Polyphenols from *Passiflora ligularis* Regulate Inflammatory Markers and Weight Gain

Abstract: Weight-related disorders affect more than half of the adult population worldwide; they are also concomitant with a state of chronic low-grade inflammation manifesting in abnormal cytokine production. The present study evaluated the effect of polyphenol and flavonoid extract from *Passiflora ligularis* (granadilla) on low-grade inflammation and body weight in overweight Wistar rats. To induce weight-gain, rats were fed a chow diet with 30% sucrose water and supplemented with 2.0, 2.5, and 3.0 g/L polyphenol extracts (*n* = 16). The design was a 3 +1 factorial model performed for 42 days (granadilla polyphenols, 3 levels of supplementation, and 1 control group). In addition to total polyphenol and total flavonoid content, the major identified and quantified polyphenol, via UHPLC, was ferulic acid. Interleukin 6 (IL-6), and cytokine tumor necrosis factor-alpha (TNF-α) were evaluated in serum. A decline in the concentration of TNF-α and in weight-gain was found in *P*. *ligularis* (granadilla) groups treated with the 2.5 g/L dose. Consumption of polyphenol extracts from granadilla inhibits interleukin-activity as an indicator of inflammation and aids in body-weight control, considering similar food intake, in overweight Wistar rats.

Keywords: *Passiflora ligularis* (granadilla); phenolic compounds; anti-inflammatory activity; overweight rats.

Introduction

Adipose tissue functions as an energy reserve for the organism and as a secretory tissue capable of producing biologically active molecules, such as resistin, leptin, tumor necrosis factor alpha (TNF-α) and interleukins (IL) [1]. These substances activate signalling pathways in the inflammation cascade, leading to macrophage infiltration in adipose tissue and M1 macrophage activation. Macrophages secrete pro-inflammatory cytokines IL-β, IL-6, and TNF-α. Interaction between adipose tissue and the defensive cells aggravates and maintains a systemic inflammatory state from which low-grade chronic inflammation originates [2,3].

There is a growing interest in studying polyphenols to reduce negative effects related to chronic low-grade inflammation present in obesity. These compounds are secondary metabolites of plants that participate in biological processes such as growth, reproduction, and protection from external threats [4]. In animals, polyphenols from the leaves of *Camellia sinensis* (green tea) have shown a positive correlation between their consumption and lowered risk of metabolic disorders related to being overweight [4,5]. Other plants with significant amounts of polyphenols could display similar effects.

Polyphenols such as flavonoids, anthocyanins, and tannins are found in fruits of the *Passiflora* family, including *Passiflora edulis* (maracuyá) and *Passiflora ligularis* (granadilla) [6]. These fruits are cultivated in tropical South American countries and are normally consumed as peeled fruit or in fresh juice form. In Colombia, granadilla is of great economic importance, with an average annual production of 9.3 tons per hectare planted [7]. Thus, *Passiflora* is a potential source of polyphenols with benefits for both animal and human health [6,8,9]. The objective of this research is to evaluate the *in vivo* effects of polyphenol extracts from granadilla on low-grade chronic inflammation in overweight rats. We hypothesized that treatment with different concentrations of polyphenol extract from the fresh pulp and seeds of granadilla could be effective at reducing weight gain or inhibiting inflammatory markers in Wistar rats.
Materials and Methods

Fruit, chemical reactants, and standard reference solutions

Fresh granadilla, a total of 10 kg, was purchased in a local market (Manizales – Colombia). Acetone, sodium carbonate, and Folin-Ciocalteu reagent were purchased from PanReac AppliChem, ITW Reagents, (Darmstadt - Germany); acetonitrile, ammonium formate, formic acid, methanol, sodium dodecyl sulphate, and hydrated sodium taurocholate were purchased from Sigma Aldrich (St. Louis, MO - USA). Sodium nitrite and aluminium chloride were obtained from LOBA Chemie (Mumbai – India), and sodium hydroxide was obtained from EMSURE Merck (Darmstadt – Germany). UHPLC reference standards apigenin, caffeic acid, caffeine, carnosic acid, (±)-catechin, cyanidin, cyanidin-3-rutinoside, (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin, (-)-epigallocatechin gallate, puranic acid, kaempferol, kaempferol 3-glucoside, luteolin, naringenin, p-coumaric acid, pelargonidin, pelargonidin-3-glucoside, pinocembrine, quercetin, quercetin 3-glucoside, kaempferol 3-glucoside, rosmaninic acid, teofiline, theobromine, ursolic acid, and vanillic acid were acquired from Sigma Aldrich (St. Louis, MO - USA). Human tumor necrosis factor alpha (TNF-α) and interleukin – 6 (IL-6) kits were obtained from BioLegend (San Diego, CA - USA).

