UNIWERSYTET MIKOŁAJA KOPERNIKA W TORUNIU

Collegium Medicum
im. Ludwika Rydygiera w Bydgoszczy

Bydgoszcz 2023

UNIWERSYTET
MIKOŁAJA KOPERNIKA
W TORUNIU
Wydział Lekarski
Collegium Medicum w Bydgoszczy

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Methods for energy metabolism modeling using queueing theory

Rozprawa na stopień doktora nauk medycznych

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Bydgoszcz 2023

„Co my wiemy, to tylko kropelka.

Czego nie wiemy, to cały ocean.”

Isaac Newton

Pragnę serdecznie podziękować

Panu prof. dr hab. n. med. Marcinowi Woźniakowi,

Panu prof. dr hab. inż. Tadeuszowi A. Wysockiemu,

Panu prof. dr inż. Tomaszowi Marciniakowi,

za poświęcony czas i pomoc podczas pisania rozprawy doktorskiej,

za każdą cenną radę, za liczne wskazówki merytoryczne,

za ukierunkowanie w zgłębianiu badanej dziedziny wiedzy

oraz wsparcie w dążeniu do wyznaczonego celu.

Table of contents

1. List of scientific papers included in the dissertation

This dissertation includes three original papers published in peer-reviewed journals included in the ministerial list of scientific journals. The total Impact Factor of the publications constituting the dissertation is 15.679 and 440 Ministry of Education and Science (Ministerstwo Edukacji i Nauki, MEiN) points.

1.1. Queueing theory model of Krebs cycle.

Sylwester Kloska*, Krzysztof Pałczyński, Tomasz Marciniak, Tomasz Talaśka, Marissa Nitz, Beata J. Wysocki, Paul Davis, Tadeusz A. Wysocki. Bioinformatics, Volume 37, Issue 18, 15 September 2021, Pages 2912–2919, https://doi.org/10.1093/bioinformatics/btab177

IF = 6.931

MEiN = 200

1.2. Queueing theory model of pentose phosphate pathway.

Sylwester M. Kloska*, Krzysztof Pałczyński, Tomasz Marciniak, Tomasz Talaśka, Marissa Miller, Beata J. Wysocki, Paul Davis, Tadeusz A. Wysocki. Scientific Reports, 12, 4601 (2022). https://doi.org/10.1038/s41598-022-08463-y

IF = 4.996

MEiN = 140

1.3. Queueing theory model of mTOR complexes' impact on Akt-mediated adipocytes response to insulin.

Sylwester M. Kloska*, Krzysztof Pałczyński, Tomasz Marciniak, Tomasz Talaśka, Marissa Miller, Beata J. Wysocki, Paul Davis, Ghada A. Soliman, Tadeusz A. Wysocki. PLoS ONE 17(12): e0279573. https://doi.org/10.1371/journal.pone.0279573

IF = 3.752

MEiN = 100

2. List of abbreviations

ATP – Adenosine triphosphate

CAC – Citric acid cycle

CME – Chemical Master Equation

DNA – deoxyribonucleic acid

$E4P$ – Erythrose 4-phosphate

$FADH_2$ – Flavin adenine dinucleotide (reduced form)

FBA - Flux balance analysis

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

GLUT4 – glucose transporter 4

GTP – Guanosine triphosphate

$G3P$ – Glyceraldehyde-3-phosphate

$G6P$ – Glucose 6-phosphate

G6PD – Glucose-6-phosphate dehydrogenase

IRS – insulin receptor substrate

$K_{S_1}, K_{S_2}, \dots, K_{S_x}$ – Kinetic constant of substrate

$K_{P_1}, K_{P_2}, \dots, K_{P_x}$ – Kinetic constant of product

mTOR – mammalian target of rapamycin

mTORC1 – mammalian target of rapamycin complex 1

mTORC 2 – mammalian target of rapamycin complex 2

$NADH$ – Nicotinamide adenine dinucleotide (reduced form)

$NADP^+$ – Nicotinamide adenine dinucleotide phosphate (oxidized form)

$NADPH$ – Nicotinamide adenine dinucleotide phosphate (reduced form)

ODE – Ordinary differential equation

6PGD – 6-phosphogluconate dehydrogenase

PGL - 6-P-gluconolactone

PGLS - 6-phosphogluconolactonase

PPP – Pentose phosphate pathway

$P_1, P_2, \dots, P_x$ – Product concentration

RBM - Rule-based Modelling

RNA – ribonucleic acid

ROS – Reactive oxygen species

RPE - Ribulose-5-phosphate-3-epimerase

RPIA - Ribose-5-phosphate isomerase A

$R5P$ – Ribose 5-phosphate

$S_1, S_2, \dots, S_x$ – Substrate concentration

S6K – S6 kinase

$S7P$ – Sedoheptulose-7-phosphate

TA – transaldolase

TCA – tricarboxylic acid cycle

TK – transketolase

v – Reaction speed

V_f– Forward reaction speed

V_r – Reverse reaction speed

$6PG$ – 6-phosphogluconate

3. Introduction

Computational biology is the application of computational methods and tools to the study of biological systems [1]. It is a rapidly growing field that is increasingly being applied to medicine, particularly in the areas of drug discovery and precision medicine [2,3]. Computational biology plays an important role in the development of new medical treatments and therapies, by providing a deeper understanding of the underlying biological mechanisms of disease and by identifying new targets for drug development.

At the intersection of computational biology and medicine, a new field is emerging – computational medicine. Computational medicine is an interdisciplinary field that utilizes computational methods and tools to better understand, diagnose, and treat diseases. It combines expertise from computer science, mathematics, and engineering with knowledge from the biomedical sciences and clinical medicine to develop new methods for analyzing large and complex biomedical data. By combining the knowledge provided by the above-mentioned fields, it is possible to mathematically track and infer the effects of research and treatment. One of the methods that make it possible to track the dynamics of metabolic reactions, changes in the concentrations of metabolically active molecules present in the cell, is modeling. This mathematical modeling can be used to understand the dynamics of metabolic pathways [4]. The ultimate goal of computational medicine is to improve patient care and outcomes by providing more accurate and personalized diagnoses and treatments.

3.1. Computational modeling of metabolism

Computational biology can be used to understand the dynamics of metabolites flow in various metabolic and signaling pathways by developing mathematical models that simulate the interactions between molecules in the cells, tissues, and organisms [5]. These models can be used to test new treatments in silico, and to identify new targets for drug development.

Modeling makes it possible to predict changes in the cell that are a consequence of interference with the biological system. An example of such interference can be the delivery of a specific biologically active molecule involved in metabolic pathways (delivery of an excessive amount of the substrate or product of a given enzyme) or the use of an enzyme inhibitor that deactivates enzyme molecules, which on a macroscopic scale will be evident by slowing down the reaction catalyzed by the enzyme. Slowing down the course of an enzymatic reaction can also be achieved by genetic knockdown of the gene encoding the enzyme in question. Following the gene knockdown, the cell will lack functionally correct molecules of

the enzyme in question, leading to a reduction in the speed of the reaction in which the enzyme participates or even a complete stop of the reaction.

In clinical settings, the inhibitor is often a therapeutic substance [6]. Predicting the changes induced by a drug as a function of its dose in theory allows for more accurate dosing to achieve the best possible therapeutic effects. This is an advantage that can be drawn upon as early as the initial molecular research stage. Having knowledge of the target molecule that a drug interacts with and the effect it is expected to have on it will enable better planning of experiments. One of the apparent benefits of modeling is the reduction in the number of laboratory animals on which experiments will be conducted. These experiments often lead to the deterioration of the health of laboratory animals or even their death [7–9]. Availability of a proper digital model for some of the studies needed to approve a drug and confirm its therapeutic ability could allow for their replacement with simulation studies.

The main difficulties faced by computational biology researchers are the limited availability of reliable data and its inconsistency. Depending on the source of the data, they can differ significantly from each other. Differences in the concentration data or kinetic parameter values of the enzymes in question can be due to, among other things, inaccurate measurements, which can be influenced by a number of different reasons, such as data acquisition methodology, equipment limitations, lack of adequate training of the scientist, or even the source of the data. Concentrations of biological molecules depend on a wide variety of factors, even as simple as the time of year in which the material for the study was collected [10], the diet, or the type, size, and location from which the tissue under study originated [11].

3.2. Methods used in computational modeling

There are several different methods that can be used for creating computational models of metabolic systems. The choice of method will depend on the specific characteristics of the metabolic system being studied and the research question being addressed. ODE-based modeling is the most commonly used method, but other methods such as chemical master equations (CMEs), constraint-based modeling, kinetic modeling [12], flux balance analysis (FBA) [13–15], Petri net modeling [16], rule-based Modelling (RBM) [17], and agent-based modeling can be useful in specific cases.

3.2.1. Ordinary differential equations

Ordinary differential equations (ODEs) are a mathematical tool commonly used in computational biology to model the dynamics of metabolic systems [18–25]. These equations describe how the concentrations of different molecules in a system change over time and can be used to simulate the interactions between different metabolic pathways.

In computational models of metabolism, ODEs are used to represent the rate of change of the concentration of a given molecule, which is determined by the balance of the influx (from other pathways) and outflux (towards other pathways or products) of that molecule. The rate of change is described by a set of ODEs one for each metabolite [26].

To construct a model of metabolism using ODEs, the first step is to identify the set of metabolic pathways and reactions that are relevant to the system being studied. Next, a set of ODEs can be written to describe the rate of change of the concentration of each metabolite in the system, taking into account the fluxes of the reactions. Once the ODEs have been written, they can be solved numerically to simulate the dynamics of the metabolic system. The results of these simulations can be used to make predictions about how the system will behave under different conditions and can be useful in understanding the underlying mechanisms of metabolic disorders. Additionally, ODE models can be used to analyze the effect of genetic mutations, environmental factors and drug interventions on metabolic pathways, and identify potential therapeutic targets.

While ODEs are a powerful tool for computational modeling of metabolic systems, there are several limitations and potential disadvantages to consider:

1. ODEs are based on the assumption of continuity and smoothness, which might not be valid for some biological systems, especially if they have a discrete, stochastic nature. In these cases, other mathematical frameworks such as stochastic differential equations or agent-based models might be more appropriate.
2. ODEs are based on the assumption of mass balance, which means that the total amount of each metabolite is conserved. This might not be true for all systems, especially in cases where the system is open to the environment or where there are significant amounts of influx or outflux.

3. ODEs require the estimation of kinetic parameters. These parameters are often difficult to measure experimentally, and the estimation process can be time-consuming and uncertain.
4. ODEs often require a large number of equations and variables, which can make the model complex and difficult to analyze. The complexity of the models increases with the number of reactions and metabolites considered, making it harder to understand the underlying mechanisms of the system.
5. ODEs can have a high computational cost, especially for large models with a large number of equations. This can make it difficult to simulate the model over a long time period or to perform sensitivity analysis to determine the impact of different parameters on the system.

It is worth noting that ODEs models are deterministic in nature, while biological systems are inherently stochastic, this lack of randomness might not reflect the real system. ODE models are a simplification of reality. Therefore, in some cases, ODEs might not capture the complexity, variability, and uncertainty of the real metabolic systems.

3.2.2. Chemical master equations

Chemical master equations (CMEs) are a mathematical tool that can be used to model the dynamics of chemical reactions, including metabolic pathways [27]. CMEs are a type of kinetic modeling, which means that they describe the kinetics of the reactions, including the rate laws and the Michaelis-Menten constants.

One of the main advantages of CMEs is that they can account for the stochastic nature of biochemical reactions, which can be important in metabolic pathways where the number of reactants is small [28]. CMEs also can take into account the discreteness of the molecules, which is a feature that ODEs lack. CMEs can be used to calculate the probability distribution of the number of molecules for each reactant in the reaction network at any given time. This can be useful in understanding the behavior of the system under different conditions and in identifying potential bottlenecks or rate-limiting steps in the pathway.

However, CMEs also have some limitations that should be considered. One of the main disadvantages of CMEs is that they can be computationally expensive, especially for large systems with many reactions and many states [29]. Additionally, the solution of CMEs can be challenging and might require approximations.

In conclusion, CMEs are a powerful tool for computational modeling of metabolic pathways, particularly in cases where the stochastic and discrete nature of the system is important. However, their computational cost and the difficulty of solving them should be taken into account when deciding whether to use CMEs for a specific problem.

3.2.3. Bypassing the limitations of the most common modeling methods

Due to the fact that the above-described methods are not without flaws, researchers are looking for other, competitive ways and methods to create more accurate computational models. There are several strategies that can be used to cope with the limitations of ODEs and CMEs models [27]. One strategy is to use a hybrid model that combines the strengths of ODEs and CMEs. For example, one can use ODEs to describe the dynamics of the system at the macroscopic level, and use CMEs to describe the dynamics of the system at the microscopic level. This can allow for the inclusion of both deterministic and stochastic behavior in the model. Another strategy is to reduce the complexity of the model by simplifying the system or by using model reduction techniques such as lumping or moment-closure approximations. This can help to make the model more computationally tractable while still capturing the essential dynamics of the system.

It is worth noting that there is no one-size-fits-all solution and the best approach will depend on the specific characteristics of the metabolic system being studied, the research question being addressed, and the available computational resources.

3.3. Queueing theory

Queueing theory is a branch of mathematical modeling that deals with the study of waiting lines (queues) and the behavior of systems that involve waiting (servers) [30]. It can be used to analyze systems where resources are limited and there is a need to wait for their availability. It was realized that queueing theory, which had been widely used in telecommunications and other fields to model systems with limited resources, could be applied to metabolic pathways [31]. It provided a new perspective on the analysis of metabolic pathways, showing that concepts and mathematical tools originally developed for other fields can be adapted and applied to biological systems [32,33]. Queueing theory can be used to create computational models of metabolic pathways by modeling the enzymes in the pathway as servers and the molecules as customers in a queue [34]. This can help to understand the behavior of the system under different conditions, such as changes in the enzyme

concentration or the substrate availability. The concept of using queueing theory in metabolism modeling has been expanded and refined by researchers over the years, and it has become a valuable tool for understanding the behavior of metabolic pathways, evaluate the impact of different parameters on the system performance, and identifying potential bottlenecks in the system and targets for drug development.

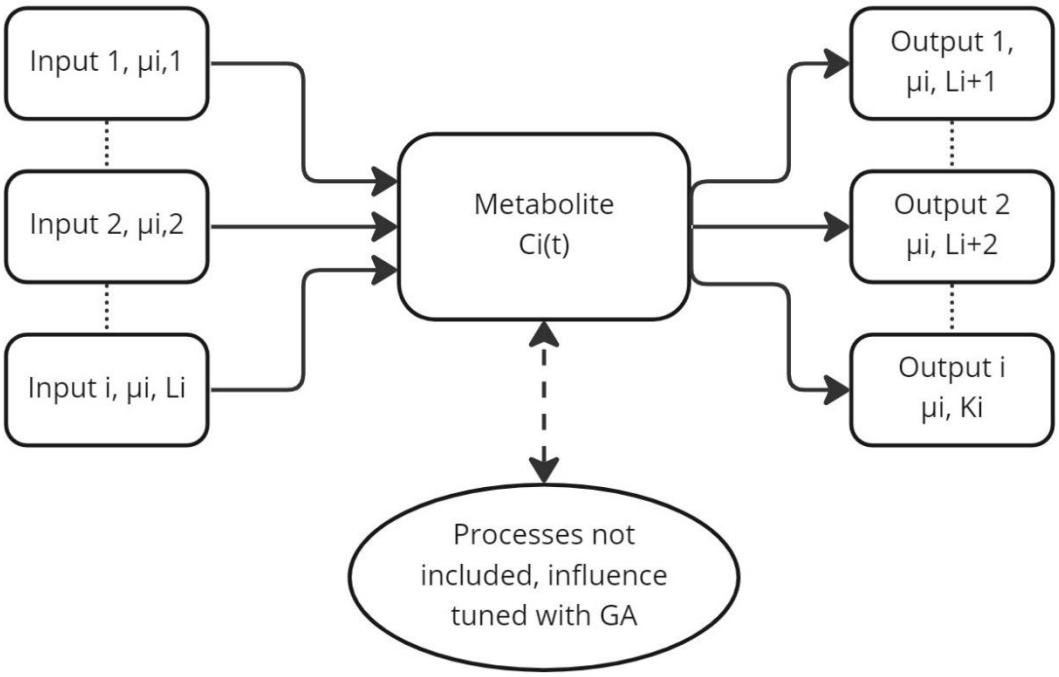

Figure 1 Example queue, which represents concentration Ci(t) of the metabolite. Arrival rates are presented as inputs, while metabolite depleting rates are outputs. Due to the complexity of the metabolic network, some simplifications were adopted. The influence of processes not included in the model were calculated using a genetic algorithm (GA).

Using the Michaelis-Menten kinetic equations, the adaptation parameter μ(t) was calculated. The behavior of metabolites of the studied metabolic pathways and reactions occurring in the model can be considered as a network of heterogeneous Poisson processes described by Equation 1:

$$P\left[\left(N(t+\tau)-N(t)\right)=k,t\right]=\frac{e^{-\mu(t)\tau}(\mu(t)\tau)^{k}}{k!} \quad (1.)$$

where:

$P\left[\left(N(t+\tau)-N(t)\right)=k,t\right]$ – probability of k arrivals in the interval $(t,t+\tau]$

$\mu(t)\tau$ – expected number of arrivals in a time interval duration of $(t,t+\tau]$

The queue processing time of metabolite increment (Eq. 2) is described by the exponential distribution of the random variable T in the terms of the rate parameter $\mu(t)$.

$$f(T;\mu(t)) = \begin{cases} \mu(t)e^{-\mu(t)T} & T \geq 0 \\ 0 & T < 0 \end{cases} \quad (2.)$$

Various metabolic pathways, which are incorporated in the presented model can be mimicked by a composition of interconnected queues based on the Michaelis-Menten equations. The flow of metabolite concentration from one queue to another is sequential, so that a decrease in concentration in one queue will cause an increase in the next queue. Thus, a network of interrelated queues can be equivalent to a set of differential equations [35].

The probability of a reaction occurring and moving to the next queue depends on factors such as metabolite-substrate concentration and kinetic constants. Each of the Michaelis-Menten kinetics equations relates to a specific substrate and affects whether a reaction occurs at a specific time point [34]. One of the advantages of basing the model on queueing theory is the possibility for its further development and addition of more reactions/metabolic pathways without interfering with the previously optimized reactions. This is particularly interesting because the model can be developed with further metabolomics discoveries or combined with pathways not included in this study.

Queueing theory-based models [17] are able to bypass some of the errors generated by models based on ODEs and CMEs. Moreover, the models based on queueing theory do not require addressing the issues that arise from using ODEs, such us dealing with negative results, which are not possible in the living cells. Such issues can be resolved quite easily, however, they require non-negative ODE solvers [36], available in e.g., MATLAB. Using queueing theory, one can analyze the average waiting time for a substrate to be processed by an enzyme, the probability of substrate being processed, the probability of substrate being blocked by another substrate and the maximum capacity of the enzyme. There are several benefits of using queueing theory in computational modeling of metabolic pathways:

1. Identification of bottlenecks: Queueing theory can be used to identify bottlenecks in the metabolic pathway by analyzing the average waiting time for a substrate to be processed by an enzyme and the probability of substrate being blocked by another substrate. This can help to understand the behavior of the system under different conditions, such as changes in the enzyme concentration or the substrate availability.

2. Evaluation of system performance: Queueing theory can be used to evaluate the performance of the metabolic pathway by analyzing the average waiting time for a substrate to be processed, the probability of substrate being processed, and the maximum capacity of the enzyme. This can help to identify potential limitations of the system and to evaluate the impact of different parameters on the system performance.
3. Modeling of the enzyme saturation: Queueing theory can be used to model the saturation of enzymes, which occurs when the enzymes are saturated by the substrate, and the substrate molecules have to wait to be processed. This can provide insights into the behavior of the system under different conditions, such as changes in the substrate availability or the enzyme concentration.
4. Analysis of the system under different scenarios: Queueing theory can be used to analyze the system under different scenarios, such as changes in the enzyme concentration or the substrate availability. This can help to understand the behavior of the system under different conditions, and to identify potential limitations of the system.
5. Computationally efficient: Queueing theory models can be computationally efficient, especially when compared to other methods, such as ODEs or CMEs, which can be more computationally intensive.
6. Provides a framework for modeling regulation mechanisms: Queueing theory can provide a framework for modeling regulation mechanisms, such as feedback inhibition or allosteric regulation, by changing the rate of the processing of the molecules by the enzymes.
7. Accounting for randomness: In modeling using queueing theory, it is easy to take into account the randomness of biological systems. This can be done by applying Gaussian noise to concentration values and kinetic parameters. Moreover, its application additionally allows one to face possible measurement errors that are consequences of various factors including apparatus errors or human factor.

It is worth noting that queueing theory is a mathematical framework that can be used to model the behavior of systems, but it is an abstraction of the real-world systems, so the

assumptions made and the parameters used in the model should be carefully considered and validated against experimental data.

In conclusion, queueing theory can be useful in creating computational models of metabolic pathways by modeling the enzymes as servers and the molecules as customers, it can provide insight into the system behavior and help identify potential bottlenecks in the pathway, but it is an abstraction of the real-world systems, so the assumptions made and the parameters used in the model should be carefully considered and validated against experimental data in order to achieve the highest possible accuracy and reality of the computation. This can help to identify any limitations or inaccuracies in the model and guide the model development process.

