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The effect of cigarette smoking on endothelial damage and atherosclerosis development – modeled and analyzed using Petri nets

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Atherosclerosis as one of the crucial causes of cardiovascular diseases (CVD) is the leading reason of death worldwide. One of the contributing factors to this phenomenon is endothelial dysfunction, which is associated with the impact of various agents and their interactions. Tobacco smoke is one of the well known factors here. For better understanding of its significance a model of its impact on atherosclerotic plaque formation has been proposed. The model contains selected aspects of the influence of tobacco smoke, dual function of nitric oxide (NO) (influence of various mechanisms on NO bioavailability), oxidative stress which promotes low density lipoproteins oxidation, macrophages significance and other mechanisms leading to an aggravation of the endothelial disturbances. The model has been built using Petri nets theory and the analysis has been based on t-invariants. This approach allowed to confirm the important role of inflammation and oxidative stress in atherosclerosis development and moreover it has shown the considerable influence of the cigarette smoke.

Key words: atherosclerosis, endothelial dysfunction, cigarette smoking, modeling, Petri nets, t-invariants

1. Introduction

Despite the progress achieved in recent years, knowledge of the mechanisms associated with atherosclerosis is still incomplete, what impede effective treatment of its complications. Atherosclerosis is a complex and dynamic chronic inflammatory process which occurs in arterial vessels. Modifications and interactions between various factors

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have influence on this disorder. One of the crucial phenomenon of this process is endothelial dysfunction accompanied by inflammatory process and oxidative stress. The latter is associated with excessive production of reactive oxygen species (ROS). It reflects an imbalance between the amount of ROS and the ability to easily detoxification. On the other hand, formation of free radicals, defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital, can act positively through transmitting signals which stimulate repairing mechanisms. Moreover, inflammation, oxidative stress (respiratory burst) and other atherosclerosis-related processes are additionally stimulated by cigarette smoking.

This network of dependencies is very interesting via its complexity and dynamics. To better understand the nature of the entire disease process the systems approach has been used, because analysis of individual/particular processes or events has been found to be not sufficient [1, 9, 16, 29]. An analysis of the presented model allowed to systematize knowledge about atherosclerosis and its key processes. Furthermore, significant components of the modeled system has been distinguished. The analysis of all mechanisms and interactions between them requires mathematical methods and computer tools. In this case healthy human organism is treated as a system in dynamic equilibrium and a disease state is a consequence of inhibition or accumulation of some molecules. To show the complexity of the presented process the mathematical model, based on Petri nets theory [5, 20, 24], has been created. The analysis of the model has been based on t-invariants, which correspond to subprocesses occurring in the modeled system [12, 18]. Thus, similarities in t-invariants allow to identify subprocesses which interact with each other and for this reason clustering of t-invariants has been done. Additionally, an analysis of MCT sets, which contain transitions corresponding to exactly the same t-invariants, may lead to identification of significant functional blocks of the proposed model.

The model which has been described in this paper is an extended version of the model presented in [3]. The first version of the model focused on endothelial damage and mechanisms which occur in human organism, but it contains only few factors related to cigarette smoking. The extended model presented in this paper includes additional processes and molecules associated with smoking. Mechanisms which have been included in the model presented in [3] promote inflammation and endothelial dysfunction. Selected additional mechanisms which have been included in the extended model are modifications of lipids profile, promotion of prothrombotic state and formation of thrombus.

