showed significant increase in fasting glucose levels at week 8 and 11 relative to the chow-sham group. 3R4F-exposed mice showed decreased trend of fasting blood glucose levels over all time points in both chow and HFD-fed groups. Similar to the observed fasting glucose levels, the HFD-sham group showed significant increase in fasting insulin levels as compared to the chow-sham group, at week 4, and 11. 3R4F and THS2.2 exposure did not affect the fasting plasma insulin levels.

Blood chemistry analysis showed that the HFD groups had higher levels of total cholesterol, triglycerides, as well as high density lipoproteins (HDL) and low density lipoproteins (LDL). Slightly higher cholesterol, triglyceride, HDL, and LDL levels were observed following 3R4F exposure relative to either sham or THS2.2-exposed animals, in both chow and HFD.

Histopathological evaluation revealed increased steatosis following HFD without aerosol exposure effects. HFD and 3R4F (but not sham or THS2.2) exposure increased the surface area of atherosclerotic plaque in the aortic arch of ApoE$^{-/-}$ mice. Morphometric measurements of the pancreas did not reveal any differences in islet area or size among any of the treatment groups.

HFD-fed mice had increased levels of plasminogen activator inhibitor 1 (PAI-1) and leptin, as compared to the chow-fed mice. 3R4F-exposed mice had a slightly but not statistically significant higher levels of PAI-1 and leptin than the sham-exposed mice, in both HFD and chow diet. The insulin-like growth factor 1 (IGF-1) was trending higher (although could not reach statistical significance) in the HFD-fed mice as compared to chow-fed mice, without any effect of 3R4F or THS2.2. There was a decreased trend for growth hormone (GH) in HFD-fed groups relative to chow-fed groups that could be attributed to reduced lean body mass as observed in human clinical studies or stress due to HFD feeding. Chronic stress has been shown to reduce anabolic hormones that promoting growth, such as growth hormone.

Consistent with our hypothesis, the HFD-sham group had increased body weights, higher fasting glucose and insulin levels, increased plasma lipid levels, increased hepatic lipid accumulation (steatosis measured by histopathology analysis) and a tendency of a greater plaque formation as compared to the chow-sham group. Contrary to our hypothesis, 3R4F in combination of HFD exposure was not associated with development of IR in this model. The 3R4F exposure in both the chow- and HFD-fed groups, was associated with reduction fasting glucose levels as compared to their respective sham-exposed. Nevertheless, consistent with our hypothesis, the impact of THS2.2 exposure in both the chow- and HFD-fed mice was not different from chow and HFD fed sham.

This study is the first scientific attempt to investigate and report the effects of 3R4F exposure on HFD-fed ApoE-/- mice. However, we observed that there was complex combination impact of HFD and CS exposure on the metabolic markers of IR. In order to elucidate the mechanisms by which 3R4F exposure showed the complex combination effect on lipid biomarkers and markers of IR in this model more comprehensive lipidomics, transcriptomics and systems biology analyses is being performed in various tissues that were collected at the end of the study. This approach will facilitate the understanding of the complex relationship between atherosclerosis and IR, as well as alteration at the molecular levels with respect to CS exposure.

3 Introduction

Smoking has been associated with reduced insulin sensitivity or increased insulin resistance (IR), as well as increased plasma glucose (hyperglycemia). Studies have reported that CS is a strong independent risk factor for non-insulin dependent diabetes mellitus (or type II diabetes mellitus, T2DM) (Cena *et al.,* 2013). T2DM is characterized by IR and subsequently, a compensatory effect resulting in hyperinsulinemia (Noto *et al.,* 2013). Although studies have indicated that CS may affect the insulin receptor signaling pathway, the mechanisms of CS-induced IR are still poorly understood,(Bergman *et al*., 2012).

One of the characteristic signs of IR is the increased fasting glucose levels, as well as increased insulin levels, as the secreted insulin becomes less effective at triggering the metabolism of circulating glucose levels. Smoking has been shown to be associated with IR in human epidemiological studies (Willi *et al.,* 2007; Xie *et al.,* 2009). However, it has been difficult to reproduce those results in murine models. First, IR in human is associated with multiple variables not only with smoking habit/history, but also body weight, blood pressure, etc. Second, Bergman and colleagues reported that smokers were less insulin sensitive, which became more sensitive upon cessation (Bergman *et al*., 2012); whereas in this study, the effect of cessation on IR was not examined. Therefore, the observation seen in human epidemiological studies cannot be completely replicated in this current study. Moreover, our observation is also aligned with other publication in which the exposure to CS reduced fasting glucose levels in male Balb/C mice (Chen *et al.,* 2007). In that study, the impact of reduced body weight was suspected to have a greater impact on the fasting glucose levels than a putative smoke-effect on IR, confirming the challenges on replicating observational study to examine the impact of CS on IR development.

Both, smoking and diabetes are risk-factors for cardiovascular disease (CVD) (Qazi and Malik, 2013). Utilization of a diabetic atherosclerosis mouse model was therefore relevant to examine the mechanism of CS-induced IR in the context of CVD development. This study was a pilot study to assess the effects of 3R4F CS and THS2.2 aerosol exposure on the development of IR, as well as to determine the duration of CS that would affect the progression of IR in the HFD-fed ApoE$^{-/-}$ mouse model. The progression of IR was monitored mainly by measuring the changes in the metabolic parameters like fasting blood glucose levels and plasma insulin levels during and at the end of CS/aerosol exposure.

The 3R4F cigarette mainstream smoke (CS) pre-test exposure resulted in final concentration of 29.9 µg/l nicotine equivalent to targeted TPM concentration of 600 µg/l therefore 3R4F CS was targeted for 29.9 µg/l of nicotine concentration and as THS2.2 aerosol exposure was matched to 3R4F nicotine concentration, so similarly targeted to 29.9 µg/l nicotine. The animals were exposed to the CS/aerosol in whole body chambers for 3 hours per day with two fresh air breaks (1h and 0.5h), 5 days per week for an 11 weeks total exposure period. A separate control group was exposed to fresh air (sham). To assess the impact of weight gain and obesity on the aerosol exposure, the mice either fed with standard chow or a HFD. The chow fed groups were exposed to fresh air, CS from 3R4F and aerosol fromTHS2.2 and were fed with pellet T2914C rodent feed, while mice from HFD groups

were exposed to fresh air, CS from 3R4F and aerosol from THS2.2, and were fed with TD.88137 rodent diet (Harland, U.K.). Longitudinal (repeated measurements for each animal at different time points) fasting whole blood glucose levels were measured at 4 weekly intervals during 11 weeks of exposure (4 times including baseline) using a handheld glucometer (Accu Check® Performa, Roche). Mice were fasted for 6 hours before blood collection. Longitudinal fasting insulin levels were measured in plasma at similar time points as that of glucose using the Low Range Assay of the Ultra-Sensitive Mouse Insulin ELISA Kit (Cat # 90080, Crystal Chem, Inc.). Similarly plasma glucagon like peptide (GLP-1) levels were measured longitudinally using GLP-1 (Active 7-36) ELISA from ALPCO Diagnostics

Cholesterol, triglyceride, HDL, and LDL levels were measured at the end of the study in the plasma samples using a Beckman Unicell® DXC 600 clinical auto-analyzer. Metabolic biomarkers were measured at the end of the study in the serum samples using multiplexed immunoassays. Atherosclerotic plaque in the aortic arch was identified as Oil Red O (ORO)-positive staining and morphometric evaluation of total plaque area was conducted using Visopharm, an image analysis software. Islet area measurements were also conducted with Visiopharm on H&E stained-slides.

Throughout the course of this study, animals were exposed to diluted CS from 3R4F cigarettes, aerosol form THS2.2 or to filtered conditioned fresh air (sham exposure group), 3 hours/day, and 5 days/week. Smoke from conventional cigarettes was generated in conformity with the Health Canada intensive smoking regime. The mainstream smoke was diluted with filtered conditioned fresh air to target TPM concentration, i.e., 600 µg total particulate matter (TPM)/l. As the TPM concentration of THS2.2 differs from that of the 3R4F reference cigarette, smoke exposure from both items was targeted to a nicotine concentration of 29.9 µg/l for both the 3R4F and the THS2.2 exposure chambers. This exposure concentration was shown to be well tolerated by the mice throughout the exposure period. In order to characterize the test atmosphere and to check the reproducibility of mainstream smoke generation and dilution, the following analytical parameters were determined at defined intervals: TPM, carbon monoxide (CO), puff volume, formaldehyde, acetaldehyde, acrolein, nicotine, particle size distribution, temperature, relative humidity and flow rate through the exposure chamber. The mice were fed either chow or HFD in order to examine the effect of diet on the progression of smoke-induced IR. The HFD composition has been reported before to induce IR development in ApoE$^{-/-}$ mice (Li *et al*., 2011). At the end of the exposure period organs and tissues (i.e., blood, liver, muscle, and fat) were collected for transcriptomic targeted protein analysis and lipidomics evaluations the results of which will be amended in the report when the evaluations will be performed. Liver and pancreas pathological and functional changes were assessed by standard histopathology. Evaluation of total plaque area was performed using Visiopharm software on the Oil red O stained aortic arch. All the experimental procedures were performed according to PMIRL-S QMS procedures, unless it is otherwise specified.

4 Objective

The objective of this arm of the study was:

- To investigate the effects of exposure to 3R4F CS or THS2.2 RRP (reduced risk product) aerosol and HFD on the development and progression of IR in ApoE$^{-/-}$ mice with and without HFD-feeding.

5 Methods

5.1 Test and Reference Items

5.1.1 Test item- THS2.2

The test item was a tobacco heating system (THS2.2) composed of a heating device (CH FDP4.2) and a sensorial media (Zurich/ZRH) with the flavor (DEL/C3/FR1/RDO). The sensorial medium (hereafter referred to as 'stick') is a special cigarette that contains different elements (tobacco plug, transfer section and mouth piece) assembled with an over-wrapping of cigarette paper. The sticks were provided by Philip Morris International (PMI), Neuchâtel, Switzerland. Aerosol specifications (Batch quality control or batch release conditions) were stored in the Project Management Folder (PMF) as batch release certificates.

The THS2.2 sticks were produced at PMI (Neuchâtel) in 2 batches, as the stability of the sticks is tested only up to 6 months (less than the 8 month study period). Proposed production volumes and delivery schedules were follows;

1. B-05879, 60,000 sticks, delivered 04 July 2013
2. B-06132, 290,000 sticks, delivered 14 August, 2013

The sticks were packaged in mini princess packs (packs of 2 collation of 10 sticks each). The packaging was labeled with product identification (Del/C3/FR1/Rdo), pack lot number and the production date. The batches of THS2.2 sticks were distinguished by cork tipping paper. All sticks were stored in the original packaging in the room 2024 with controlled room temperature (from 2 to 10 °C), but uncontrolled humidity conditions. The cigarettes were conditioned in the room 3103 according to ISO standard 3402 (1999), for between 7 to 21 days at a temperature of 21.8 ± 0.3 °C and 56.6 ± 1.1% relative humidity before being used for aerosol generation.

As part of the smoking system, the sensorial medium is inserted into a cigarette holder that heats the tobacco plug. The cigarette holder includes a battery, electronics for controlling, a heating element and the cigarette extractor. The cigarette holders were provided by Philip Morris International (PMI), Neuchâtel, Switzerland.
The daily usage of THS2.2 cigarette holders was documented by the aerosol team. A total of 600 cigarette holders were requested for use during the study exposure period.

Table 1.Identification Details for Cigarette Holders

	Cigarette Holder
Product description	FPD 4.2
Product code	DV-000067.RD
Batch Number	B-06143

5.1.2 Reference Item – 3R4F Conventional Cigarettes

The 3R4F cigarettes were purchased from the University of Kentucky (for specifications see University of Kentucky website, http//www.ca.uky.edu/refcig), packaging: soft packs of 20 cigarettes (10 soft packs/carton, and 25 cartons/box). All cigarettes were stored in the original packaging in the room 2025 with controlled room temperature (from 2 to 10 °C), but uncontrolled humidity conditions. The cigarettes were conditioned in the room 3102 for >7 to <21 days prior to smoking, at a temperature of 21.8 ± 0.3 °C and 57.6 ± 1.3% relative humidity.

5.1.3 Treatment Administration

The IR arm is a small research arm of the main study 15015, with similar 3R4F CS and THS2.2 aerosol exposure conditions on daily basis for 5 days a week. Therefore animals from the IR groups were exposed together with animals from main THS2.2 switching component of the study in the same chambers. Whole-body exposure chambers (type WBEC85) were used in this study as they are the most practical and reproducible means to expose mice to CS (3R4F) or THS2.2 aerosol. The target concentration of mainstream cigarette smoke from the 3R4F was 600 µg/l TPM, equivalent to 29.9 µg/l nicotine. The exposure concentration to the aerosol from THS2.2 was concentration-matched to the nicotine levels of the 3R4F exposure. In order to achieve these matched exposure concentrations, prior to starting the study the TPM/nicotine ratios for 3R4F were determined in the mainstream smoke, allowing to define the exact nicotine concentration to be achieved for exposure to THS2.2 aerosol. For the 3R4F, the aerosol generated from 1 smoking machine was diluted with filtered, conditioned air to the target concentration of 29.9µg/l nicotine for delivery to one exposure chamber. For the THS2.2 groups, aerosol from 2 smoking machines was combined to produce an aerosol with matched nicotine concentrations in the 3R4F exposure chambers. The aerosol from either the test or reference item were conveyed from the smoking machines to the exposure chambers via glass tubing. The total flow rate through the whole-body exposure chambers was adjusted to approximately 80 l/min. For the sham group, mice were exposed to filtered, conditioned fresh air, whilst the other exposure conditions were the same as those for the smoke-exposed mice.

5.1.4 Test Atmosphere Generation and Characterization

The 3R4F and THS2.2 sticks were smoked according to the Health Canada Intensive Smoking Protocol (1999) which is based on the ISO standards 3308 (revised in 2000) with the exceptions of the puff volume (55ml), puff

frequency (one puff every 30 seconds) and ventilation block. In addition, several minor deviations from the ISO standards 3308 (2000) were necessary for technical reasons (Table 2).

Table 2. Deviations from ISO standards 3308 (2000) for THS2.2. Deviation for puff count, relative humidity and temperature is also applicable for 3R4F.

Parameter	ISO 3308 (2000)	Recommended condition	Explanation for deviation
Puff count	rounded to the nearest one-tenth of a puff	only whole puffs	device configuration
Restricted smoking	restricted smoking	Free/ Open end smoking	device configuration
Cigarette holder	Labyrinth seal and perforated disc (washer)	Single seal and no washer	device configuration
Puff termination & counting	Butt length sensor/ 0.1 puff counting resolution	Fixed number of puffs/ only whole puffs counted	device configuration
relative humidity during smoking	60 ± 5%	The air supplied to the smoking machine meets the requirements	The conditions inside the smoking machine are unmonitored
temperature during smoking	22 ± 2 °C	The air supplied to the smoking machine meets the requirements	The conditions inside the smoking machine are unmonitored
Air velocity around sticks	170 - 230 mm/sec	The THS2.2 stick is enclosed within the cigarette holder	airflow around the stick is controlled by the device

Aerosol from 3R4F is generated on 30-port rotary smoking machines with active side stream exhaust (type PMRL-G, SM2000) and equipped with Programmable Dual-port Syringe Pump (PDSP). Aerosol from THS2.2 sticks is generated using 30-port carousel “Smoking Machines Smoking Device Docking Station Zurich (SM SDDS ZRH)” equipped with cigarette holder and a PDSP. The SDDS serves as an interface to ensure synchronization between the cigarette holder and the smoking machine. The SDDS ZRH smoking machine were developed by PMI R&D Neuchatel, Switzerland. The SDDS ZRH smoking machines also included a temperature-controlled insulation kit (Tube Warming System) in the undiluted aerosol pathway, with the goal of reducing aerosol condensation. The smoking machine and chamber layout for both 3R4F (SM-2000) and THS2.2 (SM-SDDS-ZRH) exposure conditions are depicted in Figure 1 and Figure 2, respectively.