3.4. Kinetics of enzymatic reactions

The kinetics of enzymatic reactions describes how the rate of the reaction changes with respect to the concentration of the reactants, products, and enzymes. Enzymatic reactions are catalyzed by enzymes, which are specific proteins that lower the activation energy required for a reaction to occur, thus increasing the rate of the reaction. The kinetics of an enzymatic reaction can be described by the Michaelis-Menten equation [37], which describes the relationship between the rate of the reaction and the concentration of the substrate (reactant). The Michaelis-Menten equation is given by Equation 3:

$$v(t) = \frac{V_f \frac{S_1(t)S_2(t)}{K_{S_1}K_{S_2}} - V_r \frac{P_1(t)P_2(t)}{K_{P_1}K_{P_2}}}{\left(1 + \frac{S_1(t)}{K_{S_1}} + \frac{P_1(t)}{K_{P_1}}\right)\left(1 + \frac{S_2(t)}{K_{S_2}} + \frac{P_2(t)}{K_{P_2}}\right)} \quad (3.)$$

where:

$v(t)$ – reaction speed (velocity)

V_f – forward reaction speed

V_r – reverse reaction speed

$S_1(t), S_2(t), \dots, S_x(t)$ – substrate concentration in mmol/L at time instant t

$P_1(t), P_2(t), \dots, P_x(t)$ – product concentration in mmol/L at time instant t

$K_{S_1}, K_{S_2}, \dots, K_{S_x}$ – kinetic constant of substrate

$K_{P_1}, K_{P_2}, \dots, K_{P_x}$ – kinetic constant of product

It is worth noting that the Michaelis-Menten equation is a simplification of the reality, it assumes that the enzymes are in excess, and the enzymes are not affected by the substrate concentration, which might not be true for all cases.

The parameters used in the equations were derived from the scientific literature. To obtain them, I performed an extensive literature review in article databases such as PubMed [38] and Google Scholar [39]. The sources of the parameters were scientific articles from journals with high scientific reputation. Obtaining parameters from peer-reviewed journals deemed the parameters as reliable. The data came from two types of publications, original research and review articles. The information they contained was from research and was experimental data, or (in the case of review type articles) it was a literature review done by another research team that provided the data used to calculate the enzyme kinetics equations. Another sources of kinetic parameters used in the Michaelis-Menten equations were the KEGG Pathway [40], BRENDA [41], and BioNumbers [42] databases. These sources provided information on metabolite concentrations, kinetic data of the enzyme, i.e. V_{max} and K_m. The parameters from the scientific articles also served as a source of data as a means for model validation, to verify the accuracy of the calculations.

The so-called “balancing flow” was used to determine the flow of molecules having many different roles in cellular metabolism and participating in several metabolic pathways/other biochemical reactions. These were equations that took into account, among other things, the flow of a molecule to other cellular compartments, such as from the mitochondrion to the cytoplasm.

3.5. Law of mass action

The law of mass action is a principle in chemistry that describes the equilibrium state of a chemical reaction. It states that the reaction rate is proportional to the product of the concentrations of the reactants raised to their stoichiometric coefficients (Eq. 4) [43,44]. This means that the rate of the reaction is determined by the number of reactant particles present and their likelihood of colliding and reacting with each other. The law of mass action is used to derive mathematical equations that can be used to predict the equilibrium state of a chemical reaction given the initial concentrations of the reactants and the rate constants for the forward and reverse reactions. The law of mass action is based on the assumption that the particles in a chemical reaction are in constant, random motion and that the rate of the

reaction is directly proportional to the number of collisions between the reactant particles. It is a fundamental principle that is widely used in chemical kinetics and thermodynamics.

$$rate = k[A]^m[B]^n \quad (4.)$$

where:

k – rate constant of the reaction,

$[A], [B]$ – concentrations of the reactants A and B,

m, n – stoichiometric coefficients of reactants A and B in the reaction.

The law of mass action is a useful tool for understanding and predicting the behavior of chemical reactions, but it is not always applicable in real-world situations where other factors such as enzymes or catalysts can affect the rate of a reaction. Enzymes catalyze reactions by binding to the reactants and lowering the activation energy needed for the reaction to occur [45,46]. The rate of the reaction is not solely determined by the concentration of the reactants, but also by the presence and activity of the enzymes. Therefore, the mass action law is not useful in describing enzyme-catalyzed reactions. For this reason, I used the equations described by mass action law only in developing the insulin signaling pathway model.

3.6. The Krebs cycle

The Krebs cycle, also known as the citric acid cycle (CAC) or the tricarboxylic acid cycle (TCA), is a series of chemical reactions that take place in the mitochondrial matrix of eukaryotic cells. It is the central metabolic pathway that generates energy through the oxidation of acetyl-CoA, derived primarily from carbohydrates, fats, and proteins thus linking the metabolic pathways of these compounds. The reactions of the citric acid cycle were identified in 1937 by Hans Adolf Krebs, in whose honor the cycle is commonly called the Krebs cycle after him [47].

The source of acetyl-CoA in the Krebs cycle is pyruvate formed in glycolysis, which undergoes a reaction catalyzed by pyruvate dehydrogenase [48]. The Krebs cycle starts with the condensation of acetyl-CoA and oxaloacetate to form citrate. Citrate then goes through a series of transformations, including the conversion to isocitrate, alpha-ketoglutarate, succinyl-CoA, succinate, fumarate, malate, and back to oxaloacetate, and the cycle continuously repeats (Fig. 2). These reactions are catalyzed by eight different enzymes, including citrate

synthase, aconitase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinyl-CoA synthetase, succinate dehydrogenase, fumarase, and malate dehydrogenase.

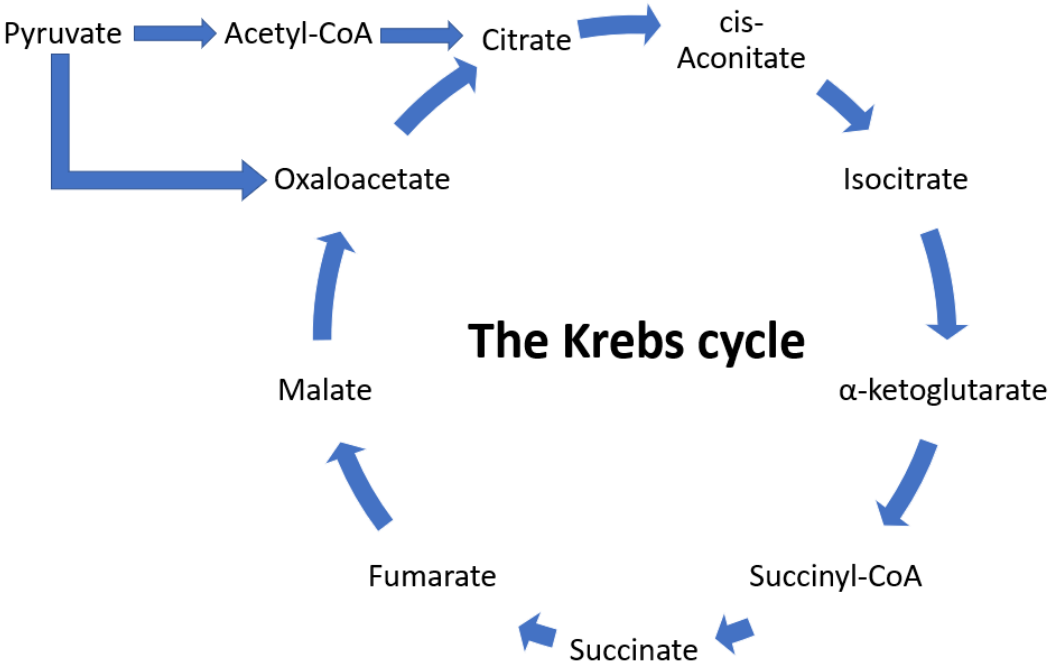

Figure 2 Overview of the Krebs cycle presenting the flow of metabolic intermediates in the cycle.

During each turn of the cycle, electrons are removed from the intermediates and transferred to the electron transport chain, ultimately leading to the production of adenosine triphosphate (ATP) through oxidative phosphorylation. Additionally, the cycle produces several important molecules, such as guanosine triphosphate (GTP), as well as reduced nicotinamide adenine dinucleotide (NADH), and reduced flavin adenine dinucleotide ($FADH_2$), which are used in the electron transport chain, where they are involved in reactions that lead to the generation of ATP. Each molecule of NADH and $FADH_2$ generated in the Krebs cycle leads to the production of 2.5 and 1.5 molecules of high-energy ATP, respectively. The Krebs cycle also produces carbon dioxide (CO_2), which is released as a waste product. Additionally, the cycle also generates important intermediates that can be used for other metabolic pathways such as gluconeogenesis, the biosynthesis of amino acids, and the synthesis of nucleotides.

To summarize, the Krebs cycle is an important source of energy for cells, as it generates GTP (which is the equivalent energy carrier as ATP) and other high-energy molecules that can be used to drive other metabolic reactions. It also plays a key role in the regulation of glucose

and lipid metabolism, and has been implicated in a wide range of physiological processes, including aging and cancer [49,50].

3.7. The pentose phosphate pathway

The pentose phosphate pathway (PPP) is a metabolic pathway that plays a crucial role in the metabolism of carbohydrates. It is also known as the hexose monophosphate pathway, the phosphogluconate pathway, or the Warburg-Dickens pathway. The main substrate of the PPP is glucose-6-phosphate (G6P), which is converted into various products such as nicotinamide adenine dinucleotide phosphate (NADPH) and ribose 5-phosphate (R5P). NADPH is used in the biosynthesis of fatty acids, while R5P is a precursor in the synthesis of nucleotides which are the building blocks of DNA and RNA. Another biologically significant molecule formed in PPP is erythrose 4-phosphate (E4P), which is used in the synthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan).

The PPP can be divided into two phases: the oxidative phase and the non-oxidative phase (Fig. 3). In the oxidative phase, NADPH is produced via the action of the enzyme glucose-6-phosphate dehydrogenase (G6PD). In the non-oxidative phase, various simple sugars are synthesized via the action of transketolase and other enzymes. 5-carbon sugars derived from the digestion of nucleic acids can be utilized in the PPP, where their carbon backbones are metabolized into intermediates for glycolysis or gluconeogenesis.

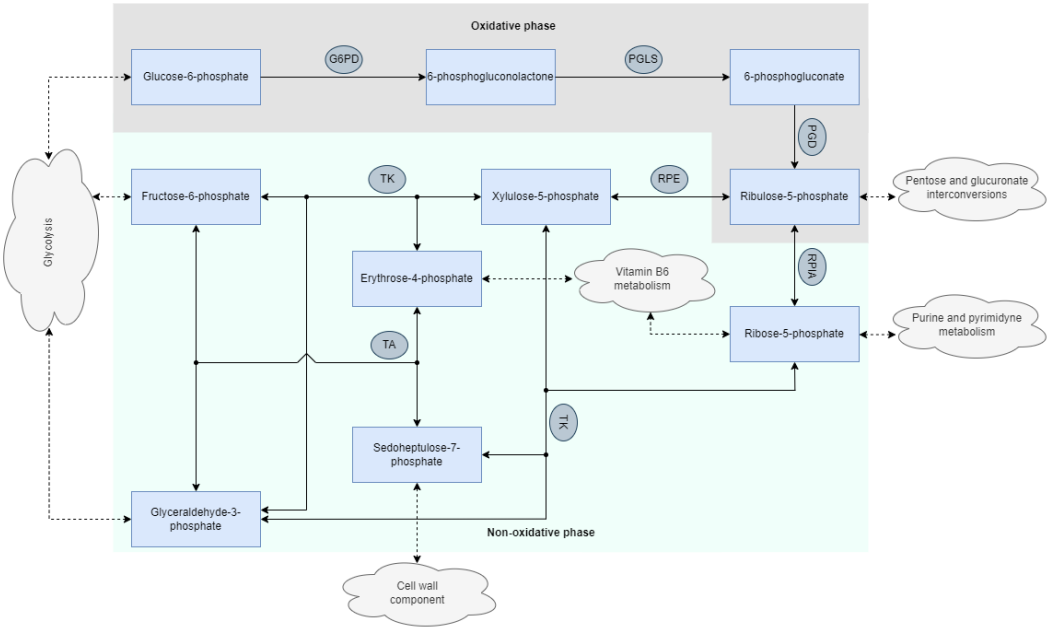

Figure 3 Overview of the pentose phosphate pathway illustrating the interconnections between metabolites and their flux to glycolysis and other metabolic pathways, where they are utilized. G6PD – glucose-6-phosphate dehydrogenase, PGLS – 6-phosphogluconolactonase, PGD – 6-phosphogluconate dehydrogenase, RPIA – ribose-5-phosphate isomerase A, RPE – ribulose-5-phosphate-3-epimerase, TA – transaldolase, TK – transketolase.

The PPP plays an important role in maintaining cellular levels of NADPH, which is necessary for maintaining cellular redox balance and preventing oxidative stress thus limiting the harmful effects of reactive oxygen species (ROS) in the cell. ROS can damage cellular lipids, proteins, and nucleic acids, and eventually cause cell death [51–53]. It is estimated that as much as 60% of NADPH comes from the PPP [54]. The pathway is active in many tissues, including the liver, adrenal cortex, and mammary glands. It is also particularly active in red blood cells, where it helps to reduce oxidative stress [55]. Due to the lack of mitochondria, the only source of NADPH in erythrocytes is PPP. NADPH is used in erythrocytes to reduce glutathione (GSH), which, in its reduced form, is crucial for normal function. When GSH levels in erythrocytes are too low, hemolysis can occur [56].

Studies have shown that the activity of the PPP is significantly increased in cancer cells compared to normal cells [57]. Elevated PPP activity is important for cancer cells to maintain their high proliferative state [58,59]. Therefore, many drugs aimed at blocking metabolic

pathways that supply cancer cells with substances necessary for proliferation target PPP [60–62].

3.8. Signaling pathway of cellular response to insulin

Insulin signaling is a process that allows cells to respond to changes in blood glucose levels by regulating the uptake, storage, and utilization of glucose. The insulin signaling pathway is a complex network of interactions between proteins (Fig. 4) [63,64]. It is initiated by the binding of insulin to its receptor on the cell surface. This binding triggers a cascade of molecular events inside the cell, ultimately leading to changes in gene expression and metabolism.

The insulin receptor is a tyrosine kinase receptor, meaning that it has an intrinsic kinase activity that phosphorylates tyrosine residues on target proteins. When insulin binds to the receptor, the receptor's intracellular domain becomes activated and phosphorylates specific tyrosine residues on intracellular proteins. This phosphorylation creates binding sites for other intracellular signaling molecules, such as insulin receptor substrates (IRS) proteins [26].

The phosphorylated IRS proteins then recruit and activate other intracellular signaling molecules such as the phosphoinositide 3-kinase (PI3K) enzyme. PI3K is activated by binding to the IRS proteins and it converts phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3), which acts as a second messenger that recruits other downstream proteins such as Akt (also known as PKB) to the membrane. Akt is a serine/threonine kinase that phosphorylates a variety of target proteins to regulate various cellular functions such as glucose uptake, glycogen synthesis, and protein synthesis. It is also responsible for activation of the downstream elements of signaling pathway that lead to changes in gene expression and glucose uptake.

One important downstream signaling pathway activated by insulin is the mTOR (mammalian target of rapamycin) pathway [65]. This pathway regulates cell growth and metabolism and is activated by the PI3K-PKB/Akt pathway [66]. mTOR ultimately leads to the activation of S6K1 (S6 kinase 1) and 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1), which are key regulators of protein synthesis and glucose uptake, respectively.

Another important downstream signaling pathway activated by insulin is the MAPK (mitogen-activated protein kinase) pathway. This pathway is activated by the IRS-PI3K-PKB/Akt pathway and ultimately leads to the activation of the transcription factors Elk-1 and c-Fos, which are key regulators of gene expression and glucose uptake. In addition, insulin also

activates other signaling pathways, such as the JAK2-STAT5 pathway, which leads to the activation of genes involved in glucose uptake and metabolism.

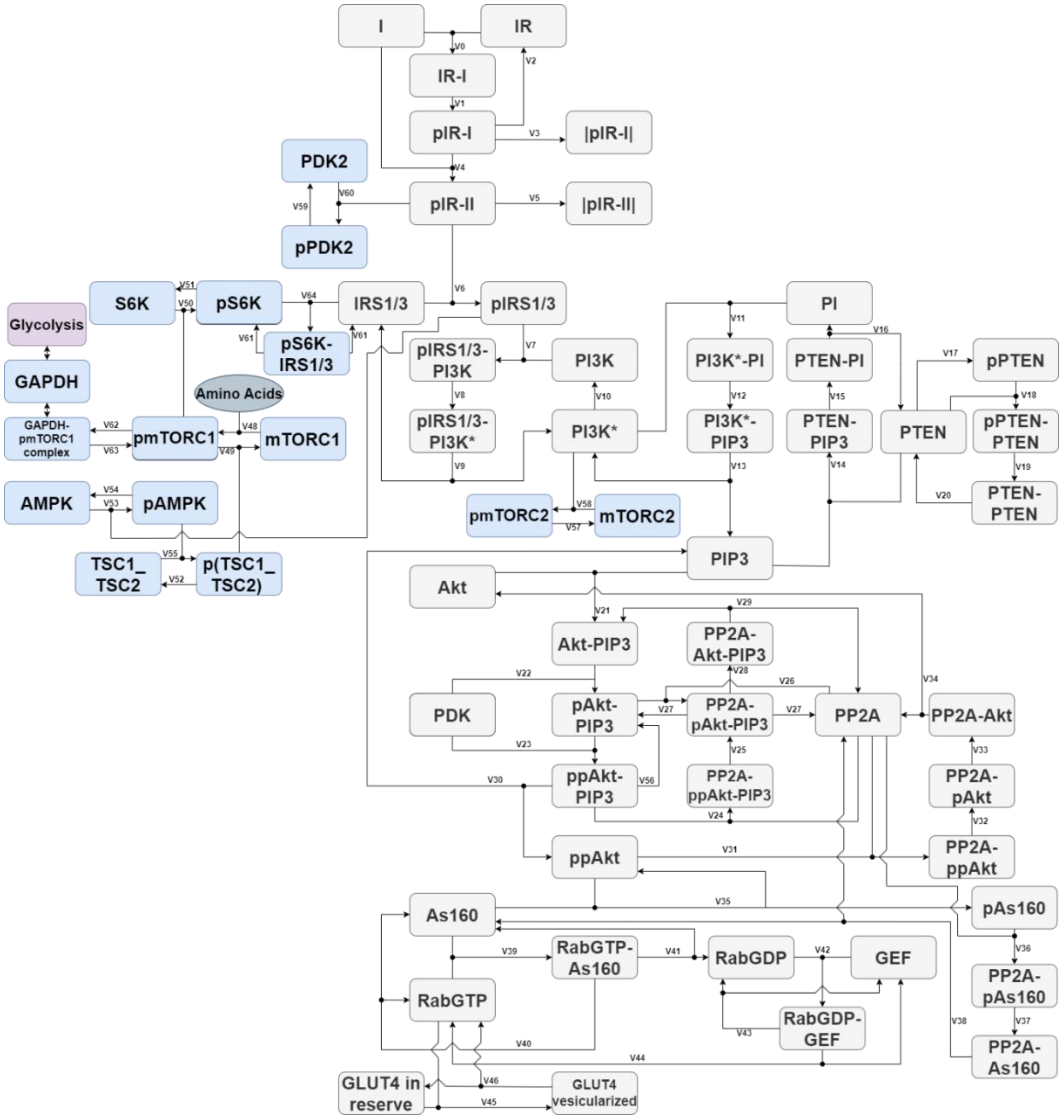

Figure 4 Insulin signaling pathway – a complex interconnected network of signaling proteins. Blocks marked in gray were characterized in [67]. Blocks marked in blue were included in an extended model that takes into account the relationship between the insulin signaling pathway and mTORC1 [68].

The activation of the insulin signaling pathway leads to an increase in the amount of activated glucose transporter 4 (GLUT4) molecules, which then translocate to the plasma membrane and increase glucose uptake into the cell [67,69,70]. The PI3K/Akt pathway, activated by insulin binding to its receptor, phosphorylates and activates the v-SNARE protein VAMP2, which in turn mediates the fusion of GLUT4-containing vesicles with the plasma

membrane. This leads to an increase in glucose uptake by the cell, which is a key component of the cellular response to insulin in terms of glucose metabolism. It also promotes the expression of genes involved in glucose transport and metabolism, and by activating enzymes involved in glucose metabolism. This results in a decrease in blood glucose levels and an increase in energy storage in the form of glycogen and fat.

One of the proteins involved in the regulation of mTORC1 activity is Ras homolog enriched in brain (Rheb). Rheb is a small GTP-binding protein that is involved in the regulation of cell growth and proliferation. One of its known interactions is with the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH is an important enzyme in both glycolysis and the pentose phosphate pathway (PPP), and it has been shown that Rheb can modulate GAPDH activity. Rheb can bind to GAPDH and activate its enzymatic activity, leading to an increase in glucose metabolism. This interaction between Rheb and GAPDH plays a critical role in cell growth, as well as in the Warburg effect, a phenomenon where cancer cells preferentially use glycolysis for energy production even in the presence of oxygen [60,71,72]. This interaction between GAPDH-Rheb-mTORC1 is not fully understood yet, however, it does have a significant impact on mTORC1 activity, thus affecting the amount of GLUT4 particles involved in the glucose transport.

4. Study aims

The primary objective of the work was to prepare and develop simulation models of cellular metabolic pathways and signaling pathway of cellular response to insulin using queueing theory and to assess the feasibility of simulating the inhibition of these pathways.

The partial objectives were:

1. Assessment of the ability of computational models to simulate and track changes in metabolite concentrations in real time.
2. Evaluation of the possibility of simulating pyruvate dehydrogenase inhibition induced by drugs used in breast cancer therapy, using the combination of Tamoxifen with Metformin or Phenformin as an example, by comparison of model concentration changes of Krebs cycle metabolites and literature data.
3. Evaluation of the feasibility of simulating 6-phosphogluconate dehydrogenase (6PGD) inhibition induced by 6PGD gene knockdown in lung cancer therapy by comparing model changes in the pentose phosphate pathway metabolite concentrations and literature data.
4. Assessment of the effect of GAPDH as a regulator of mTORC1 activity, mediated by the regulatory protein Rheb, which is an essential activator of mTORC1. Evaluation of the effect of mTORC1 activity on the amount of GLUT4 molecules used in glucose transport.

