

STA 440 Case 2

Krish Bansal, Lily Seelig, Anirudh Jain

Background

Mitochondria play a central role in cellular energy metabolism, and alterations in their molecular structures can translate into measurable physiological phenotypes. Characterizing how mitochondrial efficiency changes under different conditions provides important insights into metabolic function and disease mechanisms.

To this end, researchers often measure mitochondrial respiration under a variety of experimental settings. Multiplexed metabolic assay platforms allow simultaneous evaluation of several aspects of mitochondrial functions across substrates, redox conditions, and energetic states. One critical readout is oxygen flux (JO2), which serves as a direct indicator of mitochondrial respiratory activity. Comparing oxygen flux between experimental groups such as non transgenic and transgenic mice provides a way to evaluate whether genetic differences are associated with altered mitochondrial functions.

Our analysis focuses on modeling and testing for genotype effects on oxygen flux, while accounting for experimental design factors such as substrate type. The aim is to determine whether mitochondrial efficiency differs by genotype, and whether such effects depend on substrate choice or dose.

Data

The data for this study was collected from skeletal muscle mitochondria that are isolated from either non-transgenic or transgenic mice, measured during the mitochondrial energy transduction process. For our model we took into account two main factors: substrate type and dose. The primary factor we are interested in is genotype (non transgenic vs transgenic). Substrate and dose serve as design factors, and their interactions with genotype are of central interest in assessing whether genetic differences in mitochondrial function vary across energetic states or substrate conditions.

The data we were given originally was in a wide format, so we transformed it into a tidy long format for analysis. We also dropped the basal values since they represent the idle state. Some values were missing so we dropped them.

Model Rationale

We employed a sequence of models to evaluate the effects of genotype, substrate, and workload (Δ GATP) on oxygen flux (JO). Each model was chosen to address a specific aspect of the experimental design and to progressively incorporate complexity and biological realism.

- **Baseline model** ($\text{lm}(\text{JO2} \sim \text{Substrate} * \text{Dose})$)

This model considers only substrate and dose effects, ignoring genotype. It serves as a sanity check to establish whether fuel type and workload influence JO₂ as expected. Its main advantage is simplicity and interpretability, but it does not address the central research question concerning genotype differences.

- **Genotype-only model ($\text{lm}(\text{JO}_2 \sim \text{Genotype})$)**

This specification provides a crude comparison of JO₂ between NT and Tg mice by collapsing across all experimental conditions. It is useful as an initial test for an overall genotype effect, but it oversimplifies by ignoring the role of substrate and workload.

- **Full factorial model ($\text{lm}(\text{JO}_2 \sim \text{Genotype} * \text{Substrate} * \text{Dose})$)**

This model incorporates genotype, substrate, dose, and all interactions. It allows us to examine whether genotype effects vary across substrates or workload levels. The advantage of this approach is that it captures the complexity of the biology, including higher-order interactions. However, it treats each observation as independent and does not account for repeated measures on the same subject, which violates design assumptions.

- **Mixed-effects model ($\text{lmer}(\text{JO}_2 \sim \text{Genotype} * \text{Substrate} * \text{Dose} + (1 | \text{Subject}))$)**

The mixed model includes genotype, substrate, and dose as fixed effects, while adding a random intercept for the subject. This structure accounts for repeated measures within the same mouse and preserves independence across subjects. It balances interpretability with statistical validity, enabling us to test population-level effects while controlling for subject-level variability. Its main limitation is reduced power to detect high-order interactions, given the modest sample size (6 NT, 6 Tg).

While simpler models provided useful descriptive insights, they failed to fully respect the experimental design. The baseline and genotype-only models ignored key sources of variation, while the full factorial model captured interaction effects but treated repeated measures from the same subject as independent, inflating the risk of false positives. The mixed-effects model addressed these shortcomings by incorporating subject-level random effects, thereby accounting for within-mouse correlation while still testing the joint effects of genotype, substrate, and workload at the population level. This framework provides the most statistically rigorous and biologically meaningful analysis of our dataset, balancing model complexity with the need to respect the hierarchical structure of the data.

Model Implementation and Evaluation

We reshaped the raw worksheet into a tidy long format, and restricted analysis to active respiration by removing Basal and the lowest dose (-12.95). We converted dose to a numeric ΔGATP scale for modeling and dropped missing values. Initial checks of distribution and scale (see Figure 1 and Figure 2) informed the decision to fit models on raw JO₂ while using log-transformed views only for EDA.

We then fit a progression of regression models to quantify effects and interactions: - Baseline linear model without genotype: $\text{JO}_2 \sim \text{Substrate} * \text{Dose}$ (for design checks). - Genotype-only model: $\text{JO}_2 \sim \text{Genotype}$ (coarse group difference). - Full linear model: $\text{JO}_2 \sim \text{Genotype} * \text{Substrate} * \text{Dose}$ (all interactions). - Mixed-effects model with subject intercepts: $\text{JO}_2 \sim \text{Genotype} * \text{Substrate} * \text{Dose} + (1 | \text{Subject})$.