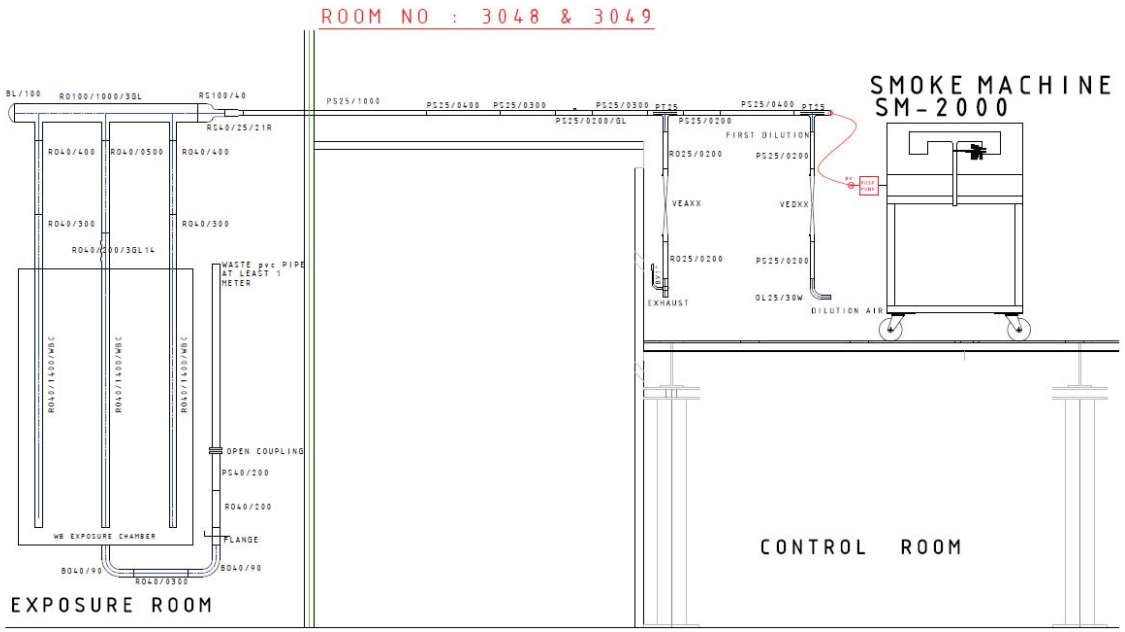


Figure 1. Smoking machine layout for 3R4F groups

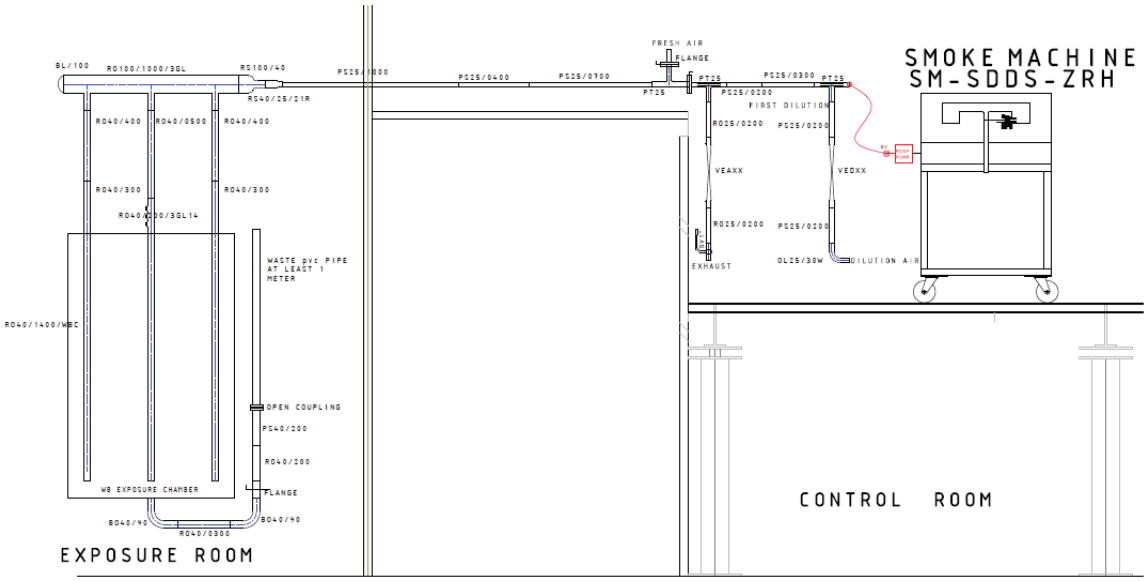


Figure 2. Smoking machine layout for THS2.2 groups

Prior to the start of the study, performance verification (PV) was performed on each of the inhalation setups by using the assigned smoking machines to achieve targeted aerosol concentration. The purpose of performing PV on the inhalation setups was:

1. To determine the process air flow rates that are required to achieve the target concentrations.
2. To determine the equivalent nicotine levels for the TPM target concentrations of the 3R4F groups.
3. To demonstrate that the inhalation setups are capable of achieving the targeted concentrations.

The inlet temperature of the test atmosphere in the exposure chamber was maintained at 22.5 ± 0.3 °C and the relative humidity between 59.5 + 2.4 %. Air flow and relative humidity through the exposure chamber was monitored continuously and recorded in Citect during the exposure period. The total flow rate through the whole-body exposure chambers was adjusted to approximately 80 l/min.Puff count for THS2.2 FPD 4.2 was set to 12 puffs due to the configuration of device. The 3R4F cigarettes were smoked to a butt length of 34.3 ± 1 mm; the butt length and puff count for 3R4F were determined monthly while static burning rate was determined at least once during the inhalation period. In order to characterize the test atmosphere and to check the reproducibility of aerosol generation and dilution, several analytical parameters were determined at the breathing area of the animals. Particle size distribution was performed using the PIXE impactor protocol.

Table 3. Analysis of Aerosol

Parameter	Assay Principle	Determination schedule SHAM	Determination Schedule 3R4F	Determination Schedule THS2.2
TPM	Gravimetry after trapping on Cambridge filters	≥ 1 time/week	During every exposure block	During every exposure block
Carbon monoxide	Nondispersive infrared photometry of gas/vapour phase	Continuously	Continuously	Continuously
Nicotine	Capillary gas chromatography after trapping on sulfuric acid-impregnated silica gel	≥ 1 time/week	During every exposure block	During every exposure block
Formaldehyde, acetaldehyde, and acrolein	Reversed phase high-performance liquid chromatography (HPLC) of 2,4-dinitrophenylhydrazine (DNPH) derivatives after trapping in DNPH solution	NA	≥ 1 time/week	≥ 1 time/week
Relative humidity	Capacitive	Continuously; only sham group	N/A	N/A
Temperature	Thermistor probe Pt100	Continuously	Continuously	Continuously
(Static) puff volume	Soap bubble flow meter	Before and after daily smoke generation	Before and after daily smoke generation	Before and after daily smoke generation
Flow rate through exposure chamber	Pressure difference over a Venturi tube	Continuously	Continuously	Continuously
Particle size distribution	Impactor	≥1 time/month	≥1 time/month	≥1 time/month

5.2 Test System

It has been previously demonstrated (PMRL Study 13882) that ApoE$^{-/-}$ mice are a suitable murine model to study atherosclerosis and emphysema development at exposure concentrations of 600 µg/l TPM 3h per day, 5 days a week for 6 months. This model system was therefore utilized in the follow-up study on exposure to CS, to THS2.2, and switching from CS exposure to THS2.2 exposure aimed at evaluating the effects on atherosclerotic and emphysema markers, as well as markers of IR using an additional experimental arm. Female ApoE$^{-/-}$ mice (TC-ApoE-F) bred under specific pathogen-free conditions were obtained from Taconic, UK. The mice were approximately 6-8 weeks old at arrival (8-10 weeks at exposure start) and their health status was verified using the health check certificate provided by the breeder. Additional health checks were performed as part of the main switching phase of the study, and are described in details in the report part 1.

The study was run in an AVA-licensed (Agri-Food & Veterinary Authority of Singapore) facility where care and use of the animals was in accordance with the National Advisory Committee for Laboratory Animal Research Guideline (NACLAR, 2004).

Following arrival of the mice to our facility, the boxes and containers were carefully examined prior to transportation into our animal holding rooms. All packaging boxes and crates arrived in good condition, with no visible signs of damage. Prior to the start of the exposure, the mice were acclimatized to the facility for a period of 2 weeks.

Mice were individually identified by subcutaneously implanted transponders. They were kept and exposed in the animal laboratory (rooms 3046 - 3051) with restricted access and under specific pathogen-free conditions. The laboratory air was filtered, conditioned fresh air. Positive pressure was maintained inside the laboratory. The room temperature was maintained at 22.9 ± 0.2 °C and the relative humidity was maintained at 59.8 ± 1.4. The light/dark cycle was 12 /12h.

A maximum of eight mice were housed per cage. Cage-enrichment (Igloo, and Nylabone™ for gnawing) were provided to each cage during the non-exposure period. The bedding material consisted of autoclaved softwood granulate. Gamma-irradiated pellet diets were provided on the cage lid's top area, and filtered tap water was supplied ad libitum to each cage. No diet was available during exposure periods.

Bedding material (Lignocel® BK 8-15, J. Rettenmaier & Sohne, GmbH + Co KG.) was analyzed by the supplier for aflatoxins, selected pesticides, heavy metals, polychlorinated biphenyls, and pentachlorophenol. There were two types of food used in this study. A gamma-irradiated pellet diet (T2914C irradiated rodent diet (standard chow), or TD.88137 for HFD groups for IR arm, both from Harlan, U.K.) were provided on the cage top from cage lids. The T2914C was screened by the supplier, with guidance from the panel of dietary components and contaminants

outlined by the NTP (NTP, 2000), which describes the analysis for levels of aflatoxins, nitrosamines, heavy metals, chlorinated hydrocarbons, organophosphates, PCB's and bacterial contaminants. The HFD (TD.88137) was screened by the supplier according to FDA and EPA recommendations (21 CFR 58, 1979, Federal Register, Part 2, 27334-27375, Part 4, 44054-44093) standards. The animal drinking water was screened once every two weeks for microbiological quality. Each batch of diet and bedding were monitored for microbiological quality before first usage and every month thereafter to endure sterile conditions.

To check the hygienic conditions within the animal laboratory rooms, samples of the laboratory air were collected every two weeks during animal housing. To check the effectiveness of laboratory disinfection and sanitation, impression samples were taken prior to the introduction of the mice and weekly thereafter. From each sample, the bacterial plate colony count were determined. The results of the environmental screening showed 2 incidences of water samples being out of specifications (INC/2013/0210), one incidence of a surface impression out of specification (INC/2013/0193). Repeated samples from both of these incidences, however, returned results within the specifications. All other parameters met specifications.

5.3 Experimental Groups and Dosing

5.3.1 Allocation of Animals to Exposure/diet groups

The 15015 study has got 2 parts, THS2.2 ApoE$^{-/-}$ switching part and insulin resistance part. The experimental set up is depicted schematically in Figure 21 (Appendix 2.) and involved 1114 animals in total. This discusses the insulin resistance part only and report for main THS2.2 switching part is done separately. The IR part of the 15015 study involved seventy five mice, allocated to 6 groups (601, 611, 602, 612, 603 and 613). The standard chow-fed groups exposed to fresh air (sham), 3R4F CS or THS2.2 aerosol (601, 602 and 603) and were allocated with 10 animals each. The HFD fed groups exposed to fresh air (sham), 3R4F CS or THS2.2 aerosol (611, 612, and 613) were allocated with 15 animals each. Animals from all 6 groups of IR part were exposed with the main ApoE-THS2.2 switching study animals due to similar daily exposure conditions (see section 16). For the HFD groups, an additional 8 mice per exposure condition were included (Figure 3). These additional 8 animals served as exposed reserves for the exposure biomonitoring (COHb, as blood collection from the main 15 animals in the group may impact the metabolic data readout), and as replacement animals in cases where there was technical or unexpected mortality in the HFD-fed groups. The allocation to groups were performed within one week of exposure start in order to obtain similar mean body weights for all groups at the start of the exposure.

Each exposure group [sham (Fresh air), 3R4F (CS) and THS2.2 (aerosol)] received normal chow diet and HFD diet for 11 weeks of exposure duration. Mice from HFD fed groups started receiving HFD from the day of exposure start (50% HFD mixed with 50% Chow diet was given for 3 days before start of exposure to acclimate them with HFD). The assessment of IR markers (fasting blood glucose and plasma insulin) was done by repeated

blood collection at 4 weekly intervals including a baseline and 3 more measurements till the end of exposure (11 weeks) to monitor IR development.

	Group name	Exposure	Duration 3 months(12 weeks) 1 2 3 4 5 6 7 8 9 10 11 12	No of mice/Group	No of Cages
Metabolic End points IR	Sham_Chow	Sham	D	10	2
	Sham_HFD	Sham	D	15	2
	3R4F_Chow	3R4F	D	10	2
	3R4F_HFD	3R4F	D	15	2
	THS2.2_Chow	THS2.2	D	10	2
	THS2.2_HFD	THS2.2	D	15	2
Exposure biomarker IR	Sham_HFD_Reserve	Sham		8	1
	3R4F_HFD_Reserve	3R4F		8	1
	THS2.2_HFD_Reserve	THS2.2		8	1
Total exposed mice with cages for Metabolic End points_IR				99	15
Pre- study Health check animals from Main study					6
Sentinel Health check animals from Main study					16

Figure 3: Schematic representation of the study set-up. Depicted are the Insulin Resistance (IR) exposure groups, dissection week (D), total mice per group, and number of cages per group. Dissection and longitudinal timings are described in section 5.4.3.1 and 5.4.3.2.

5.3.2 Exposure

Mice were whole-body exposed to fresh, conditioned air or to the aerosol generated from either the test or reference item: cigarette mainstream smoke of the reference cigarette 3R4F at concentrations of 600 µg TPM/l or to aerosol from THS2.2 to matched nicotine levels (relative to 3R4F), 3 hours per day, 5 days/week for between 1 and 8 months, depending on the necropsy time point (Table 4). Mice that were scheduled to be dissected were exposed at least two consecutive days before necropsy (therefore animals dissected on Monday or Tuesday underwent a weekend exposure prior to dissection).

The exposure period began with an adaptation phase lasting for 6 days: target 3R4F TPM concentration was 150 µg/l on study days 1 - 2, 300 µg/l on study days 3 - 4, and 450 µg/l on study days 5 - 6. From the study day 7 onwards, the target concentration was 600 µg/l (nicotine equivalent). The equivalent nicotine levels were used for the adaptation period for the THS2.2-exposed mice. During daily administration, animals were exposed to filtered fresh air for 30 minutes after completing the first 1 hour of mainstream smoke exposure, and for 60 minutes after completing the second exposure block. This was to control the increasing carboxyhaemoglobin (COHb) levels which will accumulate in the mice during the high 3R4F exposure (Figure 4).

Table 4. Exposure Groups

Short Name	Group Code	TPM Concentration (µg/l)	Daily Exposure Duration (h)	Exposure Duration (weeks)
Sham_Chow	601	fresh air or 600 µg/l CS/THS2.2 aerosol(nicotine equivalent)	3	11
Sham_HFD	611		3	11
3R4F_Chow	602		3	11
3R4F_HFD	612		3	11
THS2.2_Chow	603		3	11
THS2.2_HFD	613		3	11

Food and cage enrichment were removed during the exposure, but the animals had free access to water. The position of the cages within the exposure chamber was changed once per week (during the cage change) according to a rotation schedule. The cages were taken out from exposure chambers after exposure and put back on racks.