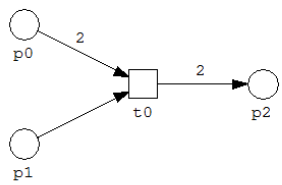
2. Methods

A structure of a Petri net is a weighted directed bipartite graph which consist of two disjoint subsets of vertices, i.e., places and transitions. Arcs in such a net connect vertices which belong to different subsets, i.e., transitions with places and places with transitions [5, 20, 24]. In the case of modeling of biological processes places (represented by circles) correspond to biological or chemical components. Transitions (represented by rectangles) correspond to elementary subprocesses (e.g., reactions between molecules)

[7, 18]. Other important elements are tokens which represent quantities of particular components included in a model. The distribution of tokens over the places corresponds to the state of the modeled system. Tokens flow through the net, and more precise, they flow between places via transitions, what is related to activation and firing of transitions. A transition is active, when the number of tokens in each place which directly precedes this transition is equal to at least the weight of the arc connecting the place with the transition. Furthermore, an active transition can be fired, what means that tokens flow from each place directly preceding the transition to each place directly succeeding it and the number of flowing tokens is equal to a weight of an appropriate arc. The flow of tokens corresponds to a flow of information, substances etc. through the modeled system [7, 28].

A graphical representation is one of the possibilities of representing Petri nets. It is very intuitive and helps to understand the structure of a modeled system but is not well suited for a formal analysis of its properties. For this purpose another representation, called incidence matrix, is used. Entry a_{ij} of such a matrix $A = [a_{ij}]_{n\times m}$, where n is the number of places, and m is the number of transitions, is an integer number equal to the difference between the numbers of tokens present in place p_i before and after firing transition t_j [20]. Figure 1 shows a graphical representation of an exemplary simple Petri net and its incidence matrix.

a)



b)

	t0
p0 (H_2)	-2
p1 (O_2)	-1
p2 (H_2O)	2

$2H_2 + O_2 \rightarrow 2H_2O$

Figure 1: a) A graphical representation of a simple Petri net modeling synthesis of water; b) an incidence matrix of the net presented in part a).

On the basis of incidence matrix A transition invariants (t-invariants) can be calculated [12, 18]. Such invariants play significant roles in an analysis of models of biological systems. A t-invariant is n-element vector x, for which equation $A \cdot x = 0$ is fulfilled. To every t-invariant x there corresponds a set of transitions, called its support, which contains those transitions which correspond to non-negative coordinates of vector x, i.e., $supp(x) = \{t_j : x_j > 0\}$. t-invariants correspond to some subprocesses which do not change the state of the system [12, 18]. An analysis of t-invariants is based on similarities among them and may lead to discoveries of some interactions between subprocesses which occur in the modeled system. The net should be covered by t-invariants what means that every transition is an element of a support of at least one t-invariant. When the number of t-invariants is high the searching for similarities among them is usually done using clustering methods. According to them similar t-invariants are grouped into set called t-clusters. Further analysis is then performed within the obtained clusters.

Moreover, every t-cluster usually corresponds to some functional module of the biological system, so its biological meaning should be determined. In addition, also transitions can be grouped into structures called Maximal Common Transitions sets (MCT sets), which correspond to functional blocks. Such a set contains transitions being elements of supports of exactly the same t-invariants [7, 8, 12, 28].

The general working scheme is shown in Figure 2.

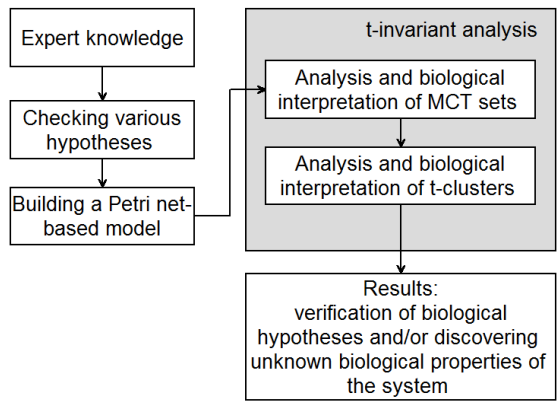


Figure 2: A scheme of work: through expert knowledge and checking various hypotheses to modeling and analysis of biological processes using Petri nets.