5. Summary of works included in the series of publications

5.1. Original paper I – Queueing theory model of Krebs cycle.

This paper describes a research project that uses computational modeling to study the Krebs cycle. The model is based on queueing theory. A detailed process description for creating a simulation model of the Krebs cycle, including a literature review, the use of a genetic algorithm to optimize kinetic constants, and a description of the model itself is presented in this paper. It was highlighted, that the benefits of using computational models include reduction of the amount of the animals used during laboratory experiments, could possibly accelerate the diagnosis and treatment of metabolic diseases, and contribute to the cost reduction of drug approval process.

The Michaelis-Menten equations of enzyme kinetics were used to create the model. These kinetic equations were combined together and formed a Krebs cycle model based on queueing theory. The metabolites of the Krebs cycle are an interconnected system of vessels; a metabolite that is a product of one reaction becomes a substrate for another enzyme in a following reaction. Therefore, using and basing the model on queueing theory seemed to be the correct approach.

Existing literature data of molecular concentrations of metabolites were taken as initial concentration values for the model [18,25,34,42,73–75]. The kinetic properties of enzymes that catalyze the reactions in the Krebs cycle were derived from the literature in order to calculate the reaction rates using Michaelis-Menten kinetics. The stability of the model was tested by simulating it for 5.5 hours and observing the difference between the model's predictions and available biological data. Further, the model was tested to emulate changes in enzyme activity associated with diseases such as cancer, which can influence metabolism. The model was used to simulate the effects of drugs used in the cancer therapy on the concentrations of individual metabolites in the cycle. The drugs used during validation process are known to affect enzyme reactions in the cycle by slowing them down as a competitive inhibitors. The experiment used existing research on substances that affect enzymes involved in the Krebs cycle reactions to reflect the effect of the drug on the rate of enzymatic reaction and the concentration of metabolites in order to understand the kinetic properties of inhibitors and predict its effect on cell metabolism.

The queueing theory proved to be an effective method of modeling the interactions between enzymes, molecules, and other biomolecules in metabolic pathway. This approach

allowed for a creation of a mathematical simulation model that resembles the biological one, and copes with the issues that would arise from using ODE-based models of biological systems. It was found that the largest relative difference between modelled and real-life data was -11.33% in the case of the combined concentrations of succinyl-CoA and succinate, while the calculated concentrations of most other metabolites differed from experimental data by less than 5%. These results were very satisfying.

During validation, the obtained computational results were compared to the experimental results published in a study of Janzer et al [76]. This study was a basis to compare the predictions of the model to the experimental data. In [76] the concentrations of several Krebs cycle metabolites were measured after administration of drugs used in anti-cancer therapy. The study tested the effects of anti-cancer drug, Tamoxifen, in combination with Metformin and Phenformin, respectively. These drugs are used to treat diabetes and have been observed to increase the anti-cancer effects of Tamoxifen.

Another improvement was the use of the so-called "balancing flow" in the model. Balancing flow imitates the drainage of metabolites due to their various uses in cell functioning, in order to stabilize the concentrations of Krebs cycle metabolites. Using a computational model, I was able to determine the percentage of inhibition induced by specific drugs used in cancer therapy. By comparing the simulation and measurement results, it was concluded that the drugs administration in the doses used in the study [76] inhibit the reaction catalyzed by pyruvate dehydrogenase by about 30%. Because reactions in the Krebs cycle are interrelated, and metabolites that are products in one reaction become substrates in the following reactions, inhibition of pyruvate dehydrogenase activity also affected changes in the concentrations of other metabolites that are not substrates of the aforementioned enzyme. It was concluded that the model accurately reflects the stochastic nature of biological reactions and provides an accurate and time-efficient representation of the Krebs cycle.

To summarize, the development of a model that mimics the conditions of metabolic reactions in living cells was presented in this paper. The model used data on metabolite concentrations and enzyme constants from different sources, but we have made efforts to ensure the data was as accurate and compatible as possible. The model can be used as a virtual laboratory to study interdependencies between substances and metabolites and their influence on cellular functions. It can also provide knowledge on how chemical compounds obtain their therapeutic efficacy and be used to improve drug development safety by

determining which reactions of the metabolic pathway are the best candidates for disturbing and what doses of the drug have a significant effect. The model can also be used to observe the dose above which the effect of the drug is imperceptible.

My contribution to the research reported in this paper has been conceptualization and realization of the study. I performed a thorough state of the art analysis, designed the structure of the algorithm. I collected the necessary numerical data for the research and model preparation regarding the concentrations of metabolites present in the pathway, as well as the kinetic constants of the enzymes catalyzing the reactions occurring in the modelled pathway. I then used these data to prepare Michaelis-Menten equations of enzyme kinetics. Moreover, I performed analysis of simulation results and compared obtained results to the literature data. Finally, I wrote the original draft of this paper.

5.2. Original paper II – Queueing theory model of pentose phosphate pathway.

The paper describes the creation of queueing theory-based computational model of a pentose phosphate pathway (PPP). PPP is a metabolic pathway that produces biologically important molecules such as nicotinamide adenine dinucleotide phosphate (NADPH), ribose 5-phosphate (R5P), and erythrose 4-phosphate (E4P). The PPP plays an important role in maintaining cellular levels of NADPH under stress, and the pathway is active in many various types of human cells, including hepatocytes, adrenal cortex, and mammary glands, as well as in red blood cells. NADPH generated by the PPP is used to prevent oxidative stress and the formation of dangerous free radicals that could harm the cell. The aim of the work was to prepare a PPP model that can track concentration changes of specific metabolites in the pathway over time.

Data on the concentrations of PPP metabolites, as well as the kinetic parameters of the enzymes in this pathway, came from a scientific publication [77]. The created model became stable within one hour and simulated the pathway using 1,000 simulations per second, averaged over 50 simulated cells. The model also used a feature called "balancing flow" to better mimic the flow of metabolites in a living cell. The accuracy of the model was assessed by comparison of the model results and literature data of PPP's metabolites concentration. We identified 6-phospho-gluconolactone (PGL) to be the bottleneck of the model, as it was found to have a high relative difference between literature and computational data. It can be explained by the fact that PGL is rapidly hydrolyzed, so the practical equilibrium between

glucose-6-phosphate (G6P) and 6-phosphogluconate (6PG) is directed towards the formation of 6PG [78].

One of the reasons we created the PPP model was that this is one of the pathways that has increased activity in cancer cells, and drugs that block this pathway can inhibit tumor growth [79]. The model was validated by comparing it to a study [79] that used small hairpin RNA (shRNA) to reduce the expression of 6-phosphogluconate dehydrogenase (*PGD*) gene. As a result of the *PGD* knockdown, an accumulation of metabolites preceding the blocked reaction occurred. It was a result of the reduced expression of the PGD enzyme. A bottleneck is created at this stage of the pathway, leading to a reduced efficiency of this stage, as there are not enough protein molecules in the cell to process all metabolite molecules. The enzyme, in turn, was followed by a decrease in metabolites, such as sedoheptulose-7-phosphate (S7P), because the reactions preceding its formation were slowed/blocked, making it impossible to preserve the natural production of S7P.

In this study we have performed several measurements to evaluate the level of inhibition of the GPD catalyzed reaction in neoplastic cells. The results show that the knockdown of *GPD* caused inhibition of 95-98%. These results are consistent with current biological knowledge and comparable to those obtained experimentally. We have also performed simulations with 100% inhibition but this led to a significant reduction in the concentration of downstream metabolites. The results from the model suggest that knockdown efficiency in vitro was likely near 95% which is common for shRNA expression knockdown. By conducting this type of study, I confirmed the potential of using queueing theory to understand the impact of gene knockdown on metabolic pathways. This model, although it uses some simplifications, is able to faithfully reproduce the shRNA-induced changes occurring in real cells.

To summarize, the model used queueing theory and took into account the effects of fluctuations in metabolite concentrations on the entire PPP. The model proved to be useful for testing the effectiveness of new drugs and predicting the impact of therapy. The study also indicated that most studies on blocking the PPP pathway in cancer patients have focused on blocking the first reaction of the pathway catalyzed by glucose-6-phosphate dehydrogenase (G6PD), but that was not very effective. The presented study explored the effects of knockdown of the *PGD*, which results in inhibition of tumor growth. The proposed model is believed to be able to predict the impact of therapy, which will lead to an increase in its effectiveness. Moreover, presented model can be used to determine the effects of the

inhibition of particular enzymes on the concentration of metabolites with considerably high accuracy.

My contribution to the research reported in this paper has been conceptualization and realization of the study. I performed a thorough state of the art analysis, designed the structure of the algorithm. I collected the necessary numerical data for the research and model preparation regarding the concentrations of metabolites present in the pathway, as well as the kinetic constants of the enzymes catalyzing the reactions occurring in the modelled pathway. I then used these data to prepare Michaelis-Menten equations of enzyme kinetics. Moreover, I performed analysis of simulation results and compared obtained results to the literature data. Finally, I wrote the original draft of this paper.

5.3. Original paper III – Queueing theory model of mTOR complexes' impact on Akt-mediated adipocytes response to insulin.

The aim of the study was to create a computational model of the effects of mammalian target of rapamycin (mTOR) complexes on the signaling pathway of the adipocyte response to insulin. The model was based on queueing theory. Using the model, it is possible to real-time track the number of glucose transporter 4 (GLUT4) molecules involved in the transport of glucose from the blood into the cell. Insulin is a hormone produced by the pancreas that helps regulate blood sugar levels in the body. It stimulates the uptake of glucose in tissues like muscle and fat and inhibits the production of glucose from non-glucose sources. The signaling network of the cellular response to glucose is complex. The cascade of responses comprising the signaling process begins with insulin attachment to the insulin receptor, which is followed by a variety of signaling molecules that lead to GLUT4 activation and glucose transport. The studies I have conducted provide a better understanding of the interactions between different signaling molecules and their effects on the cellular response to insulin. Due to the complex network of interrelationships, the multi-step nature of the whole process, research conducted using computational models can provide valuable knowledge in research related to diabetes and its treatment.

One of the protein complexes involved in this process is mTOR complex 1 (mTORC1). mTORC1 is a complex of proteins composed of mTOR, regulatory-associated protein of mTOR (Raptor), mammalian lethal with SEC13 protein 8 (mLST8), and the non-core components: PRAS40 and DEPTOR. It is a protein with a wide range of metabolic regulatory functions in tissue cells such as muscle, liver, and brown and white adipose tissue. One of the proteins that

regulates mTORC1 activity is Rheb. However, an enzyme found in the glycolytic pathway, GAPDH, has a high affinity for the Rheb protein. The combination of GAPDH with Rheb prevents mTORC1 activation. Depending on the degree of mTORC1 activity, the number of GLUT4 molecules involved in glucose transport varies. This model takes into account the random variations and fluctuations in cells that can affect signaling pathways. The model has been tested using experimental data and has shown good accuracy in predicting the number of active GLUT4 particles.

The Akt-mediated insulin signaling pathway has well-defined endpoints, which can be used to test the accuracy of the computational model. The model was developed using information from the PubMed database and was tested using simulations of 50 independent cells, mimicking human adipocytes. The concentrations of the molecules involved in the signaling pathway were randomly chosen with a range limited by 10% Gaussian noise. The simulation results were obtained using 1ms time increments, but the model allows for the use of any time increment. The simulation was performed in C# 8.0 and the results were averaged over the entire cell population. The model uses a network based on queues to model reactions whose rates change dynamically and randomly.

To test the validity of the model, the theoretical inhibition of mTORC1 activity was simulated. One inhibitor of mTOR activity is rapamycin, but it has a number of side effects, including an increased risk of infection, cancer, weight disorders, hyperlipidemia, and diabetes-like metabolic disorders. Therefore, it is important to develop drugs that selectively inhibit mTORC1 activity without significant side effects, such as astragaloside IV (As-IV). The data from the model can be used to study the kinetics of reactions in the insulin signaling pathway and to identify the most effective place for therapeutic intervention. In the absence of insulin, there are approximately 18,200 GLUT4 molecules near the cell membrane that are able to transport glucose into the cell when activated. However, insulin stimulation increases this number to approximately 195,000, or about 50% of total GLUT4.

There are two scenarios for the regulation of mTORC1 activity through the insulin signaling pathway: when the concentration of glucose in the blood is high after eating and insulin signaling is functioning correctly, and when the organism is in a state of prolonged fasting and there is a decrease in extracellular glucose and insulin secretion. In the first scenario, GLUT4 molecules are activated by insulin and move to the cell membrane to transport glucose into the cell. The glucose is then phosphorylated to form G6P, which can

either enter the glycolytic pathway or be converted into glycogen. The sequence of reactions in glycolysis produces glyceraldehyde-3-phosphate (G3P), which is converted into 1,3-bisphosphoglycerate by GAPDH. GAPDH is important for the regulation of mTORC1 activity because its concentration levels in the cell oscillate around a certain value and its state can change from processing G3P to being free to bind with Rheb protein and activate mTORC1. In the second scenario, when insulin signaling cascade is interrupted, GLUT4 remains stationary and unable to transport glucose, leading to the hydrolysis of stored glycogen and the maintenance of basic G6P levels. However, the glycolytic flux is decreased, resulting in fewer G3P molecules and more free GAPDH molecules that can bind with Rheb and inactivate mTORC1. Intermediate conditions between these two scenarios are more common in cells.

A queueing theory-based model of the insulin signaling pathway was developed and tested to understand the relationships between the levels of GLUT4, GAPDH, and mTORC1. These relationships play a significant role in how the cell responds to insulin and extracellular glucose. The model's results were consistent with current knowledge and showed that the amount of GLUT4 molecules ready to transport glucose is heavily dependent on the amount of GAPDH "occupied" with processing its substrate. The model also demonstrated that both mTORC1 activity and the amount of "occupied" GAPDH can significantly influence the amount of GLUT4 and lower the amount of GLUT4 molecules involved in glucose transport. The scenario in which all GAPDH molecules are busy processing its substrate and mTORC1 is fully active keeps the amount of GLUT4 in vesicles at the maximum level. These results suggest that drugs that can significantly decrease mTORC1 activity may be important for increasing the amount of GLUT4 directed to the cell membrane for glucose transport. The insulin signaling pathway is complex and unstable, with small changes potentially leading to altered cell responses and diseases such as type 2 diabetes. The model also showed that many elements can contribute to glucose malabsorption and that the nodes of the pathway that influence AMPK activity, specifically IRS1/3, may be important targets for therapeutic intervention.

The model I prepared shows the "big picture" and the complex interrelationships that occur between signaling molecules that lead to a differential response to insulin and consequent activation of different amounts of GLUT4 involved in glucose transport into the cell.

My contribution to the research reported in this paper has been conceptualization and realization of the study. I performed a thorough state of the art analysis, designed the

structure of the algorithm. I collected the necessary numerical data for the research and model preparation regarding the concentrations of signaling proteins involved in the process of cellular response to insulin, as well as the kinetic constants. I then used these parameters to prepare kinetic equations based on mass-action law. Moreover, I performed analysis of simulation results and compared obtained results to the literature data. Finally, I wrote the original draft of this paper.

6. Publications that are the subject of the dissertation

6.1. Original paper I – content of the publication "Queueing theory model of Krebs cycle."

Bioinformatics, 37(18), 2021, 2912–2919
doi: 10.1093/bioinformatics/btab177
Advance Access Publication Date: 16 March 2021
Original Paper

OXFORD

Systems biology

Queueing theory model of Krebs cycle

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Associate Editor: Pier Luigi Martelli

Received on December 29, 2020; revised on March 8, 2021; editorial decision on March 9, 2021; accepted on March 11, 2021

Abstract

Motivation: Queueing theory can be effective in simulating biochemical reactions taking place in living cells, and the article paves a step toward development of a comprehensive model of cell metabolism. Such a model could help to accelerate and reduce costs for developing and testing investigational drugs reducing number of laboratory animals needed to evaluate drugs.

Results: The article presents a Krebs cycle model based on queueing theory. The model allows for tracking of metabolites concentration changes in real time. To validate the model, a drug-induced inhibition affecting activity of enzymes involved in Krebs cycle was simulated and compared with available experimental data.

Availabilityand implementation: The source code is freely available for download at https://github.com/UTP-WTIiE/KrebsCycleUsingQueueingTheory, implemented in C# supported in Linux or MS Windows.

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Supplementary information: Supplementary data are available at *Bioinformatics* online.

1 Introduction

Modeling metabolic pathways can be extremely useful in the scientific world (Ederer *et al.*, 2014; Nazaret *et al.*, 2009; Theodosiou *et al.*, 2015; Wu *et al.*, 2007). The ability to predict a cell's response to changes in the surrounding environment, i.e. changes in metabolite levels or external stimulus, would greatly improve the designing of experiments. Therefore, the impact of these changes on the whole cell metabolism should be assessed in the experiment planning stage. Modeling, combined with the ever-evolving metabolomics, could help speed up the diagnosis and treatment of metabolic diseases. Often, drugs are tested on animals, given in various doses and assessed for their impact and effects. Such tests can be lethal to laboratory animals and are responsible for the majority of their deaths (Hajar, 2011; Hawkins *et al.*, 2019; Lynch and Slaughter, 2001). Developing an accurate metabolism model could reduce the need for animal studies and reduce animal cruelty. In addition, before drugs reach medical use, and after *in silico*, *in vitro* and/or *in vivo* testing, they undergo a series of long-term clinical trials during which their impact and long-term effects are carefully assessed. An effective metabolism model could accelerate the process and achieve cost reductions. Animal testing and human clinical trials are both necessary for validating the effectiveness of a drug, however, a long-term goal of our effort is to reduce our reliance on these tests and present *in silico* methods as a sufficient alternative.

Queueing theory is mainly used for issues related to telecommunications and engineering, yet queueing theory is suitable for modeling stochastic changes occurring in biological systems. Until now, the queueing theory has been used, for example, to model insulin levels and the number of insulin receptors needed. Such studies can help to understand insulin-dependent diseases (Cavas and Çavaş, 2007). Interestingly, thanks to the queueing theory, it was also possible to model the impact of ethanol consumption and remove the side effects caused by its consumption (sobering) (Guang, 1998). Such studies indicate a multitude of applications of queueing theory, also in modeling metabolic pathways. Queueing theory has been previously used to model a simple metabolism network and mimic chemical interactions between substrates and products (Evstigneev *et al.*, 2014). Recently, a model of glycolysis based on queueing theory has been presented (Clement *et al.*, 2020). The use of queueing theory is also beneficial from the computational perspective as it requires less computing power, thus accelerating computing time and allows simulations to be carried out in real time. Due to the nature of reactions in Krebs cycle, reaction products become substrates for the next reaction in the cycle. In addition, biological systems have well-organized ways to transform molecules, pass them down the pathway and transport them to where they are needed to maintain normal cell function (Tsitkov *et al.*, 2018), much like transmitting packets in the internet from one node to another one. For this

reason, we decided it would be reasonable to use queueing theory, which has been proven a useful modeling technique in communication systems, to model this metabolic cycle.

In this article, we present the entire process for creating a Krebs cycle simulation model: from a literature review to obtain empirical data on metabolites and enzymes necessary to model the reaction according to the Michaelis–Menten kinetics, through the description of the genetic algorithm used to optimize the kinetic constants found in different sources (Siess *et al.*, 1976; Singh and Ghosh, 2006), and finally the description of the model itself—the obtained concentration results and their comparison against the available data, as well as confirmation of the model's effectiveness simulating the inhibition of the Krebs cycle induced by drugs.

The Krebs cycle, also known as the tricarboxylic acid cycle (TCA) or the citric acid cycle (CAC), takes place in a mitochondrial matrix. The purpose of the Krebs cycle is to produce energy in a form of guanosine triphosphate (GTP) which also releases carbon dioxide (CO_2)(Fig. 1, Table 1) as a byproduct. GTP is the energetic equivalent of adenosine triphosphate (ATP) (Korla and Mitra, 2014; Ponizovskiy, 2016; Smith and Robinson, 2011). Summarized equation of the Krebs cycle: acetyl-CoA + 3 NAD^+ + FAD + GDP + P_i + 2 H_2O → 2 CO_2 + 3 NADH + $FADH_2$ + GTP + 2 H^+ + CoA. Formulas of Krebs cycle reactions and the enzymes catalyzing these reactions are presented in Table 1. Reaction 1 in Table 1 is the first reaction in which the cell may obtain its energy in the form of acetyl-CoA. The reaction that completes the cycle is the transfer of two acetyl groups from acetyl-CoA to a four-carbon compound—oxaloacetate and the formation of a six-carbon molecule—citrate. Citrate then undergoes a series of reactions, during which energy is produced and CO_2 released. Acetyl-CoA serves as fuel for the Krebs cycle and can be derived from various sources including fats, carbohydrates and proteins, thus connecting the metabolic pathways of these elements. Acetyl-CoA can also be derived from pyruvate, the main product of the glycolysis. Nevertheless, the Krebs cycle is also a source of amino acid precursors, as well as a molecule that is extremely important for metabolism, the reduced form of NAD—NADH, which plays a role in many other reactions in the cell like oxidative phosphorylation (Krebs and Johnson, 1937). Due to the use of individual metabolites as intermediates for the synthesis of further compounds necessary for the proper cell function, these metabolites have the ability to leave the cycle by means of transport mechanisms that move them to the appropriate site. The criticality of the Krebs cycle in mammalian physiology is the primary reason we have sought to undertake the development of the model described herein. Additionally, dysregulation of the Krebs cycle would be deleterious, and could result in large energy losses and overproduction of cofactors like NADH. Cycle regulation is based on the cellular assessment of the amount of available substrates and resulting products. A low concentration of substrates or a high concentration of products will decrease reaction rates. Cellular ADP availability and its conversion to ATP also affects the speed of reactions. Lower ADP concentrations cause accumulation of NADH, which has inhibitory properties for many enzymes. A high concentration of citrate also affects the course of the cycle because it can inhibit glycolysis reactions, thereby preventing metabolite flow.