Polyphenol extraction and quantification

Based on national agricultural industrial standards for Passifloras, granadilla at degree 5 of ripening were selected by simple random sampling by conglomerates, with a variability of 10% in size, shape, and maturing state [10]. The fruits were washed in sodium hypochlorite (50 ppm) and dried with absorbent paper [11]. The pulp and seeds were homogenized in a commercial blender (Oster® XpertSerier™ - USA). The mixture was kept at 4°C prior to the extraction process [12].

The homogeneous mixture, in equal volumes, was placed in 70% acetone, stirred for 20 minutes at 500 rpm (Dragon Lab MS-H Pro® - China), sonicated for 15 minutes (Branson® MH ™ mod 3800 series - USA), and centrifuged at 3500 rpm for 15 minutes (Hermle® Z 206 A). The solvent was rotaevaporated (Scilogex® RE 100 pro - USA), and the polyphenol-rich extract was dehydrated at 50 °C for 96 hours (Incucell® LSIS-S - Germany). Total phenolic content (TPC) was determined in triplicate according to the Folin-Ciocalteu reaction [11]. Samples of 0.50 g of dehydrated granadilla polyphenol-extracts were dissolved in 10 mL of distilled water. From this dilution, volumes of 0.50 mL were mixed with 0.50 mL of distilled water, 1.0 mL of Folin-Ciocalteu reagent with mixing for one minute, and with 2.0 mL of 3.5% sodium carbonate. The solutions were kept in the dark for 90 minutes at room temperature. The samples were read in a range of 620 to 800 nm (UV/VIS Optizen POP®). The total polyphenol content was reported as gallic acid equivalents per 100 g of dehydrated extract (mg GAE/100 g DE).

Total flavonoid content (TFC)

Flavonoids within the largest polyphenol content were quantified via aluminium chloride (AlCl₃) colorimetric assay with modifications [13]. Volumes of 0.5 mL of extract, in triplicate, were placed in test tubes containing 2.0 mL of distilled water. To each solution, 150 µL sodium nitrite (5%) was added, followed by a 5-second vortex. After 5 minutes, 150 µL AlCl₃ (10%) was added under the same agitation and reaction conditions. One mL of sodium hydroxide (1M) and 1.2 mL of distilled water were added for a total volume of 5 mL, then vortexed and incubated for a final reaction time of 5 minutes [13]. TFC was quantified as milligrams of quercetin equivalents (QE) per 100 grams of fresh fruit (mg QE/ 100 g of fresh granadilla-approximate content in one unit of fresh fruit).

UHPLC-MS analysis of phenolic compounds

Dehydrated samples of granadilla extracts were dissolved in 70% methanol (0.2% formic acid), vortexed, and sonicated for 5 min prior to chromatographic assays.
All peak separation and chromatographic analysis of *Passiflora* extract was performed using a UHPLC Dionex Ultimate 3000 (Thermo Scientific, Sunnyvale, CA - USA) equipped with binary pump (HP G3400RS). Polyphenol content was separated with the aid of a Hypersil GOLD Aq (ThermoScientific, Sunnyvale, CA - USA) 100 x 2.1 mm, 1.9 µm column at 30°C. The mobile phase A contained aqueous ammonium formate (0.2%) and B contained acetonitrile with ammonium formate (0.2%). The initial gradient was set at 100% A, switching linearly to 100% B over 8 min before being held at 100% B for 4 min, then returning to 100% A for 1 min. The total running time was 13 minutes followed by three-minute post-runs [8].