3. Informal description of the modeled process

3.1. Endothelial dysfunction

Initiation of endothelial dysfunction is related to presence of various factors, which can have direct and indirect influence on the studied phenomenon. Some of them are high blood pressure, increased glucose level in serum, high concentration of low-density lipoprotein (LDL), cytokines, toxins, etc. The changes in their level/concentration may be influenced by many agents, and cigarette smoking is one of them. The proposed model includes selected aspects of the impact of tobacco smoke [15, 19]. Indirect endothelial damage caused by cigarette smoking is understood as, inter alia: decreased quantity of tetrahydrobiopterin (BH_4 - which is a naturally occurring cofactor for the nitric oxide (NO) synthesis by the nitric oxide synthases (NOS)), decreased quantity of LDL, ROS, free radical and metals.

Damaged endothelium (caused by both direct and indirect mechanisms) expresses vascular cell adhesion protein 1 (VCAM-1) and chemokines. These chemotactic cytokines allow for monocytes adhesion and in consequence they lead to diapedesis. Monocytes movement out of the vessel wall and transformation into macrophages is typical inflammatory response [21]. One of the main function of macrophages is getting rid of damaged cells. Transformation of macrophages into foam cells is a consequence of up-

take of oxLDL through scavenger receptors. In normal condition macrophages after this activity return to blood circulation, but in case when LDL serum concentration is high, macrophages are still present in inflammatory area [27]. The modeled process includes also much more sophisticated role of these white blood cells. Macrophages secrete cytokines, what leads to VCAM-1 synthesis on the endothelial cell surface and vascular smooth muscle cells (VSMC) proliferation [21]. The presence of cytokines induces respiratory burst what results in creation of free radicals, accurately formation of superoxide anion ($O_2^{\bullet -}$) [2, 21], which participates in oxLDL formation [23]. Harmful oxLDL are absorbed by macrophages via scavenger receptors and form foam cells [10, 2], being a crucial compound of atherosclerotic plaque.

3.2. Nitric oxide

Nitric oxide is produced by a group of enzymes called nitric oxide synthases and is involved in many cellular processes, some of them are: regulation of blood pressure, angiogenesis, apoptosis, platelets aggregation, LDL oxidation etc. NO plays important role in CVD, where biochemical and molecular mechanisms which regulate NO bioavailability have significant function. These mechanisms can change NO concentration, causing opposite functions of NO: positive and negative effect. Impairment of NO availability favor CVDs. In this case it promotes atherosclerosis [19, 21, 25]. Cigarette smoke has been found to be another source that leads to decreasing of NO bioavailability [15, 19, 21].

3.3. The effect of cigarette smoking

Cigarette smoking is one of the major risk factors playing an important role in many harmful processes. This part of the model includes selected aspects of its involvement. Substances which are released, as cadmium, metals, free radicals, nicotine, polycyclic aromatic hydrocarbons, aldehydes etc. are engaged in endothelial dysfunction, inflammation, respiratory burst, LDL oxidation and have an important influence on atherosclerosis development. Increased quantity of ROS, free radicals and metals stimulate LDL oxidation, which results in reduction of NO bioavailability [15, 19, 21]. Nicotine, catecholamine, CO_2 lead to increase of blood viscosity, which promotes formation of thrombus. Other elements such as increase and activation of platelets and also increased quantity of fibrinogen, tissue factor, plasminogen activator inhibitor-1, decreased quantity of plasminogen activator and other remaining elements are additional contributors that may stimulate thrombus. Thromboxane A2 and prostacyclin I2 (which also are secreted by cigarette smoke) block the coronary blood vessels. These described processes have more direct influence on atherosclerosis. On the other hand, there are mechanisms which have indirect influence, like stimulating inflammation and endothelium dysfunction. Cigarette smoke secretes macrophages and polycyclic aromatic hydrocarbons, which stimulates chemokines - these molecules belong to inflammatory environment. Moreover, endothelial dysfunction stimulated by cigarette smoking is associated with modification of lipids profile (decreased quantity of HDL and increased quantity of LDL and triglycerides). As is widely known, high LDL level is one of the causes of endothelial damage. Therefore,

it can be noticed, that cigarette smoke is an additional source which stimulates inflammation and aggravates atherosclerotic plaque formation.