For the linear models, we report coefficients and ANOVA tables (Table 1, Table 2, Table 3). For the mixed model, we summarize fixed effects (Table 4) and compare overall model fit across candidates using AIC/BIC and log-likelihood (Table 5). Model adequacy was assessed via residual-fitted and normal Q-Q diagnostics (Figure 10 and Figure 11). To aid interpretation, we visualized fitted curves with 95% CIs from the mixed model (Figure 8) and computed estimated marginal means and Tg-NT contrasts across Substrate \times Dose cells (Figure 9; Table 6). Supporting exploratory figures show raw trajectories and group means over dose (Figure 3 and Figure 4), per-subject dose-response slopes (Figure 6), and mean JO2 heatmaps (Figure 7).

Results

Across substrates, JO2 exhibits a clear dose-dependent response and substantial substrate-to-substrate variability (see trajectories and means in Figure 3 and Figure 4, and the mean heatmap in Figure 7). The baseline linear model confirms strong effects of Substrate and Dose and their interaction (Table 2). Incorporating Genotype and its interactions in the full linear model indicates that genotype differences are not uniform; they depend on the substrate and/or dose context (Table 3).

Accounting for repeated measures with a subject-level random intercept, the mixed-effects model provides the primary inference. Fixed-effect estimates in Table 4 quantify the average genotype difference at the reference levels and how that difference varies with substrate and dose via interaction terms. The fitted curves from Figure 8 visualize these patterns: separation between Tg and NT varies by substrate and across Δ GATP, with uncertainty bands reflecting the modest sample size. The EMM contrast heatmap and table (Figure 9; Table 6) pinpoint specific Substrate \times Dose combinations where Tg differs from NT and summarize effect sizes with standard errors and p-values.

Taken together, the results support dose- and substrate-dependent mitochondrial respiration with evidence of genotype effects that are context-specific. Where significant contrasts appear in Table 6, they align with the visual separation seen in Figure 8, while diagnostics in Figure 10 and Figure 11 do not indicate major violations of linear model assumptions. Model comparison in Table 5 favors the mixed-effects specification, balancing improved fit with appropriate handling of within-subject correlation.

Limitations

There are several limitations of this analysis that should be considered when interpreting the results. First, the analysis focused exclusively on the dose-response slopes of the mitochondrial oxygen flux (JO2) after dropping basal condition. Additionally, the analysis is limited to mitochondrial oxygen flux and does not consider other relevant outcomes like reactive oxygen species, membrane potential, and redox state. Also, although the model accounted for some heteroscedasticity, the increasing variance of the JO2 with higher mean values was only partially addressed and may still affect estimates.

The study was also limited by sample size (6 non-transgenic mice and 6 transgenic mice) which reduces the statistical power, particularly for detecting complex interactions or subtle nonlinearities. To account for this, the analysis assumed a linear relationship between the dose and JO2 across the studied range. While the assumption appeared reasonable within the available data, nonlinear responses outside of the observed range cannot be ruled out. Finally, the mixed effects modeling approach included random intercepts to capture subject level variability but did not incorporate

random slopes, potentially underestimating within subject differences in dose response patterns. However, residuals were approximately normal after accounting for fixed effects, and the assumption of independence across subjects was reasonable given the experimental design.

Conclusions

Our analysis evaluated the effects of genotype, substrate and energetic workload on mitochondrial respiration in skeletal muscle using oxygen flux as the primary outcome. By applying a sequence of regression models and ultimately using a mixed effects model, we were able to account for both experimental design factors and within subject correlation to look into genotype dependent differences.

Our results found that strong dose and substrate dependent variation in mitochondrial respiration. These results suggest that the transgenic modification influences mitochondrial efficiency in a manner that depends on the energetic environment, rather than exerting a uniform effect across all conditions.

The mixed-effects model showed the most biologically and statistically relevant inference because it was able to balance complexity with the constraints of our small sample size. While there were significant contrasts that highlighted conditions where transgenic mitochondria differ from the non-transgenic controls, the broader interpretation should be take into account the limitations of the analysis, including small sample size, potential heteroscedasticity, and the restriction to oxygen flux as the only outcome we analyzed.

Overall, our analysis shows that looking at both substrate and dose together with genotype helps reveal patterns in how mitochondria function. The findings highlight the importance of considering experimental context when evaluating genetic influences on bioenergetics and provide a foundation for future studies that incorporate larger cohorts, have additional mitochondrial outcomes, and have more flexible modeling of the relationships between dose and response.