A mass spectrophotometer Exactive Plus Orbitrap (Thermo Scientific, Sunnyvale, CA - USA) was connected to an electrospray ion source (ESI) and operated in positive mode with a voltage of 4.5 kV. Spectra were recorded in the range of m/z 60-900 for full scan MS analysis with nitrogen as the nebulizing gas. The Orbitrap MS detector was calibrated with certified reference solutions Ultramark™ 1621 Mass Spec Std (ABCR GmBH & Co., Karlsruhe - Germany), sodium dodecyl sulphate and hydrated sodium taurocholate (Sigma Aldrich, St. Louis, MO - USA). For polyphenol compound identification, a full-scan acquisition and ion extraction chromatogram (EIC) mode corresponding to [M+H]^+ of polyphenols of interest was used. Mass measurements were calculated exactly and with a precision of Δppm< 0.001 using a mixed solution of external phenolic standards, based on comparing calibration curves (concentration range from 0.05 to 5.00 µg/mL).

**Animals, Overweight induction and treatments**

Sixteen adult male Wistar rats, (at least 120 days old) obtained from the same Institutional Bioterium and with an average initial weight of 288 ± 25 g, were kept under standard housing conditions (between 18 and 25°C and 50% relative humidity and 12 hours of daylight) in 800 cm² plastic boxes covered with 5 cm thick rice husk for a period of 42 days.

The 42-day experimental period consisted of a 2-week adaptation period and a 4-week supplementation period for the corresponding intervention. Prior to the polyphenol interventions, the animals (n=4 in each group) were induced to become overweight by being provided a chow diet (28% crude protein, 12% fat, 3% fibre, 10% ash and 10% moisture) and 30% sucrose water for the first two weeks [14]. The rats were weighed (from an initial mean weight of 280 ± 2 g) with an average weight-gain of 305 ± 2 g. The treatments were distributed in a 3+1 factorial model (one source of polyphenol extracts from Colombian granadilla at concentrations of 2.0, 2.5, and 3.0 g/L in drinking water, and one control group with no granadilla-polyphenol supplementation, n=4 in each group). The animals were euthanized following the recommendations for the care and use of laboratory animals of the National Research Council (2001) [15]. This investigation focused on evaluating initial blood biomarkers associated with a direct effect on weight gain by comparing the polyphenol extracts at different concentrations. Future studies using supplements based on granadilla should evaluate other parameters related to inflammation or macrophage activation in tissues.

**Ethical approval:** The research related to animals’ use has been complied with all the relevant national regulations and institutional policies for the care and use of animals. All experiments with animals were done at the Bioterium of Universidad de Caldas in Manizales (Colombia) with the approval of the Ethics Committee for Experimentation with Animals of the Faculty of Agricultural Sciences of Universidad de Caldas (protocol approved and signed February 20, 2018).

**Blood sampling and quantification of cytokines**

A 2.0 mL blood sample was collected from each rat to determine IL-6 and TNF-α concentrations. Serum was obtained by centrifugation at 4500 rpm for 5 minutes (Hettich® EBA 200 - Germany). Quantification of IL-6 and TNF-α was performed via the enzyme-linked immunosorbent assay (ELISA) technique using the RAT IL-6 and RAT TNF-α kits. Plates were read in a Multiskan™ reader (Thermofisher® - USA). Blood samples in a microtainer with anticoagulant EDTA were used for all interleukin evaluations.

**Statistical analysis**

Results were tested for analysis of variance (ANOVA), and for those variables with a significant statistical difference (p<0.05), means were compared by Duncan or Tukey tests. The statistical program STATA 12® was used for all statistical evaluation.
Results

Total Polyphenol Content (TPC) and Total Flavonoid Content (TFC) in pulp and seeds of *P. ligularis* (granadilla) Extract

Polyphenol acetone (70%) extract from fresh pulp and seeds of Colombian granadilla yielded a mean TPC of 986.02 ± 6.17 mg GAE in 100 g of fresh fruit. The equivalence in phenolic content with respect to each diluted dose (2.0, 2.5, and 3.0 g/L) in 100 g of fresh fruit corresponded to 1.97, 2.47, and 2.96 mg GAE respectively. TFC values in terms of quercetin equivalents (QE) were 785.85 ± 22.29 mg QE in 100 g of fresh granadilla corresponding to diluted doses of 1.57, 1.96, and 2.36 mg QE in 100 g of fresh fruit. Figure 2 shows comparative levels of polyphenol and flavonoid quantitation for the three different doses corresponding to the three treatments (drinking water) for Wistar rats. The polyphenol and flavonoid proportions of the different doses registered their highest value of near 80% flavonoids in the highest treatment dose (3.0 g/L).

