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Sex- and age-dependent energetic dysfunction in LRRK2 G2019S Parkinson's model revealed by structural analysis of RGC dendritic architecture and metabolic profiling of optic nerve.

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Abstract

Pathological changes in the retina of Parkinson's disease (PD) patients precede diagnostic motor and cognitive symptoms; however, the temporal and sex-specific features of this prodromal pathology remains unclear. To begin to resolve this, we investigated retinal ganglion cell (RGC) structure and optic nerve metabolism in male and female LRRK2 G2019S knock-in mice and wild-type littermates at 4, 8, and 72 weeks, using a combination of Sholl analysis and liquid chromatography–mass spectrometry. Male LRRK2 G2019S mice exhibited RGC structural abnormalities at 4 weeks, whereas comparable changes emerged in females at 8 weeks. Metabolomic profiling revealed distinct age- and sex-dependent alterations, including reduced purine and riboflavin metabolism in young males and increased nicotinate, nicotinamide, and butanoate metabolism in age-matched females. Differing sex-specific metabolic start points and trajectories converged with age, suggesting distinct prodromal mechanisms leading to a shared pathological endpoint. These retinal signatures may facilitate early PD staging and precision medicine.

Parkinson's disease (PD) is the second most common age-related neurodegenerative disorder and is projected to affect more than 25 million people worldwide by 2050¹. Despite its increasing prevalence, early diagnosis and treatment is still not possible, and understanding of the dysfunction occurring at this stage is still not completely defined. This is confounded by the observation that in sporadic PD there is a sex bias in disease manifestation with more male individuals developing the disorder². Furthermore, data suggests this interaction is even more complex in genetic forms of the disease such as LRRK2-PD where the overall sex bias is reduced^{2,3} but variants with early and prominent cognitive dysfunction again shows a male bias⁴⁻⁷. Thus, understanding why these differences occur and their early manifestation is critical for pathomechanistic identification and disease stratification allowing for appropriate diagnosis and treatment.

Although clinical diagnosis of cardinal motor signs (including resting tremor, rigidity, bradykinesia and postural instability) remains the gold standard, non-motor features are emerging as potential diagnostic tools. One putative measure is that of retinal degeneration^{8,9}. Loss of retinal ganglion cells (RGCs) has been shown to be present at early stages in rat rotenone PD models accompanied by retinal thinning as measured by optical coherence tomography¹⁰. This pathology has been shown to translate to human cohorts and scales with clinical severity and dopamine transporter loss^{11,12}. These data not only make it a potentially powerful biomarker for disease but, a tissue type useful for studying the earliest stages of disease and the differences sex may exert. Defining a prodromal phase during which disease-modifying interventions may be most effective. Identifying molecular alterations during the prodromal phases that occur before overt neurodegeneration therefore remains a major challenge in PD research and would result in the identification of important pathophysiological mechanisms and targets.

Accumulating evidence indicates that metabolic dysfunction is a key early and fundamental feature of PD and one which may yield insight into disease pathways¹³. Mitochondrial impairment, altered glucose utilisation, oxidative stress and disruption of cellular bioenergetics have been reported across experimental models, patient-derived cells and post-mortem brain tissue, suggesting that failure of metabolic homeostasis contributes to neuronal vulnerability long before clinical diagnosis¹⁴⁻¹⁶. Although these metabolic alterations are increasingly recognised, the relationship to sex and temporal evolution in the context of PD remains poorly understood.

RGCs possess exceptionally high metabolic demands owing to their continuous electrical activity and long unmyelinated axons, requiring one of the highest mitochondrial densities within the central

nervous system. Like nigral dopaminergic neurons, RGCs rely predominantly on oxidative phosphorylation to sustain ATP production and are therefore particularly susceptible to disturbances in mitochondrial function and energy metabolism¹⁷⁻¹⁹. As an accessible extension of the central nervous system, the retina shares many structural, molecular and metabolic characteristics with the brain. Thus, the retina provides a unique opportunity to investigate early pathological events in a sex and temporal specific manner.

Mutations in leucine-rich repeat kinase 2 (LRRK2), particularly the G2019S variant, represent the most common genetic cause of familial PD and are also detected in sporadic disease²⁰⁻²⁵. Increasing evidence suggests that LRRK2 regulates multiple processes central to cellular metabolism²⁰⁻²⁷, therefore, investigating metabolic trajectories in male and female LRRK2 mice may provide important insight into early adaptive responses that influence disease progression²⁸.

Here, we combined structural analysis of RGC dendritic architecture with untargeted metabolomic profiling of the optic nerve (RGC axon bundles whose changes directly correlate to RGC²⁹) to characterise metabolic changes across early development and through ageing in male and female wild-type and LRRK2 G2019S mice³⁰. By examining animals at 4, 8 and 72 weeks of age, we identify sex-specific metabolic trajectories associated with early neuronal structural changes and demonstrate that LRRK2 disrupts normal metabolic maturation before progressively converging on a common age-associated metabolic phenotype. These findings establish retinal metabolism as a sensitive readout of early Parkinson's disease-related dysfunction and highlight metabolic pathways that may represent candidate targets for interventions aimed at preserving neuronal resilience before irreversible neurodegeneration occurs. Critically these putative targets and perturbed pathways are sex dependent and thus creates a basis for stratification of aberrant biochemical pathways in PD.

Materials and methods

Animal strain and husbandry

Animals, males and females LRRK2 G2019S^{+/-} knock-in mice (B6.Cg-Lrrk2^{tm1.1Hlme}/J; strain #030961 – RRID:IMSR_JAX:030961), and their control litter mates, were co-housed in environmentally enriched cages with a 12h/12h light/dark cycle with food and water available *ad libitum*.

Ethics declaration

Experiments were performed in compliance with the Home Office Animals (Scientific Procedures) Act 1986 (PPL P49E8C976, PIL I73025511).

91 Experimental cohorts

92 The data collected were based onto an experimental cohort of 6 mice for each age point (4 weeks, 8
93 weeks and 72 weeks), sex (male and female) and genotype (wild type and LRRK2 G2019S KI). For optic
94 nerve untargeted low molecular weight metabolomics samples contaminated by blood were excluded
95 from the analysis. Metabolomics raw data provided in the supplementary materials. Power
96 calculations were performed on G*Power (version 3.1). *A priori* power calculations were performed to
97 decide the minimum sample size based on Wilcoxon-Mann-Whitney test of the means of two groups
98 for a predicted Sholl AUC change due to metabolic perturbation according to previous published
99 work²⁹. Based on a mean $\pm$ SD of group 1 (4151.87 ± 2120.55) and group 2 (2552.53 ± 1201.66), the
100 calculated effect size was 0.97. With a two-tailed comparison with α of 0.05 and power of $(1 - \beta)$ of
101 0.95, the required sample size is $N = 5$ animal/group.

102 Analysis of retinal ganglion cell dendritic structure.

103 To analyse RGC structure, mice were euthanised by cervical dislocation. Eyes were enucleated and
104 fixed in 1.5% paraformaldehyde (PFA)³¹, and kept in 1.5% PFA until dissected. For retinal dissections,
105 eyes were transferred into HBSS, retinas dissected and placed ganglion cell layer up onto a microscope
106 slide for DiOlistic labelling^{29,32}. RGC dendritic structure was assessed in flat-mounted retinas by Diolistic
107 labelling of individual retinal ganglion cell dendritic tree (Helios gene gun system, Bio-rad). 80 mg of
108 1.7 μ m M-25 Tungsten particles (Bio-rad) were coated with methylene chloride (400 μ L)-dissolved 1,1'-
109 Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (Dil, 2 mg; Invitrogen) and 3,3'-
110 Dioctadecyloxycarbocyanine Perchlorate (DiO, 4 mg; Invitrogen). Coated particles were then
111 distributed onto the interior surface of Tefzel tubing (30 cm; Bio-Rad) which was then cut into 12 mm
112 segments. Dil/DiO- coated tungsten particles were delivered (100–110 psi, helium) with the gene gun
113 to the retinal flat mount, 5 cm from the retina surface through a 3 μ m polyethylene terephthalate
114 membrane (Fisher Scientific) to prevent tungsten particle clumping. Flat mounted retinas were then
115 incubated overnight in HBSS at room temperature, fixed for 1 h in 4% paraformaldehyde (PFA), washed
116 in 1 M PBS, nuclei labelled with Hoechst (1:1000 in PBS) and washed again. Retinas were then mounted
117 with FluoroSave (Millipore) mounting media, coverslipped, and dried at room temperature for 1 h.

118 Images of RGC dendritic fields were taken with Leica SP8 confocal microscope (Leica; 20X
119 magnification, 0.345 μ m/pixel, 1 μ m z-thickness). RGCs were identified by their location in the ganglion
120 cell layer, the presence of an axon projecting to the optic nerve, and a dendritic tree extending within
121 the inner plexiform layer (IPL). Only the cells that fulfilled these selection criteria were imaged and
122 traced. Complete dendritic fields were reconstructed using Imaris software (version 9.3.1, Bitplane),

where individual retinal ganglion cell dendritic fields were manually selected as areas of interest and dendrites automatically traced using the filament tool ³². Any tracing errors were corrected manually. Statistical parameters were exported from Imaris for Sholl analysis at set intervals of 10 µm. All experimental procedures by the same investigator (GC). To avoid any bias, the genotype was masked during dendritic reconstruction and unmasked following analysis of the raw data.