Appendix

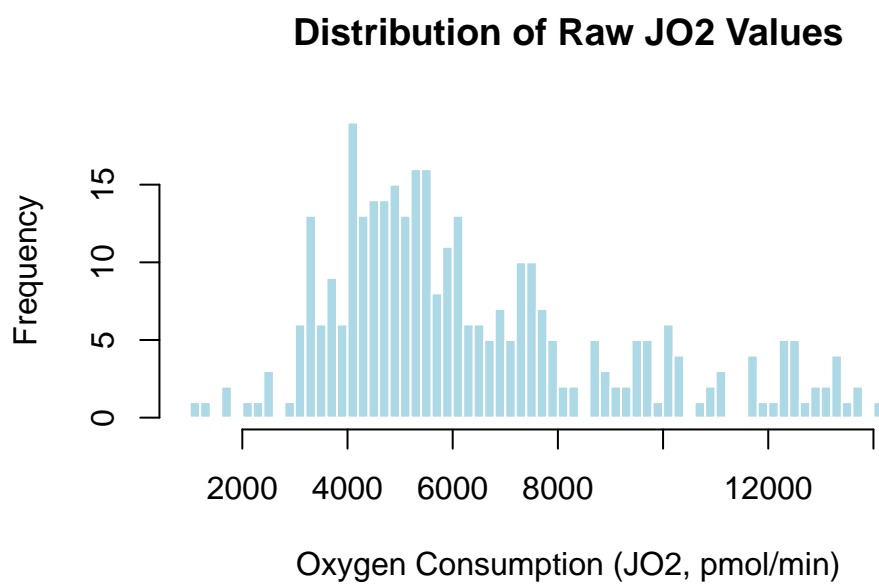


Figure 1: Distribution of raw JO2 values

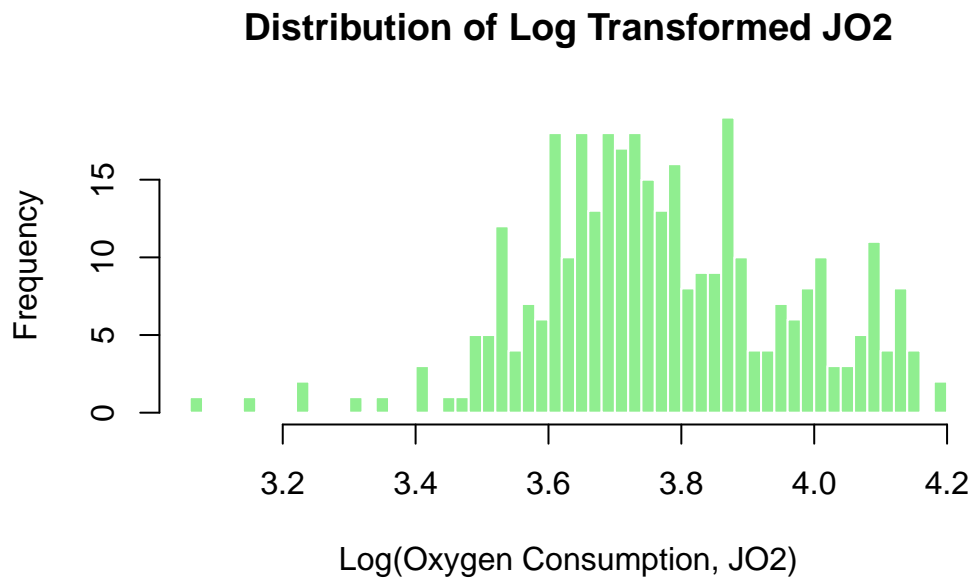


Figure 2: Distribution of log10-transformed JO2 values

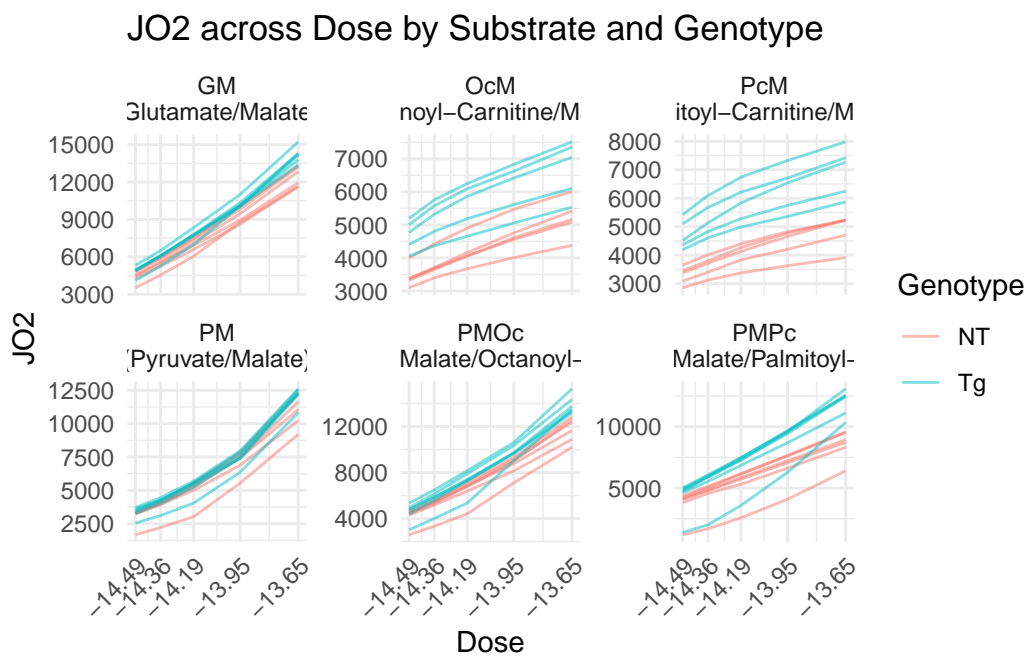