4. The model

In this section mathematical model of the analyzed biological system is proposed. The model has been expressed in the language of Petri nets theory [20, 22, 24]. It has been built using Snoopy software [13], and analyzed using R scripts.

The proposed model is shown in Fig. 3. For better transparency of the model it has been divided into two subnets. The first, upper frame presents mechanisms, reactions and molecules which are present in human organism. These processes are associated with response on endothelial dysfunction, LDL oxidation, respiratory burst, NO synthesis and in consequence they may lead to development of atherosclerosis. The second, lower frame presents mechanism, reactions and harmful molecules which are associated with cigarette smoke. These molecules are engaged in all inappropriate processes like stimulation of endothelial damage, oxidative stress promotion and LDL oxidation, reduction of nitric oxide synthesis, also increasing and decreasing quantity of various molecules - which leads to creation of thrombus, increasing blood viscous and development of atherosclerosis. Table 1 includes the names of places which correspond to the biological or chemical components and Table 2 contains the names of transitions which correspond to the reactions and interactions between molecules (represented by places).

The main complications in the modeling and analysis are caused by inhibition reactions (mechanisms decreasing quantity of some elements/molecules). This process has been modeled using Snoopy [13], which allows to use inhibitor arcs, but they are not represented in the incidence matrix and in consequence they are not taken into account in the cluster analysis. The problem of analyzing Petri nets with inhibitor arcs is still open, therefore inhibition reaction was modeled using names of transitions “inhibition” in the presented model. It might be a little unintuitive, so a two examples in Figure 4 have been presented:

- Cofactor BH_4 inhibition:
  - In normal conditions: cofactor BH_4 increases affinity eNOS to L-arginine, and it is important for NO synthesis (Figure 4 part a)).
  - In case of BH_4 inhibition: a decreased quantity of BH_4 leads to inhibition of NO synthesis (in the model this process is related with place “less NO” (Figure 4 part b)).
- eNOS inhibition:
  - In normal conditions: eNOS (nitric oxide synthase) is important molecule, which is engaged in nitric oxide synthesis (Figure 4 part c)).

– In case of eNOS inhibition: indirect inhibition of eNOS is caused by decreasing of BH_4 and direct inhibition is caused by asymmetric dimethylarginine (ADMA) (Figure 4 part d)).