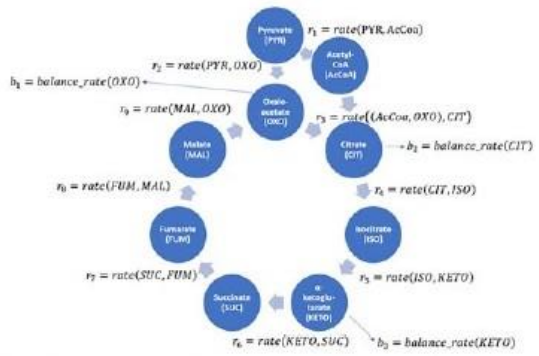

Fig. 1. Krebs cycle scheme using queueing theory. Molecules of the same type are queues. Reactions of the same type are servers. The queues are linked together, and the server output from one queue is connected to the input of the other queue

2 Methodology

2.1 Obtaining data on metabolite concentrations and characterization of enzymes catalyzing cycle reactions

Interest in metabolomics has grown rapidly due to the development of mass spectrometry (MS), which makes it possible to assess the concentrations of individual metabolites despite the fact that some of them occur in very small amounts, including those in the Krebs cycle (Ahn *et al.*, 2017; Albe *et al.*, 1990; Bennett *et al.*, 2009; Ishii *et al.*, 2007; Milo *et al.*, 2010; Mogilevskaya *et al.*, 2006; Park *et al.*, 2016). The model developed by our team is based on existing knowledge of molecular concentrations as initial values to the model (Table 2). The kinetics of enzymatic reactions are calculated according to Michaelis–Menten kinetics (1) (Singh and Ghosh, 2006). Our model enables tracking the course of reactions—both reaction speed and product growth over time. The speed of the enzymatic reaction depends on factors such as the maximum speed at which the enzyme can convert substrate into a product, the concentration of substrate and the enzymatic constant.

$$\upsilon = \frac{V_f \frac{S_1 S_2}{K_{S_1} K_{S_2}} - V_r \frac{P_1 P_2}{K_{P_1} K_{P_2}}}{(1 + \frac{S_1}{K_{S_1}} + \frac{P_1}{K_{P_1}})(1 + \frac{S_2}{K_{S_2}} + \frac{P_2}{K_{P_2}})} \quad (1)$$

where υ is the reaction speed, V_f is the forward reaction speed, V_r is the reverse reaction speed, $S_1, S_2, \ldots, S_x$ - substrate concentration in mmol/l, $P_1, P_2, \ldots, P_x$ is the substrate concentration in mmol/l, $K_{S_1}, K_{S_2}, \ldots, K_{S_x}$ is the kinetic constant of substrate and $K_{P_1}, K_{P_2}, \ldots, K_{P_x}$ is the kinetic constant of product.

Enzymatic properties of enzymes that are involved in Krebs cycle reactions are presented in Supplementary Table S1 (Singh and Ghosh, 2006). If enzymatic data was unavailable, appropriate assumptions were made. For example, if K_P is unknown, it can be calculated as $10^* K_S$; the reverse speed of reaction is 100x slower than forward reaction ($V_r = \frac{V_f}{100}$). These assumptions are based on previous research and empirical observations (Singh and Ghosh, 2006) and have been adapted in our model. The reaction equations based on the Michaelis–Menten kinetics are presented in Supplementary Table S2. The reaction rates were calculated using kinetic constants and metabolite concentrations available in literature (Ahn *et al.*, 2017; Albe *et al.*, 1990; Bennett *et al.*, 2009; Ishii *et al.*, 2007; Milo *et al.*, 2010; Mogilevskaya *et al.*, 2006; Park *et al.*, 2016; Siess *et al.*, 1976; Singh and Ghosh, 2006). The concentrations of the following metabolites were combined: isocitrate and cis-aconitate, as well as succinyl-CoA and succinate. Combined concentrations were 0.0216 and 0.73 mmol/l, respectively. Isocitrate and cis-aconitate, as well as succinyl-CoA and succinate are transient, and once produced they are immediately used in the next reaction in the cycle. Combining them for simulation purposes with the metabolites adjacent to them in the cycle accelerated the calculation processes of the model and improved its stability.

2.2 Queueing theory

There are many studies examining trials of individual metabolic pathways modeling, but the large amount of interactions between metabolites, enzymes and other biomolecules make modeling metabolic pathways an extremely difficult task. Until now, the preferred method used in systems modeling was ordinary differential equations (ODEs) (Ahn *et al.*, 2017; Cohen and Bergman, 1995; Ederer *et al.*, 2014; Foster *et al.*, 2019; Jahan *et al.*, 2016; Jeffrey *et al.*, 1999; Korla and Mitra, 2014; Kurata and Sugimoto, 2018; Mogilevskaya *et al.*, 2006). Several approaches used scenario-based modeling (SBM) in connection with already existing platforms and tools, like PlayGo (Dräger *et al.*, 2008; Lapid *et al.*, 2019; Nazaret

Table 1. Krebs cycle and related reactions—stoichiometric formulas

Number	Reaction	Enzyme
1	$Pyruvate + CoA + NAD^+ \rightarrow acetyl\text{-}CoA + CO_2 + NADH$	Pyruvate dehydrogenase
2	$Pyruvate + HCO_3^- + ATP \rightarrow oxaloacetate + ADP + P_i$	Pyruvate carboxylase
3	$Oxaloacetate + acetyl\text{-}CoA + H_2O \rightarrow citrate + CoA + H^+$	Citrate synthetase
4	$Citrate \rightarrow cis\text{-}aconitate + H_2O$	Aconitase
5	$Cis\text{-}aconitate + H_2O \rightarrow isocitrate$	Aconitase
6	$Isocitrate + NAD^+ \rightarrow \alpha\text{-}ketoglutarate + CO_2 + NADH$	Isocitrate dehydrogenase
7	$\alpha\text{-}Ketoglutarate + NAD^+ + CoA \rightarrow succinyl\text{-}CoA + CO_2 + NADH$	Ketoglutarate dehydrogenase
8	$Succinyl\text{-}CoA + P_i + GDP \leftrightarrow succinate + GTP + CoA$	Succinate thiokinase
9	$FAD + succinate \rightarrow fumarate + FADH_2$	Succinate dehydrogenase
10	$Fumarate + H_2O \rightarrow malate$	Fumarase
11	$Malate + NAD^+ \leftrightarrow oxaloacetate + NADH + H^+$	Malate dehydrogenase

Table 2. Initial concentration values of Krebs cycle metabolites

Metabolite, biomolecule	Concentration (mmol/l)	Reference
Coenzyme A	0.044	Park *et al.* (2016)
Pyruvate	0.0586	Clement *et al.* (2020)
Acetyl-CoA	0.5	Milo *et al.* (2010)
Citrate	0.19	Ahn *et al.* (2017)
Cis-aconitate	0.0016	Bennett *et al.* (2009)
Isocitrate	0.02	Milo *et al.* (2010)
α-ketoglutarate	0.54	Mogilevskaya *et al.* (2006)
Succinyl-CoA	0.66	Mogilevskaya *et al.* (2006)
Succinate	0.07	Albe *et al.* (1990)
Fumarate	0.485	Park *et al.* (2016)
Malate	0.495	Mogilevskaya *et al.* (2006)
Oxaloacetate	0.006	Mogilevskaya *et al.* (2006)
ATP	0.159	Clement *et al.* (2020)
ADP	0.0937	Clement *et al.* (2020)
GDP	0.0012	Milo *et al.* (2010)
NAD^+	0.099	Milo *et al.* (2010)
NADH	0.025	Milo *et al.* (2010)
H_2O	0.170	Milo *et al.* (2010)
H^+	5.2×10^{-6}	Milo *et al.* (2010)
P_i	0.05	Milo *et al.* (2010)
HCO_3^-	0.003	Milo *et al.* (2010)

et al., 2009; Wu *et al.*, 2007). However, despite many trials and many years of research on metabolism, it has still not been accurately represented in any model. Researchers focus on individual metabolic pathway fragments to understand the metabolites and enzymes that transform them as accurately as possible (Berndt *et al.*, 2012; Iacobazzi and Infantino, 2014; Korla *et al.*, 2015; Tretter and Adam-Vizi, 2005). Thanks to this type of research, existing knowledge can be used in the model we propose. The use of queueing theory together with the grouping of molecules of the same type (queue) and reactions of the same type (server) allow a simpler model than the use of the Gillespie algorithm (Gillespie, 1977; Voit, 2017), where each reaction and each molecule is described by a separate node in Markov chains (Massey, 1985). Queueing networks can be considered and called as hidden Markov chain. As a result, the mathematical-simulation model is identical to the biological one, as shown in Figure 2. Another advantage of using this approach is that it is impossible to achieve negative results in biological systems, as is sometimes the case with ODE-based models. There are methods forcing the system to obtain non-negative values (Shampine *et al.*, 2005), however, they could cause calculation errors. Usage of queueing theory as the basis for a Krebs cycle simulation model aims at providing a possible realization of stochastic Markovian processes representing variations in the concentration over a given metabolite. The average change in concentration can be achieved by averaging the simulation results for several simulation runs. At the heart of this stochastic model is Michaelis–Menten kinetic equations describing the relationship between quantities of substrate-product pairs and reaction velocities. In this theory, the

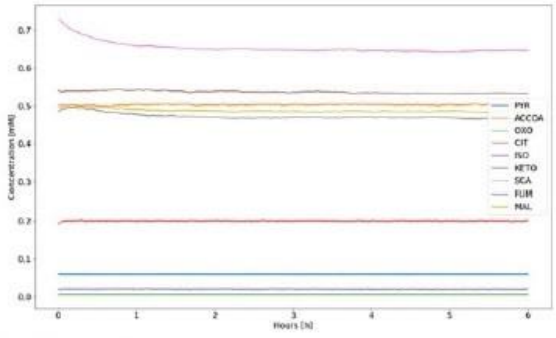

Fig. 2. Concentration level change over 24 h simulation under unperturbed conditions. 'PYR'—pyruvate; 'ACCOA'—acetyl-CoA; 'OXO'—oxaloacetate; 'CIT'—citrate; 'ISO'—isocitrate and cis-aconitate; 'KETO'—α-ketoglutarate; 'SCA'—succinyl-CoA and succinate; 'FUM'—fumarate; 'MAL'—malate

velocity of a reaction is a macroscopic representation of the aggregation of numerous microscopic reactions that may or may not exchange a fixed quantity of substances in a particular period of time. As a result, the speed of a reaction is described as a frequency of the reaction's occurrence and its relationship with the probability of increasing a certain substance by invoking reaction, in which the aforementioned metabolite is a product, and the probability of decreasing the substance by performing reaction, where it is a substrate. By describing the behavior of the Krebs cycle as probabilities of increasing and decreasing each of its substrates and correlating ones' reduction with others' accumulation, we achieved a self-regulating, stochastic process simulating the actual Krebs cycle. Michaelis–Menten kinetic equations are used to calculate the probability that a certain reaction occurs in the current time interval based on the amount of substrate, product and kinetic constants describing the reaction and the duration of the time interval. The results of these equations can be interpreted as the arrival and service rates in the Poisson processes, while an exponential distribution models the service time (the time intervals between two consecutive output events). These assumptions are consistent with classical queueing theory approaches. Therefore, the number of arrivals in any given time interval $(t, t+\tau]$ follows a Poisson distribution with a parameter $(\mu\tau)$ such that:

$$P[(N(t+\tau)-N(t))=k]=\frac{e^{-\mu\tau}(\mu\tau)^k}{k!} \quad (2)$$

where $P[(N(t+\tau)\text{-}N(t))=k]$ is the probability of k arrivals in the interval $(t, t+\tau]$ and $\mu\tau$ is the expected number of arrivals in a time interval of duration τ.

The time required for the queue to process the metabolite increment is described by the exponential distribution using the probability distribution of random variable X in the terms of the rate parameter μ as follows:

$$f(x;\mu)=\mu e^{-\mu x}max(x,0) \quad (3)$$

Therefore, the resulting arrival process at the input of a subsequent queue to which that output of the considered server is connected, follows a Poisson distribution. This a single multivariable stochastic process. All the variables are correlated. The process is described by a queueing network as shown in Figure 1, which consists of a queue describing arrivals and departures of discrete amounts of substances. For example, isocitrate (ISO), which is a product in the reaction citrate → isocitrate (CIT → ISO) and a substrate in reaction isocitrate → α-ketoglutarate (ISO → KETO). This means that the increment of ISO produced in the previous time interval adds to the queue of ISO for the ISO → KETO reaction, effectively increasing the length of ISO queue. The Krebs cycle is a looped system constructed from queues, with increments of concentration of consecutive metabolites circulating, departing from one queue and arriving at another queue. According to Michaelis–Menten kinetic equations, the probability of each packet arriving at the metabolite's queue is correlated with the amount of product and inversely correlated with amount of substrate, creating a self-regulating system, reacting to the imbalances of metabolites and equalizing the arrivals and departures from every queue.

2.3 Use of the genetic algorithm to find optimal values of kinetic constants

The genetic algorithm was used to find optimal values of kinetic constants for the Krebs cycle simulation. The genetic algorithm is a heuristic search inspired by Charles Darwin's theory of natural evolution and uses competing 'chromosomes' in order to find optimal parameters that minimize a fitness function (Man *et al.*, 1999). A 'chromosome' in this implementation is the table of constants required for reaction rates calculation. 'The chromosome' is made of 'genes', which are constants used in one reaction. For example, the first gene in the 'chromosome' consists of constant values used in PYR → AcCoA reaction. There are one hundred 'chromosomes' in the population and each of them is a candidate for table of kinetic constants. The fitness function was designed to force the genetic algorithm to find a table of kinetic constants that allows values of products' concentrations to settle at stable points and to minimize the distance between start values and stable points. The designed fitness function is expressed as:

$$f(X)=\frac{1}{9}\sum_{i=0}^{8}\left|X_{i,0}-\frac{1}{100}\sum_{j=0}^{99}X_{i,(|X|-j)}\right| \quad (4)$$

where X is the table of values of simulation product concentrations in time.

Evaluation of one 'chromosome' requires running a simulation using its set of genes as a table of kinetic constants. The simulation function returns the values of substrates' concentrations at each second. This table is used by the equation above to output the 'chromosome's' score. The function calculates an average vector of the last 100 recordings and computes absolute difference with initial simulation concentrations. In the last step, there is a calculated average of differences. The 'chromosome' minimizing this function is selected as the optimal table of kinetic constant values. Evaluation of each 'chromosome' is computed by simulating the Krebs cycle through the first one hour. There are 100 'chromosomes' in the population in each step of optimization and after evaluation only the 10 sets of constants that minimizes the fitness function are selected for reproduction. The reproductive algorithm is a variation of the standard crossover with additional mechanism preventing the finding of a trivial solution to minimize the loss function problem, which is to zero the probability of every reaction. A step-by-step description of reproduction algorithm is presented in Supplementary Data. The main disadvantage of the fitness function described above is the existence of a trivial solution for its minimization problem. If the 'chromosome' contains only zeros, then no reaction would be performed, so the settling points of concentrations of products in the Krebs cycle would have the same values as initial concentrations, thus finding a global minimum. To prevent the genetic algorithm from converging to this solution, the reproduction mechanism requires that each reaction at $t=0$ has probability of being performed between 1% and 10%. Reaction and balancing flow rates have ranges from 1 to 10% at the beginning of the simulation started from substrates concentration values described in the literature. Applying these constraints to the reaction rates prevent them from being zeroed at the start and also prevents saturation of reactions. The reproduction algorithm has a 10% chance to perform a mutation with the mutation amplitude equal to 1.0.

2.4 Krebs cycle simulation pseudocode

The pseudocode describing the computation process of the Krebs cycle simulation is included in Supplementary Data. This code assumes that:

Table 3. Comparison of concentration data: literature and model (mmol/l)

Metabolite	Initial concentration (literature)	Final concentration (model)	Standard deviation over mean	Absolute difference	Relative difference (%)
Pyruvate	0.0586	0.0586	0.0033	0.0	0.0
Acetyl-CoA	0.05	0.5028	0.0145	0.0028	0.55
Oxaloacetate	0.006	0.0059	0.0167	−0.0001	−1.5
Citrate	0.19	0.1994	0.018	0.0094	4.96
Isocitrate + cis-aconitate	0.0216	0.0216	0.0554	0.0	0.01
α-ketoglutarate	0.54	0.5346	0.0256	−0.0054	−1.01
Succinyl-CoA + succinate	0.73	0.6473	0.0167	−0.0827	−11.33
Fumarate	0.485	0.467	0.0161	−0.018	−3.72
Malate	0.495	0.4847	0.0107	−0.0103	−2.08

Note: Calculated relative difference shows similarity of obtained results and literature data.

- Kinetic constants are grouped into a table of vectors of constant values. There are 11 vectors in the table corresponding to nine different reactions and two balancing flows. Each of the reactions has a unique, four-dimensional vector and every balancing flow contains a one-dimensional table. The vector describing pyruvate carboxylase and citrate synthetase are the exceptions from this rule as they have eight and six elements, respectively. These exceptions are due to the nature of the reactions catalyzed by these enzymes. While most of the reactions require only four coefficients to be optimized the reactions catalyzed by pyruvate carboxylase and citrate synthetase involve more constants that need to be optimized.
- Concentration increment exchanged during the reactions is called 'delta' and is equal to 0.0001 mmol. 'Delta' is significantly lower than the initial value of the lowest substrate concentration. Delta value must be chosen in a way that it corresponds to a change of more than a single molecule for the rare species, in fact for rare species it should be always chosen to be a positive integer number of molecules.
- Simulation assumes that the concentration of pyruvate in the cycle is varying with 10% Gaussian noise around the constant value of 0.0586 mmol/l. Such signal-to-noise ratio depicts metabolic conditions inside the cell. Due to the various living conditions of the cell, pyruvate is consumed faster or slower. The pyruvate level is dependent on the blood glucose level, which also affects the glucose level in the cell, so the variation of 10% was assumed. It is an arbitrary choice, and the variation range can be changed if there are good reasons to do so, as was done with the drug effect simulation.

The searching for optimal kinetic constants was performed using a PC with Intel™ Core i7-7700HQ @ 2.80 GHz, RAM 16 GB. Code was written in C# 8.0. One search epoch simulating one hour for 100 different tables of kinetic constants using all 8 logic cores took approximately 10 min.

2.5 Inhibition of a specific stage of the cycle and its influence on the concentrations and kinetics of other reactions

To validate the model, we simulated the outcome of various drugs on the Krebs cycle, which are known to affect the concentrations of individual metabolites in the Krebs cycle. This approach may also emulate changes in enzyme activity associated with the progression of various diseases, including metabolic disorders or cancer (Tolstikov *et al.*, 2014); Sutendra and Michelakis, 2013; Zhang *et al.*, 2018). Drugs that affect enzyme reactions in the Krebs cycle are usually competitive inhibitors. Ultimately, the drug slows down the reaction carried out by a particular enzyme because the enzyme processes smaller amounts of substrate than it would under regular conditions, without an inhibitor. Understanding the kinetic properties of inhibitors would be sufficient to predict its effect on cell metabolism. Using the existing research on substances affecting various enzymes involved in the Krebs cycle reactions, an experiment was conducted to reflect the effect of the drug on the rate of enzymatic reaction and the concentration of metabolites.

Table 4. Comparison of concentration values during Phenformin treatment: empirical data and model based on the queueing theory

Metabolite	Concentration change after Phenformin administration in comparison to non-treatment (Janzer *et al.*, 2014) (%)	Model simulation results (%)
Pyruvate	−65	−65
Citrate	−60	−59.28
Isocitrate	−65	−63.91
α-ketoglutarate	−80	−79.38
Fumarate	−50	−50.84
Malate	−53	−53.22

3 Results

To validate the model, we have tested first its stability. The system becomes stable after approximately 5.5 h of simulation as shown in Figure 2. During this time, for every millisecond of simulation time one simulation step was performed.

In our opinion, these results are satisfactory. The largest relative difference observed in our model in comparison with available biological data is -11.33% in the case of the combined concentrations of succinyl-CoA and succinate (Table 3).

To reflect the kinetics of the Krebs cycle during inhibition induced by a drug that blocks one of the cycle reactions, we selected one of the studies based on the measurement of metabolite concentrations after administration of drugs in anti-cancer therapy (Janzer *et al.*, 2014). This study provided the most detailed information on the concentrations of several metabolites included in the Krebs cycle. Therefore, the study served as the basis for the model to check whether it achieves similar results (Table 4). This study tested the effects of Tamoxifen, already used in the treatment of breast cancer, in combination with the drugs used by diabetics—Metformin and Phenformin. The idea to use these drugs in cancer therapy resulted from clinical observations (Evans *et al.*, 2005; Jiralerspong *et al.*, 2009; Kim *et al.*, 2018; Pollak, 2012). According to these observations, cancer diagnosis incidence rate was lower in patients using the drugs, as well as the mortality rate due to cancer was lower in the diagnosed patients. This observation prompted the idea of combining Metformin and Phenformin together with the standardized Tamoxifen. Metformin and Phenformin doses were 300 and 10 μM, respectively. One of the methods of assessing the effectiveness of the therapy was the measurement of the concentration of Krebs cycle

metabolites. This metabolic cycle is extremely important for cancer cells due to the high energy demands of these cells. Therefore, a reduction in the Krebs cycle efficiency and lowering the concentration of individual metabolites, may prove the effectiveness of the used treatment method. Simulations present changes in concentration levels of each Krebs cycle metabolite during treatment (Figs 3 and 4).