Figure 3: JO2 across dose by substrate and genotype

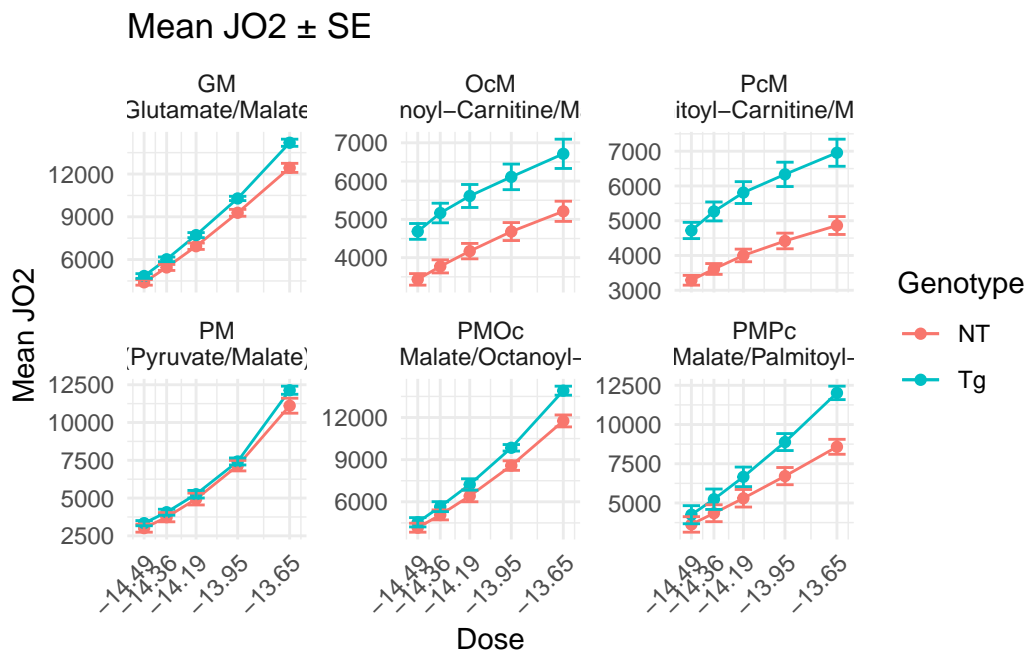


Figure 4: Mean JO2 with \pm SE by genotype and substrate

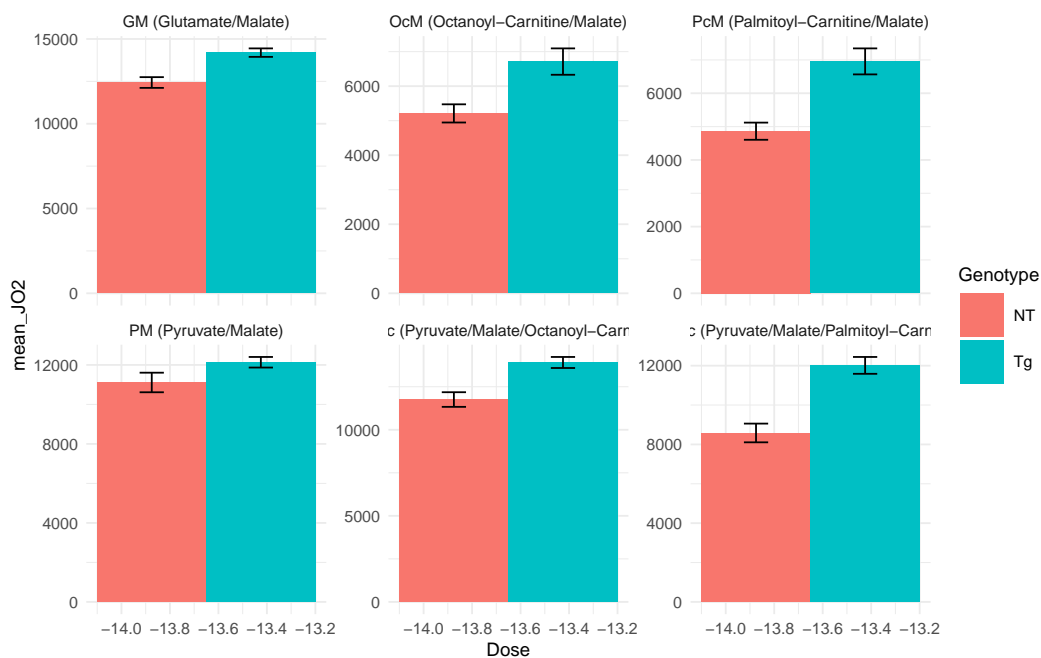


Figure 5: Mean JO2 at Basal and low dose (-13.65)