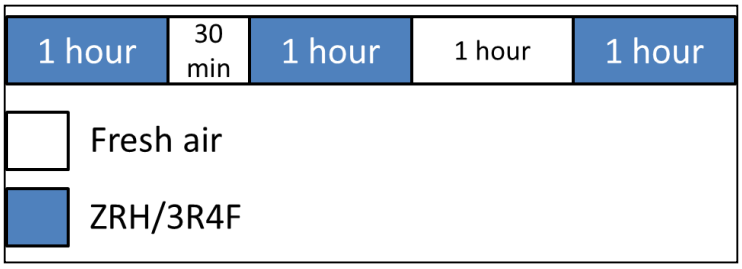


Figure 4. Exposure regime. Mice were exposed for a total of 3 hours per day, but with fresh air 'breaks' during the exposure period.

At the first month of the study, a total of 7 inhalation chambers were used to expose all of the groups including main switch arm and IR arm. The IR groups were exposed in chamber 2 (sham), chamber 3 and 4 (3R4F) and chamber 7 THS2.2 RRP (Table 5).

Table 5. Exposure chambers and numbering

Short Name	Chamber #
SHAM-1	1
SHAM-2	[i]2
3R4F-1	[i]3
3R4F-2	[i]4
3R4F-3	5
THS2.2-1	6
THS2.2-2	[i]7

[i]: Chamber used for the IR groups highlighted in blue.

5.4 Procedures

5.4.1 Parameters to Monitor General Condition and Health

General condition and health of the mice and smoke uptake/exposure were monitored throughout the study. This included body weight measurements, which were performed once per week, as well as group and individual observations. Animals with identified ailments or injuries were tracked daily on a special observations list until they were deemed fit by the study director or the attending veterinarian, or unfit and removed from the study.

Table 6. General condition and health

Parameter	Method	Frequency	Number of Mice	Remarks
Body weight	Gravimetric	2 times/week	All mice	Individually
In-life observations	Nonsystematic observation	Daily	All mice	-
	Group observations	Daily	All mice	After exposure
	Individual observations according to checklist	Daily	One cage per main group (SHAM, 3R4F, Smar, 3R4F_Sham, and 3R4F_Smar)	Immediately after removal from exposure chambers
Mortality	Observation	Daily	All mice	-

5.4.2 Parameters to Monitor Smoke Uptake and Exposure

In addition to the aforementioned analysis of the CS/aerosol in the breathing zones of the animals we also measured CS/aerosol uptake. This was monitored by measuring COHb levels in the bloodstream immediately after the 3rd exposure block (Table 7). The COHb concentration in the blood and the nicotine metabolites in the urine were tested once throughout the course of the study.

Table 7. COHb levels in whole blood

Parameter	Method	Frequency	Number of Mice	Remarks
Blood COHb	Spectrophotometric measurement of absorbance at several wavelengths of the mixed hemoglobin in hemolysate	once	8 mice from Each HFD groups	Individually

5.4.3 Biomonitoring of metabolic parameters

A limited number of parameters were monitored during the in life phase of the 'IR groups (601 - 613). They included whole blood fasting glucose, plasma insulin and GLP-1 in blood samples collected after 6 hours fasting period (but not more than 7 hours fasting). These parameters (Fasting blood glucose, plasma insulin and plasma GLP-1) are repeatedly measured at 4 weeks interval there were termed as longitudinal end points.

5.4.3.1 Longitudinal end points: Blood analysis (from 6 – 7 hour fasted animals)

On a non-exposure day, blood was collected from the facial vein of non-anaesthetized animals, and processed for assessment of glucose and protein levels (Table 8).

Table 8. Longitudinal Parameters to monitor insulin resistance

Parameter	Method	Minimum Volume	Multisite	Frequency	No of samples
Fasting Blood glucose	Enzymatic detection (glucometer)	10 µl, whole blood	PMRL, Singapore	3 times per group + pre-exposure baseline	≥10
Insulin	Plasma Insulin (ELISA)	20 µl, plasma	Cambridge Biomedical, (Boston, USA) Insulin final report GLP-1 Final report		
Plasma fasting GLP-1	Plasma GLP-1(ELISA)	20 µl, plasma			

5.4.3.2 Terminal end points at dissection (6 to 15 samples per group)

Blood samples were collected from the retro-orbital plexus and processed as follows:

- Clinical chemistry for lipid and glucose levels (plasma)
    - Blood – Glucose, HDL, LDL, (In house, PMRL Singapore)
- Multi-Analyte profile serum (raw data provided to test facility)
    - Serum: Rat Metabolic MAP®v2.0 (Myriad/Rules-based Medicine, Metabolic MAP analysis)

Necropsy – special procedures for IR study

Procedures for organ extraction was performed using standard working protocols

- Histopathological assessment of tissue samples
    - Liver (left lobe) –Histopathology (H&E, PAS, PASD)
    - Liver (left lobe) – Histopathology (Cryosections, oil red O)
- Pancreas – Histopathology (H&E)
  Morphometric analysis for plaque size and islet area
    - Aortic arch – Morphometric evaluation of total plaque area
    - Pancreas – Islet area (H&E)
- Tissue collections for Omics
    - Mesenteric fat – Transcriptomic
    - Liver (left lobe) – Transcriptomics
    - Liver (left lobe) – Protein (Western blotting for validation of biomarkers
    - Liver (left lobe) – Lipidomics
    - Plasma – Lipidomics (Zora Biosciences)
    - Muscle (soleus and gastrocnemius as single tissue piece) – Transcriptomics
    - Thoracic aorta – Transcriptomics
    - Abdominal aorta--Lipidomics
    - Heart (left ventricle, apex) – Transcriptomics

6 Statistical Methods

This study was exploratory and there was no formal hypothesis specified a priori. Nonetheless, a statistical analysis was conducted where 95% confidence intervals and unadjusted p-values were obtained.

The analysis described below was applied to the biological parameters belonging to the following categories (note that omics data analysis was not covered here and will be described in a separate study plan):

- Longitudinal end points for IR
- Terminal end points for IR

Summary statistics was provided for measurements which do not belong to the above list (test atmosphere characterization, general condition and health, smoke uptake).

6.1 Descriptive Statistics

For each treatment group, continuous variables were presented by showing the number of planned measurements, non-missing and missing observations. When applicable, the number of non-missing observations was further stratified between values within the limits of quantification, as well as values below the lower limit of quantification (LLOQ) or above the upper limit of quantification (ULOQ). Median, 25 and 75% percentiles, minimum and maximum are provided for all the groups. In addition, if all the non-missing values of a given group fall within the quantification limits, mean, standard deviation, relative standard deviation, standard error (SE), and 95% confidence intervals were provided.

For each treatment group, ordinal and categorical variables were presented by showing the number (and/or percentage) of planned measurements, non-missing observations and missing observations, as well as the number (and/or percentage) of mice falling in each level of the variable of interest (contingency tables).

6.2 Missing Values

Missing values were not substituted.

Values below the LLOQ were substituted with a value corresponding to the LLOQ/2.

6.3 Treatment Comparisons

In addition to the descriptive statistics, exploratory comparisons between treatment groups were performed.

6.3.1 Continuous variables

For continuous variables, the comparison was done by an analysis of variance (ANOVA) assuming that all the non-missing values fell within the quantification limits. Model assumptions were examined and non-parametric methods applied where necessary. In the presence of LLOQ or ULOQ values, non-parametric methods were directly applied. Values above the ULOQ were assigned the same and maximum rank. Finally, the proportion of

values below the LLOQ can be compared across treatment groups by means of the Fisher's exact test. Ordinal and categorical variables are analyzed by classical statistical tests associated with contingency tables and/or logistic regression.

6.4 Extreme Values

If extreme values were identified, they were discussed with the study designers. These values were included in the analysis except if clear measurement errors were identified.

7 Administrative Aspects

7.1 Study Timelines

Date of arrival of animals:	16 August 2013
Starting date of exposure:	02 September 2013
End of exposure (IR groups):	21 November 2013
Experimental completion date:	31 December 2013
Date of insulin and GLP data report (Cambridge Biomedical)	4 Jan 2014
Proposed date for final study report:	30 April 2014

7.2 Names and Addresses

Sponsor	Peitsch, Manuel VP Biological Systems Research PHILIP MORRIS INTERNATIONAL CH-2000 Neuchâtel, Switzerland
Test Facility	Philip Morris International Research Laboratories Pte Ltd 50 Science Park Road, #02-07 The Kendall Singapore Science Park II Singapore 117406
Test Facility Management	Boll, Yin. Manager Support & Technology Philip Morris International Research Laboratories Pte Ltd 50 Science Park Road, #02-07 The Kendall, Singapore Science Park II, Singapore 117406 Tel: +65-66314524 Fax: +65 64130948 Email: Yin.Boll@pmi.com
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Test site 6	Barbara Osband Cambridge Biomedical 1320 Soldiers Field Road Boston, MA 02135 Tel: +1-617-456-0765 Fax:+1-617-456-0766 Email: avanderpot@cambridgebiomedical.com

Study Director	Phillips, Blaine Philip Morris International Research Laboratories Pte Ltd 50 Science Park Road, #02-07 The Kendall Singapore Science Park II, Singapore 117406 Tel.: +65-6631-4510 Fax: +65 64130948 Email: Blaine.Phillips@pmi.com
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7.3 Multi-Site Arrangements

This study includes analytical endpoints at both Singapore (PMIRL-S) and outsourced to external contract research organization. The separation of activities is as follows:

1) PMIRL (Singapore)

Aerosol generation and dilution

- Animal housing and husbandry
- Blood carboxyhemoglobin
- Longitudinal end points
    - Blood collection and shipment samples to Cambridge Biomedical for insulin and GLP-1 analysis
    - Fasting whole blood glucose levels
- Terminal end points Dissection and necropsy
    - Blood collection for (plasma and serum)
    - Shipment of serum samples to Rules- based medicine
    - Clinical chemistry for cholesterol (lipoprotein) and glucose levels (plasma)
    - Tissue collection for histopathological, morphometric and 'omics analysis
    - Histoprocessing of tissue samples
    - Pathological assessment of tissue samples
    - Morphometric analysis for plaque size and islet area

2) Test site 4 (Rules-based Medicine)

- Multi-Analyte profile serum (raw data provided to test facility)

3) Test site 6 (Cambridge Biomedical)

- Plasma Insulin and GLP-1 analysis

8 Study plan amendments and deviations

Table 9. Amendments and deviations

Type	Change No.	Date of Study Director's Approval	Change	Author of Change
Amendment	1	30 Aug 2013	Longitudinal blood glucose determination work flow following facial vein blood collection	D. Sharma
Amendment	2	16 September 2013	Explanatory algorithm of fasting and blood collection steps	D. Sharma
Amendment	3	16 September 2013	Explanatory algorithm of fasting and blood collection steps	D. Sharma
Amendment	4	01 Oct 2013	Update to the dissection plan	D. Sharma
Amendment	5	11 Nov 2013	Week end exposure change before the dissection and blood collection activity	D. Sharma
Amendment	7	18 Nov 2013	Tissue collection for additional end points	D. Sharma
Deviation	1	11 Sept 2013	HFD food consumption discontinuation	B. Phillips

9 Results

9.1 Parameters to monitor the General condition and Health of the Animals

9.1.1 Mortality

A total of 3 animals were declared moribund during the study period, and were consequently removed from the study, and are described in the Table 10. These removals were not attributable to exposure to any of the test aerosols.

Table 10 List of dead animals during the study exposure phase

UAN	CAN	Date of death	Study day	Replacement mouse	Cause of death
6022009	920581	31 Aug 2013	-2	9992017	Technical (blood collection)
6132018	920537	12 Sep 2013	11	NA	Moribund (no cause found on necropsy)
6132013	920502	24 Oct 2013	54	6132013	Moribund (Xanthoma growth on jaw)

9.1.2 In life Observations

The animals were observed throughout the study on a daily basis. The observations included group and individual observations, and were performed shortly after each exposure period. These observations revealed only incidental abnormalities, as expected from the large population size of the study. A summary of findings is as follows:

Group observations:

Abnormal grooming was frequently flagged in the animals just following exposure, particularly in the HFD groups (who had oil coats mainly from the diet, which is normal and frequently observed in HFD-fed mice).

- Chow, sham: 18 days out of 99, Maximum score 3 out of 10 mice.
- Chow, 3R4F: 38 days out of 99, Maximum score 10 out of 10 mice.
- Chow THS2.2: 29 days out of 99, Maximum score 6 out of 10 mice
- HFD sham: 32 days out of 99, Maximum score 4 out of 23 mice
- HFD 3R4F: 54 days out of 99, Maximum score 9 out of 23 mice
- HFD THS2.2: 48 days out of 99, Maximum score 7 out of 48 mice.

Piloerection: There were sporadic cases of piloerection, but this phenomenon did not persist in the individual animals (single occurrences). It was observed on 5 occasions in the 3R4F groups, and 2 cases in the THS2.2 groups. Diet had no impact on this observation.

- There were no observations of the other group observation categories (breathing difficulty, stereotypic behaviour, tremor, pallor).

Individual observations:

There was a single occurrence of the following:

- Irritability
- Decreased spontaneous activity
- Startle response
- Vocalization

Those occurred in different groups and different days, and can be considered as incidental.

9.1.3 Food Consumption

The food consumption was determined for each group by calculating the weekly weight differential of the food provided to the group (food in) and the remaining food after 7 days (food out). This measurement was performed only in the standard chow groups because the HFD pellets were very crumbly, and therefore food was lost in powder and crumbs, rendering the food weights for the HFD to be unreliable (Deviation 1).

In the first week of the study, during the concentration adaptation period, there was a slightly increased food consumption in the 3R4F-exposed chow fed mice. However, food consumption in all subsequent weeks showed no obvious exposure-related trends. Note, the small sample sizes precluded a meaningful statistical evaluation.

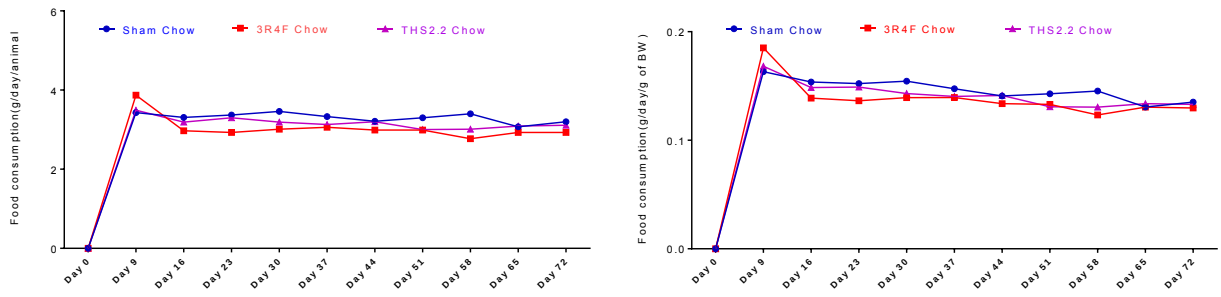


Figure 5. Food consumption of the chow-fed animals.

9.1.4 Body Weight

The body weight of the animals was measured on a weekly basis. The mice that were fed the regular chow showed the typical body weight progressions over time in response to their exposures seen in other PMI's studies, where the 3R4F-exposed animals gained weight at a much slower rate compared to the sham-exposed or the THS2.2-exposed animals (Figure 6). The sham and THS2.2-exposed animals on the chow-fed diet showed very similar body weight throughout the study.

The animals on the HFD had an overall higher body weight gain (and body weight values) relative to their standard chow counterparts throughout the study period starting at approximately day 23 onwards. Unexpectedly,

the HFD 3R4F-exposed animals had a large increase in group mean body weight, identified on study day 9 (during the concentration adaptation period). The reason for weight gain of more than 2 grams (on average) is unclear and may be considered incidental. Following this initial weight gain, the body weight of the animals exposed to 3R4F remained relatively stable from day 9 until day 58, at which time the sham- and THS2.2-exposed mice body weights finally caught up with the 3R4F weights until the end of the study (day 72).