Due to the difficulty of obtaining measurements, the article (Janzer *et al.*, 2014) did not include all the Krebs cycle metabolites. The data presented in Tables 4 and 5 confirm the accuracy of the proposed solution. The prepared model based on the queueing theory was designed to accurately reflect the stochastic nature of biological reactions. For this reason, training and adapting it to the conditions and phenomena that may occur during biochemical reactions in the cell, also under the influence of pharmaceuticals, allowed to obtain results similar to the experimental values. Tables 6 and 7 present the results of a six-hour simulation of the concentrations (with 10% gaussian noise) of all Krebs cycle metabolites for Tamoxifen + Phenformin and Tamoxifen + Metformin treatment. To obtain the presented results, the so-called 'balancing flow' was used in the model. It imitates the drainage of metabolites due to their various uses in cell functioning (e.g. being precursors for other compounds). Balancing flow was used to stabilize the concentrations of oxaloacetate and citrate. Oxaloacetate is used in gluconeogenesis, in the urea cycle and in the synthesis of fatty acids to create citrate in the form of which it is transported; this happens when there is no demand for energy at the moment. Citrate is transported beyond the mitochondria to the cytoplasm, then broken down into acetyl-CoA and oxaloacetate for the synthesis of fatty acids. Probability rates of balancing flows for oxaloacetate and citrate are linearly correlated with the concentrations of respective metabolites. Such a composition of arriving, departing and balancing rates forms a stochastic representation of the Krebs cycle and provides an accurate and time-efficient model. By comparing the simulation and measurement results, we stipulate that the drugs administration in previously mentioned doses inhibits the reaction catalyzed by pyruvate dehydrogenase by about 30%. α-ketoglutarate and malate have been shown as examples of the metabolites which concentration was measured during the studies on the effects of these drugs (Figs 5 and 6). The model allows for the observation of pharmaceutical influence on the kinetics of the cycle reactions and their influence on metabolite levels.

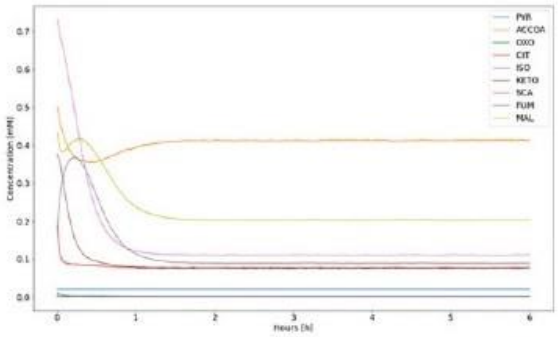

Fig. 3. Concentration levels change during inhibition: reflection of Tamoxifen + Phenformin treatment. 'PYR'—pyruvate; 'ACCOA'—acetyl-CoA; 'OXO'—oxaloacetate; 'CIT'—citrate; 'ISO'—isocitrate and cis-aconitate; 'KETO'—α-ketoglutarate; 'SCA'—succinyl-CoA and succinate; 'FUM'—fumarate; 'MAL'—malate

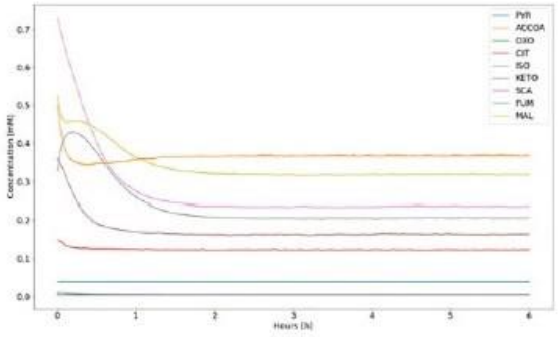

Fig. 4. Concentration levels change during inhibition: reflection of Tamoxifen + Metformin treatment. 'PYR'—pyruvate; 'ACCOA'—acetyl-CoA; 'OXO'—oxaloacetate; 'CIT'—citrate; 'ISO'—isocitrate and cis-aconitate; 'KETO'—α-ketoglutarate; 'SCA'—succinyl-CoA and succinate; 'FUM'—fumarate; 'MAL'—malate

4 Discussion

The presented results demonstrate the preparation of a model capable of mimicking the conditions of metabolic reactions in living cells. A disadvantage of our model is that the data on metabolite concentrations and enzyme constants come from different sources. Measurements carried out on different measuring devices by different research teams may not be fully compatible. However, to the

Table 5. Comparison of concentration values during Metformin treatment: empirical data and model based on the queueing theory

Metabolite	Concentration change after Metformin administration in comparison to non-treatment (Janzer *et al.*, 2014) (%)	Model simulation results (%)
Pyruvate	−35	−35
Citrate	−15	−16.18
Isocitrate	−40	−39.17
α-ketoglutarate	−55	−54.47
Fumarate	−37	−37.03
Malate	−39	−39.43

Table 6. Comparison of concentration data: literature and model (mmol/l) under inhibition caused by Tamoxifen and Phenformin

Metabolite	Initial concentration (literature)	Final concentration (model)	Standard deviation over mean	Absolute difference	Relative difference (%)
Pyruvate	0.0205	0.0205	0.0039	0.0	0.03
Acetyl-CoA	0.5	0.4129	0.0161	−0.0871	−17.42
Oxaloacetate	0.006	0.0025	0.0239	−0.0035	−58.7
Citrate	0.1899	0.0773	0.0267	−0.1126	−59.28
Isocitrate + cis-aconitate	0.0056	0.002	0.0636	−0.0036	−63.91
α-ketoglutarate	0.3764	0.0776	0.042	−0.2988	−79.38
Succinyl-CoA + succinate	0.73	0.1113	0.0308	−0.6187	−84.75
Fumarate	0.1825	0.0897	0.0315	−0.0928	−50.84
Malate	0.4335	0.2028	0.0164	−0.2307	−53.22

Table 7. Comparison of concentration data: literature and model (mmol/l) under inhibition caused by Tamoxifen and Metformin

Metabolite	Initial concentration (literature)	Final concentration (model)	Standard deviation over mean	Absolute difference	Relative difference (%)
Pyruvate	0.0381	0.0381	0.0036	0.0	0.0
Acetyl-CoA	0.5	0.3676	0.0139	−0.1324	−26.49
Oxaloacetate	0.006	0.0044	0.015	−0.0016	−27.46
Citrate	0.1466	0.1229	0.0232	−0.0237	−16.18
Isocitrate + cis-aconitate	0.0081	0.0049	0.0709	−0.0032	−39.17
α-ketoglutarate	0.3596	0.1637	0.0274	−0.1959	−54.47
Succinyl-CoA + succinate	0.73	0.2346	0.0228	−0.4954	−67.86
Fumarate	0.3272	0.206	0.0227	−0.1212	−37.03
Malate	0.5235	0.3171	0.0139	−0.2064	−39.43

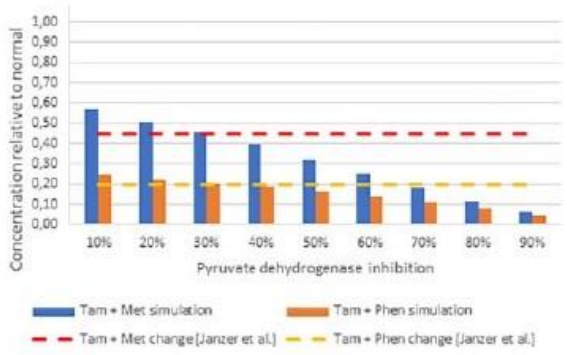

Fig. 5. α-Ketoglutarate concentration change in regards to pyruvate dehydrogenase inhibition. 'Tam'—Tamoxifen; 'Met'—Metformin; 'Phen'—Phenformin

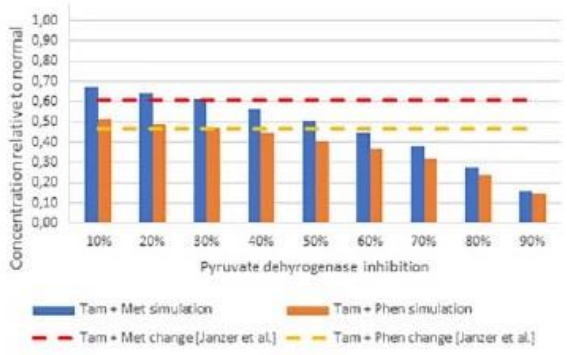

Fig. 6. Malate concentration change in regard to pyruvate dehydrogenase inhibition. 'Tam'—Tamoxifen; 'Met'—Metformin; 'Phen'—Phenformin

best of our knowledge there is no publication which presents metabolic data for every Krebs cycle metabolite derived from one research group. We have made every effort to ensure that the data used are as accurate and compatible with each other as possible. The models can be a kind of virtual laboratory where one can consider interdependencies between specific substances and metabolites and their influence on basic cellular functions. The developed model may provide knowledge on how chemical compounds obtain their therapeutic efficacy, which may result in improved safety from the early stages of drug development, e.g. setting up experiments before tests on living organisms, and between preclinical testing and clinical trials. The model allows to determine which reactions of the metabolic pathway are the best candidates for disturbing the metabolic pathway. In addition, we can observe what doses of the drug have a significant effect on the metabolic pathway, as well as the dose above which the effect is imperceptible, something like 'maximum effective dose'. There are many studies proving the usefulness of various drugs affecting cell metabolism. These drugs are used, in conjunction with others, in bacterial infections and in neoplastic diseases. The available literature data on the kinetic properties of enzymes and the concentration of metabolites can be used in this model. As mentioned before in the inhibition modeling section, Metformin and Phenformin are drugs that lower the level of metabolites in the Krebs cycle. This is associated with a reduction in the supply of pyruvate that could be transformed and enter the Krebs cycle, as well as an increase in the amount of lactate produced under anaerobic conditions. Previous studies have suggested that the Krebs cycle is not inhibited by metformin and changes the source of cellular 'fuel' (Janzer *et al.*, 2014). However, the possibility that this apparent difference in inhibition of the Krebs cycle may be due to the analysis of stably transformed cancer cells as opposed to cells at an early stage of transformation was considered. However, biguanide treatment of the stably transformed CAMA-1 breast cancer cell line leads to a reduction in the concentrations of cycle intermediates. This suggests that metabolic reduction of the Krebs cycle by biguanides may be important for inhibiting transformation (Janzer *et al.*, 2014).

5 Conclusions

The combination of knowledge available in the literature and the programming of the model provided a tool capable of mimicking Krebs cycle-related metabolic processes in living cells in real time. We demonstrated that metabolic pathways can be effectively simulated using methods based on queueing theory and affected by simulated application of drug. However, in self-criticism we found a place where the described model could be improved. We assume that access to data obtained under the same conditions on specific cells could potentially improve the obtained results, but due to limited access to such data, our model was prepared based on the most accurate available data. Future research efforts will be devoted to combining the Krebs cycle model with the previously developed glycolysis model (Clement *et al.*, 2020) and adding the pentose phosphate pathway to obtain a comprehensive model of cellular carbohydrate metabolism.

Funding

This work was funded by the National Science Center (NCN) of Poland in terms of Opus-17 Program [2019/33/B/ST6/00875].

Conflict of Interest: none declared.

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6.2. Original paper II – content of the publication "Queueing theory model of pentose phosphate pathway."

scientific reports

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Queueing theory model of pentose phosphate pathway

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Due to its role in maintaining the proper functioning of the cell, the pentose phosphate pathway (PPP) is one of the most important metabolic pathways. It is responsible for regulating the concentration of simple sugars and provides precursors for the synthesis of amino acids and nucleotides. In addition, it plays a critical role in maintaining an adequate level of NADPH, which is necessary for the cell to fight oxidative stress. These reasons prompted the authors to develop a computational model, based on queueing theory, capable of simulating changes in PPP metabolites' concentrations. The model has been validated with empirical data from tumor cells. The obtained results prove the stability and accuracy of the model. By applying queueing theory, this model can be further expanded to include successive metabolic pathways. The use of the model may accelerate research on new drugs, reduce drug costs, and reduce the reliance on laboratory animals necessary for this type of research on which new methods are tested.

In recent years, there has been significant progress in metabolomics. New and improved test methods allow for the measurement of many important biochemical parameters. The acquired data can be used to create simulation models of biochemical reactions and entire metabolic pathways. Queueing theory can successfully model metabolic processes, as demonstrated by the example of the glycolysis pathway[1] and Krebs cycle[2]. The preparation of an accurate model simulating the course of PPP could potentially reduce the time needed for drug testing and reduce the number of laboratory animals on which new drugs are tested[3].

The PPP is a metabolic pathway whose main substrate is glucose-6-phosphate (G6P). Throughout the reactions that make up this pathway, numerous molecules are formed, such as: nicotinamide adenine dinucleotide phosphate (NADPH), which is used in the biosynthesis of fatty acids, ribose 5-phosphate (R5P), which is a precursor in the synthesis of nucleotides, and erythrose 4-phosphate (E4P), which is used in the synthesis of aromatic amino acids[4,5]. Products of the PPP are essential for the formation of new cells. However, under stress, cell growth is slows down and the PPP is responsible for maintaining cellular levels of NADPH. In fact, such conditions increase the reliance of the PPP in the cell over glycolysis to maintain the needed ratio between $NADP^+$ and NADPH[6]. In most living organisms, this pathway takes place in the cell cytosol.

There are two phases in the PPP: the oxidative phase and the non-oxidative phase. During the oxidative phase, NADPH is produced[7]. In the non-oxidative phase, various simple sugars are synthesized. 5-carbon sugars derived from the digestion of nucleic acids can be utilized in the PPP, where their carbon backbones are metabolized into intermediates for glycolysis or gluconeogenesis. In the non-oxidative phase, one of the enzymes- transketolase—is responsible for catalyzing two different reactions, with two sets of substrates. Therefore, these substrates act as inhibitors to each other, since they are competing for the same active site of the enzyme.

It is estimated that as much as 60% of NADPH comes from the PPP[8]. The PPP is most active in the liver, adrenal cortex, and mammary glands. The activity for this pathway is high in red blood cells, making it extremely important in erythrocytes[9]. NADPH formed by the PPP is used in the cell to prevent oxidative stress and the formation of dangerous free radicals that could harm the cell[10]. Reactive oxygen species (ROS) can damage cellular lipids, proteins, and nucleic acids, and eventually cause cell death[11]. It is worth noting that ROS are associated with many diseases[12–14]. Since erythrocytes do not have mitochondria, they have no other source of reducing oxidative stress other than the PPP. For example, large amounts of NADPH generated in erythrocytes are used to reduce glutathione (GSH). This reduced form of GSH is essential for maintaining the proper state of the cell. If GSH level is lowered in erythrocytes, hemolysis may occur[15].

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Metabolite	Initial conc. (literature)	Final conc. (model)	Standard deviation over mean (%)	Absolute difference	Relative difference (%)
Glucose-6-P (G6P)	0.0026	0.0026	3	0	0
$NADP^+$	0.001	0.001	3	0	0
NADPH	0.0002	0.0002	3	0	0
6-P-gluconolactone (PGL)	5×10^{-6}	9.3×10^{-6}	36	4.3×10^{-6}	86
6-P-gluconate (6PG)	0.018	0.019	2	0.001	5.5
Ribulose-5-P (Ru5P)	0.012	0.012	2	0	0
Ribose-5-P (R5P)	0.009	0.009	1	0	0
Xylulose-5-P (X5P)	0.018	0.018	1	0	0
Glyceraldehyde-3-P (G3P)	0.00234	0.00242	3	0.00008	3.4
Sedoheptulose-7-P (S7P)	0.068	0.062	1	0.006	8.8
Erythrose-4-P (E4P)	0.004	0.004	3	0	0
Fructose-6-P (F6P)	0.083	0.079	0	0.004	4.8

Table 1. Comparison of concentration data: literature and model (mmol/L). Calculated relative difference shows similarity of obtained results and literature data.

The most common approach used to model metabolic changes in a cell is to use Ordinary Differential Equations (ODE). For metabolic reactions, ODEs provide quantitative information on interactions that occur between metabolites in specific reactions taking place in the cell. Previously, ODEs have been successfully used in simulation studies of biochemical kinetics and biochemical connections[16–18]. The authors in[19] presented a PPP model based on ODEs. This approach was beneficial because it did not require complicated operations that strained the capabilities of computers in the past, resulting in lower computing power. However, the simplifications and assumptions made when using ODEs in metabolic simulations do not reflect the stochastic nature of cell biochemistry[20]. The Chemical Master Equation (CME) was another approach used to model the stochasticity of biological reactions[21]. However, due to the complexity and computing requirements, networks based on these models cannot be too extensive. A relatively new approach to computational metabolic modeling is the use of queueing theory. Queueing theory has wide applications in telecommunications, but also in biological and medical science topics, such as modeling drug pharmacokinetics[22] or HIV infectivity[23]. Using this method, it was possible to accurately model a simple metabolic network and mimic the interactions between metabolites[24], as well as the Krebs cycle[2]. A genetic algorithm was used to optimize the kinetic coefficients. A variety of AI methods can be used for this purpose, but genetic algorithm was chosen because it was used with success when modeling the Krebs cycle.

The aim of this work was to prepare a PPP model capable of tracking concentration changes of specific metabolites occurring in this pathway over time. Additionally, the usefulness of the genetic algorithm for finding values of the kinetic constants used in the model was confirmed[2]. A genetic algorithm was used to find values corresponding to those in the literature.

Results

The generated model becomes stable within approximately one hour. Every second, there are 1000 simulations of each pathway reaction (or 1 simulation step per millisecond), averaged over 50 simulated cells. This number has been selected experimentally. However, the model is designed to vary this number depending on the needs of the researcher. Figure 1 shows concentration changes of individual metabolites over time. Due to the various conditions of the living cell, G6P and NADP are consumed faster or slower depending on the blood glucose level, since glucose is phosphorylated to G6P to stay inside the cell and prevent diffusion out of the cell. This affects the glucose level in the cell, so the variation of 10% was assumed. The variation level is an arbitrary choice; meaning it can be changed. The purpose for the use of variation is to represent the concentration fluctuations in the cell. For this model to reflect the flow of metabolites in the cell as accurately as possible, the so-called "balancing flow" was used[1,2]. This feature allows for proper simulation of metabolite flow depending on the current needs of the cell (Fig. 2). Thus, the level of metabolites that occur in more than one metabolic pathway, e.g. F6P and G3P being part of the PPP and glycolysis, better mimics biological conditions. Table 1 presents the comparison of model generated data and literature data regarding concentration of individual metabolites.

PGL is rapidly hydrolyzed, so the practical equilibrium between G6P and 6PG is directed towards the formation of 6PG[25]. Any existing PGL is almost immediately converted to 6PG, therefore the variance is very high. The relative difference of PGL is high because it depends on the measurement time. In the future, we intend to combine the PPP with the already developed Krebs cycle and glycolysis models, so the results of the PPP model are likely to be closer to the experimental results.

Due to the high demand of glucose and its metabolites by cancer cells, many drugs are aimed at blocking metabolic pathways that supply cancer cells with substances necessary for proliferation. The PPP is one of the pathways with significantly increased activity in neoplastic cells. Compared to healthy cells, the activity of the PPP

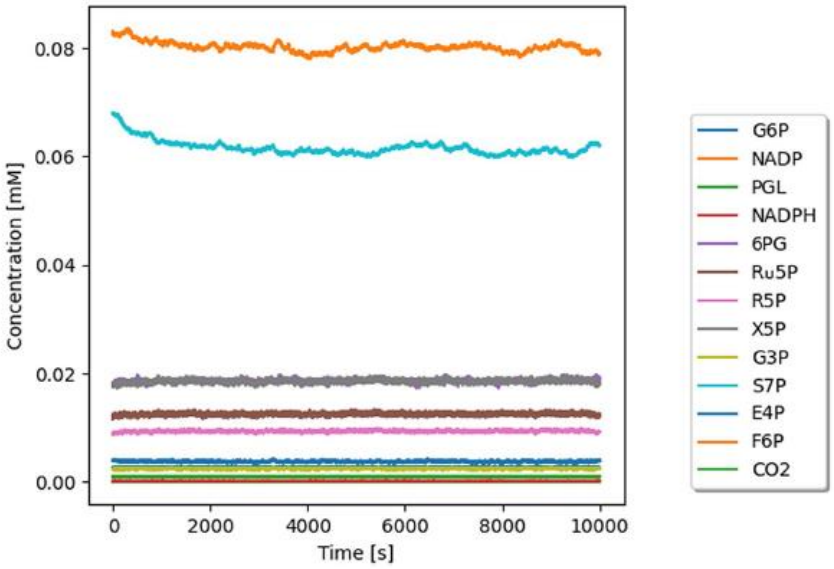

Figure 1. Concentration level change over time under unperturbed conditions. *G6P* glucose-6-phosphate, *NADP* NADP+, *PGL* 6-P-gluconolactone, *6PG* 6-phosphogluconate, *Ru5P* ribulose-5-phosphate, *R5P* ribose-5-phosphate, *X5P* xylulose-5-phosphate, *G3P* glyceraldehyde-3-phosphate, *S7P* sedoheptulose-7-phosphate, *E4P* erythrose-4-phosphate, *F6P* fructose-6-phosphate.

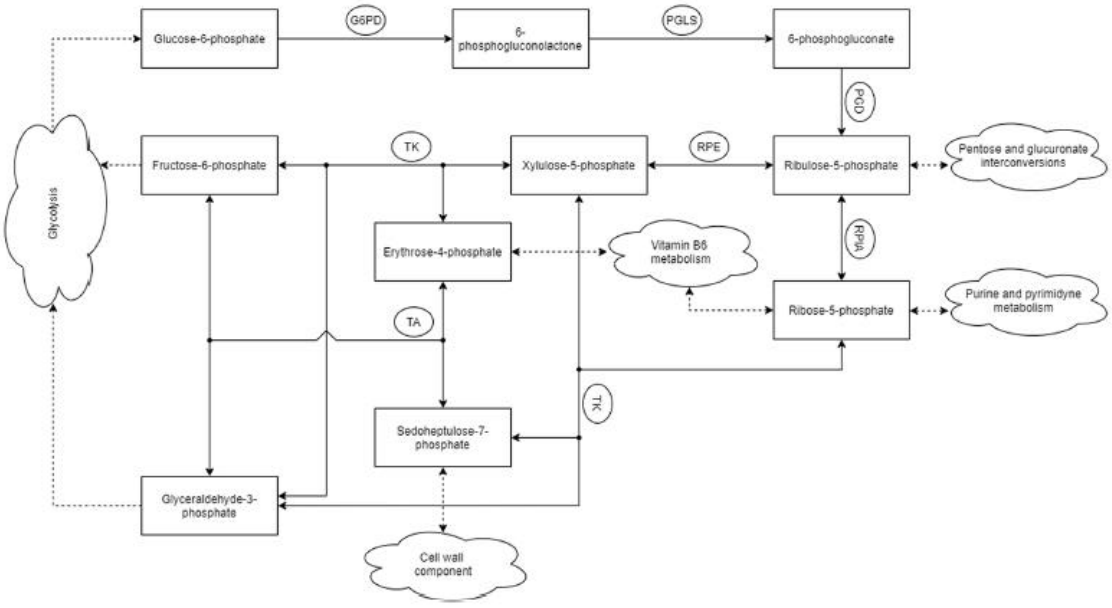

Figure 2. PPP scheme; the graph shows the main carbohydrate products, their relations with other metabolic pathways, and enzymes that catalyze reactions. *G6PD* glucose-6-phosphate dehydrogenase, *PGLS* 6-phosphogluconolactonase, *PGD* 6-phosphogluconate dehydrogenase, *RPIA* ribose-5-phosphate isomerase A, *RPE* ribulose-5-phosphate-3-epimerase, *TA* transaldolase, *TK* transketolase.