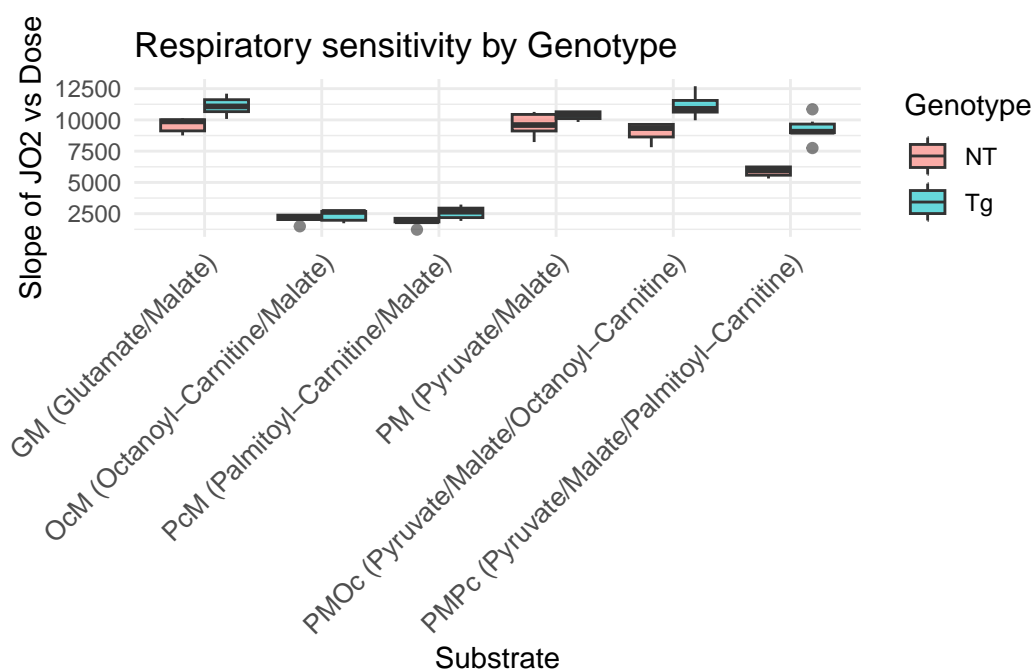


Figure 6: Distribution of JO2-dose slopes by genotype and substrate

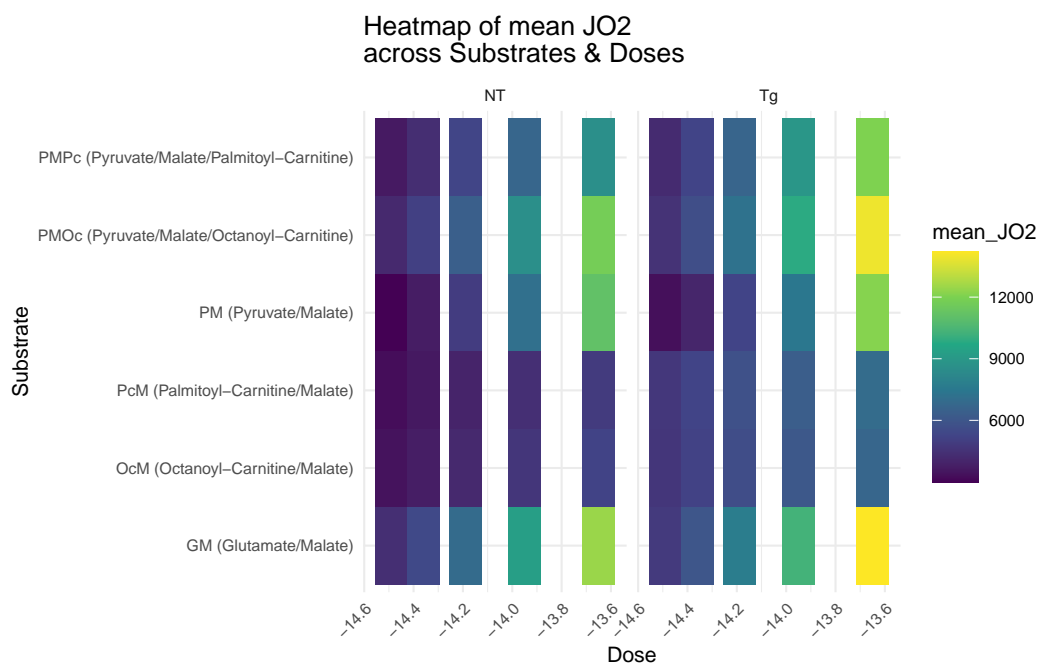


Figure 7: Heatmap of mean JO2 across substrates and doses

Model Tables

Table 1: Linear model (JO2 ~ Substrate * Dose) coefficients

term	estimate	std.error	statistic	p.value
(Intercept)	154427.081	6618.435	23.333	0.000
SubstrateOcM (Octanoyl-Carnitine/Malate)	-117987.489	9816.726	-12.019	0.000
SubstratePcM (Palmitoyl-Carnitine/Malate)	-118080.399	9816.726	-12.028	0.000
SubstratePM (Pyruvate/Malate)	-7154.046	9359.881	-0.764	0.445
SubstratePMOc (Pyruvate/Malate/Octanoyl-Carnitine)	-4072.084	9359.881	-0.435	0.664
SubstratePMPc (Pyruvate/Malate/Palmitoyl-Carnitine)	-40862.833	9359.881	-4.366	0.000
Dose	10353.285	468.357	22.106	0.000
SubstrateOcM (Octanoyl-Carnitine/Malate):Dose	-8124.780	694.686	-11.696	0.000
SubstratePcM (Palmitoyl-Carnitine/Malate):Dose	-8129.319	694.686	-11.702	0.000
SubstratePM (Pyruvate/Malate):Dose	-369.061	662.357	-0.557	0.578
SubstratePMOc (Pyruvate/Malate/Octanoyl-Carnitine):Dose	-257.125	662.357	-0.388	0.698
SubstratePMPc (Pyruvate/Malate/Palmitoyl-Carnitine):Dose	-2779.600	662.357	-4.197	0.000

Table 2: Type II ANOVA for JO2 ~ Substrate * Dose

	Term	Sum Sq	Df	F value	Pr(>F)
Substrate	Substrate	501456649	5	84.879	0
Dose	Dose	1654342978	1	1400.103	0
Substrate:Dose	Substrate:Dose	362539320	5	61.365	0
Residuals	Residuals	387560330	328	NA	NA

Table 3: Type III ANOVA for JO2 ~ Genotype * Substrate * Dose

	Term	Sum Sq	Df	F value	Pr(>F)
(Intercept)	(Intercept)	276876709	1	392.648	0.000
Genotype	Genotype	3352741	1	4.755	0.030
Substrate	Substrate	170806542	5	48.445	0.000
Dose	Dose	248030403	1	351.740	0.000
Genotype:Substrate	Genotype:Substrate	8560933	5	2.428	0.035
Genotype:Dose	Genotype:Dose	3085222	1	4.375	0.037
Substrate:Dose	Substrate:Dose	162166791	5	45.995	0.000
Genotype:Substrate:Dose	Genotype:Substrate:Dose	8399558	5	2.382	0.038
Residuals	Residuals	222828039	316	NA	NA

Table 4: Mixed model fixed effects (lmer)

	Term	Estimate	Std. Error	df	t value	p-value
(Intercept)	(Intercept)	143278.423	1551.721	308.850	31.478	0.000
GenotypeTg	GenotypeTg	22297.316	6437.105	308.850	3.464	0.001
SubstrateOcM	SubstrateOcM	-	6734.222	305.904	-	0.000
(Octanoyl-Carnitine/Malate)	(Octanoyl-Carnitine/Malate)	109528.826			16.265	
SubstratePcM	SubstratePcM	-	6734.222	305.904	-	0.000
(Palmitoyl-Carnitine/Malate)	(Palmitoyl-Carnitine/Malate)	113298.516			16.824	
SubstratePM (Pyruvate/Malate)	SubstratePM (Pyruvate/Malate)	-	6420.797	305.904	-	0.835
		1342.392			0.209	
SubstratePMOc	SubstratePMOc	-	6420.797	305.904	-	0.212