Low molecular weight enriched metabolomics

Metabolomics were performed as previously described^{29,33}. Briefly, mice were euthanised by cervical dislocation, and eyes with attached optic nerves were enucleated. Optic nerves were severed at the optic nerve head and immediately snap frozen. The tissue was stored at -80° C and shipped on dry ice to the Karolinska Institutet Small Molecule Mass Spectrometry Core Facility (Solna, Sweeden) for processing. Prior analysis, samples were divided into extraction batches keeping samples from the same group together. The samples were first placed on a tray in dry ice. Then, a volume of 400 µL of ice-cold LC-MS methanol was added to each tube. Tubes were then closed, and samples were quickly vortexed to ensure that the optic nerve was in solution. Then, after an additional vortex step of 5 seconds, samples were placed in an ultrasound bath with ice (so that the temperature does not increase above 10 °C) and sonicated for 15 minutes (Sweep mode). Samples were afterwards centrifuged at 12000 g for 12 minutes (8°C). Finally, 200 µL of the supernatant was transferred to an LC-MS vial equipped with a 300 µL insert, ready for injection on the polar platforms. Additionally, around 30 µL of each supernatant from the first batch were pooled together in one Eppendorf tube. The tube was then capped and vortexed and 200 µL of the mix was transferred to five LC-MS vials equipped with a 300 µL insert to be used as a quality control of injection (QCInj).

Data were collected in polar positive and negative ionization mode. For polar positive collected data: chromatographic column, ACQUITY Premier BEH Amide Vanguard FIT, 2.1 x 100 mm, 1.7 µm; guard column, Vanguard FIT (integrated in column); mobile aqueous phase (A), 20 mM ammonium formate + 0.1% formic acid in Milli-Q H₂O; mobile organic phase (B), 0.1% Formic acid in LC-MS acetonitrile. Chromatographic gradient (minute, %B, flowrate µl/min), 0.0, 95, 400; 1.5, 95, 400; 12.0, 61.5, 400; 12.2, 52, 400; 13.2, 52, 400; 13.5, 95, 400; 14.2, 95, 475, 18.5, 95, 475; 18.7, 95, 400. Column temperature 30° C, injection volume 2 µl. For polar negative collected data: chromatographic column, Atlantis Premier BEH Z-HILIC Vanguard FIT, 2.1 x 100 mm, 1.7 µm; guard column, Vanguard FIT (integrated in column); mobile aqueous phase (A), 10 mM ammonium acetate at 9.2 pH (adjusted with NH₄OH) in double deionized water; mobile organic phase (B), LC-MS acetonitrile. Chromatographic gradient (minute, %B, flowrate µl/min), 0.0, 85, 450; 3, 79, 450; 6, 60, 450; 7.0, 45, 450; 7.5, 40, 450; 10, 40, 450; 10.5, 85, 450; 12.8, 85, 450. Column temperature 30° C, injection volume 1.5 µl. Hundred

fifty-six low molecular weight metabolites that could be verified with standards were detected. Metabolites were quantified as area under the curve of the mass spectrometry peak. Since the optic nerves were collected to have all the same weight and internal standard data for AMP showed a stable intensity suggesting that normalisation was not necessary, samples data were not furtherly normalised to tissue weight. Data were analysed using MetaboAnalyst³⁴⁻³⁷. Groups were compared by two-sample *t*-test with an adjusted *p* value cut off of 0.05 considered significant. Metabolic analyses were undertaken using Pareto scaling. Pathway analysis was performed in MetaboAnalyst using the *Mus musculus* Kyoto Encyclopaedia of Genes and Genome (KEGG) library.

Statistical analysis

Statistical analyses were performed in GraphPad Prism (version 11.0.2(92)). The Normality of the data sets was tested using the *Shapiro-Wilk* test. Two group comparisons were evaluated by *t*-test or *Mann-Whitney U* test as appropriate. To compare three or more groups one-way *ANOVA*, two-way *ANOVA*, or *Kruskal-Wallis* one-way *ANOVA* were used as appropriate, followed by *Tukey post-hoc* or *Dunn's* test respectively.

Results

Retinal ganglion cell changes over time are sex and genotype dependent

First, we sought to establish whether retinal degeneration seen in chemically induced models of PD and PD patients was observed in our genetic model^{9,38-43}. Using retinal explants taken from LRRK2 mice we performed DiOlistics and assessed the resultant stained cells using Sholl analysis. Data showed no major significant difference compared to littermate controls among the groups (4w, 8w, and 72w; no sex split, supplementary Fig. 1). However, when separated by sex, a reduction in neuritic arborization was observed in male LRRK2 mice at 4 weeks (Fig. 1 A and B) and this was still present at 8 weeks (Fig. 2 A and B). In contrast, a similar reduction in arborization was observed in female LRRK2 mice only at 8 weeks (Fig. 2 C and D). These differences were not apparent at 72 weeks due to a reduction in wild type arborisation in both sexes. Direct comparison of sexes showed a generally more complex architecture in the female compared to the males, at 4 weeks (Fig. 1 E). At 8 weeks this greater complexity of the female LRRK2 group was lost (Fig. 2 C and D). Although the Sholl curves of both male and female cohorts looks similar (WT vs LRRK2), we observed the opposite trend, with male RGCs acquiring dendritic complexity at this age point. At an advanced age point (72 weeks), all the differences that characterised each age point were lost (Fig. 3 A - D), with no major differences between the groups (Fig. 3 E).

Temporal metabolic analysis revealed genotype sex specific differences.

Having confirmed a genotype specific early RGCs degeneration with sex and age dependence, we next sought to determine whether any specific metabolic changes accompanied the degeneration. First, we verified if similar sex specific differences could be observed in the metabolomic analysis (Fig. 5 A – F). At each age point both WT and LRRK2 male and female groups differed metabolically, which confirmed our RGC structural observations. At 4 weeks in the WT group the abundance of more than 30 metabolites was significantly decreased in the females compared to the males (e.g. NAD, choline; Fig. 5 A). However, in the female LRRK2 group an increased abundance of 15 metabolites (e.g. pyruvate, choline, tyrosine; Fig. 5 D) was observed. At 8 weeks the WT metabolic profile reversed (Fig. 5 B). In contrast with the metabolic fingerprint at 4 weeks, at 8 weeks point around 20 metabolites were more abundant in the females (e.g. nicotinamide, glycerol-3-phosphate; Fig. 5 B), with no metabolites appearing in the left side of the volcano plot. For the LRRK2 (Fig. 5 E), as suggested by the Sholl analysis, we observed a more homogeneous metabolic profile between the two sexes, as shown by the alignment in dendritic complexity at 8 weeks. At 72 weeks female and male WT groups did not show any great imbalance in their energetic profile, with only 3 metabolites less abundant in the females (Fig. 5 C). In contrast, at 72 weeks in the LRRK2 G2019S model we started to observe again metabolic differences between the two sexes (Fig. 5 F). At that specific late time point, female LRRK2 showed both increased and decreased metabolic changes, with 3 upregulated metabolites involved in energy forming cascades (e.g. NAD).

Thus, given the sex dependent differences observed both in the RGC arborization and metabolism, we decided to assess the metabolic status at each age point separately.

Age and sex specific metabolic analysis revealed sex-related energy changes in LRRK2.

To define the differences that could determine a pathological fingerprint, we analysed the metabolic differences caused by the G2019S mutation versus controls within the same sex, at the specific time points (Fig. 6-8). Metabolic analysis of female mice at 4 weeks (Fig. 6 A-C), showed a significant metabolic upregulation in the LRRK2, with 47 over abundant metabolites (e.g. NAD, nicotinamide, nicotinamide riboside, tyrosine; Fig. 6 A) creating a significant impact on 25 pathways, among which tyrosine biosynthesis and metabolism, nicotinamide and glutathione metabolism had the biggest shift compared to the control, along with changes in the TCA cycle (Fig. 6 B and C). In contrast, at the same age point, LRRK2 male metabolic snapshot, showed a downregulation of 9 metabolites (e.g. glycerol-3-phosphate, choline; Fig. 6 D), with a general significant effect on only three pathways, mainly lipid metabolism (Fig. 6 E and F).

At 8 weeks, we could not observe any energetic discrepancy when female LRRK2 were compared to control, which resulted in no major pathways imbalance (Fig. 7 A-C). A similar tendency was observed for the male groups, with just the caffeine metabolism pathway slightly affected by the metabolic differences (Fig. 7 D-F). With aging the metabolic scenario shifts again (Fig. 8). At 72 weeks, female LRRK2 displayed a mixed metabolic imbalance (metabolic up- and down-regulation) compared to control (Fig. 8 A), significantly affecting 13 pathways with pathological relevance as the tyrosine metabolism pathway (Fig. 8 B and C). In contrast, metabolomics analysis of the LRRK2 male, identified a less prominent metabolic phenotype with 5 downregulated (e.g. NAD) and 2 upregulated (e.g. benzoic acid) metabolites compared to controls (Fig. 8 D), with an overall influence on only two pathways (Fig. 8 E-F).

Metabolomics changes overtime show a clear sex-specific metabolic phenotype linked to LRRK2

Although we could evidence metabolic changes between sex and genotype at specific time points, we undertook a specific set of metabolic analysis to pinpoint the metabolic changes overtime, by mean of progressive changes based on sex and genotype conditions. Starting with females (Fig. 9 and 11), we observed several metabolic shifts in the progression from 4 to 8 weeks (Fig. 9 A and 11 A). Analysis showed, around 70 downregulated metabolites in WT (Fig. 11 A) compared to 16 in the LRRK2 group (Fig. 9 A). However, none of those metabolites were similar between the two group, suggesting a different energetic developmental path, with only three common pathways, glycolysis and gluconeogenesis, fatty acid degradation and nicotinamide metabolism (Fig. 9 B and C, Fig. 11 B and C).