UHPLC-MS analysis of *P. ligularis* (granadilla) extracts

Colombian granadilla polyphenols were identified by UHPLC-MS in electrospray ionization mode through comparison to several reference standards. Targeted quantification was used to characterize the phytochemical profiles of extracts from *P. ligularis* (granadilla). Sixteen polyphenol compounds were detected and quantified as shown in Table 1.

Concentrations of phytochemicals in the fruit extracts were determined for compounds detected above the LOQ (0.05 µg/mL) (Table 2). The main components detected were phenolic acids (with ferulic acid being the major quantifiable compound), a hydroxycinnamic acid, and several flavonoids. Representative chromatograms in Figure 3 illustrate peaks, retention times, and m/z values for ferulic acid, the most concentrated phenolic compound identified in granadilla. Other predominant compounds detected in granadilla extracts were caffeine, luteolin, apigenin, ursolic acid, quercetin 3-glucoside, and pelargonidin.

Weight gain and food intake

After a 14-day adaptation period, supplementation with polyphenol extracts lasted 28 days (termed the “intervention period”). Table 2 provides the dose equivalence in mg of polyphenols per kg of body weight (BW) per day for the supplemented rats (drinking water with polyphenols), considering a mean extract volume of 1273.5 mL throughout the treatment.
Table 1: Standard reference compounds, with their retention time and mass spectrum MS responses and concentrations for polyphenols in *P. ligularis* (granadilla) extract.

| Reference Standards | Retention Time (min) | [M+H]+ (\(m/z\)) | *P. ligularis* (granadilla) µg/mL |
|---------------------|----------------------|------------------|---------------------------------|
| theobromine         | 3.63                 | 181.07109        | ≤0.02                           |
| epigallocatechin    | 3.80                 | 307.07966        | ≤0.02                           |
| cyanidin 3-rutinoside | 3.90              | 595.16072        | ≤0.02                           |
| (+)-catechin        | 3.97                 | 291.08484        | ≤0.02                           |
| caffeine            | 4.09                 | 195.08672        | 0.02 - 0.05                     |
| (-)-epicatechin     | 4.13                 | 291.08776        | ≤0.02                           |
| epigallocatechin gallate | 4.15            | 459.08990        | ≤0.02                           |
| quercetin 3-glucoside | 4.45              | 465.09987        | 0.02 - 0.05                     |
| cyanidin            | 4.55                 | 287.05416        | ≤0.02                           |
| p-coumaric acid     | 4.63                 | 165.05377        | ≤0.02                           |
| ferulic acid        | 4.71                 | 195.06422        | 0.94                            |
| rosmarinic acid     | 4.78                 | 361.08999        | ≤0.02                           |
| pelargonidin        | 4.82                 | 271.05928        | 0.02 - 0.05                     |
| luteolin            | 5.24                 | 287.03566        | 0.02 - 0.05                     |
| apigenin            | 5.53                 | 271.05874        | 0.02 - 0.05                     |
| ursolic acid        | 9.46                 | 457.36631        | 0.02 - 0.05                     |

Data are concentrations for extracted compounds from pulp and seeds of granadilla. LOQ (limit of quantification, based on a calibration curve ranging from 0.05 to 5.00 µg/mL).

Figure 3: Total Ion Chromatograms (TIC) for extract of dehydrated pulp and seeds of granadilla in 70% acetone and its main comparative standard, ferulic acid. (a) Granadilla extract. (b) Ferulic acid, reference standard.
Average weight at the initiation of the supplementation was 350 g. At the end of the diet adaptation period (14 days), body weight increased 17.7%. The animals received food ad libitum, which contained 28% crude protein, 12% fat, 3% fibre, 10%, ash, and 10% moisture, satisfying the nutritional requirements for Wistar rats, along with 30% sucrose water to induce weight gain [16]. Water containing 2.0, 2.5, and 3.0 g/L polyphenol-rich extract was offered at will during the supplementation period of 28 days. Figure 4 represents food consumption during the study.