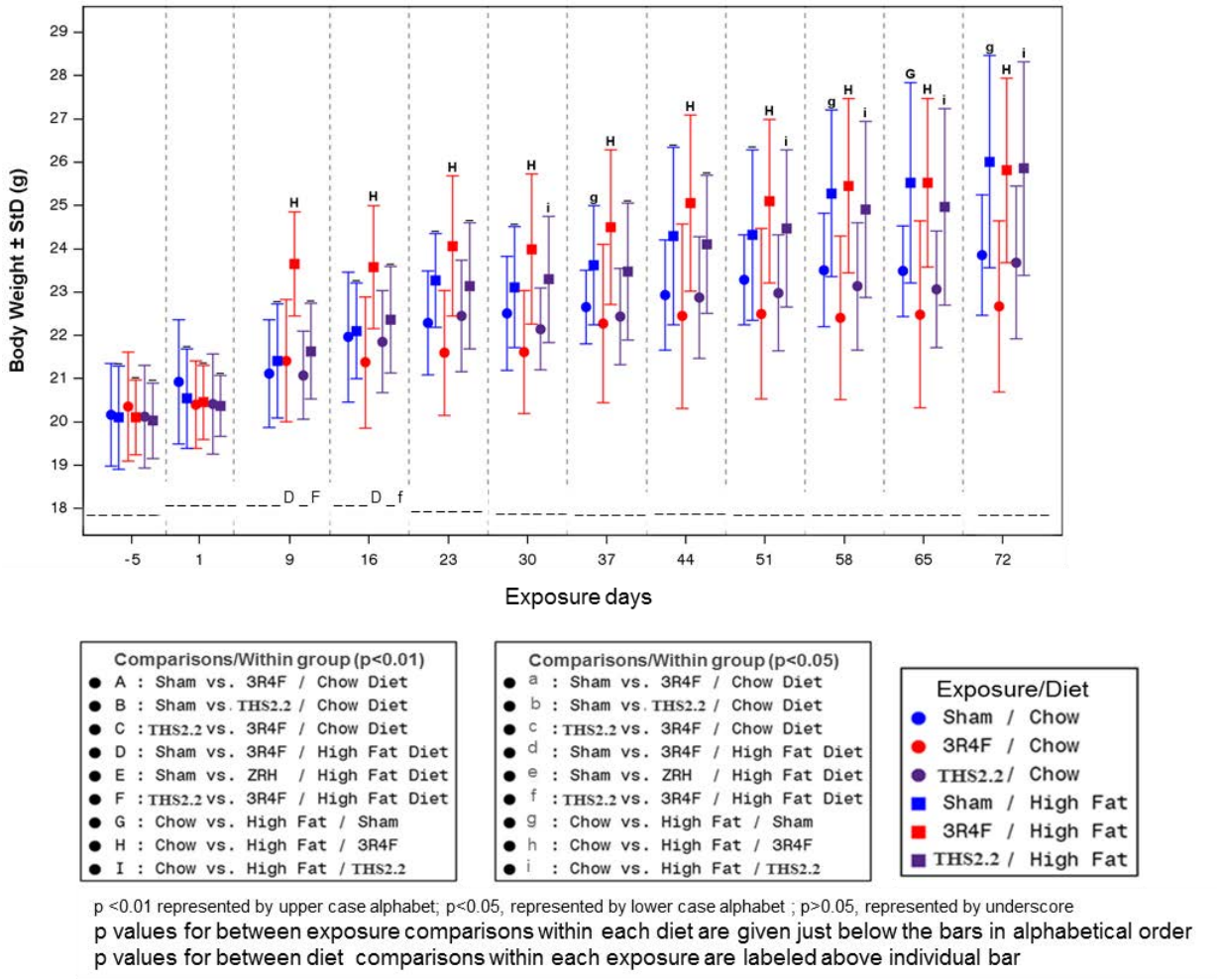


Figure 6. Weekly body weight values of the animals over the course of the study. Significance levels were measured by a linear model adjusting for the variability between groups.

9.2 Parameters to monitor exposure and aerosol delivery

9.2.1 Exposure concentrations

Following the 6-day adaptation period, the aerosol target concentrations in the exposure chambers were targeted to 29.9 µg/l nicotine (equivalent to 600 µg/l TPM based on the 3R4F pre-test smoking) for both the 3R4F and the THS2.2 groups. In order to monitor the nicotine levels, nicotine concentrations were measured once during each exposure block (3 times per exposure day). The average nicotine concentrations (for the entire exposure period) were within the specified range of +/- 1% of the target concentrations (Table 11).

In both THS2.2 and the 3R4F exposure chambers the daily administration of test aerosols showed a characteristic daily fluctuation observed in both the nicotine and TPM graphs (Figure 7 and Figure 8). The THS2.2 smoking machines are high maintenance for operation, and there are occasional periods of jams or technical faults, further resulting in daily variability relative to the 3R4F exposures.

Table 11. Analysis of test atmosphere

Exposure Chamber	TPM		Carbon monoxide (ppm)		Nicotine (µg/l)	
	mean	SD	mean	SD	mean	SD
SHAM-2	0	0	0.06	0.08	0	0
3R4F-1	578.55	28.57	643.30	26.30	29.79	2.16
3R4F-2	613.64	28.90	643.75	26.28	29.79	2.19
THS2.2-2	321.02	30.25	13.40	0.57	28.41	3.07

Remarks: Mean ± SD

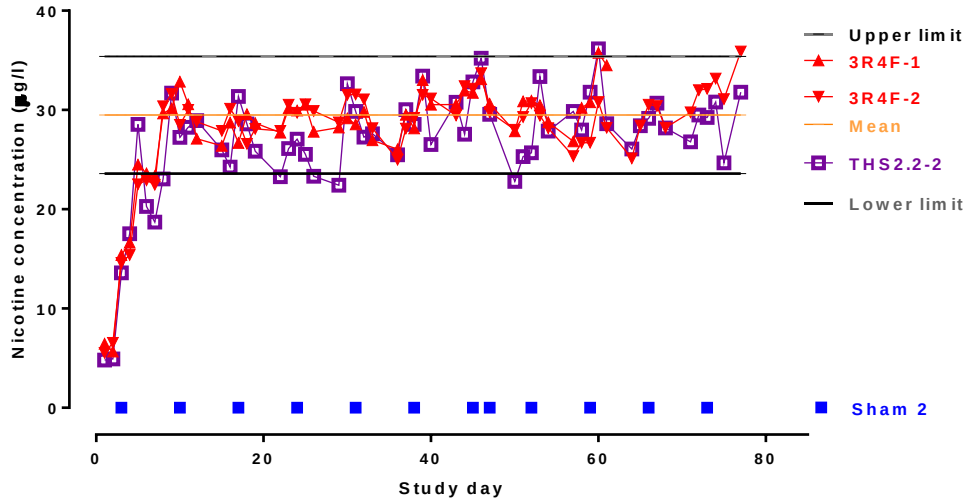


Figure 7. Mean daily nicotine levels (number behind the group refers to exposure chamber).

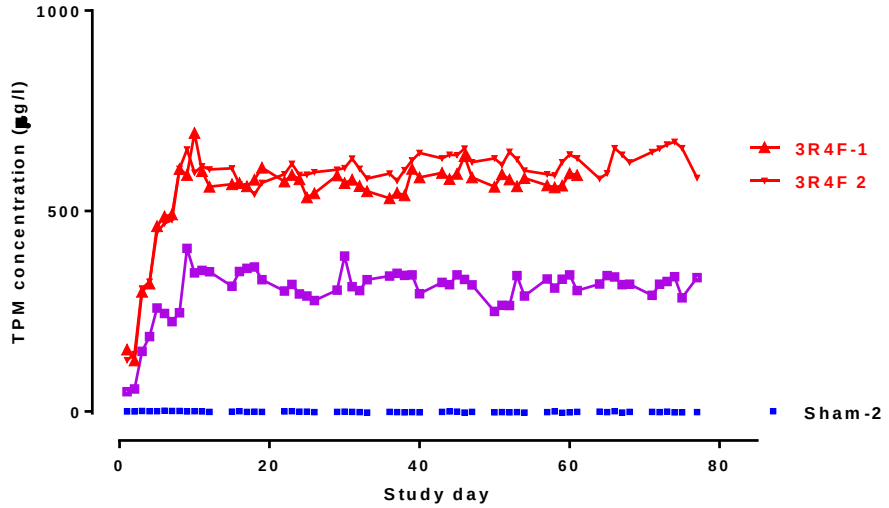


Figure 8. Mean daily TPM concentrations (number behind the group refers to exposure chamber)

Aldehyde concentrations were measured in each exposure chamber (excluding sham) on a weekly basis. The results show that the levels of aldehydes (formaldehyde, acetaldehyde and acrolein) were all within the expected levels of historical data.

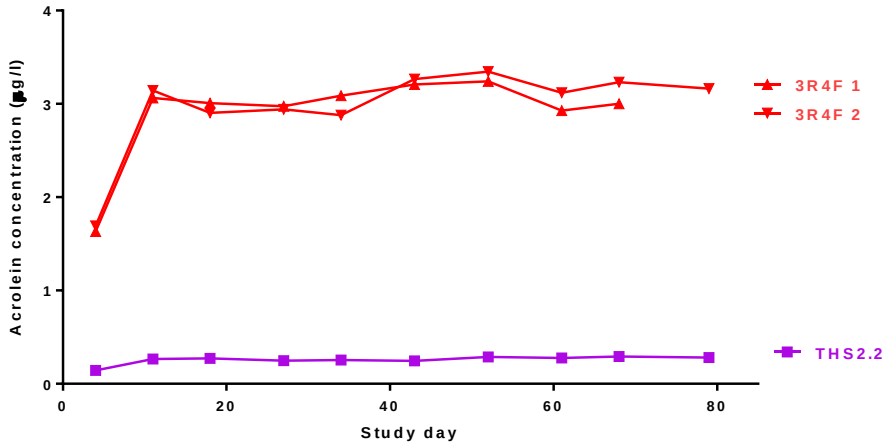


Figure 9. Weekly aldehyde concentrations.

9.3 Parameters to Monitor Smoke Uptake and Exposure

9.3.1 Carboxhaemoglobin in whole blood

Blood (COHb) carboxyhaemoglobin was measured a single time for the IR groups from the HFD groups only. For comparative purposes, chow-fed mice from the ApoE-THS2.2 switching study (main study) were included in the graph (Figure 10). Blood was collected from different animals than those used for the metabolic marker analysis

(glucose, insulin, etc.) to minimize any effect the blood collection, which might affect the measured metabolic parameters.

The detected COHb levels were higher in the 3R4F-exposed animals, averaging 23.9% for the HFD-exposed animals and 27.8% for the chow-fed. The blood COHb % is measured within half an hour, immediately after the exposure is over for the day (the half-life of COHb in blood is 0.5h). The Lower COHb % in HFD fed 3R4F relative to Chow fed 3R4F (not significant) may be due to delay in blood collection activity after exposure. The sham-exposed animals had COHb levels at the baseline level of the analytical instrument (Radiometer), while the THS2.2-exposed animals showed a slightly elevated COHb level compared to sham, expected from the low levels of CO present in the THS2.2 aerosol (though higher than fresh air).

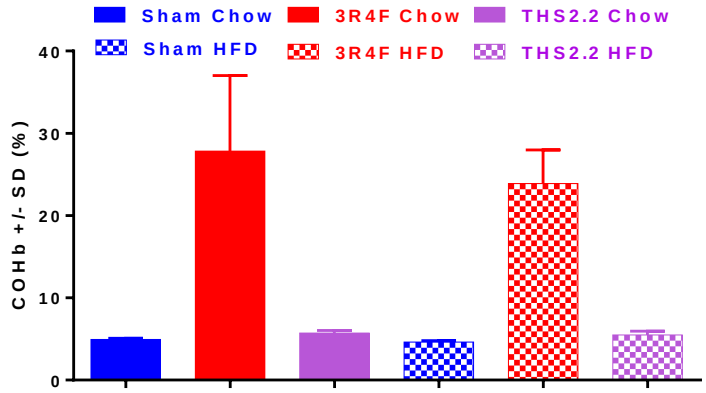


Figure 10. Carboxyhaemoglobin levels in whole blood.

9.4 In life assessment of IR

9.4.1 Longitudinal end points: Blood analysis (from 6h fasted animals)

9.4.1.1 Whole blood fasting glucose

Fasting glucose levels (6h fasting) were assessed on study day 0 (baseline measurement prior to the exposure), on week 4, week 8 and week 11. The procedure was performed on a non-exposure day (Saturday), to avoid additional exposure related stress to animals and to have sufficient time for blood collection and glucose measurements.

The baseline difference in fasting blood glucose between HFD and chow fed animals in 3R4F CS exposure group may be incidental as no exposure or diet treatment was started by then. Throughout the course of the study, only the sham group showed any diet-related changes– in which the HFD feeding was associated with increased fasting glucose levels relative to the standard chow-fed mice at the week 8 and week 11 sampling. The fasting glucose levels in the other groups exposed to THS2.2 aerosol and 3R4F were unaffected by diet. Notably, 3R4F-

exposure was associated with a statistically significantly lower fasting glucose relative to both the sham and THS2.2-exposed animals in both the chow and HFD conditions. Throughout the study period, only the sham HFD-fed animals group displayed an increased fasting glucose over time, though this increase was relatively lower than expected based on published literature (Chen *et al.*, 2007). The 3R4F-exposure was linked to a decreasing fasting glucose trend over time. Potential mechanisms by which 3R4F resulted in a lower fasting glucose levels would be further explored using the omics approach.

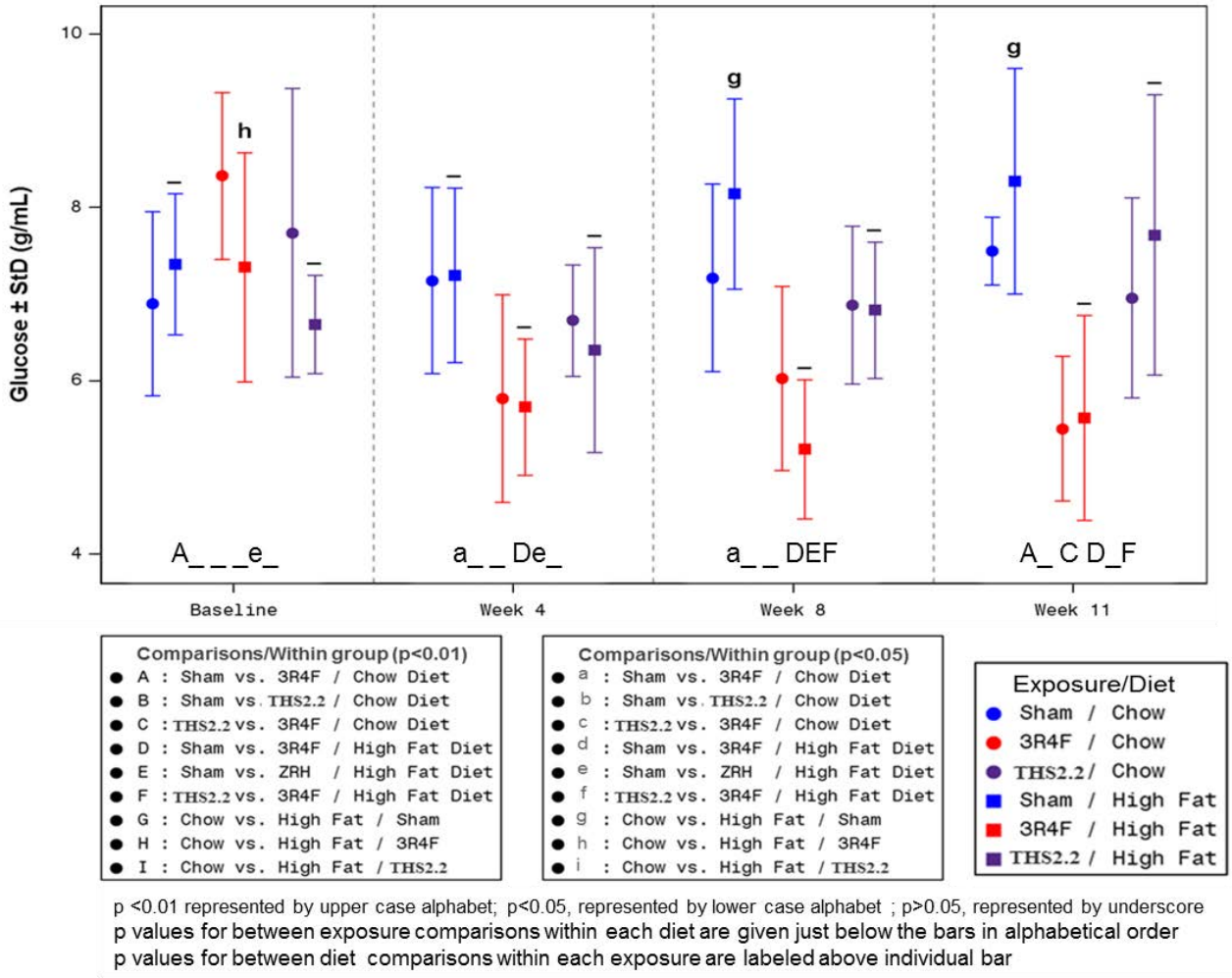


Figure 11. Fasting glucose levels

Statistical comparisons were done using a linear model adjusting for the variability between groups.