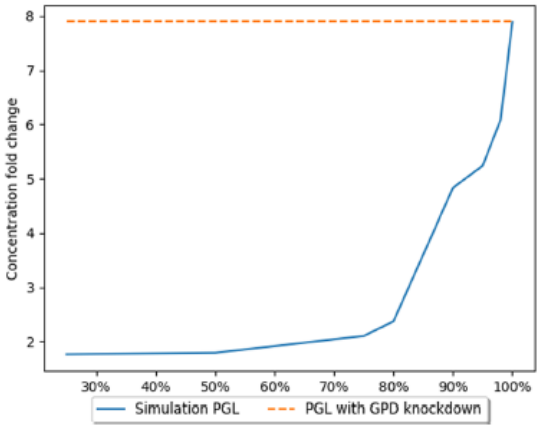

Figure 3. The effects of GPD gene expression knockdown on PGL concentration[26]. The X axis presents level of simulated GPD inhibition. The Y axis presents fold change in concentration in comparison to the natural state (without inhibition).

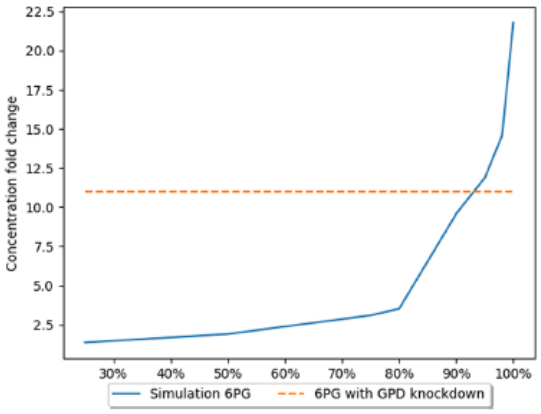

Figure 4. The effects of GPD gene expression knockdown on 6PG concentration[26]. The X axis presents level of simulated GPD inhibition. The Y axis presents fold change in concentration in comparison to the natural state (without inhibition).

in cancer cells can be increased up to 8 times. The oxidative part of the pathway provides cells with a large amount of NADPH, helping the cell can effectively fight excess oxidative stress. Effects that reduce the effectiveness of the production of NADPH in the cell, in combination with factors that induce this stress, such as radiotherapy or chemotherapy, can kill cancer cells.

To validate the model, model results were compared to those obtained empirically. The paper[26] serving as the benchmark for our model described the effect of a third PPP enzyme, PGD, in lung cancer cells. Inhibition of this enzyme's activity does not significantly affect the level of NADPH, but inhibits tumor growth. The gene encoding PGD is characterized by increased expression in neoplastic cells. ShRNA molecules were used to reduce PGD expression. This approach resulted in inhibition of tumor growth , indicating an important role for PGD in cancer cell metabolism. Concentrations of several PPP metabolites were measured, however, not all of them had significant changes. Metabolites of the oxidative phase of the PPP, such as 6-phosphogluconolactone (PGL) and 6-phosphogluconate (6PG) had concentrations 7.9 and 11 times higher than their regular concentrations, respectively (Figs. 3 and 4). These metabolites accumulated due to the absence/decreased activity of PGD. The concentrations of metabolites of the non-oxidative phase of the pathway such as S7P or X5P were not measured, but no significant changes in the concentrations of ribose phosphate and nucleotide triphosphate were detected.

The accumulation of metabolites preceding the blocked reaction is because the expression of the PGD enzyme has been reduced. A bottleneck is created at this stage of the pathway, leading to a reduced efficiency of this stage, as there are not enough protein molecules in the cell to process all metabolite molecules. As a further consequence, a decrease in the concentration of metabolites occurring further down the pathway, e.g., G3P, can be

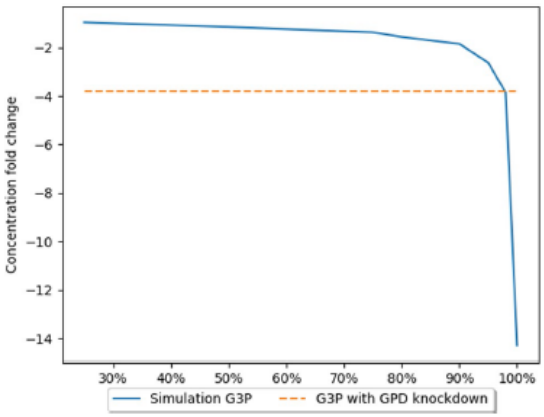

Figure 5. The effects of GPD gene expression knockdown on G3P concentration[26]. The X axis presents level of simulated GPD inhibition. The Y axis presents fold change in concentration in comparison to the natural state (without inhibition).

Metabolite	Experimental data concentration change[26]	Model data concentration change using 90% inhibition	Model data concentration change using 95% inhibition	Model data concentration change using 98% inhibition	Model data concentration change using 100% inhibition
G6P	1.8	1.8	1.8	1.8	1.8
PGL	7.9	4.83	5.24	6.08	7.89
6PG	1	9.59	11.88	14.56	21.8
G3P	− 3.8	− 1.85	− 2.63	− 3.85	− 14.29

Table 2. Comparison of metabolite concentration changes (fold changes) caused by knockdown of the PGD gene.

observed (Fig. 5). For the validation of the model, measurements of the concentrations of metabolites obtained empirically were used. The model makes it possible to simulate and track the changes in the concentrations of the metabolites.

Several measurements were performed to evaluate the level of inhibition of the GPD catalyzed reaction. The obtained results show that the GPD knockdown caused inhibition at the level of 95–98%. These assumptions are based on the results presented in Table 2. The results for these inhibition levels are the closest to the empirical results. The paper[26] used shRNA to achieve expression knockdown, which is an incomplete mechanism to reduce (but not eliminate) expression. This form of knockdown is not expected to achieve 100% silencing. Indeed, 80–99% knockdown of expression is normal and expected. The calculated results are comparable to those obtained experimentally and are consistent with current biological knowledge. Another point to consider is that the glucose metabolism of neoplastic cells remains unknown in some aspects and these cells may possibly bypass a blocked reaction in the metabolic pathway. Simulations using 100% inhibition were also performed, but this led to a significant reduction in the concentration of metabolites downstream of the bottleneck of the pathway. However, it can be observed that due to the bidirectional character of reactions of the second phase of the PPP and the flux of metabolites from other pathways, e.g., F6P generated in glycolysis, we do not observe a complete 'zeroing' of metabolite concentration.

The results generated in our model (Table 2) follows the trend of changes in concentration observed in vitro, and suggests that knockdown efficiency in vitro was likely near 95%, which is common for shRNA expression knockdown.

Discussion

As mentioned in the introduction, most previous models simulating metabolic pathways, not only PPP, have been based on the use of ODEs. However, due to the advantages offered by queueing theory, it seems reasonable to use this method in modeling. The preparation of a quantitative model of a biological pathway such as the PPP requires the necessary information on starting concentrations and kinetic data of the enzymes that catalyze the pathway reactions. The presented model can be viewed as a 'virtual laboratory'. This model tracks the relationships between individual metabolites formed at different stages of the pathway. It is possible to observe changes caused by fluctuations in metabolite concentrations and their impact on the entire pathway.

It can also be used to test the effectiveness of new drugs if their influence on the kinetics of the reaction they affect is known. In this way, one can also theoretically get answers to questions such as which reactions are worth blocking to obtain the best possible therapeutic result. Most studies aimed at blocking the PPP pathway

Number	Reaction	Enzyme
1	$G6P + NADP^+ \rightarrow PGL + NADPH + H^+$	Glucose 6-phosphate dehydrogenase
2	$PGL + H_2O \rightarrow 6PG + H^+$	6-Phosphogluconolactonase
3	$6PG + NADP^+ \rightarrow Ru5P + NADPH + H^+ + CO_2$	6-Phosphogluconate dehydrogenase
4A	$Ru5P \rightarrow R5P$	Ribose-5-phosphate isomerase
4B	$Ru5P \rightarrow X5P$	Ribulose 5-phosphate 3-epimerase
5	$R5P + X5P \rightarrow G3P + S7P$	Transketolase
6	$X5P + E4P \rightarrow G3P + F6P$	Transketolase
7	$G3P + S7P \rightarrow E4P + F6P$	Transaldolase

Table 3. Stoichiometric reactions of the PPP. Reactions 1-3 form the oxidative branch of PPP, reactions 4-7 are in the non-oxidative branch.

in cancer patients have focused on blocking the first reaction of the pathway catalyzed by G6PD[27,28]. However, clinical data indicate that this therapy is not very effective without additional exposure to oxidative stress[29,30]. For this reason, the results of studies on the knockdown of the gene encoding PGD in this paper were used[26]. Even though the knockdown of the G6PD gene does not affect the amount of NADPH, which is important for tumor development, the knockdown of this gene alone results in inhibition of tumor growth. Perhaps the metabolites that accumulate in the cell prior to the blocked reaction are responsible for this situation. Their concentration in cells reaches values significantly greater than their natural concentrations. The exact mechanism of tumor growth inhibition is unknown, however, the effect achieved is important.

The proposed model obtained stability based on the data from the above-mentioned paper. We believe that this type of model can be used to predict the impact of therapy, which in turn will lead to an increase in its effectiveness.

Thanks to the use of experimental data together with a computational process based on the queueing theory, a model was obtained that can track the metabolic pathway that takes place in the cells of living organisms. In this paper, we present a separate PPP model without detailed analysis of the relationship between PPP and glycolysis. The metabolites common to both pathways have been identified and several principles have been adopted to create a functional PPP model. In the future, our plan is to connect the existing glycolysis, Krebs cycle, and PPP models together. We believe that such a procedure may also positively affect the consistency of simulation and experimental results.

The presented results indicate that the model can be used to predict changes in metabolite concentrations. For this purpose, it is sufficient to enter the concentration value of one of the metabolites. In this way, the entire study can prove to be more cost-effective—no need to determine each metabolite separately, which also saves time.

As demonstrated by the knockdown of one of the genes encoding the enzyme catalyzing the PPP reaction, this model is adapted to follow the trend of metabolite changes. Moreover, it can determine the specific effect of the inhibition of particular reactions on the concentration of metabolites with relatively high accuracy. Further research providing data on how inhibition of a particular pathway step may affect kinetic constants could contribute to an increase in the accuracy of the presented model.

Methods

Obtaining metabolic data and the use of enzymatic reaction kinetics. This work focuses on the reflection of changes in PPP metabolite concentrations over time. For this purpose, a literature review was carried out to provide data on these concentrations (Table 1). Presented concentrations were measured with the use of mass spectrometry[31]. Several kinetic constants, and enzymatic properties, like maximum velocity (V_{max}), necessary for the correct operation of the model were used to calculate the speed of chemical reactions[31]. Reaction rates were calculated using equations based on Michaelis–Menten kinetics (for more information please check Supplementary Information).

NADPH is formed from 2 $NADP^+$ molecules in the oxidative phase. The energy generated during the conversion of G6P into ribulose 5-phosphate (Ru5P) is used in the reaction. The overall reaction of the first phase of the pathway is as follows:

$$G6P + 2NADP^+ + H_2O \rightarrow Ru5P + 2NADPH + 2H^+ + CO_2$$

Ru5P, which is one of the products of the first phase of the PPP, is the first substrate for the non-oxidative phase. Ribose-5-phosphate isomerase can convert Ru5P to R5P. On the other hand, ribulose 5-phosphate epimerase converts Ru5P to xylulose 5-phosphate (X5P). The next reactions involve changing the length of the carbon chain in the carbohydrates. These two five-carbon sugars then undergo a transketolase-catalyzed reaction. The result is production of glyceraldehyde 3-phosphate (G3P) and sedoheptulose 7-phosphate (S7P). Then G3P and S7P undergo a transaldolase-catalyzed reaction, which produces E4P and fructose 6-phosphate (F6P) (Fig. 2; Table 3).

Queueing theory. The complicated nature of metabolic pathways, in which there are huge amounts of biochemical substances constituting the substrates and reaction products, makes modeling metabolism extremely

challenging. Methods commonly used to model metabolic pathways require supervision and the use of appropriate constraints, like forcing ODEs not to reach negative values. Such treatments may cause small calculation errors which could accumulate in long-term modeling and result in incorrect calculations. Biological systems are organized to pass the products of individual metabolic reactions further down the pathway, so that they become substrates for downstream reactions or are used by the cell to support necessary life processes[32]. For this reason, the use of queueing theory in metabolic pathway modeling seems to be the right approach.

Queueing networks can be thought of as hidden Markov chains, similar to Gillespie's modelling technique[20,21]. The advantage of using queueing theory to model metabolic pathways is that they do not require enhanced computing power. Therefore, the results can be obtained close to real time. Networks based on queueing theory can be applied with ease to a significantly greater number of molecules, grouped into the queues representing different molecular species. Due to the nature of this approach, it is capable of combining individual pathways into larger, more complex groups of metabolic pathways.

Averaging the results from several simulation runs provides information on the average changes in the concentrations of the individual pathway metabolites. The proposed model is based on calculations of the kinetics of Michaelis–Menten enzymatic reactions, which focus on the relationship between the concentrations of the substrate and the product, and the velocity of the reaction. According to this theory, the macroscopic concept of enzymatic reaction speed is the sum of many microscopic reactions that can exchange specific amounts of substances per time unit. The description of the PPP as the probability of decreasing and increasing the concentration of each of the substances present in the pathway and the correlation of their reduction with the accumulation of other substrates results in a self-regulating, stochastic process that imitates the actual course of the PPP. The Michaelis–Menten kinetic equation was used to calculate the probability of the reaction. A detailed description of the methodology used is described in the work describing the Krebs cycle model[2].

The probability of the reaction can be converted to an average amount of arrivals when measured for a significant amount of time. Therefore, the kinetic equations can be used to calculate the adaptive parameter $\mu(t)$ utilized for modelling PPP behavior by a network of inhomogeneous Poisson processes described by equation (1):

$$P[(N(t+\tau) - N(t)) = k, t] = \frac{e^{-\mu(t)\tau}(\mu(t)\tau)^k}{k!} \tag{1}$$

Where:

$P(N(t+\tau) - N(t)) = k, t]$—probability of k arrivals in the interval $(t, t+\tau)$
$\mu(t)\tau$—expected number of arrivals in a time interval duration of $(t, t+\tau)$

The queue processing time of metabolite increment is described by the exponential distribution of the random variable T in the terms of the rate parameter $\mu(t)$ as follows (2):

$$f(T; \mu(t)) = \begin{cases} \mu(t)e^{-\mu(t)T} & \text{when } T \geq 0 \\ 0 & \text{when } dT < 0 \end{cases} \tag{2}$$

Therefore, the PPP is modelled by the composition of interconnected queues. Departure of substrate's increment from one queue is followed by the arrival at the successive queue. It is worth noting that the network of interconnected queues is equivalent to the set of ODEs as proven by[33].

Probability of substrate's increment departure from each queue depends on the current concentration of the substrates and the kinetic constants of the reaction causing that departure. Every queue uses its individual Michaelis–Menten kinetic equation with kinetic constants normalized according to the method based on the formula described in[1], to determine the likelihood that in this time step the reaction occurs. Since the reaction rates depend on the current concentration of molecules that change from step to step, the resulting inhomogeneous Poisson process implements the feedback loop, which results in a system with memory.

Use of a genetic algorithm to optimize model parameters. Values of enzyme kinetic constants were found with the use of a genetic algorithm starting from literature data. Every 'gene' in the 'chromosome' is a vector of kinetic constants describing each Michaelis–Menten kinetic equation. The new values of kinetic constants are found by randomly selecting from which 'parent' 'offspring' inherits 'gene' (set of kinetic constants for a particular reaction). However, mutation occurs on each kinetic value regardless to which parent it belongs. The loss function optimized by the algorithm is the sum of the squared distances between PPP state described by the literature and the current optimization step of the simulation using kinetic constants that makes an individual 'chromosome'. The formula of loss function is as follows (3):

$$f(X_l; X) = \sum (X_l - X)^2 \tag{3}$$

Where: X_l—vector of substrates described by a literature; X—vector of substrates describing stable state of simulation.

The loss function described above has a trivial solution. If all kinetic constants that are used in Michaelis–Menten reactions as multipliers (instead of dividers) are zeroed, then the results of these equations are equal to zero. As a result, no reactions occur, so the simulation's stable point is equal to the original literature vector. To prevent such a solution, the genetic algorithm sets a constraint on newly generated 'chromosomes'. Each reaction parametrized by values of the 'chromosome' for a literature vector of substrates must have a probability of occurrence between 0.00005 and 0.05.

The first set of 'chromosomes' are made of Michaelis–Menten kinetic constants defined in the literature with added gaussian noise. Given the selected starting point, the genetic algorithm is set on finding the optimal value in the proximity of the already established values. This reduces the risk of the algorithm generating an output that minimizes the loss function, but produces kinetic constants significantly different from the literature values.

Pseudocode of the PPP model. The pseudocode describing the computational processes can be found in the Supplementary Information. This code assumes that:

- Kinetic constants are grouped into a table of vectors of constant values. There are 14 vectors in the table corresponding to eight different reactions and six balancing flows. Each of the reactions has a unique vector of dimension equal to the number of kinetic constants used in the reaction rate computation and every balancing flow contains a one-dimensional vector.
- Concentration increment exchanged during the reactions is denoted 'delta' and is unique for each reaction. It ranges from 2.3×10^{-6} mM to 5.0×10^{-5} mM. 'Delta' is significantly lower than the initial value of the lowest substrate concentration. The 'delta' value must be chosen in a way that corresponds to a change of more than a single molecule for the rare species; in fact, for rare species, it should always be a positive integer number of molecules.
- the concentrations of G6P and NADP in the cycle vary with 10% Gaussian noise around the constant values of 0.001 mM and 0.0026 mM, respectively. This signal-to-noise ratio aims to reflect metabolic conditions inside the cell.

The search for optimal kinetic constants was performed using a PC with AMD Ryzen 7 3800X 8-Core Processor, 3900 MHz, RAM 32 GB. Code was written in *C#* 8.0. One search epoch simulating one hour for 50 different tables of kinetic constants using all 8 logic cores, took approximately 7 hours.

Model validation based on the use of experimental data. G6P dehydrogenase is the enzyme that catalyzes the first reaction of the pathway[34]. Therefore, it is the enzyme that controls the starting velocity of the pathway. This enzyme is strongly inhibited by NADPH[35]. Drugs aimed at reducing the intensity of the reaction mainly focus on reducing the activity of this enzyme, which leads to a reduction in the velocity of the entire pathway[27]. However, clinical results indicate that inhibiting this enzyme is not an effective therapeutic approach[26]. For this reason, data obtained from the study of knockdown expression of the 6-phosphogluconate dehydrogenase (PGD) enzyme were selected for model validation[26].

Data availability

The dataset supporting the conclusions of this article is available in the GitHub repository, https://github.com/UTP-WTIiE/PPPQueueingTheory.

Received: 30 November 2021; Accepted: 8 March 2022
Published online: 17 March 2022

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Acknowledgements
This work was funded by the National Science Center (NCN) of Poland in terms of Opus-17 Program with grant number 2019/33/B/ST6/00875, and NIH GM103427.

Author contributions
S.K., T.W. and B.W. conceived the idea for the model and described the theoretical background. K.P. and M.M. implemented the algorithms, under the supervision of T.T. and T.M. T.W., T.M. and P.D. supervised the project and coordinated the research team. S.K. and K.P. wrote the paper. All authors reviewed and approved the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-08463-y.

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6.3. Original paper III – content of the publication "Queueing theory model of mTOR complexes' impact on Akt-mediated adipocytes response to insulin."

PLOS ONE

RESEARCH ARTICLE

Queueing theory model of mTOR complexes' impact on Akt-mediated adipocytes response to insulin

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OPEN ACCESS

Citation: Kloska SM, Pałczyński K, Marciniak T, Talaśka T, Miller M, Wysocki BJ, et al. (2022) Queueing theory model of mTOR complexes' impact on Akt-mediated adipocytes response to insulin. PLoS ONE 17(12): e0279573. https://doi.org/10.1371/journal.pone.0279573

Editor: Irina U. Agoulnik, Florida International University, UNITED STATES

Received: July 6, 2022

Accepted: December 11, 2022

Published: December 27, 2022

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: https://doi.org/10.1371/journal.pone.0279573

Data Availability Statement: All relevant data are within the paper and its Supporting Information files. The source code of the described model can

Abstract

A queueing theory based model of mTOR complexes impact on Akt-mediated cell response to insulin is presented in this paper. The model includes several aspects including the effect of insulin on the transport of glucose from the blood into the adipocytes with the participation of GLUT4, and the role of the GAPDH enzyme as a regulator of mTORC1 activity. A genetic algorithm was used to optimize the model parameters. It can be observed that mTORC1 activity is related to the amount of GLUT4 involved in glucose transport. The results show the relationship between the amount of GAPDH in the cell and mTORC1 activity. Moreover, obtained results suggest that mTORC1 inhibitors may be an effective agent in the fight against type 2 diabetes. However, these results are based on theoretical knowledge and appropriate experimental tests should be performed before making firm conclusions.