(Pyruvate/Malate/Octanoyl-Carnitine)	(Pyruvate/Malate/Octanoyl-Carnitine)	8026.608			1.250	
SubstratePMPc	SubstratePMPc	-	6420.797	305.904	-	0.000
(Pyruvate/Malate/Palmitoyl-Carnitine)	(Pyruvate/Malate/Palmitoyl-Carnitine)	54350.934			8.465	
Dose	Dose	9596.474	321.289	305.904	29.869	0.000
GenotypeTg:SubstrateOcM	GenotypeTg:SubstrateOcM	-	9523.628	305.903	-	0.067
(Octanoyl-Carnitine/Malate)	(Octanoyl-Carnitine/Malate)	17511.829			1.839	
GenotypeTg:SubstratePcM	GenotypeTg:SubstratePcM	-	9523.628	305.903	-	0.287
(Palmitoyl-Carnitine/Malate)	(Palmitoyl-Carnitine/Malate)	10158.270			1.067	
GenotypeTg:SubstratePM	GenotypeTg:SubstratePM	-	9080.378	305.903	-	0.201
(Pyruvate/Malate)	(Pyruvate/Malate)	11623.308			1.280	
GenotypeTg:SubstratePMOc	GenotypeTg:SubstratePMOc	7909.048	9080.378	305.903	0.871	0.384
(Pyruvate/Malate/Octanoyl-Carnitine)	(Pyruvate/Malate/Octanoyl-Carnitine)					
GenotypeTg:SubstratePMPc	GenotypeTg:SubstratePMPc	26976.201	9080.378	305.903	2.971	0.003
(Pyruvate/Malate/Palmitoyl-Carnitine)	(Pyruvate/Malate/Palmitoyl-Carnitine)					
GenotypeTg:Dose	GenotypeTg:Dose	1513.622	454.371	305.903	3.331	0.001
SubstrateOcM	SubstrateOcM	-	476.548	305.903	-	0.000
(Octanoyl-Carnitine/Malate):Dose	(Octanoyl-Carnitine/Malate):Dose	7485.712			15.708	
SubstratePcM	SubstratePcM	-	476.548	305.903	-	0.000
(Palmitoyl-Carnitine/Malate):Dose	(Palmitoyl-Carnitine/Malate):Dose	7737.110			16.236	
SubstratePM (Pyruvate/Malate):Dose	SubstratePM (Pyruvate/Malate):Dose	26.091	454.371	305.904	0.057	0.954
SubstratePMOc	SubstratePMOc	-	454.371	305.904	-	0.242
(Pyruvate/Malate/Octanoyl-Carnitine):Dose	(Pyruvate/Malate/Octanoyl-Carnitine):Dose	532.464			1.172	
SubstratePMPc	SubstratePMPc	-	454.371	305.904	-	0.000
(Pyruvate/Malate/Palmitoyl-Carnitine):Dose	(Pyruvate/Malate/Palmitoyl-Carnitine):Dose	3706.611			8.158	
GenotypeTg:SubstrateOcM	GenotypeTg:SubstrateOcM	-	673.941	305.903	-	0.059
(Octanoyl-Carnitine/Malate):Dose	(Octanoyl-Carnitine/Malate):Dose	1278.135			1.897	
GenotypeTg:SubstratePcM	GenotypeTg:SubstratePcM	-	673.941	305.903	-	0.245
(Palmitoyl-Carnitine/Malate):Dose	(Palmitoyl-Carnitine/Malate):Dose	784.418			1.164	
GenotypeTg:SubstratePM	GenotypeTg:SubstratePM	-	642.578	305.903	-	0.220
(Pyruvate/Malate):Dose	(Pyruvate/Malate):Dose	790.304			1.230	
GenotypeTg:SubstratePMOc	GenotypeTg:SubstratePMOc	550.678	642.578	305.903	0.857	0.392
(Pyruvate/Malate/Octanoyl-Carnitine):Dose	(Pyruvate/Malate/Octanoyl-Carnitine):Dose					
GenotypeTg:SubstratePMPc	GenotypeTg:SubstratePMPc	1854.022	642.578	305.903	2.885	0.004
(Pyruvate/Malate/Palmitoyl-Carnitine):Dose	(Pyruvate/Malate/Palmitoyl-Carnitine):Dose					