9.4.1.2 Insulin in plasma

Fasting insulin levels were measured in the plasma samples on study day 0 (baseline measurement prior to exposure), week 4, week 8 and week 11 (longitudinal measurement as that of blood glucose). The mice were fasted for 6-7 hours before blood collection (the blood collection was done simultaneously for the glucose measurement discussed above). Insulin levels in the plasma were assayed using low range assay of the ultra-sensitive mouse insulin ELISA kit (Cat # 90080, Crystal Chem, Inc.) by Cambridge Biomedical. There were no apparent differences in the fasting insulin levels between the exposure groups in the weeks 8 and 11. Note, the insulin measurements were from diluted samples, and the measurements were close to the instrument detection limits.

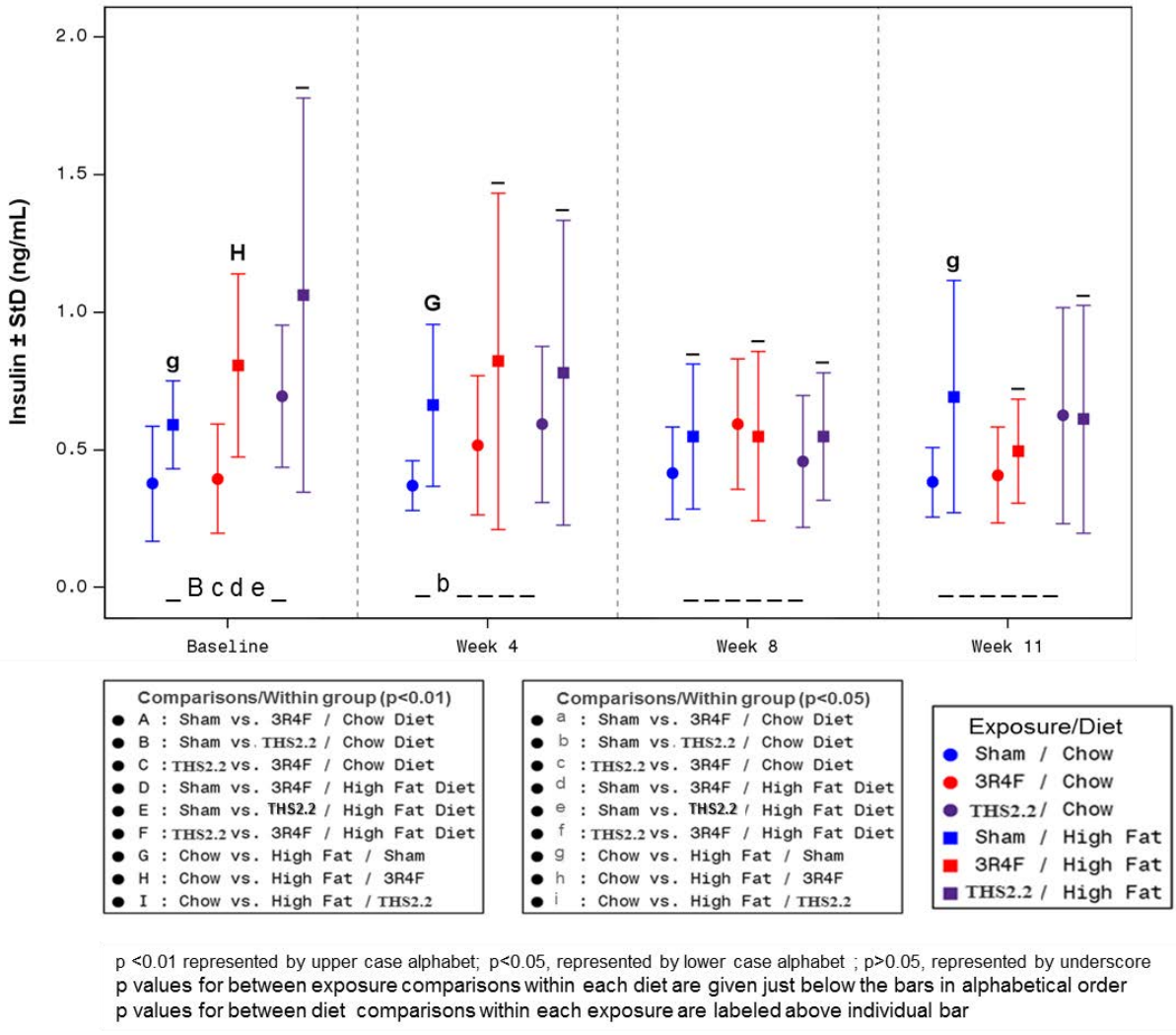


Figure 12. Plasma insulin levels

Statistical comparisons were done using a linear model adjusting for the variability between groups.

9.4.1.3 Glucagon like peptide 1 (GLP-1) in plasma (longitudinal measurement)

Fasting glucagon like peptide 1 (GLP-1) levels were measured in plasma on study day 0 (baseline measurement prior to exposure), week 4, week 8 and week 11 by Cambridge Biomedical. Mice were fasted for 6 hours before blood collection. GLP-1 levels in plasma were then assayed using GLP-1 (Active 7-36) ELISA from ALPCO Diagnostics. All the measurements were below the levels of quantification (formal report and optimization of the assay conducted by Cambridge Biomedical can be found in 15015_CVD_Resp_ApoE_SW_SR_Part2 App4. This observation of low GLP-1 levels is aligned with the GLP-1 measurement levels in the serum samples that were analyzed using Multi Analyte Profiling by RBM.

9.4.1.4 Clinical chemistry for plasma lipid and glucose levels.

Just prior to necropsy, a limited clinical chemistry panel was performed on the serum from the animals following 6-7 hours fasting, focusing on cholesterol and basic metabolic parameters. A direct measurement of cholesterol and lipoproteins was performed for total cholesterol, as well as HDL and LDL. Note, direct measurements of VLDL and IDL could not be done using the Unicell DX800 clinical analyzer.

Both experimental variables, i.e. aerosol exposure and diet, impacted the cholesterol measurements. The consumption of a HFD resulted in a significant increase in total cholesterol, as well as the HDL and LDL fractions. HFD resulted in an approximately 2.5 fold increase in total cholesterol levels, with a similar impact on HDL observed (Figure 13). The LDL fraction showed a higher increase in response to the HFD, with >10-fold increased LDL serum concentration. The cholesterol levels also showed an effect from the exposure conditions. Exposure to 3R4F CS resulted in a slight (but not significant) increased total cholesterol in the chow-fed mice. The total cholesterol levels were slightly amplified in the HFD-fed mice: exposure to a 3R4F aerosol resulted in an increased total cholesterol serum concentration ($p<0.05$). This trend was similarly observed for the HDL and LDL cholesterol components, where the CS resulted in increased lipoprotein particle concentrations, amplified by the HFD.

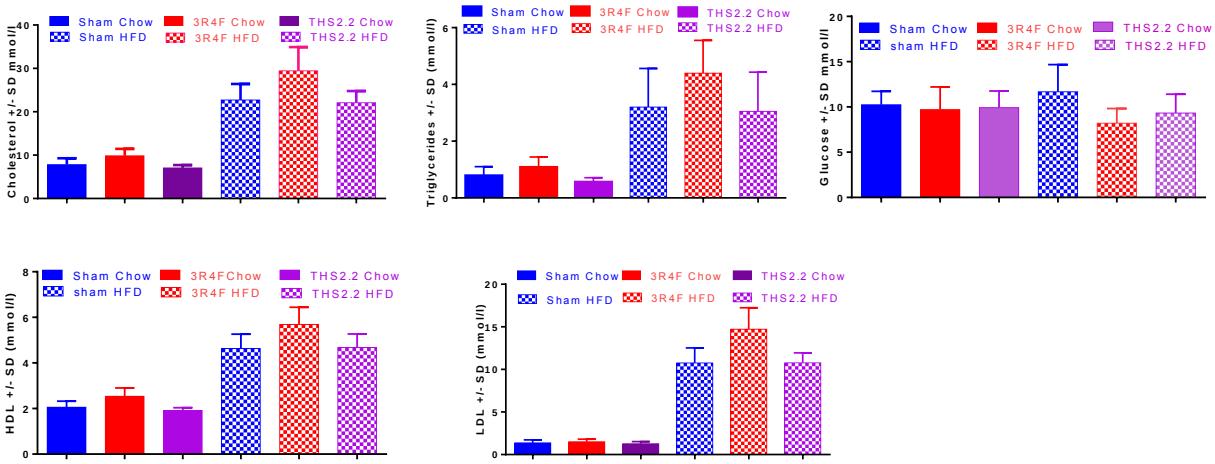


Figure 13. Cholesterol, lipoprotein, glucose and triglycerides concentrations in the serum of exposed ApoE$^{-/-}$ mice.

In addition to cholesterol levels, the concentration of serum glucose and triglycerides were also evaluated. In the regular chow-fed animals, the glucose levels were unchanged regardless of the exposure conditions. The HFD-fed animals showed a likely exposure effect, with 3R4F exposure associated with a lower glucose serum concentration ($p<0.05$). Triglycerides were affected by the HFD, in which its levels increased approximately by 4 fold, irrespective of the exposure. Similar to the cholesterol levels, the levels of triglycerides were further increased with 3R4F exposure in both the chow-fed mice (not significant) and in the HFD-fed animals ($p<0.05$).

In all of the above clinical chemistry parameters, the THS2.2-exposed mice showed similar levels with the sham-exposed. Thus, the differences were likely affected by 3R4F- exposure.

9.4.1.5 Multi-Analyte profile in serum

An analysis of common metabolic analytes was performed at the end of the exposure on serum samples from each of the diet and exposure groups by RBM. Biological effects were observed in multiple analytes, generally clustered into either a HFD effect, and/or an exposure effect (Figure 14 and 13).

These can be summarized as follows:

Multiple analyte profiling; HFD effect:

- Growth hormone – lower concentration in HFD groups(not significant)
- Insulin-like growth factor – increased concentration in HFD groups (not significant)
- Leptin - increased concentration in HFD groups
- Plasminogen activated inhibitor -1 - increased concentration in HFD groups

Multiple analyte profiling; CS effect:

- Cortisol – higher concentration in 3R4F-exposed group (regular chow only)

- Leptin – higher concentration in 3R4F-exposed groups (regular chow and HFD)

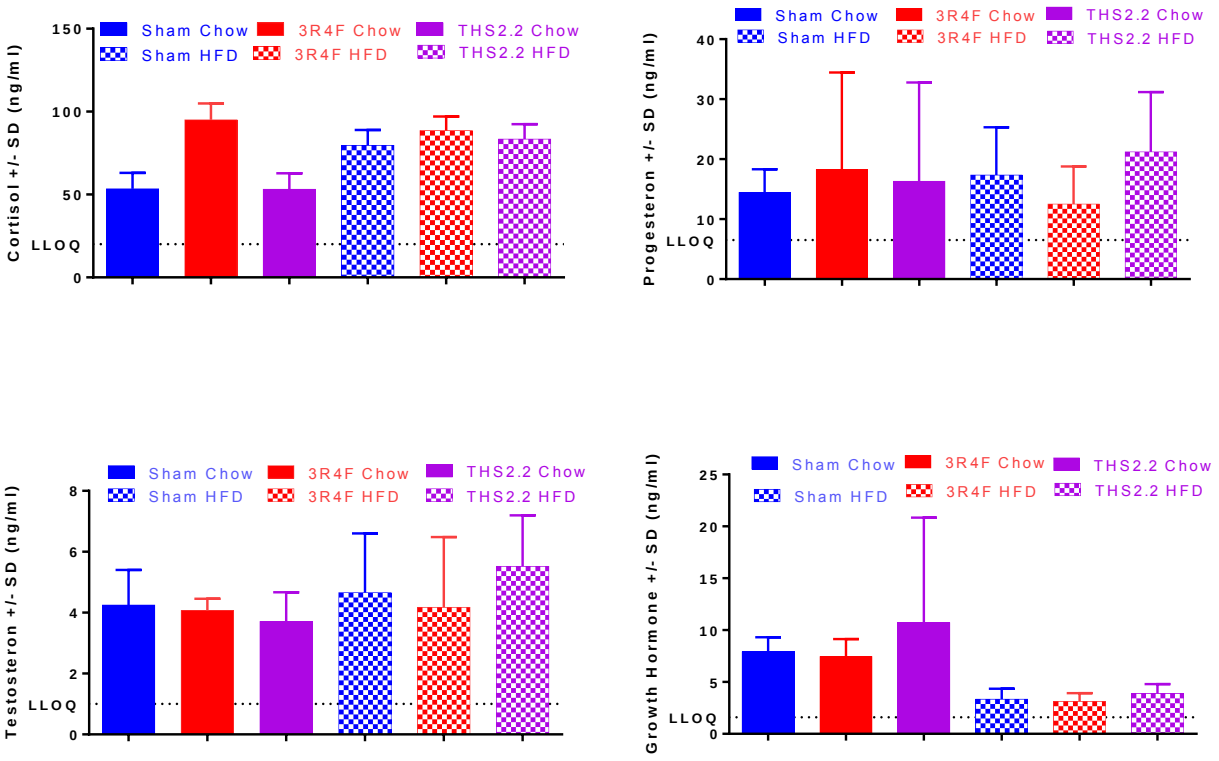


Figure 14. Metabolic Multiple Analyte Profiling, steroids

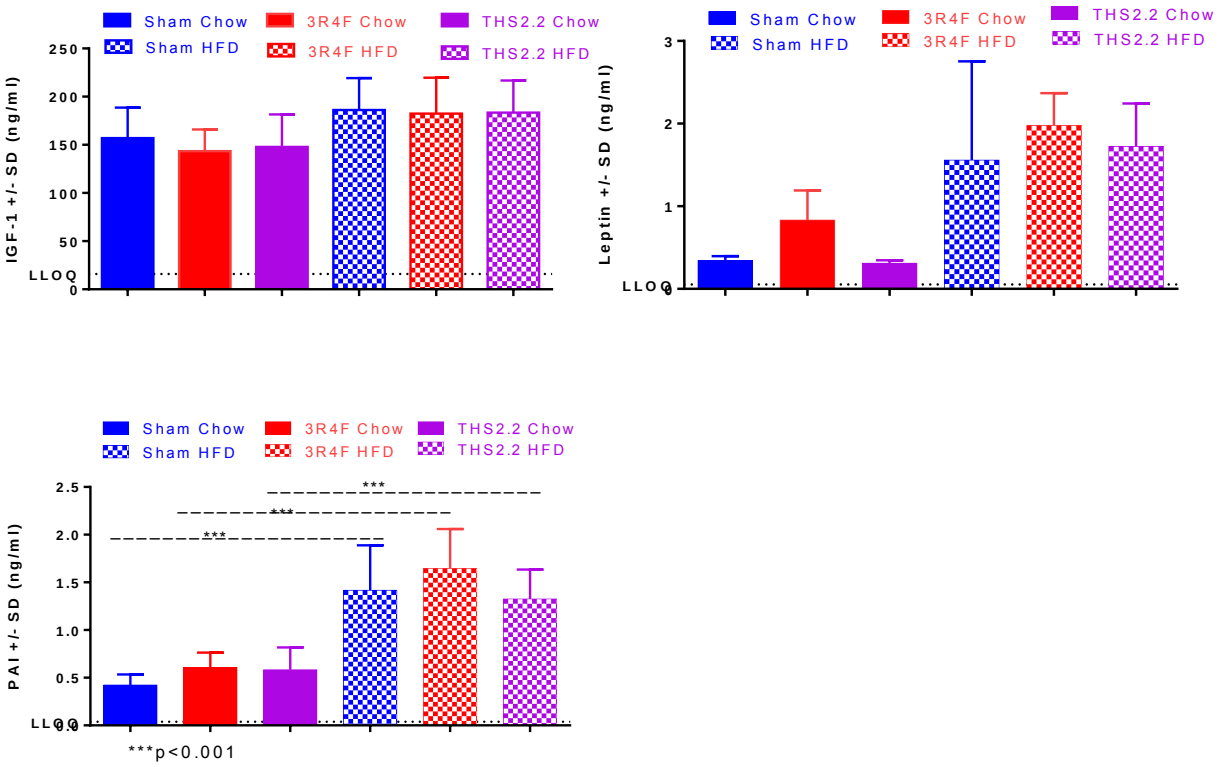


Figure 15. Metabolic Multiple Analyte Profiling, hormones

***p<0.001(ANOVA, Tukey adjustment for multiple comparisons)

Of the parameters assessed, only leptin, PAI-1 and cortisol have demonstrated a CS exposure effect (either in both or one of the different diet groupings).