In contrast, 8 to 72 weeks transition addressed almost no changes in the WT (Fig. 11 D-F), with nicotinamide metabolism and fatty acid degradation pathways still relevant, whereas the changes in LRRK2 female showed a more dysregulated metabolic profile (Fig. 9 D-F). This late-stage progression addressed an upregulation of the main biochemical components of the glycolytic pathways (e.g. glucose-6-phosphate and fructose-6-phosphate; Fig. 9 D) suggesting a compromised energy catabolism (cellular respiratory pathway). Indeed, ingenuity pathway analysis revealed significant disruption in all the main energy forming pathways, as TCA cycle and glycolysis, with a shift to fatty acid degradation as a compensatory mechanism (Fig. 9 E and F).

Male metabolic temporal progression showed a different tendency compared to the females (Fig. 10 and 12). In the WT, 4 to 8 weeks transition was defined by a quite homogeneous metabolic profile, with no major shifts (up or down), and no impact on any pathway, except for caffeine metabolism (Fig. 12 A-C). In the LRRK2, temporal progression analysis showed a consistent downregulated metabolic

profile, with 18 significantly downregulated metabolites (e.g. pyruvate, fructose, and phenylalanine; Fig. 10 A), which overall influenced dopamine related pathways (e.g. tyrosine metabolism, phenylalanine metabolism and tyrosine biosynthesis), and TCA cycle (Fig. 10 B and C). Proceeding to the 8 to 72 weeks metabolic transition, the impact of age was defined by a metabolic readjustment in the WT (Fig. 12 E and F), with changes mainly affecting the fatty acid degradation pathway, which was upregulated at 72 weeks. Similarly, LRRK2 aging metabolic transition shifted the energy production toward fatty acids as suggested by carnitine increase at 72 weeks (downregulation 8 weeks means upregulation at 72; Fig. 10 D) coupled with the significant functional increase in the fatty acid degradation pathway (Fig. 10 E and F).

Discussion

This study demonstrates that retinal pathology associated with LRRK2 G2019S is age- and sex-dependent and is detectable early in life. By integrating RGC morphology with optic nerve metabolomic profiling across three age cohorts, we show that structural and metabolic alterations emerge during early postnatal development, follow distinct trajectories in males and females, and become more similar with aging. These findings identify the retina–optic nerve system as a sensitive model of early LRRK2-associated dysfunction and indicate that biological sex influences its temporal evolution.

Maintenance of neuronal structure requires efficient energy metabolism, and metabolic impairment is increasingly recognised as a central feature of Parkinson's disease^{22,44-46}. RGCs have high energetic demands because of their continuous electrical activity and long unmyelinated axons, and they rely heavily on oxidative phosphorylation to maintain dendritic and axonal integrity¹⁹. This energetic burden may make the retina particularly sensitive to early bioenergetic disturbance and therefore useful for examining disease-associated changes before overt neuronal loss is apparent^{47,48}.

Consistent with this vulnerability, male LRRK2 mice exhibited reduced dendritic complexity together with metabolic suppression at four weeks of age. Reductions in purine and riboflavin metabolism, alongside changes in metabolites associated with mitochondrial energy production, suggest impaired oxidative metabolism, diminished cofactor availability and reduced antioxidant capacity during a critical developmental period^{49,50}. Their association with reduced dendritic complexity supports a relationship between early bioenergetic disruption and altered neuronal structure, although the present data do not establish causality. However, previous published data showed direct metabolic influence on RGCs structural preservation as a measure of neuronal functional integrity^{29,33}.

In contrast, female LRRK2 mice demonstrated preserved, dendritic complexity at four weeks despite carrying the same pathogenic mutation. The comparative difference to the male LRRK2 model is seemingly the different metabolic state. This showed a broad upregulation of pathways involved in mitochondrial metabolism, antioxidant defence, nucleotide synthesis and cellular maintenance. Rather than representing enhanced basal metabolism, these changes may reflect an early adaptive response that temporarily preserves neuronal homeostasis under metabolic stress^{46,51-53}. Consistent with this interpretation, the early metabolic advantage observed in females was no longer evident at eight weeks, when reduced dendritic complexity emerged. Thus, the principal early effect of sex may lie not in protection from LRRK2-associated dysfunction, but in the timing and nature of the response to it.

Longitudinal profiling indicated a biphasic pattern of metabolic changes, with extensive developmental reprogramming between four and eight weeks followed by more selective changes between 8 and 72 weeks. Wild-type animals showed metabolic maturation consistent with the establishment of adult homeostasis, whereas LRRK2 G2019S altered these age-related trajectories. The mutation therefore appears to perturb metabolic maturation rather than simply reproduce an accelerated aging profile. Importantly for understanding sex differences in LRRK2-associated PD, this early disruption was expressed differently in males and females before the metabolic profiles became more similar with age.

Despite their distinct early responses, aged male and female LRRK2 mice shared features consistent with impaired mitochondrial fatty acid oxidation, reduced tricarboxylic acid cycle activity, altered one-carbon and polyamine metabolism, and decreased antioxidant buffering. These changes suggest an age-related loss of metabolic resilience extending beyond mitochondrial dysfunction alone. Nevertheless, convergence was incomplete. Female mice retained evidence of enhanced glycolytic and pentose phosphate pathway activity, potentially supporting glucose utilisation and NADPH-dependent redox homeostasis, whereas aged males showed a more globally suppressed profile with less evidence of compensatory adaptation.

These persistent differences suggest that females may retain greater metabolic plasticity in the presence of progressive mitochondrial dysfunction. The early metabolic activation observed in females was associated with delayed dendritic abnormalities but did not prevent their later emergence. Metabolic resilience, rather than the abundance of any single metabolite or pathway at one age, may therefore influence the timing of neuronal dysfunction. This interpretation offers a testable framework for sex differences in PD. Specifically, female adaptive responses may shift the

temporal onset of pathology without providing permanent protection. Direct manipulation of the implicated pathways will be required to determine whether these responses are protective, compensatory or secondary to altered development, before testing would be possible.

Interestingly, similar metabolic perturbations have been reported in a two-hit mouse model of PD. Using the same LRRK2 knock-in mice as the presented study, and exposing the animals to a high fat diet, Ma *et al.* observed perturbations similar to our own in 16 months old mice⁵⁴. This work not only validates the metabolic alterations as a key part of PD biology, but highlights the strength that redouts from the retina offer as early diagnostic and the value of metabolomics investigation for early interventions.

The correspondence between RGC dendritic remodelling and optic nerve metabolic changes suggests that combined structural and molecular profiling can reveal genotype-, sex- and age-dependent disease features. These changes may be evident due to retinal neurons possessing limited capacity for metabolic compensation, thus subtle genotype-dependent disturbances become detectable before widespread neurodegeneration develops. The retinal structure is clinically accessible, but further work in brain is required to determine whether the optic nerve metabolic signatures identified here are reflected in measurable retinal or systemic biomarkers and whether they correspond to pathology elsewhere in the nervous system.

Whilst there may be limitations, such as samples being representative of cross-sectional cohorts rather than repeated measurements in the same animals, our data demonstrate that LRRK2 G2019S disrupts normal metabolic maturation in a sex-dependent manner long before overt neurodegeneration. Early metabolic adaptation delays, but does not prevent, structural pathology in females, whereas males exhibit earlier metabolic failure and neuronal vulnerability. With aging, both sexes converge toward a common state characterised by impaired mitochondrial function and loss of metabolic resilience. These findings identify metabolic changes as an early determinant of LRRK2-associated retinal pathology, emphasise the importance of considering sex as a biological variable, and support retinal metabolic profiling as a promising strategy for identifying biomarkers and therapeutic targets during the prodromal stages of Parkinson's disease. Further work looking at the connection between pathway level metabolic changes and dendritic architecture is required in future work. In addition, future work should look at regional (brain area) and spatial (different areas of the cell) to establish temporality of metabolic dysfunction and provide a fingerprint for neuronal dysfunction.

344 Conclusions

345 These findings identify sex as a determinant of the timing of early LRRK2-associated retinal pathology.
346 Female mice showed a transient metabolic response associated with delayed dendritic abnormalities,
347 whereas males exhibited earlier structural and metabolic disruption; with aging, the two sexes
348 developed increasingly similar features of impaired mitochondrial function and reduced metabolic
349 resilience. Defining the mechanisms that support the early female response may reveal strategies for
350 prolonging neuronal homeostasis. More broadly, the results demonstrate that both sex and disease
351 stage should be incorporated into the investigation and development of metabolism-based
352 interventions for Parkinson's disease.