Table 5: Model fit statistics

Model	AIC	BIC	logLik	DF_Residual	N
lm_marginal	6383.189	6394.676	-3188.594	338	340
lm_raw	5732.667	5782.443	-2853.333	328	340
lm_multi	5568.486	5664.210	-2759.243	316	340
lmm	5002.741	5102.293	-2475.370	NA	340

Results

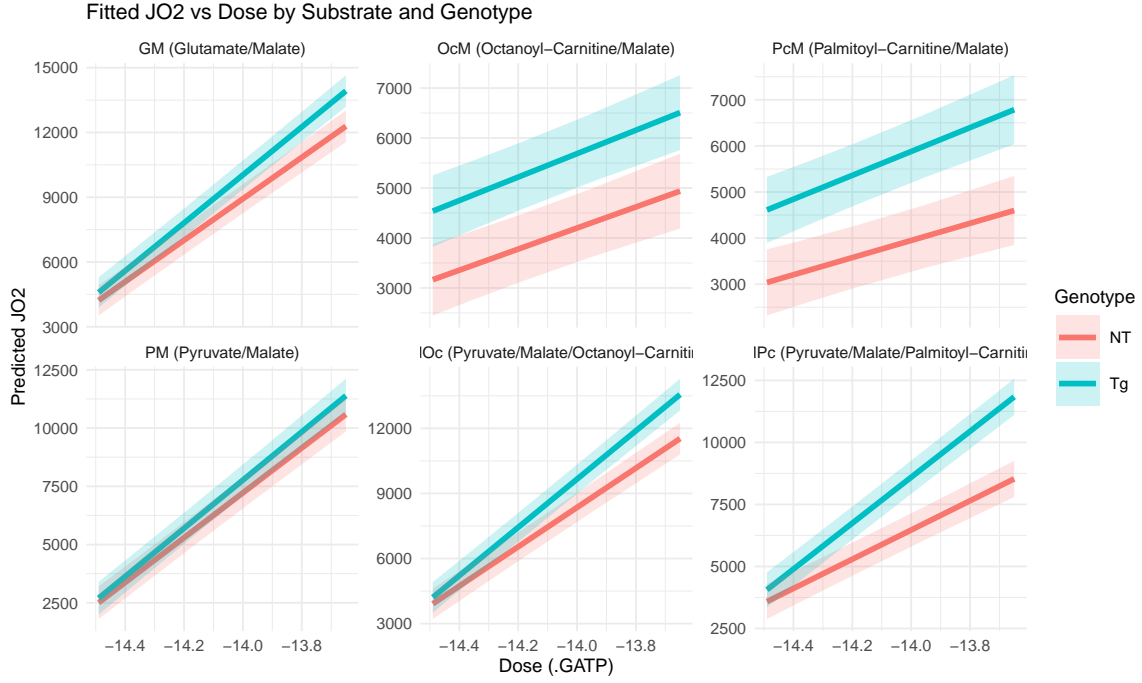


Figure 8: Predicted JO2 vs dose by substrate and genotype from mixed model

Table 6: Estimated genotype differences (Tg – NT) by substrate and dose

Substrate	Dose	Estimate	SE	df	t	p
GM (Glutamate/Malate)	-14.128	912.865	477.733	11.470	1.911	0.081
OcM (Octanoyl-Carnitine/Malate)	-14.128	1458.532	482.546	11.934	3.023	0.011