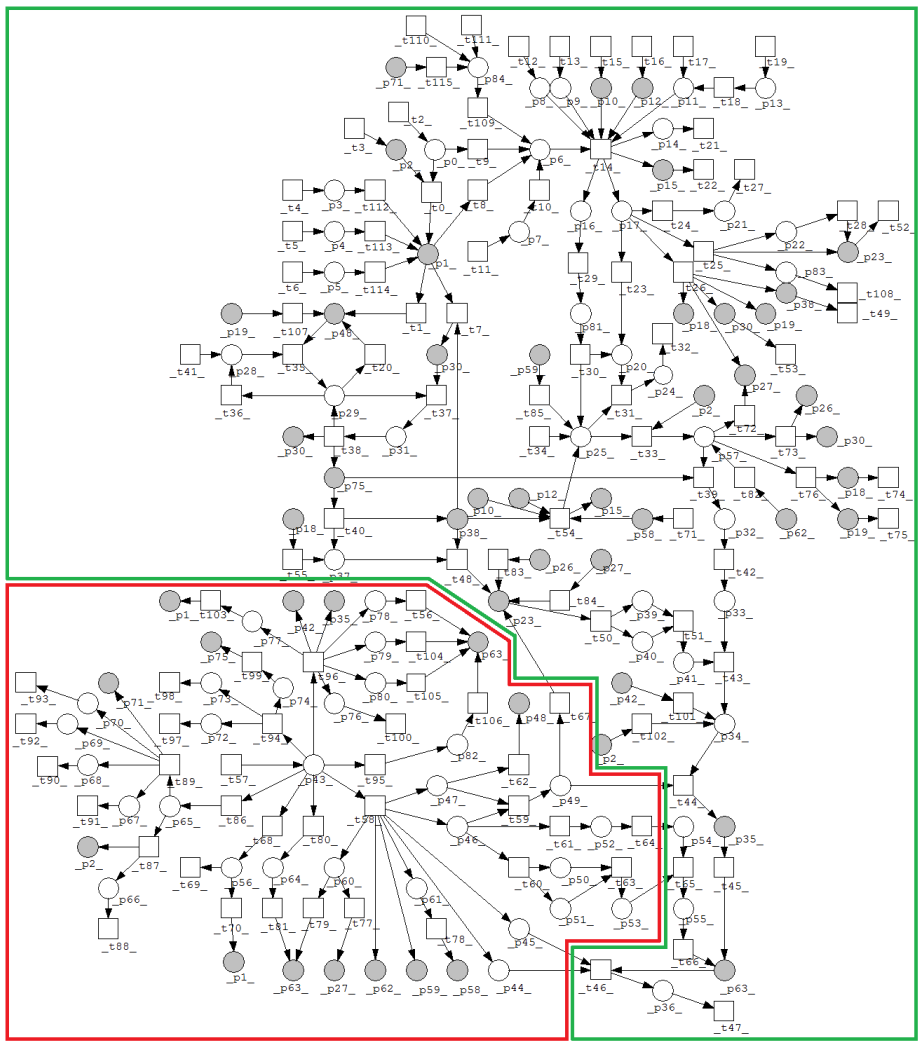


Figure 3: The proposed model is divided into two subnets: the upper frame presents mechanisms and molecules which are present in human organism and the lower frame presents mechanisms and harmful molecules which are associated with cigarette smoke.

Other simplifications included in the model resulted from lack of precise data. Therefore, the model includes transitions with names: “increase”, “decrease”, “less NO”, “NO”. The place “less NO” is related with inhibition of nitric oxide synthesis, and the place “NO” is related with correct NO synthesis. The model also includes additional

arcs which represent reversible reactions, for example: creation of active monocytes. Chemokines and inactive monocytes are involved in creation of active monocytes which can form a complex with VCAM-1 protein. In this process chemokines and VCAM-1 protein can be reused.