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357 Author contributions

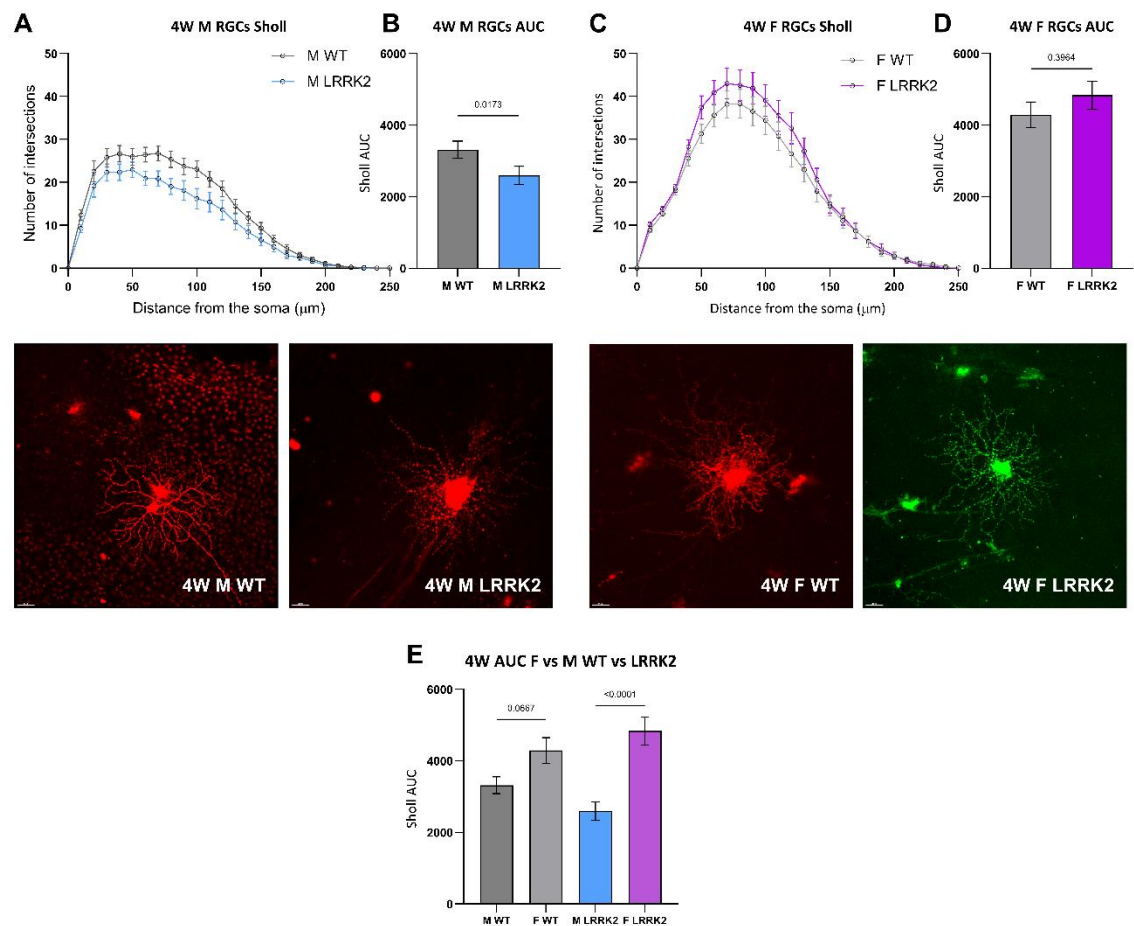
358 GC – conceived, designed and performed the experiments, analysed the data and wrote the
359 manuscript; JRT – provided resources; PAW – edited the manuscript, provided funding and resources;
360 JEM – part funding acquisition; MV – conceptualisation, part funding acquisition, writing – review and
361 editing; DBK – was involved in conception, edited the manuscript, provided funding and resources.

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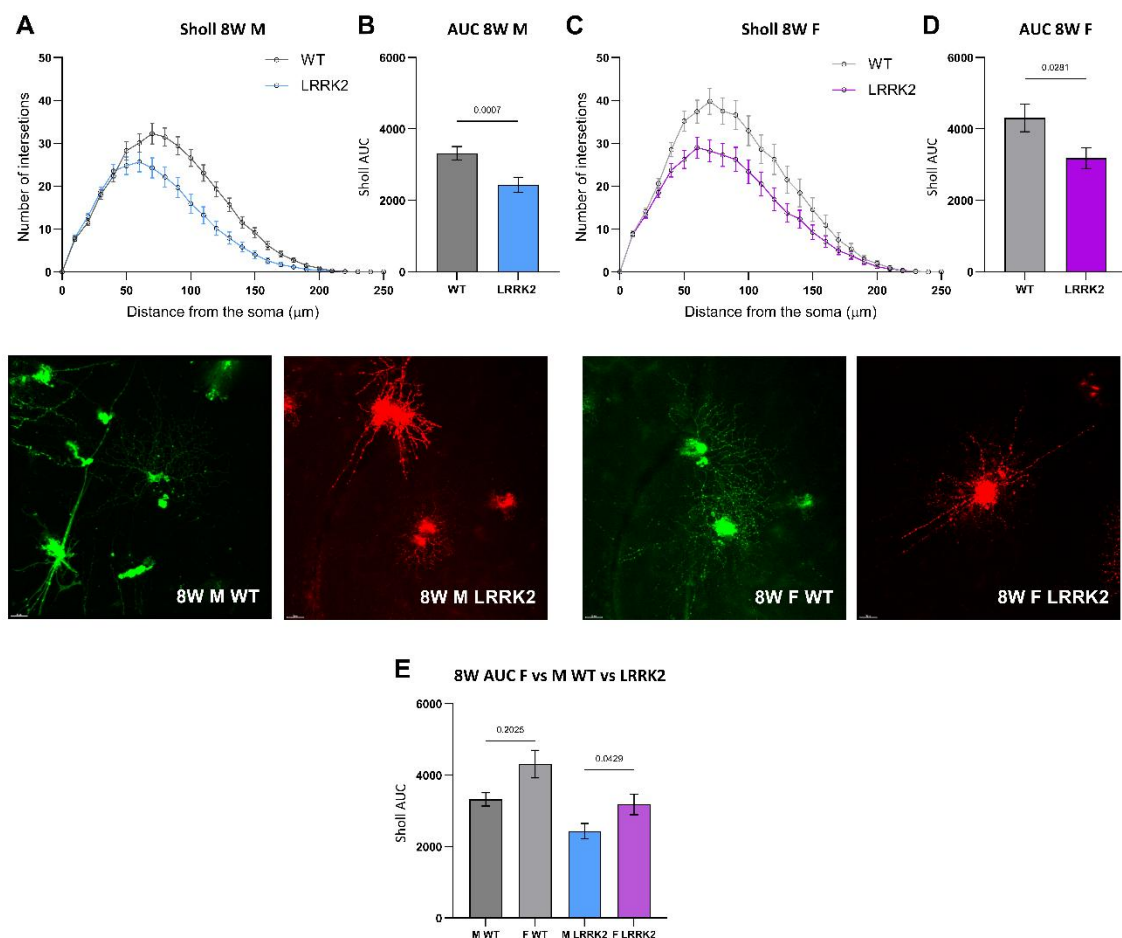
367 Availability of data and materials

368 All data generated or analysed during this study are included in this published article. Raw
369 metabolomics data provided in the supplementary information.



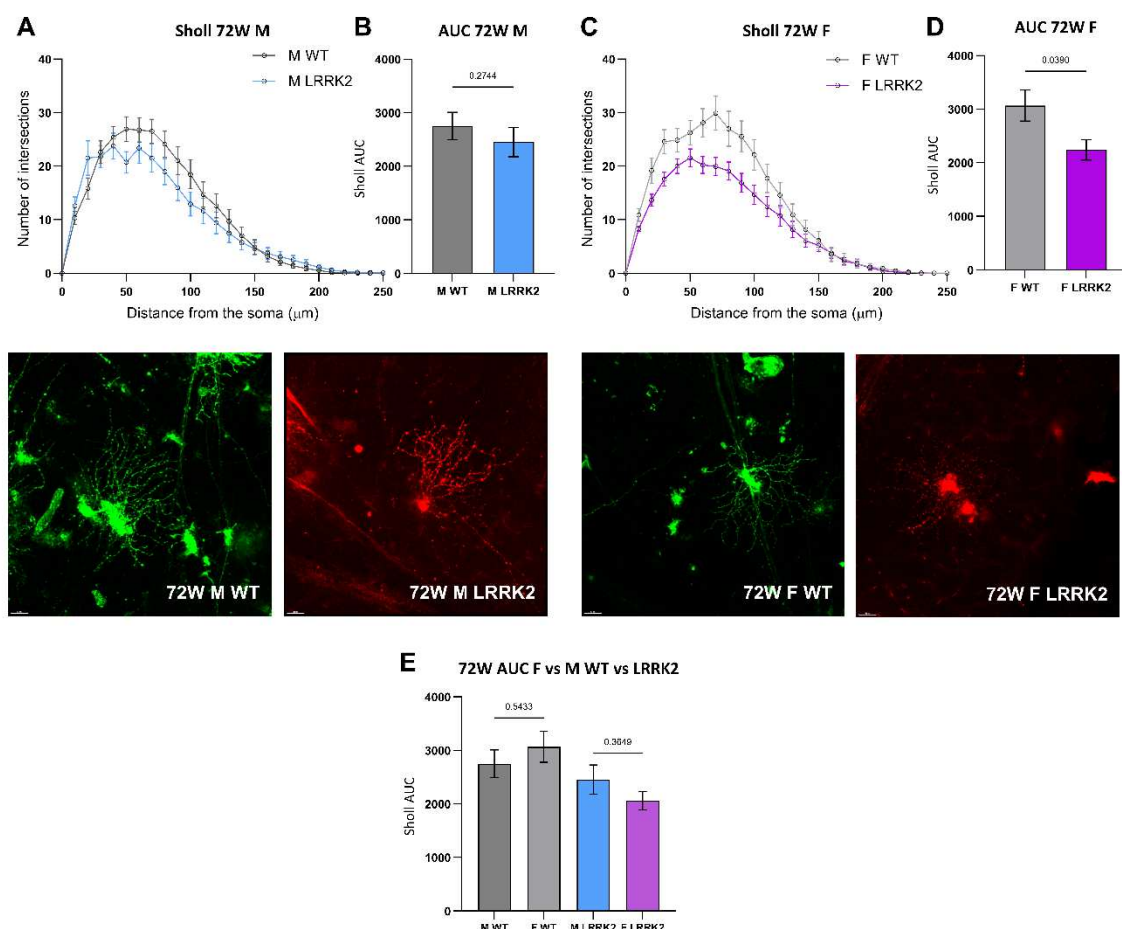
371

372 *Figure 1. Retinal ganglion cell dendritic morphology at 4 weeks. Sholl analysis of Diolistically labelled RGCs of A) male with B)*
373 *corresponding Sholl AUC. Similarly, data for female C) Sholl analysis and D) Sholl AUC are also presented. Representative RGCs*
374 *are shown below each Sholl. E) Sholl AUC comparison at 4 weeks. For Sholl curves, bars $\pm$ SEM. For Sholl AUCs, bars $\pm$ SEM. To*
375 *compare two groups Mann-Whitney U test was used. To compare three or more groups Kruskal-Wallis one-way ANOVA*
376 *followed by Dunn's test was performed. Representative images: scale bar 30 μm , image pixel size 2048 x 2048. Sample size*
377 *N=6 for each group (sex and genotype).*