9.4.1.6 Histopathological assessment of tissue samples

Histopathological evaluation of the liver and pancreatic tissue from all mice from each group was performed in-house to characterize whether any of the treatments (exposure or diet) were related to toxicological effects on these following tissues: pancreas and liver. Histoprocessing of the pancreas and liver were performed according to the PMIRLS internal working instructions applying score based on severity as explained in appendix 3. Liver (left lateral lobe) and pancreas, 4µm thick tissue slices, paraformaldehyde fixed, processed and stained with hematoxylin and eosin (HE). Two additional liver sections were stained with periodic acid-Schiff (PAS) and PAS with diastase (PASD). PAS stain typically gives a magenta color in the presence of glycogen. When PAS and diastase are used together, a light pink color replaces the deep magenta. The diastase (or a-amylase) act on glycogen to de polymerize it into smaller sugar units, maltose and glucose, that are washed out of the section. Glycogen will be stained magenta on the PAS stained slide and will be absent on the PAS/D stained slide. Differences in the intensities of the two stains (PAS and PAS-D) can be attributed to different glycogen

concentrations and can be used to semi quantify glycogen in samples. Another 8µm thick frozen OCT embedded slices of liver (left lateral lobe) were stained with oil red O to evaluate steatosis. The routine histopathological evaluation of the pancreas and liver is done using H&E while determination of hepatic steatosis was done using ORO and determination of liver glycogenosis was done using PAS and PASD staining.

9.4.1.6.1 Liver (left lobe) –Histopathology (H&E, PAS, PASD).

Hepatocellular vacuolation in the H&E stained tissues was observed across all the animals from all groups; however, there were no differences among the groups. The degree of steatosis observed in the liver was significantly higher in the HFD groups, relative to the chow-fed, without significant effect from the exposure (3R4F or THS2.2). There was higher trend of portal fibrosis in the HFD fed mice groups especially the HFD fed 3R4F group had significantly higher portal fibrosis than the chow fed 3R4F group. Glycogen content of the liver slices stained with PAS and PASD showed an increased trend in the chow fed groups than the HFD fed mice groups (not significant). There was minimal inflammatory cell infiltration across all the groups, which is likely not to have any biological significance.

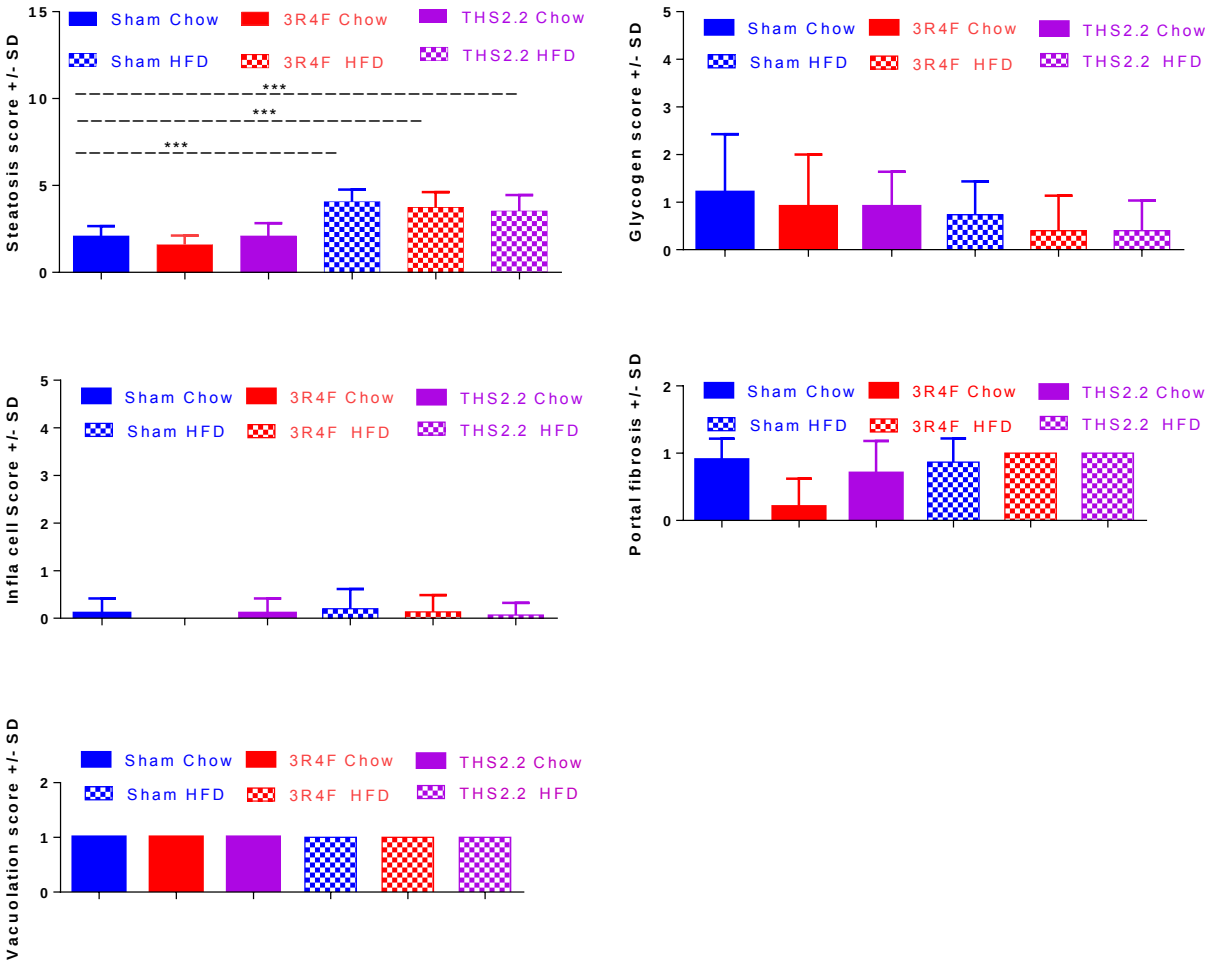


Figure 16. Histopathological evaluation of the liver.

***p<0.001, (ANOVA, Tukey adjustment for multiple comparisons).

Pancreatic tissue showed an increased vacuolation in the islets of Langerhans of the 3R4F chow fed and sham chow fed animals as compared to 3R4F HFD. There was minimal inflammatory cell infiltration across all the groups least in HFD fed 3R4Fgroup, which likely do not result in any biological significance.

9.4.1.6.2 Pancreas –Histopathology (H&E)

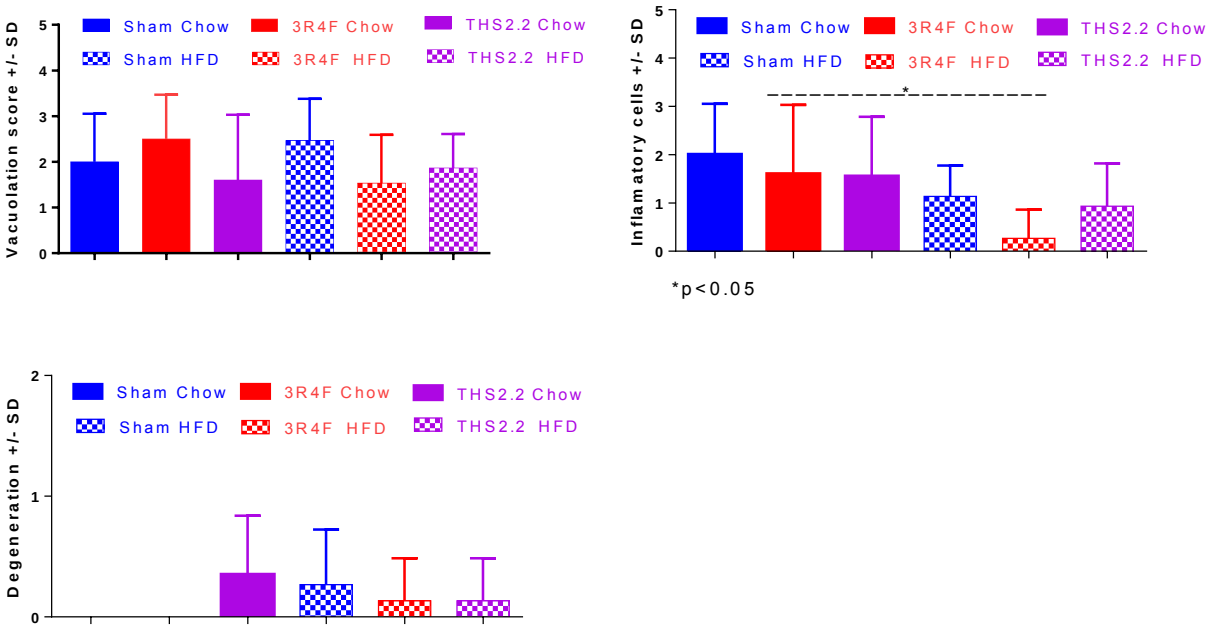


Figure 17. Histopathological assessment of the pancreas

*p<0.05, ***p<0.001, (ANOVA, Tukey adjustment for multiple comparisons)

9.4.1.7 Liver weight

The absolute liver weights in HFD-fed sham group were higher than the chow-fed sham group ($p<0.05$), however there was no difference when the liver weights were normalized to body weights between these groups. There were also no exposure effects seen in any of the groups.

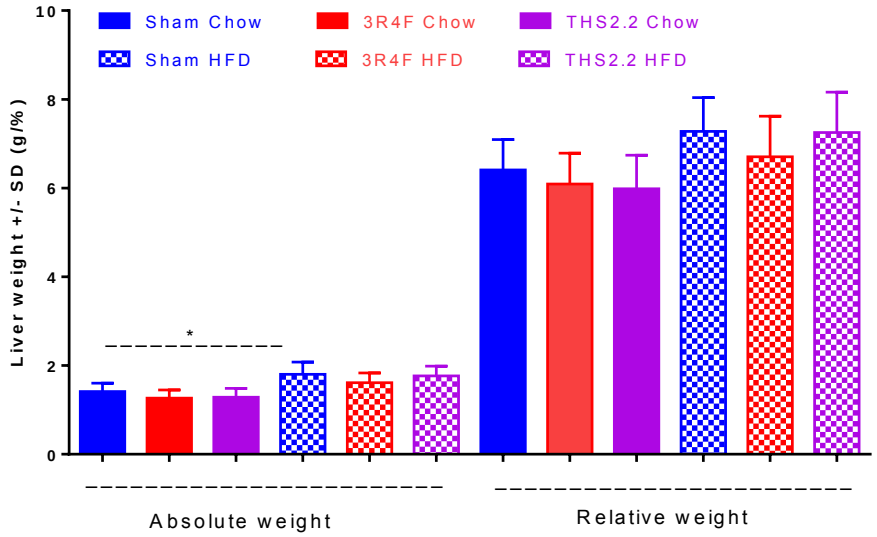


Figure 18. Liver weights (absolute and relative to body weight).

*p<0.05, (ANOVA, Tukey adjustment for multiple comparisons)

9.4.1.8 Morphometric analysis for plaque size and islet area

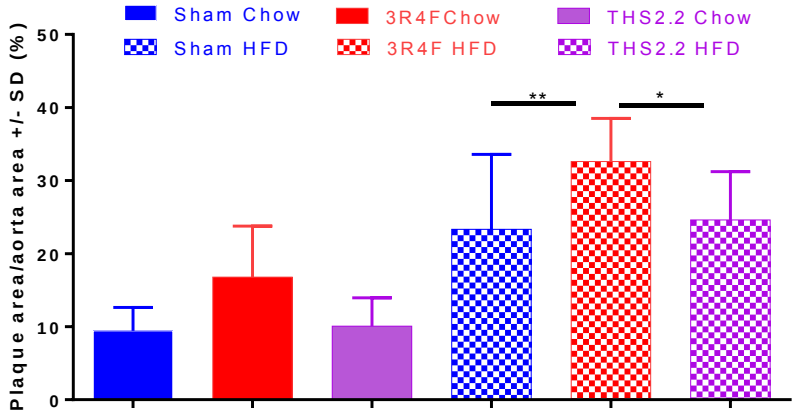


Figure 19. Plaque area measurements were conducted at the aortic root

*p<0.05, **p<0.01, (ANOVA, Tukey adjustment for multiple comparisons)

The plaque area was altered with diet and smoke exposure. There was an increase in plaque area in HFD-fed mice as compared to chow-fed mice at the end of exposure (~11 weeks). Furthermore, exposure of CS was linked to significantly increased plaque area in the HFD-fed mice.

Total area of Islets of Langerhans was measured relative to the total area of the pancreas (Figure 20).

The islets of Langerhans were evaluated in each of the exposure groups, though limited to a single cross-section per pancreas. Thus, the results of this analysis should be interpreted with caution because the islets may be randomly distributed throughout the tissue, and the single section evaluation may not be representative for the full tissue). The relative islet area did not show any obvious trends related to exposure. There was a slightly reduced islet area in the THS2.2-exposed chow-fed mice group, a phenomenon not observed in the HFD-fed animals.