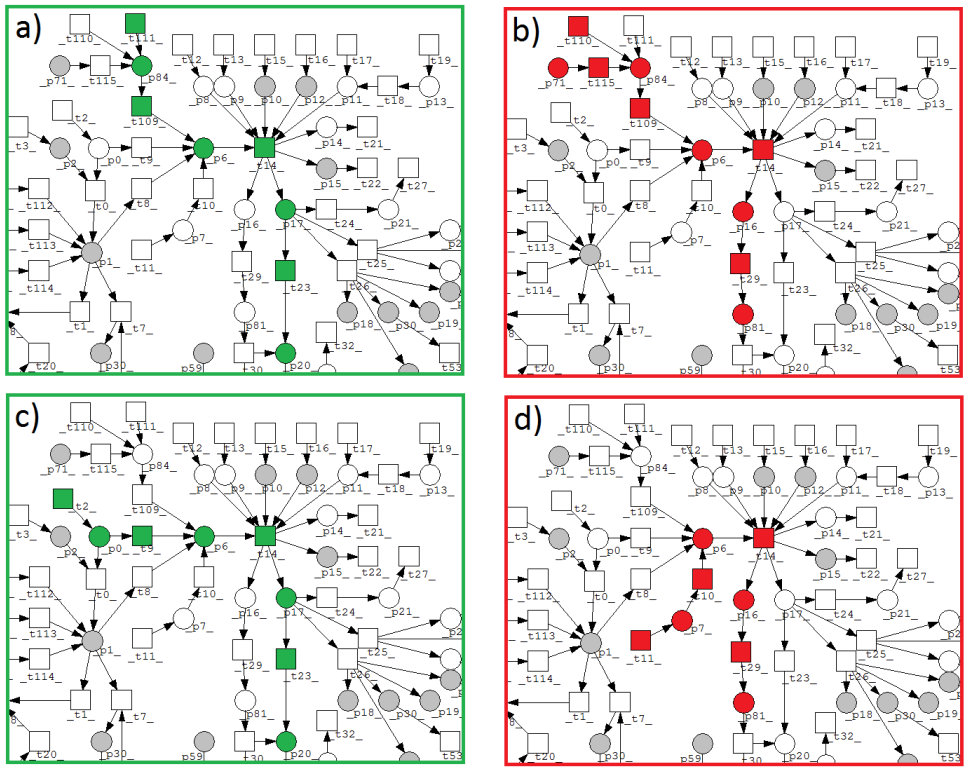


Figure 4: Simplifications caused by inhibition reactions: the left frames a) and c) correspond to situations in normal conditions and the right frames b) and d) correspond to inhibition reactions.

Table 1: List of places. The column "No." includes the place numbers and the column "Biological meaning" contains the names of biological or chemical components.

No.	Biological meaning	No.	Biological meaning
p_0	healthy endothelium	p_{43}	cigarette smoke
p_1	damaged endothelium	p_{44}	thromboxane A2
p_2	LDL	p_{45}	prostacyclin I2
p_3	high blood pressure	p_{46}	nicotine
p_4	toxins	p_{47}	polycyclic aromatic hydrocarbon
p_5	other factors	p_{48}	chemokines
p_6	eNOS	p_{49}	matrix metalloproteinases (MMPs)
p_7	asymmetric dimethylarginine (ADMA)	p_{50}	adrenaline

No.	Biological meaning	No.	Biological meaning
p_8	iNOS	p_{51}	noradrenaline
p_9	nNOS	p_{52}	catecholamine
p_{10}	NADPH	p_{53}	CO
p_{11}	L-arginine	p_{54}	alpha receptors
p_{12}	O_2	p_{55}	high blood viscosity
p_{13}	L-NMMA	p_{56}	impairment of fibromuscular dysplasia (FMD)
p_{14}	citrulline	p_{57}	oxLDL
p_{15}	NADP	p_{58}	ROS
p_{16}	NO if endothelium is damaged	p_{59}	free radical
p_{17}	NO if endothelium is healthy	p_{60}	cadmium
p_{18}	macrophage colony stimulating factor (MCSF)	p_{61}	aldehydes
p_{19}	monocyte chemotactic protein 1 (MCP-1)	p_{62}	metals
p_{20}	NO	p_{63}	thrombus
p_{21}	blood pressure	p_{64}	platelets
p_{22}	platelet aggregation	p_{65}	lipid profile
p_{23}	amplified cell caused by proliferation of VSMC	p_{66}	triglycerides
p_{24}	peroxynitrite	p_{67}	HDL
p_{25}	superoxide anion	p_{68}	FMD
p_{26}	intercellular adhesion molecule 1 (ICAM-1)	p_{69}	apolipoprotein A1
p_{27}	adhesion molecules	p_{70}	selenium
p_{28}	inactive monocyte	p_{71}	less quantity of BH4
p_{29}	active monocyte	p_{72}	lymphocytes
p_{30}	vascular cell adhesion molecule 1 (VCAM-1)	p_{73}	neutrophils
p_{31}	complex VCAM-1 and monocyte	p_{74}	auxiliary place
p_{32}	foam cell	p_{75}	macrophage
p_{33}	necrosis core	p_{76}	oxidative stress markers
p_{34}	plaque	p_{77}	endothelin 1 (ET-1)
p_{35}	tissue factor	p_{78}	plasminogen activator inhibitor 1
p_{36}	atherosclerosis	p_{79}	fibrinogen
p_{37}	growth factor	p_{80}	von Willebrand factor
p_{38}	cytokines	p_{81}	less NO
p_{39}	matrix glycoproteins	p_{82}	plasminogen activator
p_{40}	collagen	p_{83}	other mechanisms
p_{41}	fibrous cap	p_{84}	BH4 cofactor of eNOS
p_{42}	white blood cells		

Table 2: List of transitions. The column "No." includes the transition numbers while the column "Biological meaning" contains their biological functions.