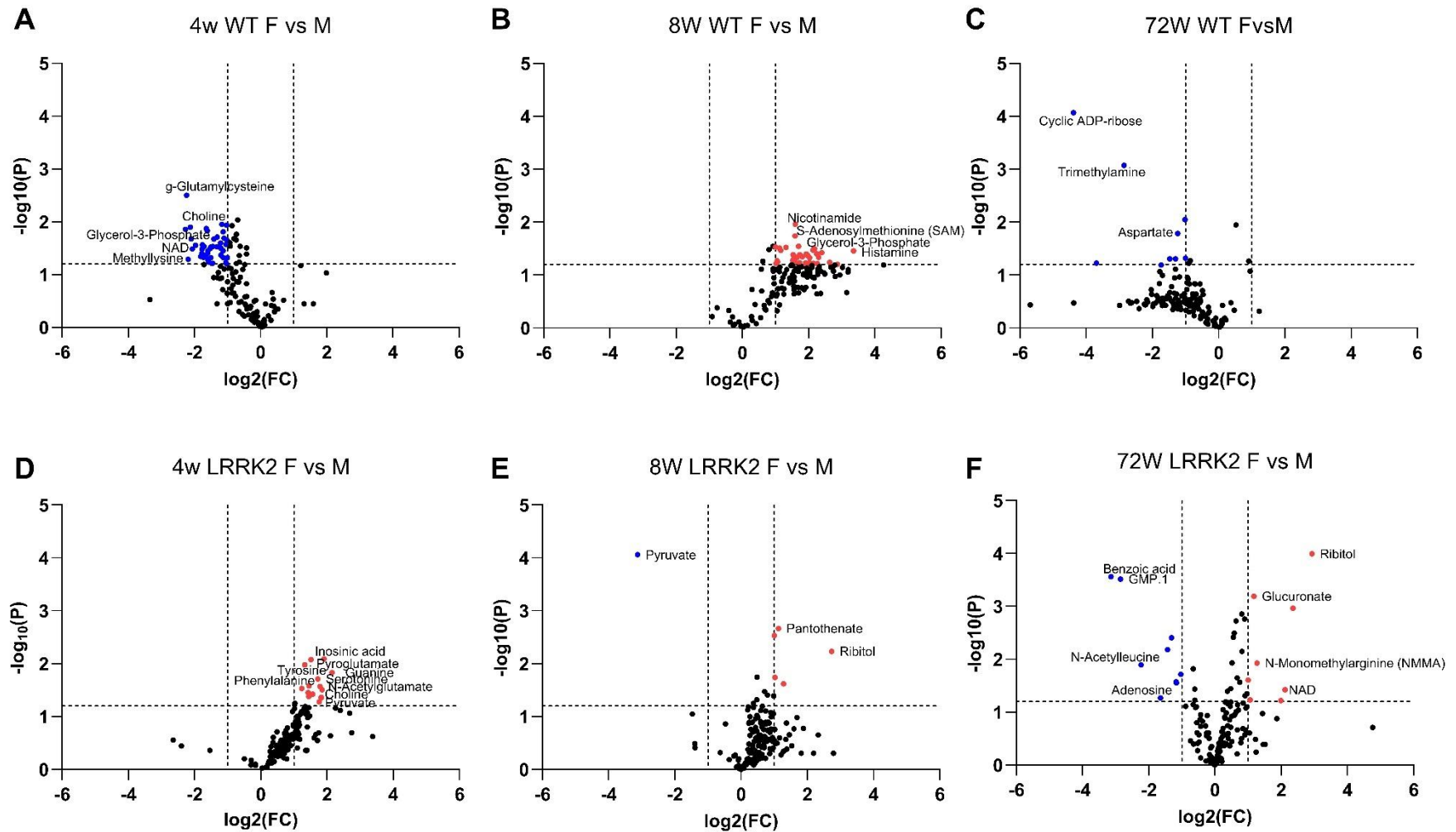
378

379 *Figure 2. Retinal ganglion cell dendritic morphology at 8 weeks. Sholl analysis of Diolistically labelled RGCs of A) male with B)*
 380 *corresponding Sholl AUC. Similarly, data for female C) Sholl analysis and D) Sholl AUC are also presented. Representative RGCs*
 381 *are shown below each Sholl. E) Sholl AUC comparison at 8 weeks. For Sholl curves, bars $\pm$ SEM. For Sholl AUCs, bars $\pm$ SEM. To*
 382 *compare two groups Mann-Whitney U test was used. To compare three or more groups Kruskal-Wallis one-way ANOVA*
 383 *followed by Dunn's test was performed. Representative images: scale bar 30 μ m, image pixel size 2048 x 2048. Sample size*
 384 *N=6 for each group (sex and genotype).*



385

386 *Figure 3. Retinal ganglion cell dendritic morphology at 72 weeks. Sholl analysis of Diolistically labelled RGCs of A) male with*
 387 *B) corresponding Sholl AUC. Similarly, data for female C) Sholl analysis and D) Sholl AUC are also presented. Representative*
 388 *RGCs are shown below each Sholl. E) Sholl AUC comparison at 72 weeks. For Sholl curves, bars $\pm$ SEM. For Sholl AUCs, bars $\pm$*
 389 *SEM. To compare two groups Mann-Whitney U test was used. To compare three or more groups Kruskal-Wallis one-way*
 390 *ANOVA followed by Dunn's test was performed. Representative images: scale bar 30 μ m, image pixel size 2048 x 2048. Sample*
 391 *size N=6 for each group (sex and genotype).*



392

393 Figure 5. Sex dependent metabolomic differences in WT and LRRK2 optic nerves at different time points. Volcano plots showing sex-dependent metabolomic differences in wild type at A) 4 weeks,
 394 B) 8 weeks, and C) 72 weeks, and in LRRK2 G2019S KI at D) 4 weeks, E) 8 weeks, and F) 72 weeks. Blue dots metabolite abundance significantly decreased, red dots metabolite abundance
 395 significantly increased.