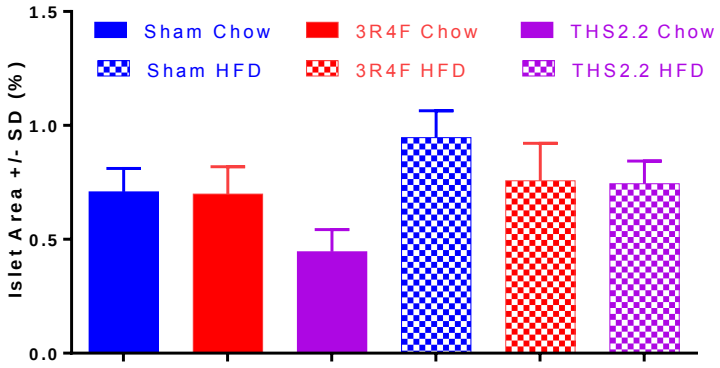


Figure 20. Total Islet area relative to total pancreatic area

10 Discussion

In the current study, which is a supplement to study 15015 ApoE-THS2.2 switching study, we evaluated the impact of exposure to 3R4F CS or RRP (THS2.2) aerosol in the development of IR in female ApoE$^{-/-}$ mice. HFD feeding in ApoE$^{-/-}$ mice induces type 2 diabetes. In this study, the mice were randomized to chow and HFD-feeding to observe whether the impacts of exposure (3R4F or THS2.2) on IR-related metabolic profiles were different between the diabetic atherosclerosis (i.e. the HFD-fed mice) and the non-diabetic atherosclerosis mice (i.e. the chow-fed mice). The HFD-fed ApoE$^{-/-}$ mouse has been used for a model of diabetic atherosclerosis (Li *et al*., 2011; Wu and Huan, 2007). In IR condition, following increase of food intake (fuel loading), the pancreas releases more insulin to compensate for the increased blood glucose until the beta-cells can no longer secrete sufficient levels of insulin, which situation eventually leads to type 2 diabetes. Therefore, to monitor the development of IR, we measured the circulating levels of fasting glucose and insulin every month. We hypothesized that the HFD-fed mice, which exhibit IR, would progressively had greater fasting glucose and insulin levels. Furthermore, we hypothesized that 3R4F exposure would exacerbate the development of IR; whereas THS2.2 exposure would be comparable to that of the sham exposure (in both chow and HFD-fed animals).

At the end of the study (week 11), we observed a significant higher levels of fasting glucose in the HFD-sham group as compared to the chow-sham group. This change was not accompanied by alterations in insulin levels, which remained unaltered throughout the study. These results are in agreement with a previous published study where ApoE$^{-/-}$ mice showed hyperglycaemia after HFD feeding for 12 weeks without obvious signs of IR measured and reported previously (Li *et al*., 2011). The observed higher fasting glucose levels at the end of the study suggested an impaired fasting glucose, which level often defines a pre-diabetic state (Ford *et al*., 2010). Contrary to our hypothesis, we observed a significant reduction of fasting glucose levels in the HFD-3R4F group at the 11 week period as compared to the HFD-sham without alteration in insulin levels. Therefore, based on the fasting glucose levels, our results suggest that 3R4F did not promote the development of IR. Additionally, the levels of fasting glucose of the HFD-THS2.2 group were not different than the HFD-sham group. Potential molecular mechanisms accounting for the association of 3R4F with reduced fasting glucose (and the possible molecular changes associated with THS2.2 exposure) will be examined using the omics approach—including lipidomics, transcriptomics and systems biology analyses that provides more powerful and robust approach in detecting subtle changes at the molecular levels that might not be phenotypically observable.

Our study is the first to examine the impact of CS and THS2.2 exposure on the HFD-ApoE$^{-/-}$ mice, which was different from previous HFD-fed ApoE$^{-/-}$ mouse studies, in which the model has been used (Li *et al*., 2011; Wu and Huan, 2007). The exposure of the aerosols was administered for 4.5 h daily for 11 weeks without access to food. . The exposure of the aerosols was monitored by measuring the increased COHb levels in the circulation of the reserved animals (specifically reserved for COHb monitoring). In general, increased COHb levels were mainly in 3R4F-exposed animals because THS2.2 aerosols resulted in lower CO concentrations. In-life observations

indicated the exposure concentrations of both 3R4F and THS2.2 were well tolerated. In the chow groups, exposure to 3R4F was associated with the predicted lower body weight gain relative to sham group (as compared to our previous Study-3452). The food consumption, measured only in the chow-fed groups, was not significantly affected by the different exposure. The THS2.2-exposed animals showed a similar body weight as the sham-exposed mice at all-time points with chow and HFD fed groups. All HFD fed exposure groups showed increased body weight gain than chow fed exposure groups, interestingly during the adaptation of exposure period, the 3R4F exposed HFD-fed mice showed a rapid increase in body weight gain relative to HFD fed sham and THS2.2 groups, which levelled-off gradually by week 11. Towards the end of the study (week 5 to week 11), there were no significant differences of the body weights among the different exposure groups within the same diet.

IR is characterized not only by reduced glucose uptake but also altered lipid metabolism (Hue and Taegtmeyer, 2009). The ApoE$^{-/-}$ mice are known to exhibit greater plasma cholesterol levels compared to the wild type (C57Bl6/J), resulting in the formation of arterial plaques. Clinical evidence show that IR can promote atherogenesis and advanced plaque progression (Bornfeldt and Tabas, 2011). The mechanisms are thought to be related to dyslipidemia, hypertension, and a proinflammatory state (Bornfeldt and Tabas, 2011). The higher cholesterol levels were evident in the present study, which was slightly exacerbated in the 3R4F-exposed mice, but not in the THS2.2-exposed. This 3R4F effect was more substantial in the HFD-group, where 3R4F-exposed animals showed significantly higher total cholesterol levels, as well as HDL and LDL serum concentrations relative to chow-fed animals. In humans, diet high in saturated fat and cholesterol increased the levels of HDL-cholesterol. This phenomena was also observed in another mouse model, i.e. transgenic mice expressing the human apolipoprotein A-I; HuAITg (human Apolipoprotein transgenic). The mechanism by which HFD increased HDL levels in humans may be related to the promotion of reverse cholesterol transport through the HDL pathway (perhaps an adaptation of the metabolic load of a high fat diet as reported by (Hayek *et al*., 1993)). Aligned with these clinical chemistry results (i.e. the blood lipid profiles), the HFD-fed mice had increased plaque development, as measured by relative plaque area of the aortic root (%), which was further increased by the exposure to 3R4F. On the other hand, the effects of THS2.2 exposure on the serum cholesterol, and atherosclerotic plaque development was similar to the fresh air exposure (sham group). Therefore, these changes in lipid levels and plaque formation were consistent with our hypothesis, in which 3R4F exacerbates the perturbed lipid metabolism.

Additionally, the observed increase of steatosis score and proportional portal fibrosis in the HFD fed ApoE$^{-/-}$ mice exposed to 3R4F was comparable to a published study which showed that the combination of high cholesterol diet and CS exposure increased hepatic fat accumulation through alterations in lipoprotein and triglyceride processing (Mensenkamp *et al*., 2001). All the HFD fed groups showed decreased trend for liver glycogen content (though not significant) relative to chow fed groups. Possible mechanisms to explain the histological changes discussed here cannot be derived at this point and remain unclear without further investigation.

Additionally, selected metabolic parameters (Rodent metabolic MAP, Myriad/Rules-based medicine, USA) were measured in the serum of the mice. This analysis identified that several analytes were affected by the diet supplied, including the expected increase in serum leptin, as well as PAI-1, and the decreased levels of growth hormone. CS moderate affected the analyte panel assessed, and did appear to affect leptin levels, as well as subtle effects on cortisol (chow diet only) and PAI-1.

The elevated levels of leptin in the HFD-exposed animals relative to chow-fed mice, can be explained as adipose tissue effect because leptin is released from adipocytes and serum leptin concentrations increase with adipocyte mass. It has been reported that previously that HFD evokes a sustained increase in circulating leptin in both normal and transgenic mice (Frederich *et al.,* 1995) without decreasing their caloric intake, suggesting that a high content of dietary fat changes the 'set point' for body weight, at least in part by limiting the action of leptin. Therefore, it is not surprising that in our study serum leptin levels are elevated in HFD fed mice with an increase in body weight. However, the increase following smoke-exposure is unexpected, particularly in the chow-fed animals, as the CS resulted in a slightly lower body weight, with accompanying reduced adiposity. These results are in disagreement with previous studies where smoke exposure was associated with decreased leptin levels in Wistar rats (Tomoda *et al*., 2012) and Balb/c mice (Chen *et al.,* 2007). Strain-related variation in biochemistry may be responsible for the differences observed.

Cortisol has been shown to be elevated in the plasma of adult human smokers (Chiodera *et al*., 1984; Seyler *et al*., 1984; Wilkins *et al*., 1982). CS exposure was shown to cause elevated plasma levels of cortisol in a mouse model of chronic lung inflammation, tumorigenesis and emphysema (Stinn *et al.,* 2013). Elevated cortisol levels increases glucose production in the liver, thus resulting in hyperglycemia (Kruyt *et al*., 2012). Cortisol also inhibits secretion of insulin from the pancreatic beta-cells (Kruyt *et al*., 2012). We observed that cortisol levels were higher in the HFD-animals, without obvious effects of the CS/aerosol exposure. It has been reported that different methods of CS exposure, various mice strain, TPM concentration, as well as CS exposure duration (2–5 h daily) and the age of mice during the exposure period may all have an impact on the observed plasma levels of cortisol (Sundar *et al*., 2014). Therefore, whether the increase in cortisol levels in chow-fed 3R4F group was related to the exposure-associated stress cannot be excluded. However, CS exposure with HFD did not affect cortisol levels suggesting that these mice were not experiencing stress from the CS exposure during high fat feeding (Hui Chen *et al*., 2005). Chronic stress also reduces anabolic hormones that promoting growth, such as growth hormone (Kruyt *et al*., 2012). The observed decrease (although not significant) in growth hormone levels in HFD fed groups relative to chow fed groups could be attributed to the increased cortisol levels or to the reduced lean body mass due to HFD feeding as observed in human clinical studies (Frystyk *et al*., 1995)

The high levels of PAI-1 in HFD-fed mice as compared with the chow-fed sham, CS and aerosol exposed mice was comparable to the observations of human atherosclerosis as PAI-1 is expressed at sites of vascular disease and has been proposed to play an important role in the pathogenesis of human atherosclerosis (Frystyk *et al*., 1995; Juhan-Vague *et al*., 2003). Importantly, it is well established that greater levels of plasma PAI-1 is a central

feature of metabolic syndrome and that the higher levels of PAI-1 remain increased at the continuum of IR [from impaired glucose tolerance to pre-diabetes to diabetes]. Pharmaceutical drugs (e.g. metformin, TZD) as well as lifestyle modification (e.g. weight loss) have been shown to reduce plasma PAI-1 levels (Lee *et al*., 2013).

Insulin reduces hepatic production of IGF-binding protein-1 (IGFBP-1), an inhibitor of IGF bioactivity, and it has been suggested that the obesity-related hyperinsulinemia may increase free (bioactive) IGF *in vivo* by reducing the concentration of IGFBP-1(Frystyk *et al*., 1995). In this study the high levels of IGF-1 in HFD-fed sham, 3R4F and THS2.2 groups relative to chow-fed sham, 3R4F and THS2.2 groups are in accordance of the proposed insulin dependent effect partially as only HFD fed, sham group showed slight increase in insulin levels. 3R4F and THS2.2 exposure were not associated with alterations in PAI-1 and IGF-1 levels.

11 Summary and Conclusions

We evaluated the impact of exposure of ApoE$^{-/-}$ mice to either CS or to the aerosol from THS2.2 RRP on metabolic parameters associated with IR. IR is linked to obesity, atherogenic lipid abnormality, hyperglycemia and hyperinsulinemia. To study IR-metabolic changes, the diabetic atherosclerotic HFD-fed ApoE$^{-/-}$ mice was used in this study. Alteration of metabolic changes were also examined in chow-fed ApoE$^{-/-}$ mice that do not exhibit diabetes. Development of IR was monitored by measuring the levels of fasting glucose and insulin every 3 weeks (for 11 weeks). The impacts of 3R4F and THS2.2 on the IR-related metabolic changes were investigated in both the chow-fed and HFD-fed mice.

Consistent with our hypothesis, the HFD-sham group had increased body weights as compared to the chow-sham group. Additionally, at the end of the study, the HFD-sham group had higher fasting glucose and insulin levels, increased plasma lipid levels, increased hepatic lipid accumulation (steatosis measured by histopathology analysis) and a tendency of a greater plaque formation, without any change of fasting insulin levels as compared with the chow-sham group.

Contrary to our hypothesis, 3R4F exposure was not associated with promotion of IR development in this model. The 3R4F exposure in both the chow- and HFD-fed groups, was associated with reduction fasting glucose levels as compared to their respective sham-exposed. Interestingly, 3R4F exposure in both the chow- and HFD-fed groups was linked to the greater plasma lipid levels (i.e. triglycerides, cholesterol, LDL, VLDL, and HDL), as well as tendency of greater plaque formation and IR-associated metabolic analytes (PAI-1, leptin, and cortisol), as compared to their respective sham-exposed. Therefore, although 3R4F did not promote IR-associated increased fasting plasma glucose, it worsened the lipid accumulation and seemed to increase the IR-related metabolic analytes. Consistent with our hypothesis, the impact of THS2.2 exposure in both the chow- and HFD-fed mice were not different from their respective sham-exposed.

This study is the first to report and investigate the effects of 3R4F exposure on HFD-fed ApoE$^{-/-}$ mice. Further lipidomics, transcriptomics and systems biology analyses will be carried out to elucidate the mechanisms by which 3R4F exposure in this model (i.e. alteration in lipid and reduction fasting plasma glucose). Systems biology approach will facilitate the understanding of the complex relationship between atherosclerosis and IR, as well as alteration at the molecular levels.

12 Archiving

The study plan, PMF, raw data (from procedures performed in Singapore only), copies of phase reports 1 through 4, the reference item (60 sticks, 3R4F), the test item (60 sticks per batch, THS2.2) and the final report will be stored at the test facility for a period of up to 2 years after the study completion date. Thereafter, they will be transferred for long-term storage at an external GLP-certified archive center (Iron Mountain Deutschland GmbH, Harpeer Hellweg, 4408 Bochum Germany). Electronic records will be stored on the 'electronic secure storage share' at Philip Morris Research Laboratories, Singapore (#02-07 Science Park Road, Singapore, 117406). Electronic and paper records will be stored for at least 15 years after study completion date according to local archiving SOPs and WKIs. Histopathological samples will be processed to paraffin blocks within 2 weeks of collection. The blocks will be stored at the test facility (room temperature) for up to 2 years after the study completion date, thereafter, they will be transferred for long-term storage at an external GLP-certified archive center (Iron Mountain Deutschland GmbH, Harpeer Hellweg, 4408 Bochum Germany). Tissue samples will be frozen and stored (-80^{o}C) after collection, and will be processed within 2 year of collection, or sent for external storage (site and storage period to be determined, and is defined in a study plan amendment). RNA, DNA and protein will similarly be stored locally at -80^{o}C, before long-term storage (site and storage period to be determined, and is defined in a study plan amendment).

At the test sites of delegated phases 1 – 3, all raw data of the delegated phases, remainders of the specimen sent to the test site, original phase reports and all delegated phase-related records are stored at the test site for a maximal period of one year after the finalization of the study, thereafter these records and materials will be transferred to the external GLP-certified service archive Iron Mountain Deutschland GmbH, Harpeer Hellweg, 4408 Bochum Germany. Upon completion of the study phase 4 and 5, any remaining samples at test site 4 or 5 will be returned to the test facility or destroyed, at the discretion of the study director. Electronic records generated during the study phases will be archived on the central archiving server. Electronic and paper records will be archived for at least 15 years after the finalization of the final report according to the local archiving SOPs.