A significant statistical difference in weight (Figure 5) with respect to the control group was observed \((p<0.05)\). (a) Average food consumption for the duration of the study (42 days). (b) Daily average grams of food intake per animal. GRA: Granadilla at doses of 2.0, 2.5 and 3.0 g/L. No significant differences were detected for food consumption.

### Table 2: Polyphenol-supplementation per kilogram of body weight per day and equivalence to a daily dose for a 60-kg human adult.

| Dose of Extract (g/L) | 2.0 | 2.5 | 3.0 |
|-----------------------|-----|-----|-----|
| Polyphenol content/dose | 1.97 | 2.47 | 2.96 |
| Polyphenol human dose  | 1183.23 | 1479.03 | 1774.84 |
| Number of fresh fruits | 1.20 | 1.50 | 1.80 |

Data correspond to TPC (mg) in each dose and daily average volume of extract consumed by each animal during the intervention period (28 days). Data also represent TPC values for comparative daily intake of fresh granadilla for a human adult.

### Tumor necrosis factor alpha (TNF-α)

Serum TNF-α was statistically reduced in animals supplemented with 2.5 g/L of polyphenols from *P. ligularis* (granadilla) compared to both the lower-dose and control groups \((p=0.03)\) (Table 3). For animals treated with granadilla polyphenol-extracts at a concentration of 2.5 g/L (1.75 mg/kgBW/day), TNF-α concentration was lowered more than 2%.

### Interleukin 6 (IL-6)

The concentration of serum IL-6 was not different between different doses of polyphenol extract, nor did it show a statistically significant effect relative to the control group. The highest IL-6 decrease was registered at over 6% in rats treated with *P. ligularis* (granadilla) extracts with a TPC of 2.96 mg per kg of body weight, highlighting a flavonoid proportion of 79.9%.

### Discussion

### Total polyphenol content in *P. ligularis* (granadilla)

The TPC of *P. ligularis* (granadilla) in this study agrees with research in which TPC was quantified in different
Ecuadorian fruits; this study found *P. ligularis* to contain 91 mg GAE per 100 g of fresh fruit, equivalent to a range of 325 to 455 mg GAE per 100 g of dehydrated Colombian granadilla, depending on humidity [17]. These authors classified granadilla as a fruit with low phenolic content.

On the contrary, in an experiment with 24 Colombian fruits, *P. ligularis* was classified as a fruit with a high TPC, with one study reporting 664.8 mg GAE per 100 g of fruit in freeze-dried powder and another showing 637.7 mg GAE per 100 g of dry matter (results in the present study are all reported per 100 g of fresh fruit) [18]. The polyphenol doses used in the present study (2.0, 2.5, and 3.0 g/L) correspond respectively to 1.6, 2.0, and 2.4 mg per kg of body mass per day in each animal, based on a dissolved polyphenol-rich extract consumption of less than 1300 mL throughout the intervention.

Weight gain and food consumption

Treatment with 3.0 g/L granadilla polyphenol extract statistically reduced weight gain in Wistar rats despite showing no significant difference in food consumption from the control group. A study using a similar dose (3.2 g/L) of polyphenol extracts from green tea reported significant differences in weight gain from week nine [19]. Similarly, other researchers that treated rats with green tea extract at 5.0 g/L in drinking water found results consistent with those of the present study [20]. The relationship between polyphenol consumption and weight gain reduction has been explained from multiple physiological perspectives [21,22]. Researchers have proven that polyphenols can diminish dietetic lipid absorption; moreover, there is evidence that polyphenols can modulate genes involved in lipid metabolism and hinder formation of adipose tissue [23,24,20].

Considering that being overweight is a determinant factor contributing to metabolic syndrome and has a prevalence of more than 30% in the USA and China, the...
results of the present work show promising alternative approaches with regard to weight gain control [25,26,27]. More studies in humans, including research of high-polyphenol content in regular diets, suggest the possibility of preventive approaches for metabolic syndrome [28].

**Inflammatory markers**

A significant decrease in serum TNF-α was observed in overweight rats supplemented with polyphenols. Rats treated with the 3.0 g/L dose showed decreased IL-6 levels by about 6%, but this change was not statistically significant. Lu et al. reported that IL-6 levels decreased by the action of green tea polyphenol-supplementation using a 5.0 g/L dose in rats fed a high-fat diet [20]. These authors also found a decrease in circulating IL-1β, showing that these polyphenols reduce the chronic inflammation generated by high-fat diets. The highest granadilla polyphenol extract dose used in the present investigation was 3.0 g/L, and supplementation with higher polyphenol contents could suggest statistically significant inhibition of IL-6.