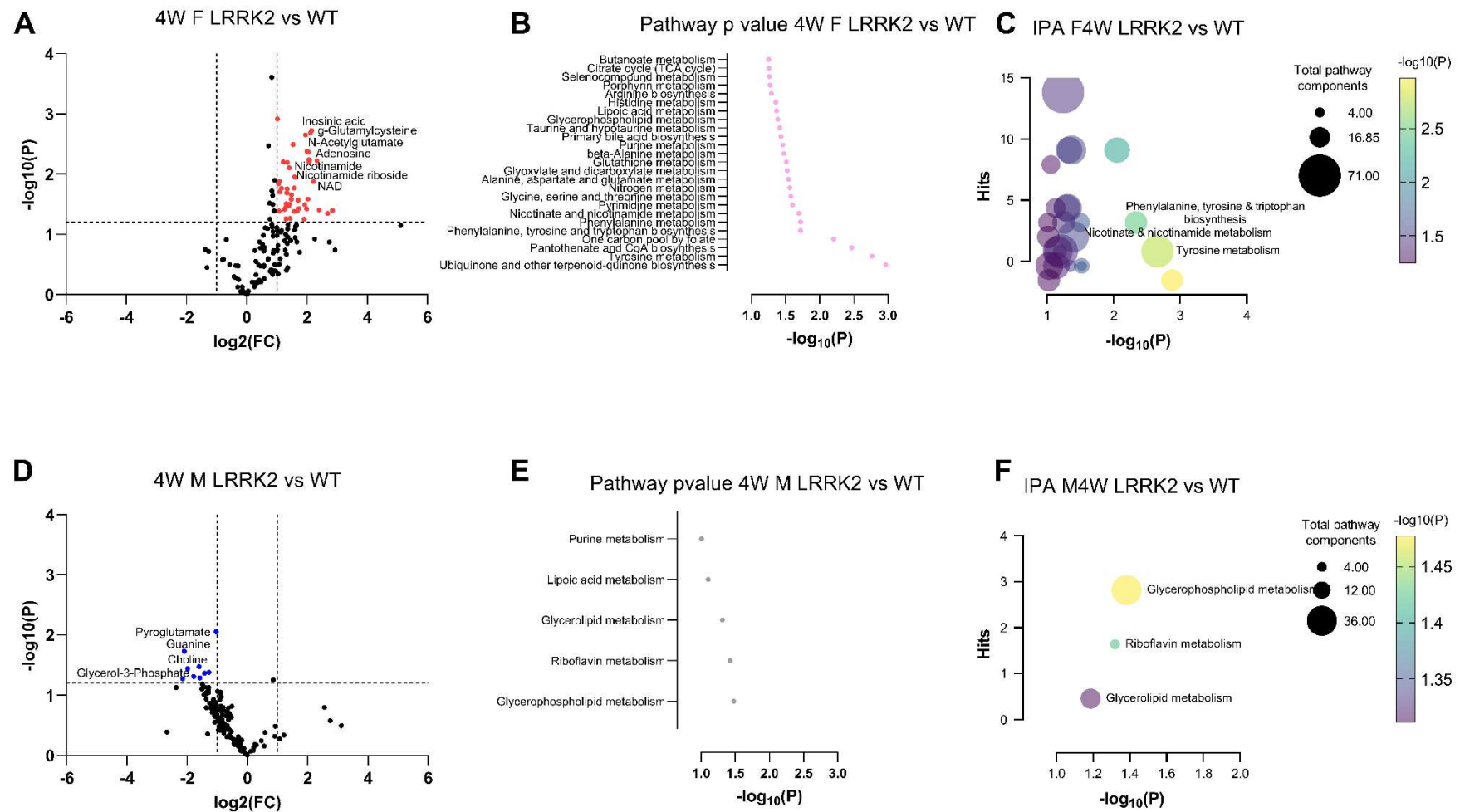
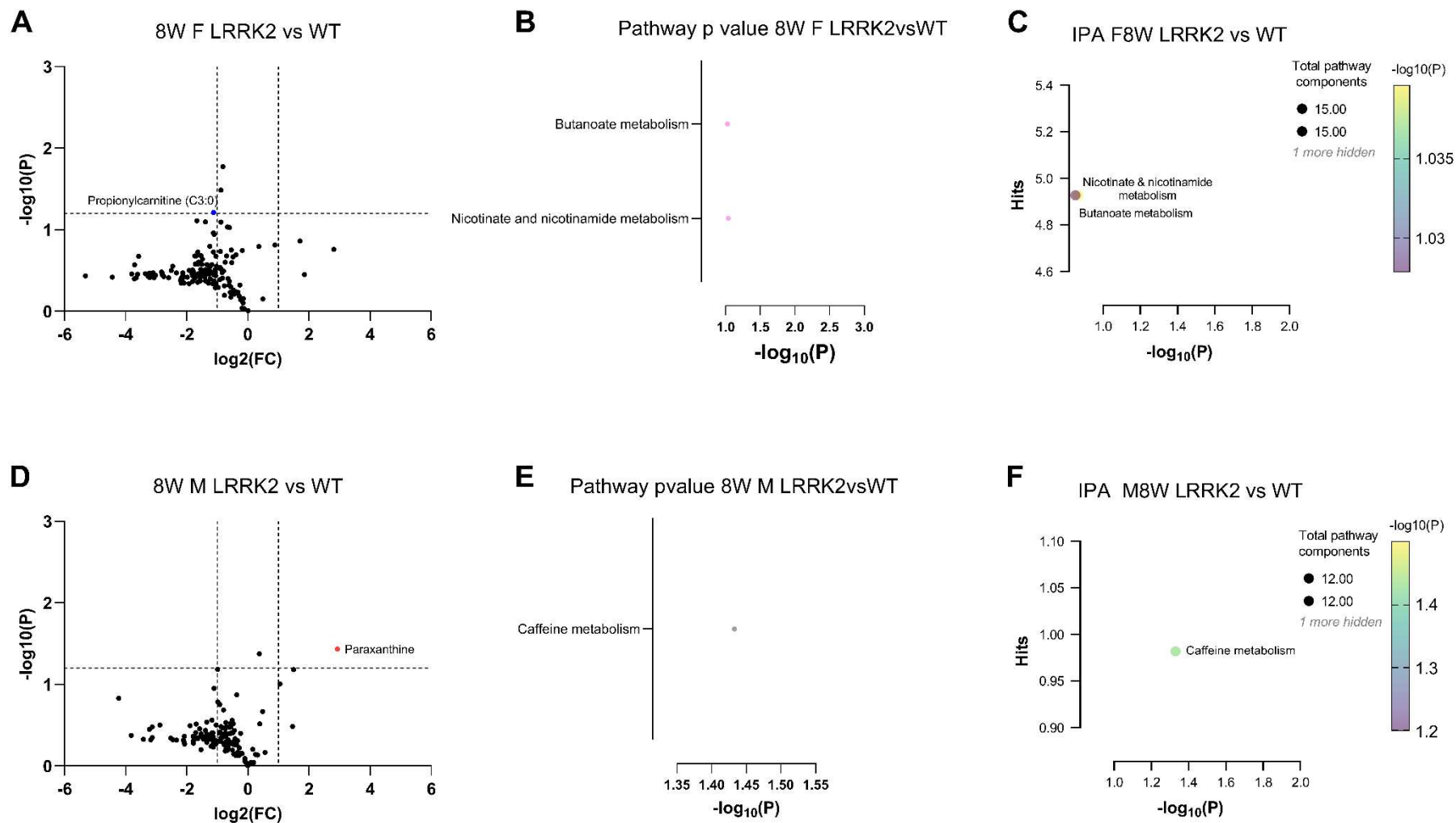


Figure 6. Sex and genotype-dependent metabolic differences at 4 weeks. Volcano plots showing metabolic differences in A) female and D) male between LRRK2 G2019S and wild type, and the respective pathways significantly influenced by those differences in B) female and E) male. Ingenuity pathway analysis (IPA) in C) female and F) male showing the number of metabolites changes (hits) in each pathway according to their significance as listed in B) and E); labels in C) added for the most relevant. For volcano plots, blue dots metabolite abundance significantly decreased, red dots metabolite abundance significantly increased.



401

402 *Figure 7. Sex and genotype-dependent metabolic differences at 8 weeks. Volcano plots showing metabolic differences in A) female and D) male between LRRK2 G2019S and wild type, and the*
 403 *respective pathways significantly influenced by those differences in B) female and E) male. Ingenuity pathway analysis (IPA) in C) female and F) male showing the number of metabolites changes*
 404 *(hits) in each pathway according to their significance. For volcano plots, blue dots metabolite abundance significantly decreased, red dots metabolite abundance significantly increased.*

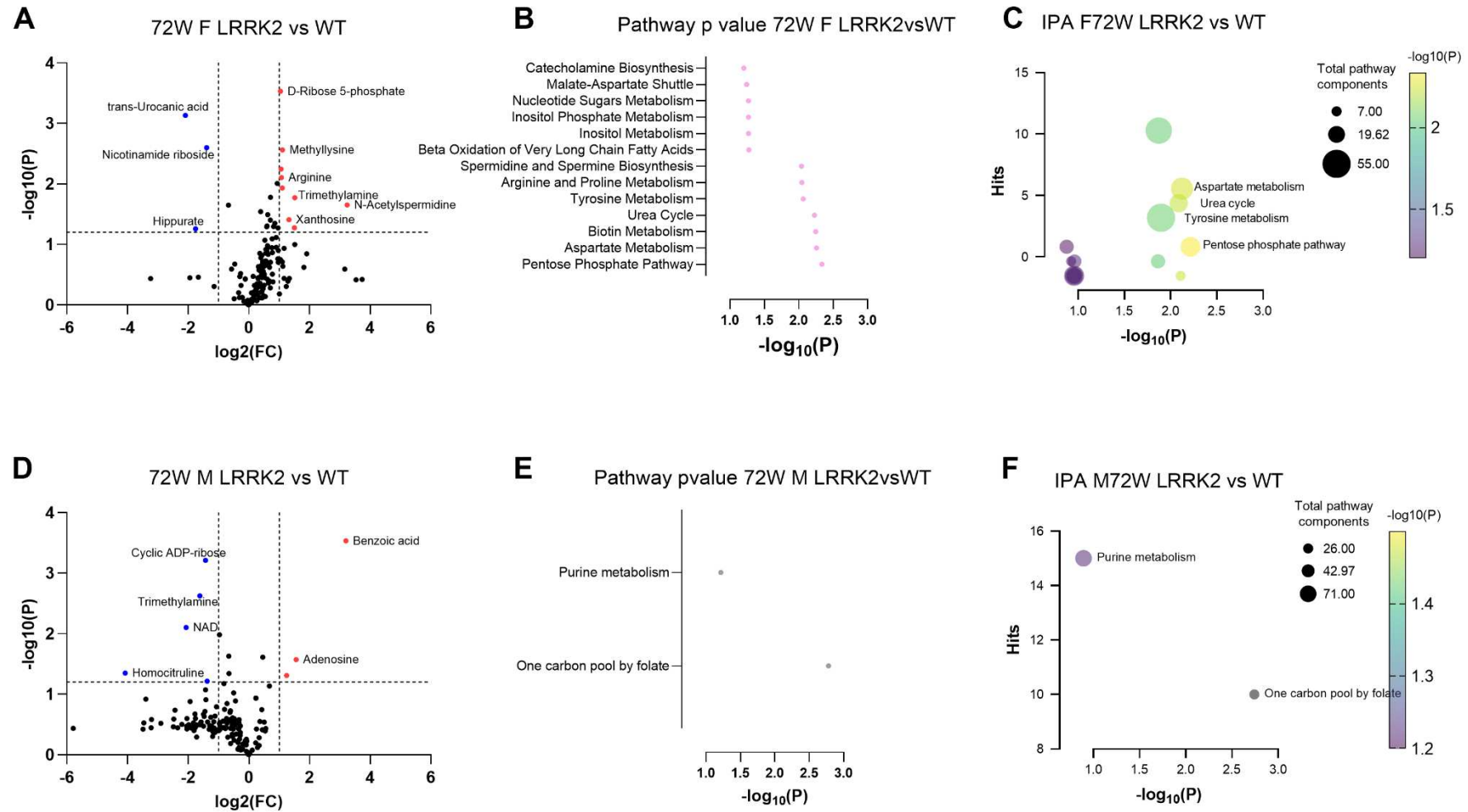


Figure 8. Sex and genotype-dependent metabolic differences at 72 weeks. Volcano plots showing metabolic differences in A) female and D) male between LRRK2 G2019S and wild type, and the respective pathways significantly influenced by those differences in B) female and E) male. Ingenuity pathway analysis (IPA) in C) female and F) male showing the number of metabolites changes (hits) in each pathway according to their significance as listed in B) and E); labels in C) added for the most relevant. For volcano plots, blue dots metabolite abundance significantly decreased, red dots metabolite abundance significantly increased.