Introduction

Biological importance

A key hormone that controls blood glucose levels is insulin. This hormone is secreted by the β-cells of pancreatic islets. Insulin facilitates glucose uptake in peripheral tissues including the muscle, and adipose tissue [1]. It inhibits glucose production from non-glucose sources by inhibiting gluconeogenesis and glycogenolysis, while stimulating glycogen synthesis. The hormone with the opposite effect of insulin is glucagon [2]. Both of these hormones together are primarily responsible for the maintenance of glucose homeostasis in mammals.

The attachment of insulin to the insulin receptor starts a cascade of reactions responsible for the absorption of glucose inside the cell [3]. One of the main effects of this cascade is the translocation of glucose transporter 4 (GLUT4) from the center of the cell towards the cell

be accessed by: https://doi.org/10.5281/zenodo.7117138.

Funding: This work was supported by the National Science Center (Narodowe Centrum Nauki, NCN) of Poland (https://www.ncn.gov.pl/) in terms of Opus-17 Program [2019/33/B/ST6/00875 awarded to TAW]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

membrane. GLUT4 is a protein that facilitates the diffusion of glucose along a concentration gradient–from a higher concentration in the blood to a lower concentration in the cell. The participation of GLUT4 in the transport of glucose inside the cell increases the amount of transported glucose molecules by 30 times [4, 5].

Adequate management of glucose levels in the cell is crucial to maintain a healthy environment in the cell and its function. One of the mechanisms that supervise the maintenance of adequate blood glucose levels is through mammalian target of rapamycin (mTOR) kinase. mTOR links with other proteins and forms two protein complexes described as mTORC1 and mTORC2. These complexes are responsible for regulation of various important processes inside the cell, including cell growth regulation, cell proliferation, cell motility, cell survival, protein synthesis, autophagy, DNA transcription, and metabolism [6]. The dysregulation and incorrect activity of mTOR complexes can lead to diseases such as obesity, diabetes and even cancer [7, 8]. One of the proteins that regulate the mTORC1 complex is the Rheb protein [9, 10]. It is one of the key mTORC1 activating proteins. However, one enzyme in the glycolytic pathway–glyceraldehyde 3-phosphate dehydrogenase (GAPDH), has a high affinity for the Rheb protein [11]. When GAPDH enzyme molecules are not involved in the reaction that produces 1,3-bisphosphoglycerate (1,3-BPG) from glyceraldehyde 3-phosphate (G3P), they combine with Rheb protein molecules, depriving the mTORC1 complex its key activator, leading to inactivity of the mTORC1. When the cell has normal/high concentrations of G3P, GAPDH molecules are busy processing G3P, so Rheb can freely bind to mTORC1 and activate it. Depending on the above-described mTORC1 activation process, the amount of GLUT4 particles varies. For this reason, we decided to prepare a computational model capable of predicting the number of active GLUT4 particles that are capable of participating in glucose transport.

Queueing theory

Typically, cellular signaling networks have been modeled using a set of ordinary differential equations (ODEs) [12]. Using these equations, it is possible to demonstrate the changes that occur in the cell during rest and in response to external stimuli causing upstream signals. However, when using ODEs, the fluctuations in the cell leading to local changes (e.g., temperature) are not taken into account, which influences the values of the kinetic constants that affect the way the cell responds. To map the intracellular environment more accurately, as well as the random variation, a model based on the queueing theory can be useful. Queueing theory was mainly used in telecommunications and engineering [13–16]. Additionally, it is suitable for modeling stochastic processes in cells. The idea to use a method commonly used in telecommunications comes from the fact that signaling paths, similar to the transmission of internet packets, transmit information from node to node. Likewise, in a cell, signaling molecules are passed on, activating subsequent elements (proteins) of the cascade. To date, the queueing theory approach has been used to model simple metabolic networks [17], metabolic pathways such as glycolysis [18] and the Krebs cycle [19]. The presented model is an extension of the work [20] to include loops related to the regulation of cellular metabolism by mTOR complexes and mTORC1 regulation via GAPDH availability, or more precisely–'occupancy'. In the case of models such as the one presented here, which use a large number of variables, the application of the queuing theory seems to be more optimal than the use of ODEs. The model is capable of achieving stability. Another advantage of using queueing theory to model signaling pathways is that they require significantly less computing power compared to ODE models. For this reason, simulation can be carried out practically in real time. Due to the short duration of the simulation, it can be used to learn about the relationships caused by manipulations of specific kinetic constants or concentrations, which also has its advantages when

considering the reactions that are not well studied/established. To confirm the correctness of the obtained simulation results, the simulated data were compared with the results of laboratory experiments [21]. Finally, using the queueing theory gives the possibility of expanding the model with further reactions, without major interference in the course of the previously described, due to the fact that they are based on empirically obtained values. Therefore, the model is adapted to be supplemented with the development of the state of knowledge about the given signaling or metabolic pathways. Moreover, it can be used to theoretically test the kinetic changes brought about by potential mTORC inhibitors [22].

The aim of this work is to present a comprehensive model of cellular response to insulin, which leads to GLUT4 translocation and mTORC activation as a part of processes responsible for maintaining proper cellular glucose concentration. Fig 1 shows the links between molecules involved in the insulin signaling pathway. The research hypothesis of this work is the ability to simulate the cellular response to insulin and track changes in the concentrations of proteins involved in this response using queueing theory based simulation model. The presented model shows the mechanism of mTORC1 influence on mobilization of GLUT4

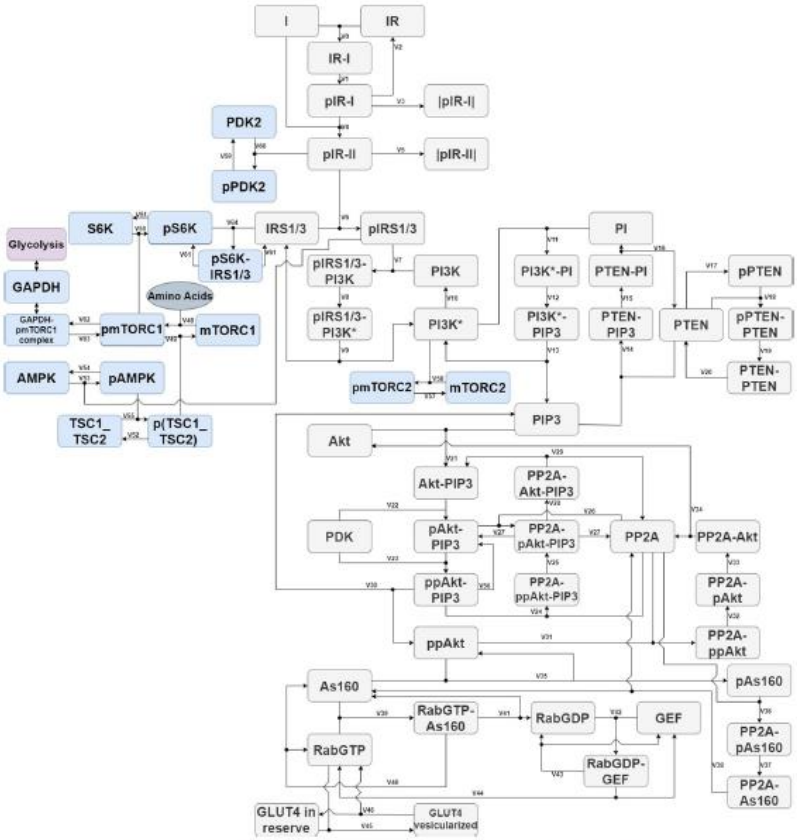

Fig 1. Diagram illustrating the computational model of the insulin signaling pathway. The cascade of reactions begins when insulin binds to the insulin receptor. The scheme includes the most important proteins in the PI3K/Akt/mTOR pathway which play a role in the cellular response to insulin.

https://doi.org/10.1371/journal.pone.0279573.g001

particles. Since mTORC1 has been reported in literature as having an impact on glucose uptake [23, 24].

The presented model is an extension of previously described simulation of insulin mediated GLUT4 translocation [20]. Since then the mTORC1 signaling pathway connections with Akt-mediated insulin response has been described [25, 26]. This work presents a model where those connections have been included. Moreover, the paper studies regulation of mTORC1 activity by the glycolytic enzyme GAPDH, which has high affinity for the mTOR activator protein–Rheb. To train the model we have used genetic algorithm (GA) to optimize the kinetic coefficients. The achieved results allow to conclude that artificial intelligence (AI) algorithms, in this case the genetic algorithm, can be effective tools for optimizing computational models. In order to validate the obtained results, we present multiple variants of mTORC1 activity that can be practically obtained through the administration of an mTORC1 inhibitor, such as rapamycin [27].

Methodology

The endpoints of the Akt-mediated insulin signaling pathway are well characterized [28–30]. Therefore, by comparing the experimental and computational results, it can be assessed whether the model works properly. The values of kinetic constants and concentrations of signaling molecules were obtained by searching the PubMed database. Simulations were performed separately for 50 independent cells, which mimic human adipocytes. This type of cell was chosen because of the availability of literature data, which was used in the development of the model. For each of the cells, the concentrations of all molecules participating in the signaling pathway were randomly chosen from the given range, limited by 10% Gaussian noise. According to the queueing theory, the current concentrations of individual molecules in each cell are separate 'stores'–queues [18, 31]. The speed of the response determines the probability of passing from one queue to the next. The simulation results are averaged over the entire cell population. A network based on queues can be used to model reactions whose rates change dynamically and randomly. The simulation was performed in C# 8.0. All the results were obtained using 1ms time increments; however, the simulation allows the choice of any user-selected time increment. While changing the time increment, one should pay attention to the fact that the probabilities of the reaction occurrence are <1. Detailed information on the equations, kinetic constants, and initial concentrations can be found in the S1 File.

The Genetic Algorithm [32, 33] was used to tune the model of interconnected queues realizing Michaelis-Menten equations. Each 'chromosome' consisted of linear coefficients for selected group of queues scaling their probability of reaction occurrence. The population of GA consists of ten 'chromosomes'. In each epoch, every 'chromosome' is evaluated and the two 'chromosomes' with the best scores are chosen. The process of 'chromosome' evaluation consists of performing three simulations with a set of kinetic constants, linear coefficients stored in each 'chromosome', and a value of available GAPDH. Each simulation used a different value of available GAPDH taken from a set {0%, 20%, 50%, 100%}. One simulation was formed emulating 50 cells working in parallel to each other. The evaluation step was added to measure, 1) how many cells reached the maximum value of GLUT4 in vesicles for available GAPDH equal to 100%, 2) how many cells reached the minimum value of GLUT4 in vesicles for available GAPDH equal to 0%, and 3) how distant is the number of cells that reached the maximum value of GLUT4 in vesicles for available GAPDH equal to 50% from aforementioned results for GAPDH equal to 100% and 0%.

To validate the model, theoretical inhibition of mTORC1 was used to test the effects of changes in reduction of its activity. One of the inhibitors of mTOR complexes' activity is

rapamycin. Previous studies show that rapamycin causes a number of side effects, including increased risk of infection [34], increased incidence of cancer [35], weight disorders, hyperlipidemia, and diabetes-like metabolic disorders [36]. For this reason, it seems necessary to develop drugs that selectively affect mTORC1 activity, while at the same time not having such significant side effects, like astragaloside IV (As-IV) [37]. As-IV was proven to be effective mTORC1 inhibitor and reduced mTORC1 signaling in mice. The data obtained from the presented model can be used in the study of the kinetics of reactions in the insulin signaling pathway, which will help to select the appropriate place where the influence of therapeutics could have the best effect.

Without insulin activating the cascade and mobilizing GLUT4 to move towards the cell membrane, there are approximately 18,200 GLUT4 molecules proximate to the cell membrane [38], ready to transport glucose inside the cell. This number increases to approximately 195,000 as a result of insulin-stimulated activation [38, 39]. However, these are not total GLUT4 stocks. In fact, the cell has a large reservoir that it can use in extreme cases. The said number 195,000 accounts for approximately 50% of total GLUT4 [21].

To illustrate the changes caused by the influence of GAPDH molecules on mTORC1 activation, two varying scenarios are described below (Fig 2). These scenarios focus on different cellular conditions such as glucose levels and the intensity of glycolysis.

Scenario I–the concentration of glucose in the blood is elevated after eating and the insulin signaling pathway works correctly. As a result, GLUT4 molecules are mobilized to migrate to the cell membrane, where they facilitate the flow of glucose from the blood to inside the cell. The glucose level in the blood drops, while the cellular level of glucose rises. To avoid the situation where glucose molecules leave the cell, glucose is phosphorylated and becomes G6P. There are two destinations for G6P molecules: 1) the glycolytic pathway or 2) glycogenesis, the formation of glycogen.

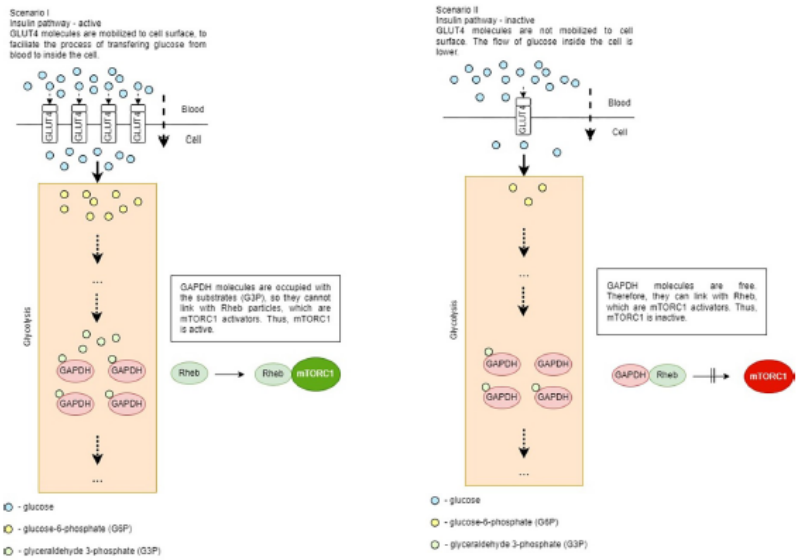

Fig 2. Various scenarios of mTORC1 activity depending on GAPDH 'occupancy'.

https://doi.org/10.1371/journal.pone.0279573.g002

When G6P enters glycolysis, the sequence of reactions takes place and glyceraldehyde 3-phosphate (G3P) molecules are formed. G3P is converted into 1,3-bisphosphoglycerate (1,3BPG) by GAPDH.

GAPDH is particularly important because it is involved in regulation of mTORC1 activity. GAPDH concentration levels in the cell do not change drastically rather they oscillate around the same values. However, what changes is their state–they can be either 'occupied' with processing G3P molecules, or if there are more enzyme molecules than substrate molecules, the excessive amount of enzyme molecules is free. Those free GAPDH molecules connect with Rheb protein and activate mTORC1. It remains unknown how Rheb stimulates the activity of mTORC1.

Scenario II–the organism is in state of prolonged fasting causing a decrease in the supply of extracellular glucose and ceasing insulin secretion. Without the release of insulin from the blood, the reaction remains inactivated and GLUT4 remains stationary and unable to transport glucose. In this situation, the stored amounts of glycogen are hydrolyzed and the basic levels of G6P are maintained. As previously described, glycolysis runs as normal. However, the amount of formed G3P molecules is lower than in Scenario I. In fact, there is larger amount of GAPDH molecules than G3P molecules. Therefore, the free GAPDH molecules can freely bind with Rheb protein, resulting in mTORC1 inactivation.

To conclude, increased extracellular supply of glucose activates insulin signaling. The glycolytic flux is increased and the GAPDH molecules are occupied with processing G3P molecules. As a result, Rheb molecules are floating freely and can bind to and activate mTORC1.

However, the conditions presented in both scenarios are extreme and practically unrealistic in the cell, as the probability of such extreme conditions as 0 or 100% 'occupancy' of GAPDH is low. In a cell, most often intermediate conditions prevail.

Results

Effect of GAPDH and mTORC1 on the amount of GLUT4 involved in glucose transport

A working, stable queueing theory-based model of the insulin signaling pathway was obtained. The presented study was aimed at illustrating the interrelationships between the levels of GLUT4, GAPDH, and mTORC1. These relationships have a significant impact on how the cell responds to insulin and extracellular glucose supply. The results obtained with the use of the model are consistent with the current state of knowledge [10, 40]. The amount of GLUT4 particles ready to take part in the glucose transport process is significantly dependent on the amount of 'occupied' GAPDH. When the system is not inhibited, less than 200,000 GLUT4 molecules are involved in the transport of glucose to the cell. However, depending on the level of activity that is influenced by both GAPDH and indirectly by mTORC1, this number fluctuates. Fig 3 shows the relationship between the level of GLUT4 in the vicinity of the cell membrane and the level of 'occupied' GAPDH. Depending on the condition of the cell, as well as mTORC1 activity, the amount of GLUT4 mobilized can vary considerably (Figs 4 and 5). The greater amount of GAPDH involved in substrate processing allows Rheb to link freely with mTORC1. mTORC1 activity and GLUT4 level are correlated with each other [41, 42]. The same conclusions can be drawn by analyzing the obtained results on the charts.

We also tested the effect of lowering mTORC1 activity, e.g., through the use of drugs, on the amount of GLUT4 particles, while assuming different levels of GAPDH 'occupancy' (Fig 4). Analogous studies were performed for different levels of GAPDH with respect to mTORC1 activity (Fig 5). Both mTORC1 activity and the amount of 'occupied' GAPDH significantly influences the amount of GLUT4 and can contribute to lowering the amount of GLUT4

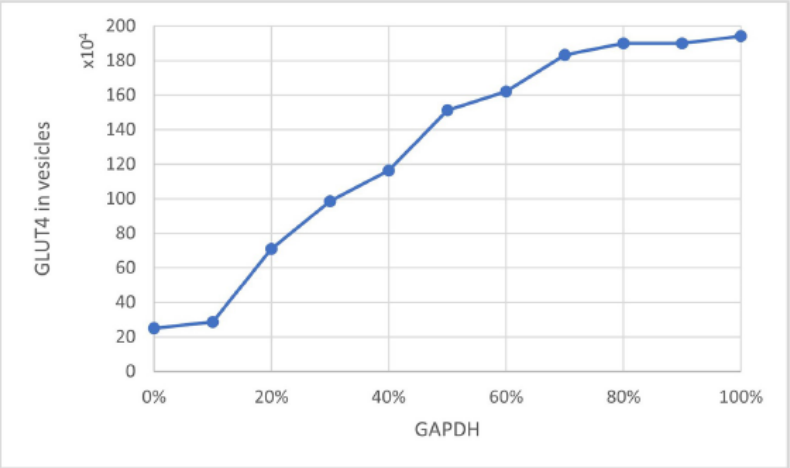

Fig 3. The relationship between the amount of GLUT4 particles in the cell membrane area and the level of 'occupied' GAPDH.

https://doi.org/10.1371/journal.pone.0279573.g003

particles involved in glucose transport (Fig 5). The scenario in which all the GAPDH particles present in the cell are busy processing its substrate so that the mTORC1 can be fully active, keeps the amount of GLUT4 in vesicles at the maximum level (Fig 5). The presented results indicate that drugs that can significantly decrease mTORC1 activity (at least 50% inhibition) are of great importance for the amount of GLUT4 particles directed to the cell membrane for glucose transport inside the cell. Similar conclusions can be drawn from the results presented

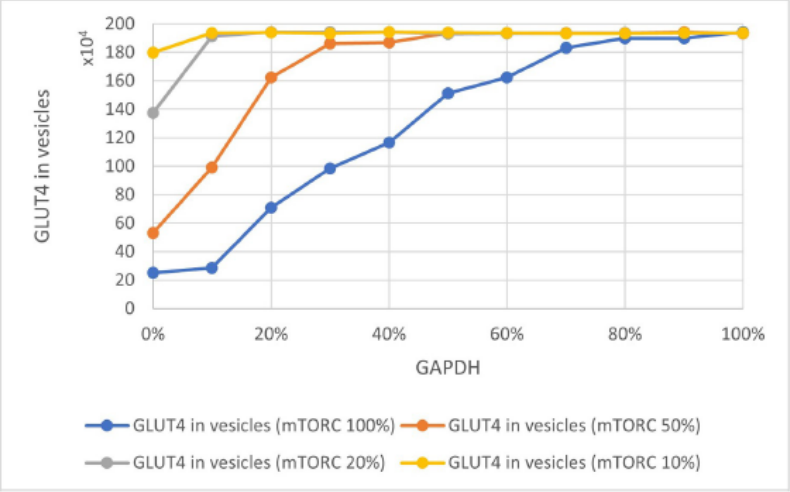

Fig 4. Relationship of GLUT4 in vesicles and 'occupied' GAPDH. Colored lines indicate different levels of mTORC1 activity.

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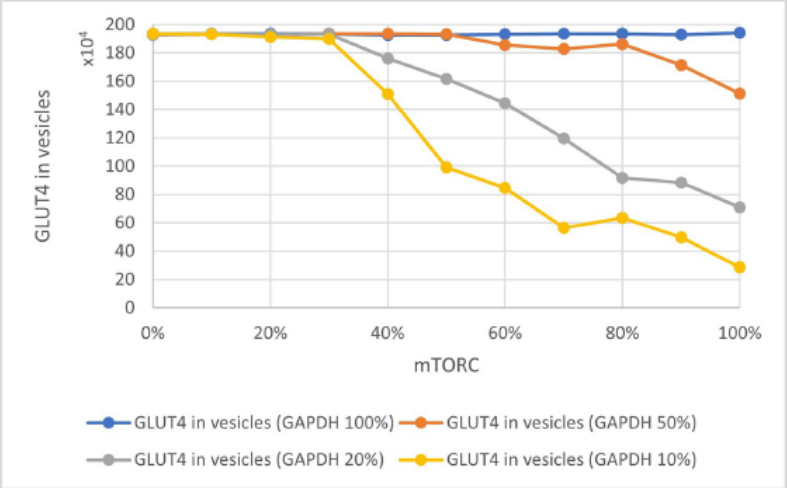

Fig 5. Dependence of GLUT4 level in vesicles in relation to mTORC1 activity. The colored lines indicate the different levels of 'occupied' GAPDH.

https://doi.org/10.1371/journal.pone.0279573.g005

by Rajan et al. [43] and Veilleux et al. [44] which confirms the validity of the method we presented.