Our results are consistent with those of prior studies reporting decreased IL-6 and TNF-α following supplementation of rats with 200 mg/kg/day green tea [20,30]. These authors attributed their results to the capacity of polyphenols to protect animals from fatty liver disease, as the cytokines IL-6 and TNF-α manifest during disease progression and with the appearance of fibrotic lesions [31,32]. Results of the present study concur and are complementary to in vitro assays evaluating the inhibitory action of polyphenols from *Passifloras* on inflammatory markers tested for transepithelial/endothelial electrical resistance (TEER) [8]. Different phenolic compounds, as identified by UHPLC/MS, showed positive and statically inhibitory action against an inflammatory cocktail containing IL-1β, TNF-α, IFN-γ, and pro-inflammatory lipopolysaccharides [8].

Studies with *Citrus unshiu* supplementation in db/db mice resulted in significantly lowered IL-6 and TNF-α, as well as increased adiponectin [33,34,35]. The relationship between the effect of adiponectin and the anti-inflammatory activity of polyphenols was reported by Tian et al. (2013) [19], who suggested that these compounds either increase expression of the peroxisome proliferator-activated gamma receptor (PPARγ) or inhibit its phosphorylation, stimulating production of adiponectin and regulating production of IL-6 and TNF-α [19,36,37]. Polyphenols are anti-inflammatory agents that inhibit nuclear factor kappa B (NF-kB) and inhibit the induction of expression of pro-inflammatory genes, such as TNF-α, IL-1β or IL-8 [36]. Other results in our research suggest that polyphenols in *P. ligularis* (granadilla) can regulate in vitro specific nuclear mechanisms responsible for low-grade inflammation and weight gain. With respect to a healthy digestive system, the inflammatory markers TNF-α and IL-1β stimulate cell signalling routes that affect membrane integrity and are normally registered as lower transepithelial/endothelial electrical resistance (TEER) values [8]. Positive TEER effects, based on treatment of Caco-2 cells with different doses of polyphenol-extracts from Colombian *Passifloras*, suggest a direct correlation with the results of in vivo assays in the present work [8].

**Conclusion**

The pulp and seeds of Colombian *P. ligularis* (granadilla) are an important source of polyphenols, with percentages of total flavonoid content reaching around 80%. Polyphenol doses of 2.5 and 3.0 g/L have the ability to reduce weight gain in rats. This dietary content of polyphenols suggested as beneficial for human consumption is found in two fresh Colombian granadillas. The evaluated extracts supplemented in the daily diet of Wistar rats were associated with no differences in food consumption, but were statistically correlated with weight gain. Polyphenol treatments inhibited weight gain throughout the study. Results in overweight rats shown in this study illustrate a promising inhibiting action with respect to weight control during consumption of high-calorie diets supplemented with different doses of polyphenol extracts.

Polyphenol extract treatments also statistically decreased serum concentrations of the pro-inflammatory cytokine TNF-α and reduced IL-6 levels in overweight rats, suggesting an ability to reduce the risk of low-grade chronic inflammation by acting as inhibitory agents during interleukin activity. Transepithelial/endothelial electrical resistance in vitro assays in Caco-2 cells corroborate the inhibitory action of polyphenols and flavonoids from *Passiflora* extracts. More in vivo and human studies would help to evaluate the dietary benefits associated with inclusion of *Passifloras*, either as fresh fruit or as extracts. Exploration of inhibitory activity against additional cytokines and plasma lipid regulation studies are recommended.

It is also advised to propose further in vivo studies, ultimately culminating in human trials, using *P. ligularis* (granadilla) extracts to evaluate other parameters related to inflammation or macrophage activation in different metabolic pathways. Considering the high presence of
flavonoids in the total polyphenol content of *P. ligularis* (granadilla) and the positive *in vitro* and *in vivo* results indicating inhibition of low-grade inflammatory stages, this study highlights the benefits of the consumption of *Passifloras* as part of a regular diet.

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**Conflicts of Interest:** Authors state no conflict of interest.

**Data Availability Statement:** The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

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