No.	Biological meaning	No.	Biological meaning
t_0	damage caused by LDL	t_{58}	secretion caused by cigarette smoke
t_1	secretion caused by damaged endothelium	t_{59}	activation caused by nicotine and polycyclic aromatic hydrocarbon
t_2	auxiliary transition	t_{60}	release caused by nicotine
t_3	auxiliary transition	t_{61}	induction caused by nicotine
t_4	auxiliary transition	t_{62}	induction caused by polycyclic aromatic hydrocarbon
t_5	auxiliary transition	t_{63}	decrease O_2 increase CO
t_6	auxiliary transition	t_{64}	activation caused by catecholamine
t_7	expression caused by damaged endothelium	t_{65}	increase the viscosity of blood
t_8	inhibition of eNOS caused by damage endothelium	t_{66}	stimulation caused by highly viscous blood
t_9	secretion caused by healthy endothelium	t_{67}	stimulation caused by MMPs
t_{10}	inhibition of eNOS caused by ADMA	t_{68}	direct damage caused by cigarette smoke
t_{11}	auxiliary transition	t_{69}	remodeling tissue caused by impairment of FMD
t_{12}	auxiliary transition	t_{70}	damage caused by impairment of FMD
t_{13}	auxiliary transition	t_{71}	auxiliary transition
t_{14}	synthesis of NO	t_{72}	expression adhesion molecules caused by oxLDL
t_{15}	auxiliary transition	t_{73}	expression caused by oxLDL
t_{16}	auxiliary transition	t_{74}	auxiliary transition
t_{17}	auxiliary transition	t_{75}	auxiliary transition
t_{18}	inhibition caused by LNMMA	t_{76}	stimulation caused by oxLDL
t_{19}	auxiliary transition	t_{77}	expression caused by cadmium
t_{20}	auxiliary transition	t_{78}	increase caused by aldehydes
t_{21}	auxiliary transition	t_{79}	role in endothelial cell death
t_{22}	auxiliary transition	t_{80}	increase and activation caused by cigarette smoke
t_{23}	high quantity of NO	t_{81}	prothrombotic procoagulative states caused by increase of platelets
t_{24}	regulation caused by NO if healthy endothelium	t_{82}	stimulation of oxidation caused by metals
t_{25}	inhibition caused by NO if healthy endothelium	t_{83}	stimulation caused by ICAM-1
t_{26}	inhibition adhesion molecule	t_{84}	stimulation caused by adhesion molecules
t_{27}	auxiliary transition	t_{85}	stimulate respiratory burst caused by free radical