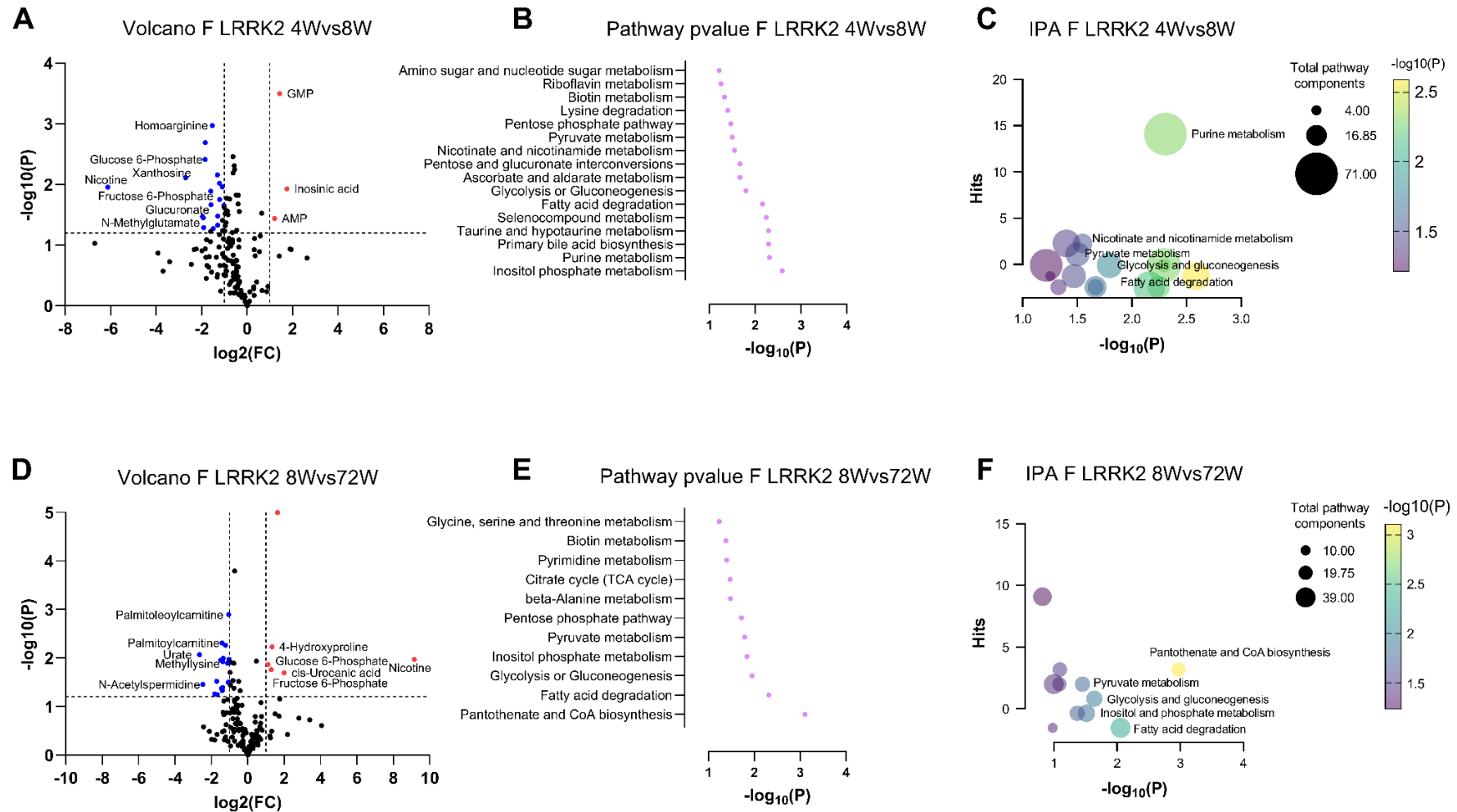


Figure 9. Longitudinal metabolic analysis of female LRRK2 G2019 KI. Volcano plots showing metabolic differences in A) early/juvenile transition (4w vs 8w) and D) late/aging transition (8w vs 72w), and the respective pathways significantly influenced by those differences in B) early/juvenile time points and E) late/aging time points. Ingenuity pathway analysis (IPA) in C) early/juvenile and F) late/aging showing the number of metabolites changes (hits) in each pathway according to their significance as listed in B) and E); labels in C) and F) added for the most relevant. For volcano plots, blue dots metabolite abundance significantly decreased, red dots metabolite abundance significantly increased.