PcM (Palmitoyl-Carnitine/Malate)	-14.128	1836.859	482.546	11.934	3.807	0.003
PM (Pyruvate/Malate)	-14.128	454.965	477.733	11.470	0.952	0.361
PMOc (Pyruvate/Malate/Octanoyl-Carnitine)	-14.128	1041.937	477.733	11.470	2.181	0.051
PMPc (Pyruvate/Malate/Palmitoyl-Carnitine)	-14.128	1695.440	477.733	11.470	3.549	0.004

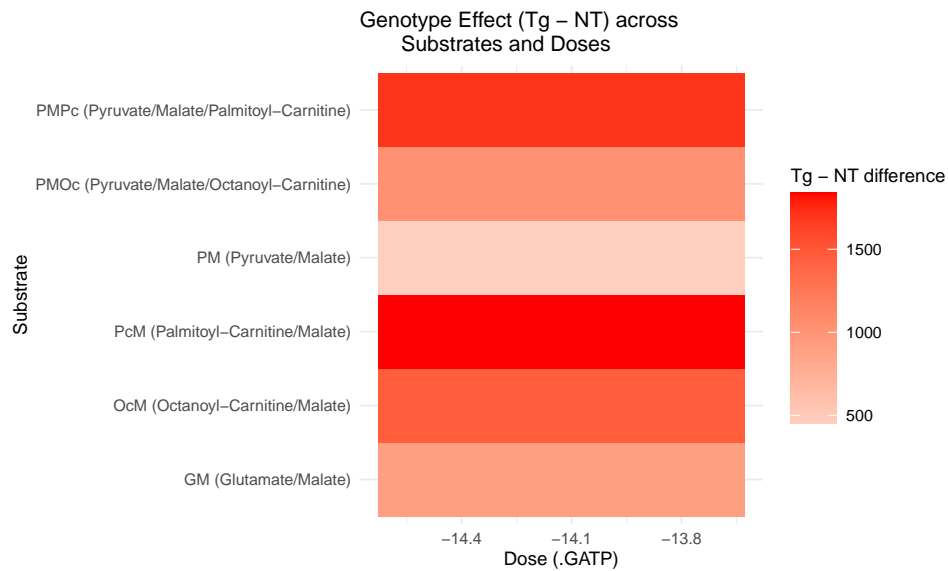


Figure 9: Genotype effect ($T_g - NT$) across substrates and doses

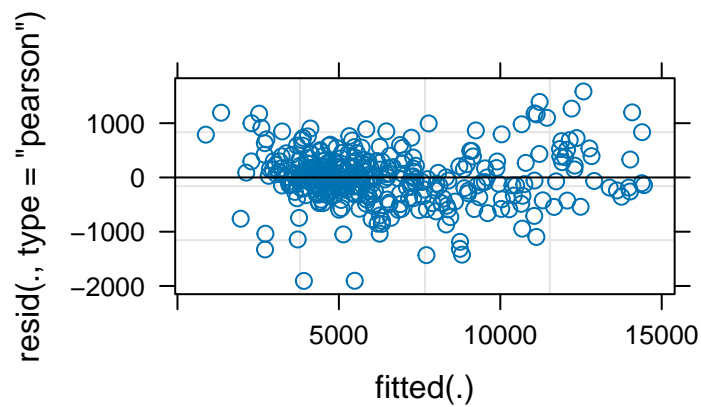


Figure 10: Residuals versus fitted values for mixed model

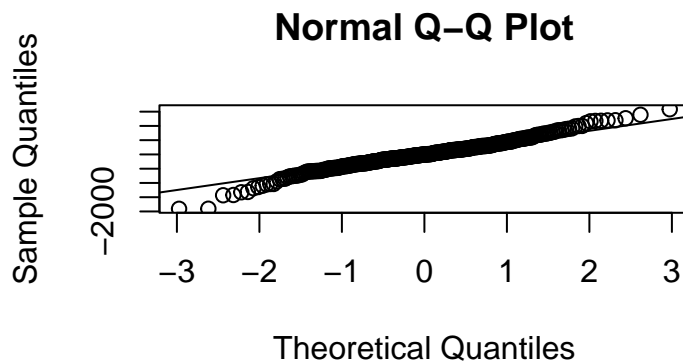


Figure 11: QQ plot of mixed model residuals