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14 Abbreviations

Abbreviation	
ANOVA	Analysis of Variance
AVA	Agri-Food & Veterinary Authority of Singapore
BSR	Biological Systems Research
CO	Carbon Monoxide
COPD	Chronic Obstructive Pulmonary Disease
CS	Cigarette Smoke
CW	Calendar Week
FTC	Federal Trade Commission
GLP	Good Laboratory Practice
H&E stain	Hematoxylin and Eosin stain
COHb	Carboxyhemoglobin
HFD	High Fat Diet
Histophathologic	Histological/pathological
HPLC	High-Performance Liquid Chromatography
IGF	Insulin Like Growth Factor
Lm	Mean Cord Length
RRP	Modified Risk Tobacco Product
NACLAR	National Advisory Committee for Laboratory Animal Research
NGP	Next Generation Product
NTP	National Toxicology Program
ORO	Oil Red Orange
PAI	Plasminogen Activator Inhibitor
PAS	Periodic Acid-Schiff
PAS	Periodic acid -Schiff

PASD	Periodic acid –Schiff with Diastase
PMIRL-S	Philip Morris International Research Lab in Singapore
PMRL	Philip Morris Research Laboratories
PUC	Project Unique Code
PV	Pressure Volume
p-value (p)	Probability of receiving the value of the test statistic or a more extreme value of the test statistic, if the null hypothesis holds
QMS	Quality Management System
RBM	Rules-Based Medicine
RNA	Ribonucleic Acid
SOP	Standard Operating Procedure
TBD	To Be Determined
THS	Tobacco Heating System
TPM	Total Particulate Matter
WHO	World Health Organization
WKI	Work Instruction
ZRH	Zurich

15 Appendix 1 data tables

15.1 COHb raw data

	SHAM Chow	3R4F Chow	THS2.2 Chow	SHAM HFD	3R4F HFD	THS2.2 HFD
1	5.1	25.9	5.6	4.5	30.9	6.5
2	4.9	24.4	5.8	4.7	21.5	5.1
3	4.9	22.4	5.8	4.8	25.8	5.2
4	5.0	20.0	6.1	4.4	19.4	5.2
5	4.7	28.4	6.4	4.4	24.9	5.5
6	4.8	19.4	5.7	4.8	20.5	5.4
7	5.0	21.2	5.8	4.7	27.6	5.5
8	5.0	19.0	5.8	4.7	20.5	
9	5.1	26.5	5.3			
10	4.9	42.4	5.5			
11	4.8	42.3	5.4			
12	5.1	42.1	5.3			
Mean	4.94	27.8	5.71	4.63	23.89	5.49
SD	0.13	9.18	0.32	0.17	4.08	0.47
SEM	0.038	2.65	0.09	0.06	1.44	0.18

15.2 Summary of body weights

Days	SHAM Chow (n=10)		3R4F Chow (n=10)		THS2.2 Chow (n=10)	
	Mean	SD	Mean	SD	Mean	SD
Day -5	20.17	1.19	20.36	1.26	20.13	1.18
Day 1	20.93	1.44	20.40	1.01	20.42	1.16
Day 9	21.12	1.24	21.42	1.42	21.08	1.02
Day 16	21.97	1.50	21.38	1.51	21.86	1.18
Day 23	22.30	1.20	21.60	1.44	22.45	1.29
Day 30	22.51	1.32	21.62	1.42	22.15	0.94
Day 37	22.66	0.85	22.28	1.83	22.44	1.12
Day 44	22.94	1.28	22.45	2.14	22.88	1.41
Day 51	23.29	1.04	22.50	1.97	22.99	1.33
Day 58	23.51	1.31	22.41	1.89	23.14	1.47
Day 65	23.49	1.05	22.49	2.16	23.07	1.35
Day 72	23.86	1.39	22.67	1.98	23.69	1.76
Days	SHAM HFD (n=15)		3R4F HFD (n=15)		THS2.2 HFD (n=15)	
	Mean	SD	Mean	SD	Mean	SD
Day -5	20.11	1.19	20.11	0.86	20.04	0.87
Day 1	20.55	1.15	20.46	0.86	20.37	0.70
Day 9	21.42	1.32	23.66	1.20	21.64	1.10
Day 16	22.11	1.11	23.59	1.42	22.37	1.23
Day 23	23.28	1.08	24.07	1.62	23.15	1.46
Day 30	23.12	1.40	24.00	1.73	23.30	1.46
Day 37	23.63	1.38	24.51	1.79	23.48	1.58
Day 44	24.30	2.04	25.06	2.03	24.11	1.60
Day 51	24.33	1.97	25.10	1.89	24.47	1.81
Day 58	25.29	1.92	25.46	2.01	24.91	2.04
Day 65	25.53	2.31	25.53	1.95	24.97	2.27

table continues

Day 72	26.02	2.45	25.82	2.13	25.86	2.47

15.3 Summary of longitudinal fasting whole blood glucose

Time point	SHAM Chow (n=10)		3R4F Chow (n=10)		THS2.2 Chow (n=10)	
	Mean	SD	Mean	SD	Mean	SD
Baseline	6.89	1.06	7.35	0.81	8.37	0.96
Week 4	7.16	1.07	7.22	1.01	5.80	1.20
Week 8	7.19	1.08	8.16	1.10	6.03	1.06
Week 11	7.50	0.39	8.31	1.31	5.45	0.84
Time point	SHAM HFD (n=15)		3R4F HFD (n=15)		THS2.2 HFD (n=15)	
	Mean	SD	Mean	SD	Mean	SD
Baseline	7.31	1.32	7.71	1.66	6.65	0.57
Week 4	5.70	0.79	6.70	0.64	6.36	1.18
Week 8	5.21	0.80	6.88	0.91	6.82	0.78
Week 11	5.57	1.18	6.96	1.15	7.69	1.62

15.4 Summary of longitudinal plasma Insulin

Time point	SHAM Chow (n=10)		3R4F Chow (n=10)		THS2.2 Chow (n=10)	
	Mean	SD	Mean	SD	Mean	SD
Baseline	0.38	0.21	0.40	0.20	0.26	0.38
Week 4	0.37	0.09	0.52	0.25	0.28	0.37
Week 8	0.42	0.17	0.59	0.24	0.24	0.42
Week 11	0.38	0.13	0.41	0.17	0.39	0.38
Time point	SHAM HFD (n=15)		3R4F HFD (n=15)		THS2.2 HFD (n=15)	
	Mean	SD	Mean	SD	Mean	SD
Baseline	0.59	0.16	0.81	0.33	1.06	0.72
Week 4	0.66	0.29	0.82	0.61	0.78	0.57
Week 8	0.55	0.26	0.55	0.31	0.55	0.23
Week 11	0.69	0.42	0.50	0.19	0.61	0.41

15.5 Summary of terminal fasting glucose and lipid levels (plasma)

Parameter	SHAM Chow (n=10)		3R4F Chow (n=10)		THS2.2 Chow (n=10)	
	Mean	SD	Mean	SD	Mean	SD
Cholesterol	7.81	1.49	9.86	1.58	7.07	0.66
Triglycerides	0.79	0.32	1.08	0.36	0.57	0.15
HDL	2.06	0.26	2.54	0.37	1.92	0.12
LDL	1.38	0.34	1.51	0.29	1.28	0.25
Glucose	10.24	1.49	9.71	2.50	9.92	1.83
Parameter	SHAM HFD (n=15)		3R4F HFD (n=15)		THS2.2 HFD (n=15)	
	Mean	SD	Mean	SD	Mean	SD
Cholesterol	22.71	3.71	29.46	5.46	22.07	2.71
Triglycerides	3.20	1.36	4.40	1.16	3.05	1.38
HDL	4.63	0.63	5.69	0.75	4.68	0.59
LDL	10.75	1.77	14.73	2.50	10.77	1.17
Glucose	11.67	3.00	8.22	1.61	9.33	2.09

15.6 Summary of Metabolic end points (Serum)

Parameter	SHAM Chow (n=10)		3R4F Chow (n=10)		THS2.2 Chow (n=10)	
	Mean	SD	Mean	SD	Mean	SD
Cortisol	53.40	30.69	95.10	31.27	53.20	30.59
Progesterone	14.44	3.88	18.28	16.17	16.31	16.49
Testosterone	4.25	1.15	4.07	0.39	3.71	0.96
Growth hormone	7.90	4.46	7.45	5.32	10.71	10.13
IGF-1	157.00	31.71	143.50	22.50	147.80	33.76
Leptin	0.34	0.17	0.83	1.13	0.31	0.11
PAI-1	0.42	0.11	0.61	0.15	0.58	0.23
Parameter	SHAM HFD (n=15)		3R4F HFD (n=15)		THS2.2 HFD (n=15)	
	Mean	SD	Mean	SD	Mean	SD
Cortisol	79.67	35.87	88.53	32.83	83.47	34.51
Progesterone	17.35	7.97	12.51	6.28	21.20	9.99
testosterone	4.66	1.94	4.18	2.30	5.53	1.67
Growth hormone	3.35	3.89	3.13	3.08	3.88	3.56
IGF-1	186.10	33.20	182.40	37.30	183.50	33.28
Leptin	1.56	1.19	1.98	1.53	1.72	2.01
PAI-1	1.42	0.47	1.65	0.41	1.33	0.31

15.7 Summary of morphometric analysis (plaque area and islet area)

Parameter	SHAM Chow (n=9)		3R4F Chow (n=8)		THS2.2 Chow (n=9)	
	Mean	SD	Mean	SD	Mean	SD
Islet area	0.71	0.31	0.70	0.34	0.44	0.29
Parameter	SHAM HFD (n=15)		3R4F HFD (n=14)		THS2.2 HFD (n=15)	
	Mean	SD	Mean	SD	Mean	SD
Islet area	0.95	0.46	0.76	0.61	0.74	0.38
Parameter	SHAM Chow (n=10)		3R4F Chow (n=9)		THS2.2 Chow (n=10)	
	Mean	SD	Mean	SD	Mean	SD
Plaque area	9.44	3.21	16.80	6.96	10.08	3.89
Parameter	SHAM HFD (n=15)		3R4F HFD (n=15)		THS2.2 HFD (n=15)	
	Mean	SD	Mean	SD	Mean	SD
Plaque area	23.31	10.27	32.62	5.89	24.60	6.62

15.8 Summary of Histopathological scoring

Liver histopathology	Type	SHAM Chow (n=10)		3R4F Chow (n=10)		THS2.2 Chow (n=10)	
		Mean	SD	Mean	SD	Mean	SD
Liver Inflammatory cell infiltrate	Score	0.10	0.32	0.00	0.00	0.10	0.32
Liver Hepatocellular vacuolation	Incidence	1.00	0.00	1.00	0.00	1.00	0.00
Liver Portal fibrosis	Incidence	0.90	0.32	0.20	0.42	0.70	0.48
Liver Steatosis (ORO)	Score	2.10	0.57	1.60	0.52	2.10	0.74
Liver Glycogen (PAS&PASD)	Score	1.20	1.23	0.90	1.10	0.90	0.74
Liver histopathology	Type	SHAM HFD (n=15)		3R4F HFD (n=15)		THS2.2 HFD (n=15)	
		Mean	SD	Mean	SD	Mean	SD
Liver Inflammatory cell infiltrate	Score	0.20	0.41	0.13	0.35	0.07	0.26
Liver Hepatocellular vacuolation	Incidence	1.00	0.00	1.00	0.00	1.00	0.00
Liver Portal fibrosis	Incidence	0.87	0.35	1.00	0.00	1.00	0.00
Liver Steatosis (ORO)	Score	4.07	0.70	3.73	0.88	3.53	0.92
Liver Glycogen (PAS&PASD)	Score	0.73	0.70	0.40	0.74	0.40	0.63
Pancreas histopathology	Type	SHAM Chow (n=10)		3R4F Chow (n=10)		THS2.2 Chow	

table continues

						(n=10)	
		Mean	SD	Mean	SD	Mean	SD
Interstitial Fibrosis	Score	0.00	0.00	0.00	0.00	0.00	0.00
Inflammatory cell infiltrate	Score	0.70	0.64	0.60	0.49	0.50	0.67
Exocrine Degeneration	Score	0.00	0.00	0.00	0.00	0.10	0.30
Endocrine-Islets of Langerhans	Score	2.00	1.00	2.50	0.92	1.60	1.36
Pancreas histopathology	Type	SHAM HFD (n=15)		3R4F HFD (n=15)		THS2.2 HFD (n=15)	
		Mean	SD	Mean	SD	Mean	SD
Interstitial Fibrosis	Score	0.00	0.00	0.00	0.00	0.00	0.00
Inflammatory cell infiltrate	Score	1.13	0.62	0.27	0.57	0.93	0.85
Exocrine Degeneration	Score	0.27	0.44	0.13	0.34	0.13	0.34
Endocrine-Islets of Langerhans	Score	2.47	0.88	1.53	1.02	1.87	0.72

16 Appendix 2.

16.1 Study set up

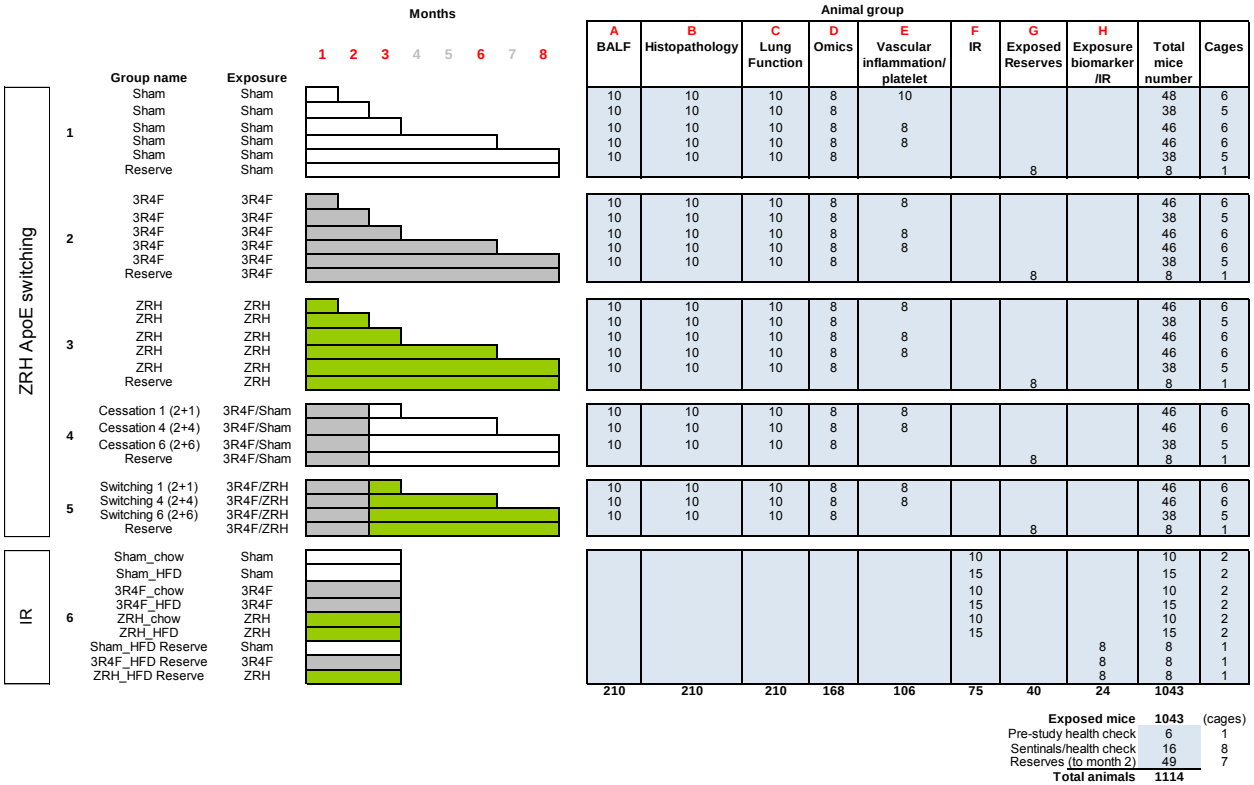


Figure 21. **Schematic representation of the study set-up.** Depicted are the study arms THS2.2 ApoE$^{-/-}$ Switching, and Insulin Resistance (IR), exposure groups, the dissection timepoints, the distribution of the mice to each dissection point, total mice per group, and number of cages per group.

17 Appendix 3.

17.1 Histopathological grading scheme

Severity Codes		Incidence Codes		Miscellaneous Codes	
0	Within normal limits	P	Present	---	Missing
1	Minimal	N	Not Present	x	Not Evaluated
2	Mild				
3	Moderate				
4	Marked				
5	Severe				