Discussion

Identification of key nodes in insulin signaling

Practical application of the conclusions of the described scenarios for GAPDH and mTORC1 allowed the identification of key nodes for the appropriate cell response to insulin, and confirmed previous experimental results described in [45]. In [45] the authors explained and proved the important role of S6 kinase (S6K). mTORC1 participates in its phosphorylation. S6K is crucial because it is the link between the mTORC1 loop and the rest of the proteins responsible for insulin signaling. Signaling between mTORC1 and S6K causes a negative-feedback loop which lowers cellular sensitivity for insulin. The activation of the mTORC1/S6K loop leads to increased degradation of insulin receptor substrate 1/3 (IRS1/3) and therefore influences the amount of GLUT4 in vesicles. This entire process affects how many glucose molecules enter the cell from the bloodstream.

The experimental results, as well as those obtained in the presented model, indicate that the insulin response system is very complex and depends on many elements that regulate it. It is characterized by high instability, small changes that can lead to a greatly altered cell response, causing disease such as type 2 diabetes, where the cells become insensitive to insulin. As shown in the above model, there are many elements that can cause glucose malabsorption.

Sonntag et al. [46] focused on determining which of the 'nodes' of the insulin signaling pathway influences AMP-activated protein kinase (AMPK) activity. The equations described in the [46] are based on the mass action law. The obtained results state that IRS1/3 is the 'node' influencing AMPK. The model proposed by Sonntag et al. focused on simplifying the insulin signaling pathway and it does not take into account several 'nodes' that play a significant role in this process. Therefore, the combination of the data and results presented by [46] was a

valuable source in the preparation of the model based on the queueing theory. GA was used to find an appropriate scaling of the values so that the model as a whole would work properly.

The presented model has several limitations. It does not take into account other signaling pathways or individual reactions that are also connected to and influence signaling proteins. This is especially true for the Akt protein, which is the central node in the presented signaling model. Moreover, a model based on literature data will only be as good as the available data. However, we do not question the reliability of other research teams and their published results. Another of the limitations is that in queueing theory, each simulation gives one realization of the stochastic process, while ODE gives an averaged solution. Therefore, a limitation is that depending on the number of cells for which one runs simulations and then averages them, this is how accurate the result will be. Therefore, the model presented here is for averaged results for 50 cells.

mTORC1 activity and related treatment strategies

The results of the described model could be used as a suggestion in the process of developing new drugs, including drugs that increase insulin-sensitivity in peripheral tissues such as the muscle and adipose tissue (e.g., Metformin). Identifying key 'nodes' throughout the signaling pathway could guide researchers in helping cells regain their original insulin sensitivity. However, due to the complexity of connections between all signaling molecules, this task is very difficult.

mTORC1 plays an important role in the maintenance of an adequate level of glucose in the blood. When necessary, i.e., in a nourished state, mTORC1 activity stimulates pancreatic β-cells to secret insulin, thus maintaining adequate glucose tolerance. However, studies in mice [47, 48] show that mTORC1 overactivity may cause a faster deterioration in β-cell function and consequently complications with glucose homeostasis. Therefore, the use of mTORC1 inhibitors to improve glucose tolerance has been considered. Previous studies in mice have shown that S6K knockdown or inhibitors that reduce S6K phosphorylation make cells more insulin sensitive [49, 50]. The results obtained with the use of the queueing theory model confirm earlier reports [45] that mTOR/S6K inhibition could be a therapeutic target in type 2 diabetes.

One of the most common prototype mTOR inhibitors is rapamycin. However, the use of rapamycin has been counterproductive, inducing insulin resistance and disrupting glucose homeostasis in the body [51]. Rapamycin is an effective inhibitor of mTORC1. Most researchers agree that rapamycin does not inhibit mTORC2 at least in the acute stimulation [52]. Few researches suggest that rapamycin inhibits mTORC2 only in some cell types and only with chronic administration due to inhibiting to mTORC2 assembly [53, 54]. Knowing the function and the importance of this complex in signaling pathway, it is no wonder that long-term mTOR inhibition interferes with the body's response to insulin. Due to the fact that rapamycin affects both mTORC1 and mTORC2, it can be concluded that it is worth testing substances that act selectively on only one of these complexes.

Research by Tao et al. [22] provided useful information on the influence of inhibitors on mTORC kinetics and activity. mTORC1 activity can be completely inhibited by ATP competitive inhibitors, like BEZ235 or PI103, while non-competitive ATP inhibitors, like rapamycin, inhibits mTORC1 activity only partially by interacting with the FRB (FKBP-rapamycin-binding) domain. By affecting kinetic properties of mTOR, they influence the process of glucose absorption in the cell. These types of results and information can provide data that can be complemented by the presented model. In this way, it will be possible to characterize changes in the entire signaling pathway induced by the use of mTORC1 inhibitors and evaluate the effect of this inhibition on the amount of GLUT4 in vesicles.

Increased mTORC1 activity has been also reported in many types of cancer [8]. mTOR is one of the factors influencing the development and growth of cells. Its excessive activity encourages cancer cells to further grow, divide and invade other healthy tissues. For this reason, it was decided to test mTORC1 inhibitors in cancer therapy [27], as they appeared to be an effective tool for coercing cancer cells into apoptosis. Although many mTORC inhibitors have been tested, some of them have been approved for therapy, however, their therapeutic capacity is relatively low. For this reason, they are most often used in combination with other anticancer drugs. In addition, their side effects must be considered. Palm et al. [55] demonstrated on mouse model of pancreatic cancer that rapamycin may even promote cell proliferation at poorly vascularized sites of the tumor. In view of all this information, it remains vital to study mTOR more thoroughly because its participation in cancer metabolism is undeniable [56], which is why it seems to be such an important research direction. The presented model can be used for this type of research, during the theoretical phase, where the likely results of their use can be determined using the data on the influence of new drugs on mTOR kinetics.

Conclusions

A queueing theory model of mTORC1 and mTORC2 impact on Akt-mediated cell response to insulin was prepared. The presented results show that queuing theory can effectively model the manipulation of mTORC1 kinase activity influences the amount of GLUT4 used to transport glucose inside the cell, and therefore influences the concentration of glucose in the cell. The work shows suggestions of alternative targets for treating type 2 diabetes. Due to the number of people with diabetes and the existing methods of relieving symptoms, without treating the disease, any new therapeutic target may prove to be crucial. However, it should be noted that due to the nature of the studies performed, our findings must be confirmed in clinical trials.

Supporting information

S1 File. Additional supporting information may be found in the online version of this article. Supporting Information file contains values of literature concentrations used in the model and reaction equations and kinetic constants used in the model. The source code is freely available for download at https://github.com/UTP-WTIiE/IrsMtorcQueuesSimulation, implemented in C# supported in Linux or MS Windows.
(DOCX)

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7. Conclusions

The main and secondary objectives of the work have been achieved. Using the queueing theory, I developed simulation models of the Krebs cycle, PPP, and signaling pathway of cellular response to insulin. In my research, by comparing the calculated values with literature data, I showed that these models can be effective in assessing and predicting the concentration of individual metabolites in real time, both in natural conditions and under the influence of inhibitors. The development of biological computational models could have a major impact on the development of personalized medicine. By understanding an individual's unique metabolic pathway, doctors can tailor treatment plans to optimize efficacy and minimize side effects. However, the accuracy of the models heavily depends on the accuracy of the input data and assumptions made during the modeling process, so it is important to carefully validate the models before drawing any firm conclusions.

The models may help researchers to better understand the behavior of these systems under different conditions and to identify potential targets for therapeutic interventions. This can speed up the drug discovery process and improve the success rate of drug development.

Computational models of the Krebs cycle can have several practical applications in biology and medicine. The Krebs cycle is a central metabolic pathway that is involved in the production of ATP and the generation of metabolic intermediates that are required for many cellular processes. One potential application of computational models of the Krebs cycle is to study the metabolism of cancer cells. Cancer cells have altered metabolic pathways, and the Krebs cycle is often dysregulated in these cells. Computational models of the Krebs cycle can help to identify the specific alterations in this pathway that occur in cancer cells, and can aid in the development of new cancer treatments that target these altered metabolic pathways. Krebs cycle models can also be used to study the effects of drugs and other compounds on cellular metabolism. By simulating the effects of different compounds on the Krebs cycle, these models can help to identify potential drug targets and optimize the pharmacological properties of drugs. The studies carried out on the Krebs cycle model performed on the purpose of this dissertation, confirmed that it can be used in the assessment of the effect of drugs used in anticancer therapy, such as Tamoxifen in combination with Metformin or Phenformin. Thanks to this model, it is possible to assess the effect of a specific drug dose on the concentration of Krebs cycle metabolites, which can be one of the methods of assessing their effectiveness.

Another potential application of Krebs cycle models is in the field of metabolic engineering. By simulating the activity of the Krebs cycle in different organisms or under different conditions, these models can help to identify strategies for optimizing metabolic pathways for the production of biofuels, chemicals, and other bioproducts.

Overall, computational models of the Krebs cycle can have a wide range of practical applications in biology and medicine, from cancer research to metabolic engineering and drug discovery. By providing insights into the regulation of this central metabolic pathway and its role in health and disease, these models can help to identify new therapeutic targets and develop more effective treatments for a range of diseases.

The developed PPP model is able to calculate the concentration of pathway metabolites and track changes in their concentration both under normal conditions and after knocking out the 6PGD gene. Computational models of the PPP can have several practical applications in medicine. Due to the fact that PPP is an important metabolic pathway that generates several biologically important molecules, which are necessary for many cellular processes, including biosynthesis and antioxidant defense, dysregulation of the PPP has been implicated in various diseases, such as cancer, neurodegeneration, and metabolic disorders.

One potential application of computational models of the PPP in medicine is to aid in the development of new drugs that target this pathway. For example, by simulating the effects of different compounds on the PPP, potential drug candidates that can modulate PPP activity in a specific way can be identified. This can help the process of new drug development and optimize the pharmacological properties of drugs to enhance their efficacy and reduce side effects.

Another potential application of PPP models in medicine is in the field of pharmacokinetics. Computational models of the PPP can help predict how drugs will be metabolized and eliminated from the body, which is important for optimizing drug dosing and reducing the risk of adverse effects. By integrating data on the activity of the PPP and other metabolic pathways, these models can predict drug metabolism and clearance in different tissues and under different conditions, which can help to optimize drug dosing and reduce the risk of toxicity.

Overall, computational models of the PPP can have a wide range of practical applications in medicine, from drug discovery to pharmacokinetics. By providing insights into the regulation of the PPP and its role in disease, these models can help to identify new drug

targets, optimize drug efficacy and safety, and improve our understanding of cellular metabolism in health and disease.

The model of the signaling pathway of the cellular response to insulin shows the influence of the interdependence between GAPDH, Rheb, and mTORC1, on the amount of active GLUT4 molecules involved in intracellular glucose transport. This area is not fully known by the world of science, however, the developed model, due to their nature, can be modified with the emergence of new scientific knowledge, without the risk of losing its previous achievements.

The Akt protein present in this model plays a crucial role in regulating a wide range of cellular processes, including cell growth, metabolism, and survival. In particular, this pathway has been linked to the aging process, as studies have shown that reducing Akt activity can extend lifespan in various model organisms, such as worms and mice. The PI3K/Akt pathway is known to influence several mechanisms that are thought to contribute to aging, such as oxidative stress, inflammation, and cellular senescence. For example, Akt activation can promote cell survival by inhibiting pro-apoptotic factors, which can protect cells from stress-induced damage. On the other hand, excessive Akt activity can lead to an overgrowth of cells, which can contribute to the development of cancer and other age-related diseases.

Therefore, I believe that given the important role of Akt-mediated signaling in aging, computational models of aging can be useful for understanding the mechanisms involved in Akt signaling and its impact on the aging process. By integrating data from various sources, such as genetic and epigenetic factors, environmental exposures, and lifestyle factors, these models can provide insights into the complex interplay between different factors that contribute to aging. Computational models of aging can also be used to identify potential interventions that can modulate Akt signaling to promote healthy aging. For example, by simulating the effects of drugs or lifestyle modifications on Akt activity, these models can help researchers to identify potential interventions that can extend lifespan and reduce the risk of age-related diseases.

In summary, the impact of Akt-mediated signaling in the aging process highlights the importance of understanding the mechanisms involved in this pathway, and developing computational models of aging can be a useful tool for investigating these mechanisms and identifying potential interventions to promote healthy aging.

Hereby, I was able to confirm the usefulness of the methods of modeling metabolic and signaling pathways, which allows for better understanding, learning, and conducting in silico

research. In addition, apart from the scientific papers described in this dissertation, two more are under review in reputable scientific journals. They concern the model of beta-oxidation of fatty acids and the comprehensive model of metabolism integrating glycolysis, Krebs cycle, PPP, and beta-oxidation of fatty acids.

One potential direction for further research involving the application of queueing theory to computational biology is to assess the effects of environmental stressors on metabolic pathways. In my study, I addressed the impact of different concentrations of metabolites or the availability of enzymes present in a given pathway. However, metabolic pathways are affected by many factors. External conditions or environmental stressors can significantly affect the level of a cell's metabolic activity. Therefore, an extension of my research may contribute to the state of the art in this area.

Moreover, metabolic models can be used to optimize the pharmacokinetics, by providing a better understanding of the metabolic pathways involved in the production of bioproducts such as drugs and other biologically active products. By developing accurate models of these pathways, researchers can identify potential bottlenecks and limitations in the process, and identify opportunities to increase yields, reduce costs, and improve efficiency. For example, in the pharmaceutical industry, metabolic models can be used to optimize the production of drugs by identifying the most efficient metabolic pathways for the production of drug intermediates or precursors. This can help to reduce the cost and time required to produce the drug, and ensure a consistent and reliable supply of the drug.

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9. Statements of co-authors of publications included in the series

9.1. Attachment No. 1

Prof. dr hab. inż. Tadeusz A. Wysocki
[1]Wydział Telekomunikacji, Informatyki i Elektrotechniki
Politechnika Bydgoska im. J. i J. Śniadeckich w Bydgoszczy
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Oświadczam, że w artykule „Queueing theory model of Krebs cycle" opublikowanym w czasopiśmie *Bioinformatics* w 2021r.; https://doi.org/10.1093/bioinformatics/btab177, którego współautorami są Sylwester Kloska, Krzysztof Pałczyński, Tomasz Marciniak, Tomasz Talaśka, Marissa Nitz, Beata J. Wysocki, Paul Davis oraz Tadeusz A. Wysocki, mój wkład merytoryczny polegał na opiece nad badaniami i współredagowaniu artykułu (senior author).

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Sylwestra Michała Kloska jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

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(Podpis)

Oświadczam, że w artykule „Queueing theory model of pentose phosphate pathway" opublikowanym w czasopiśmie *Scientific Reports* w 2022r.; https://doi.org/10.1038/s41598-022-08463-y, którego współautorami są Sylwester Kloska, Krzysztof Pałczyński, Tomasz Marciniak, Tomasz Talaśka, Marissa Nitz, Beata J. Wysocki, Paul Davis oraz Tadeusz A. Wysocki, mój wkład merytoryczny polegał na opiece nad badaniami i współredagowaniu artykułu (senior author).

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Sylwestra Michała Kloska jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

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(Podpis)

Oświadczam, że w artykule „Queueing theory model of mTOR complexes' impact on Akt-mediated adipocytes response to insulin" opublikowanym w czasopiśmie *PLOS One* w 2022r.; https://doi.org/10.1371/journal.pone.0279573, którego współautorami są Sylwester Kloska, Krzysztof Pałczyński, Tomasz Marciniak, Tomasz Talaśka, Marissa Nitz, Beata J. Wysocki, Paul Davis, Ghada A. Soliman oraz Tadeusz A. Wysocki, mój wkład merytoryczny polegał na opiece nad badaniami i współredagowaniu artykułu (senior author).

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Sylwestra Michała Kloska jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

.....

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I hereby declare that in the paper "Queueing theory model of Krebs cycle" published in the journal *Bioinformatics* in 2021; https://doi.org/10.1093/bioinformatics/btab177, whose co-authors are Sylwester Kloska, Krzysztof Pałczyński, Tomasz Marciniak, Tomasz Talaśka, Marissa Nitz, Beata J. Wysocki, Paul Davis and Tadeusz A. Wysocki, my substantive contribution was to supervise the research and co-edit the paper (senior author).

At the same time, I agree to submit the above-mentioned work by Sylwester Michał Kloska, M.Sc., as part of a doctoral dissertation in the form of a thematically coherent collection of scientific papers published in scientific journals.

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(Signature)

I hereby declare that in the paper "Queueing theory model of pentose phosphate pathway" published in the journal *Scientific Reports* in 2022; https://doi.org/10.1038/s41598-022-08463-y, whose co-authors are Sylwester Kloska, Krzysztof Pałczyński, Tomasz Marciniak, Tomasz Talaśka, Marissa Miller, Beata J. Wysocki, Paul Davis, and Tadeusz A. Wysocki, my substantive contribution was to supervise the research and co-edit the paper (senior author).

At the same time, I agree to submit the above-mentioned work by Sylwester Michał Kloska, M.Sc., as part of a doctoral dissertation in the form of a thematically coherent collection of scientific papers published in scientific journals.

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9.2. Attachment No. 2

Prof. Tomasz Marciniak

Faculty of Telecommunications, Computer Science and Electrical Engineering

Bydgoszcz University of Science and Technology

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Dr inż. Tomasz Marciniak, prof. PBŚ

Wydział Telekomunikacji, Informatyki i Elektrotechniki

Politechnika Bydgoska im. J. i J. Śniadeckich w Bydgoszczy

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Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Sylwestra Michała Kloska jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

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(Podpis)

9.3. Attachment No. 3

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Faculty of Telecommunications, Computer Science and Electrical Engineering

Bydgoszcz University of Science and Technology

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Tomasz Talaśka

(Podpis)

Streszczenie

Rozprawa omawia zastosowanie teorii kolejek jako metody modelowania ścieżek biologicznych takich jak cykl Krebsa, szlak pentozofosforanowy (PPP) i szlak odpowiedzi komórkowej na insulinę. Modele biologii obliczeniowej mogą być wykorzystywane do symulacji zachowania systemów biologicznych i przewidywania wyników różnych ingerencji/interwencji w badany układ. Metoda teorii kolejek jest wykorzystywana do śledzenia zależności pomiędzy poszczególnymi metabolitami powstającymi na różnych etapach szlaków metabolicznych oraz do obserwacji zmian spowodowanych fluktuacjami stężeń metabolitów i ich wpływem na cały szlak. Tego typu model może być wykorzystany do przewidywania wpływu terapii, co może przyczynić się do zwiększenia jej skuteczności. W rozprawie wykazano również, że model uzyskał stabilność na podstawie danych pochodzących z literatury naukowej.

Modele biologii obliczeniowej mogą być niezwykle przydatne w medycynie precyzyjnej, ponieważ mogą pomóc przewidzieć, jak pacjent odpowie na konkretne leczenie. Symulując biologię pacjenta, modele te mogą zidentyfikować konkretne geny, białka i ścieżki, które napędzają chorobę i przewidzieć, które leki lub inne metody leczenia będą najbardziej skuteczne. Może to prowadzić do poprawy wyników pacjentów, zmniejszenia skutków ubocznych i obniżenia kosztów opieki zdrowotnej. Modele te mogą być wykorzystywane do symulacji interakcji pomiędzy metabolitami, białkami i innymi biomolekułami.

Opracowane modele zostały oparte na równaniach kinetyki, które opisują szybkość reakcji katalizowanych przez enzymy. W modelach cyklu Krebsa i PPP wykorzystano równania kinetyki Michaelisa-Menten, które są powszechnie stosowane do opisu kinetyki enzymów i uwzględniają stężenia substratów i produktów oraz właściwości kinetyczne danych enzymów. Natomiast model szlaku sygnalizacyjnego insuliny oparty był na prawie zachowania mas, które opisuje szybkość reakcji na podstawie stężeń reagentów i produktów. Taki wybór równania kinetyki odzwierciedla specyficzne cechy każdego ze szlaków oraz cele prezentowanych badań.

Summary

The text discusses the use of queueing theory methods in computational biology, specifically in the modeling of biological pathways like the Krebs cycle, pentose phosphate pathway (PPP), and the insulin signaling pathway. Computational biology models can be used to simulate the behavior of biological systems and predict the outcomes of different treatments or interventions. The queueing theory method is used to track the relationships between individual metabolites formed at different stages of the pathway and to observe changes caused by fluctuations in metabolite concentrations and their impact on the entire pathway. This type of model can be used to predict the impact of therapy, which in turn will lead to an increase in its effectiveness. The text also mentioned that the model obtained stability based on the data derived from scientific papers.

Computational biology models can be extremely useful in precision medicine, as they can help predict how a patient will respond to a particular treatment. By simulating the patient's biology, these models can identify the specific genes, proteins, and pathways that are driving a disease and predict which drugs or other treatments will be most effective. This can lead to improved patient outcomes, reduced side effects, and reduced healthcare costs. These models can be used to simulate the interactions between metabolites, proteins, and other biomolecules.

The developed models were based on different kinetics equations that describe the rate of enzyme-catalyzed reactions. The models of the Krebs cycle and PPP used Michaelis-Menten kinetics equations, which are commonly used to describe enzyme kinetics and take into account the substrate and product concentrations and kinetic properties of given enzymes. On the other hand, the model of the insulin signaling pathway was based on mass action law, which describes the rate of reactions based on the concentrations of the reactants and products. This choice of kinetics equation reflects the specific characteristics of each pathway and the goals of the presented research.