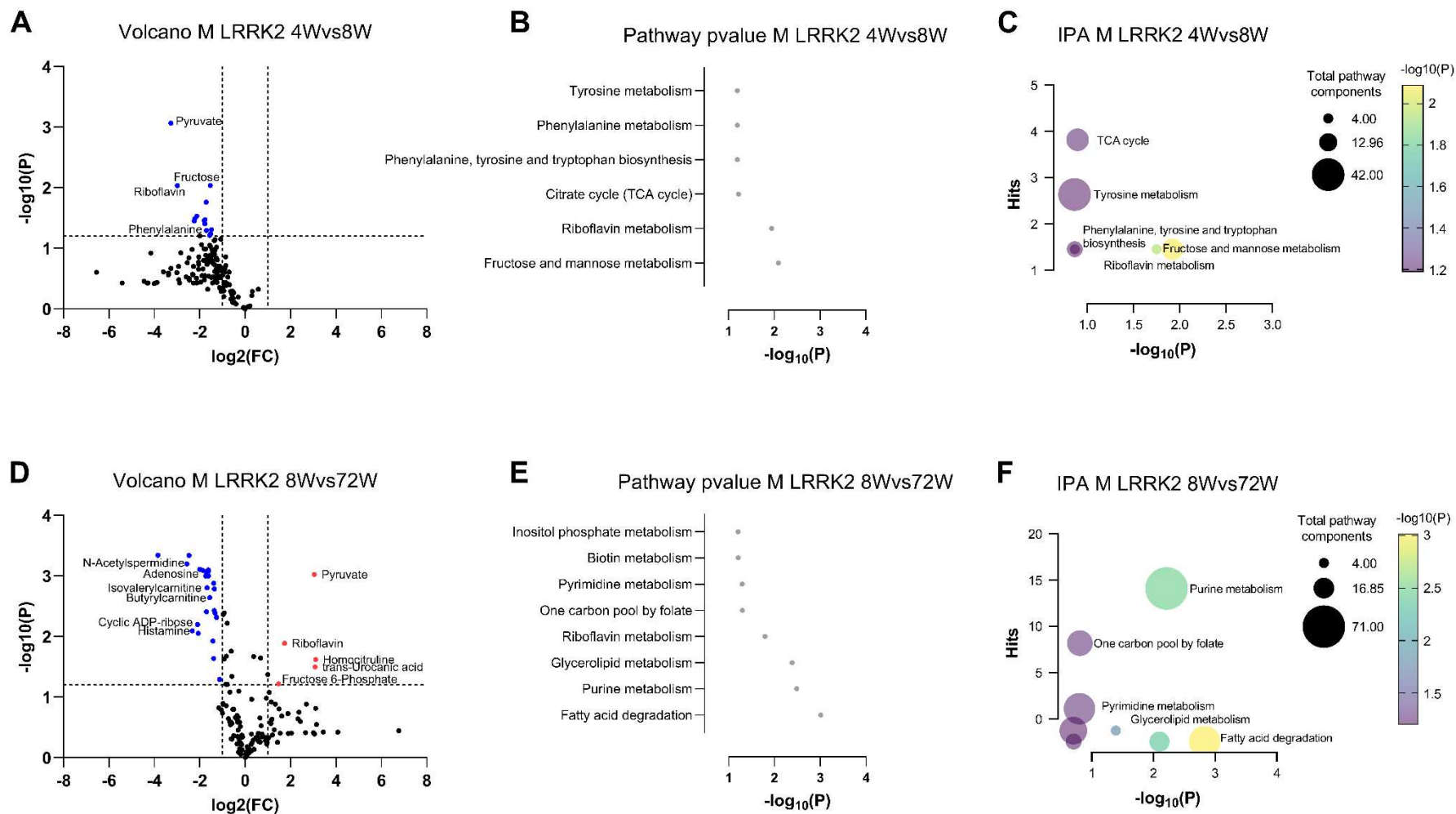
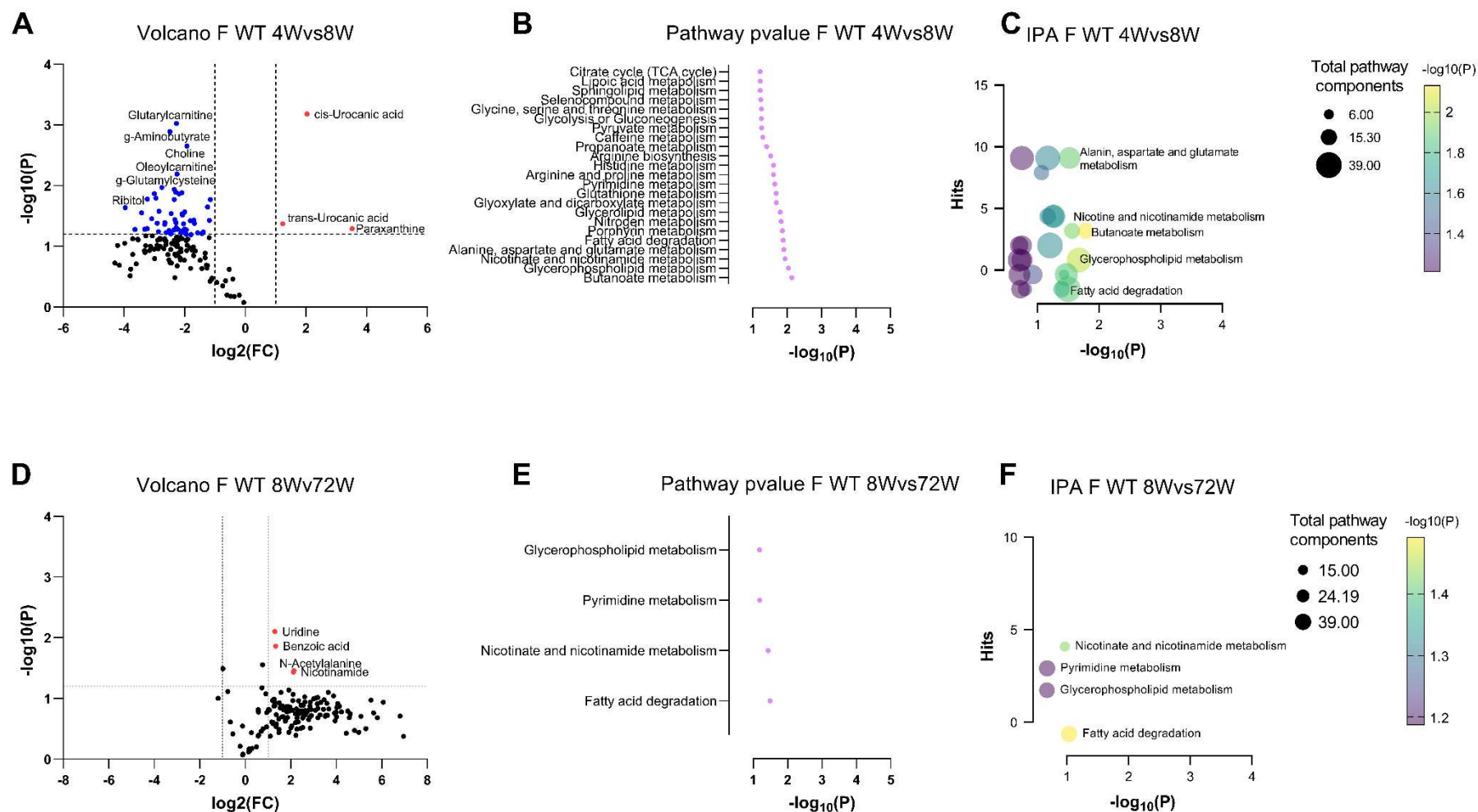
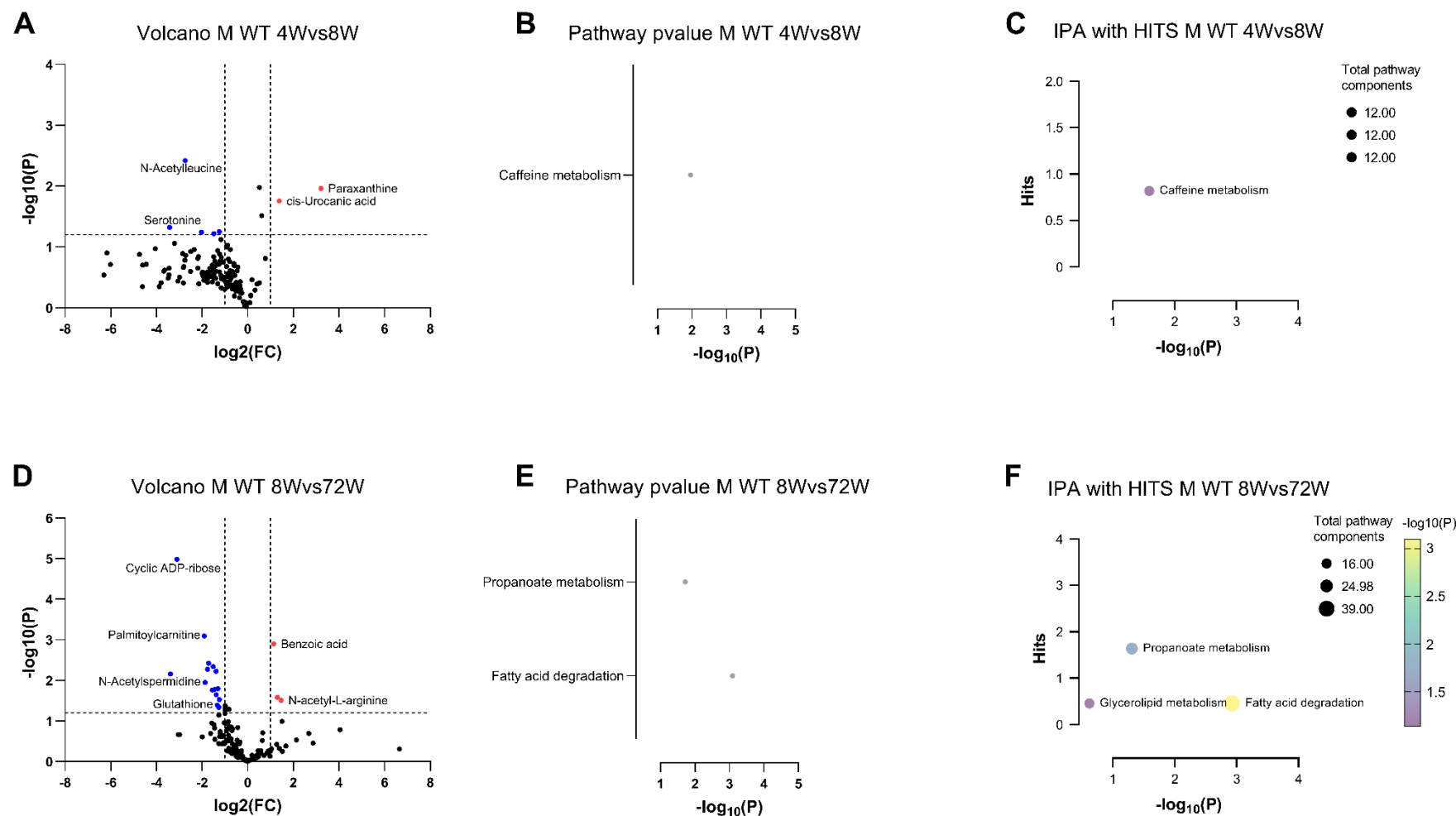


Figure 10. Longitudinal metabolic analysis of male LRRK2 G2019 KI. Volcano plots showing metabolic differences in A) early/juvenile transition (4w vs 8w) and D) late/aging transition (8w vs 72w), and the respective pathways significantly influenced by those differences in B) early/juvenile time points and E) late/aging time points. Ingenuity pathway analysis (IPA) in C) early/juvenile and F) late/aging showing the number of metabolites changes (hits) in each pathway according to their significance as listed in B) and E); labels in C) and F) added for the most relevant. For volcano plots, blue dots metabolite abundance significantly decreased, red dots metabolite abundance significantly increased.



420

421 *Figure 11. Longitudinal metabolic analysis of wild type female. Volcano plots showing metabolic differences in A) early/juvenile transition (4w vs 8w) and D) late/aging transition (8w vs 72w),*
 422 *and the respective pathways significantly influenced by those differences in B) early/juvenile time points and E) late/aging time points. Ingenuity pathway analysis (IPA) in C) early/juvenile and*
 423 *F) late/aging showing the number of metabolites changes (hits) in each pathway according to their significance as listed in B) and E); labels in C) added for the most relevant. For volcano plots,*
 424 *blue dots metabolite abundance significantly decreased, red dots metabolite abundance significantly increased.*



425

426 *Figure 12. Longitudinal metabolic analysis of wild type male. Volcano plots showing metabolic differences in A) early/juvenile transition (4w vs 8w) and D) late/aging transition (8w vs 72w), and*
 427 *the respective pathways significantly influenced by those differences in B) early/juvenile time points and E) late/aging time points. Ingenuity pathway analysis (IPA) in C) early/juvenile and F)*
 428 *late/aging showing the number of metabolites changes (hits) in each pathway according to their significance as listed in B) and E). For volcano plots, blue dots metabolite abundance significantly*
 429 *decreased, red dots metabolite abundance significantly increased.*

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