No.	Biological meaning	No.	Biological meaning
t_{28}	auxiliary transition	t_{86}	modification caused by cigarette smoke
t_{29}	low quantity of NO	t_{87}	increase caused by modification of lipid profile
t_{30}	high quantity of superoxide anion radical	t_{88}	auxiliary transition
t_{31}	reduction	t_{89}	decrease caused by modification of lipid profile
t_{32}	inhibition of oxLDL	t_{90}	tissue remodeling caused by FMD
t_{33}	oxidation	t_{91}	auxiliary transition
t_{34}	auxiliary transition	t_{92}	auxiliary transition
t_{35}	auxiliary transition	t_{93}	auxiliary transition
t_{36}	auxiliary transition	t_{94}	increase caused by development environment inflammatory
t_{37}	adhesion of monocyte	t_{95}	decrease caused by cigarette smoke
t_{38}	transformation	t_{96}	increase caused by cigarette smoke
t_{39}	uptake	t_{97}	auxiliary transition
t_{40}	secretion caused by macrophage	t_{98}	auxiliary transition
t_{41}	auxiliary transition	t_{99}	stimulation caused by inflammatory environment
t_{42}	destruction foam cell	t_{100}	auxiliary transition
t_{43}	formation of plaque composed of necrosis core and fibrous cap	t_{101}	formation caused by white blood cells
t_{44}	plaque rapture	t_{102}	creation caused by LDL
t_{45}	activation of blood platelets	t_{103}	damage caused by endothelin 1
t_{46}	block of the coronary blood vessels	t_{104}	prothrombotic states caused by fibrinogen
t_{47}	auxiliary transition	t_{105}	prothrombotic states caused by von Willebrand factor
t_{48}	proliferation caused by growth factor and cytokines	t_{106}	prothrombotic states caused by plasminogen activator
t_{49}	auxiliary transition	t_{107}	stimulate chemokines
t_{50}	secretion caused by amplified cells	t_{108}	auxiliary transition
t_{51}	formation of fibrous cap	t_{109}	increases the affinity of eNOS to L-arginine
t_{52}	auxiliary transition	t_{110}	inhibition of BH4 cofactor
t_{53}	auxiliary transition	t_{111}	auxiliary transition 120
t_{54}	respiratory burst	t_{112}	damage caused by high blood pressure
t_{55}	simulation growth factor	t_{113}	damage caused by toxins
t_{56}	prothrombotic states caused by plasminogen activator inhibitor 1	t_{114}	damage caused by other factor
t_{57}	auxiliary transition	t_{115}	inhibition of BH4 caused by smoking

5. Analysis

5.1. MCT sets

The proposed Petri net includes 84 places, 115 transitions, 21 non-trivial (i.e., containing more than one transition) MCT sets and is covered by 5344 t-invariants. For all of the MCT sets its biological meaning has been determined and described in Table 3.

Table 3: List of non-trivial MCT sets. The column "MCT set" contains names of these sets, while the column "Transitions" includes names of transitions contained in a given MCT set. The column "Biological meaning" includes biological interpretation (functions and mechanisms) of MCT sets.

MCT set	Transitions	Biological meaning
m_1	t_{12}, t_{13}, t_{14}, t_{15}, t_{16}, t_{21}, t_{22}, t_{29}, t_{30}, t_{31}, t_{32}	Nitric oxide synthesis without L-arginine leads to reduction of NO (L-arginine is necessary to proper NO synthesis). This MCT set includes two opposite processes: promotion of LDL oxidation and inhibition of LDL oxidation by superoxide anion reduction to peroxynitrite. This second process acts despite inhibition of NO synthesis.
m_2	t_{56}, t_{96}, t_{100}, t_{101}, t_{103}, t_{104}, t_{105}	Increased of various elements caused by cigarette smoke. These elements have influence on endothelial damage and development of prothrombotic states (which stimulate thrombus formation and promote atherosclerosis).
m_3	t_{60}, t_{61}, t_{62}, t_{63}, t_{64}, t_{65}, t_{66}	Nicotine induces catecholamine and releases adrenaline and noradrenaline, which lead to increased of the blood viscosity. This MCT set includes stimulation of inflammatory response which is caused by polycyclic aromatic hydrocarbon through stimulation of chemokines.
m_4	t_{46}, t_{47}, t_{58}, t_{78}, t_{82}, t_{85}	Secretion of aldehydes (it leads to increased quantity of ROS, which stimulate respiratory burst), metals and free radicals (they have influence on LDL oxidation and respiratory burst), thromboxane A2 and prostacyclin I2 (they promote atherosclerosis by narrowing of the coronary blood vessels).
m_5	t_{89}, t_{90}, t_{91}, t_{92}, t_{93}, t_{115}	Modification of lipid profile (caused by cigarette smoke) stimulate decreased quantity of HDL, FMD, apolipoprotein A1, selenium and BH4 (decreased quantity of BH4 leads to inhibition of NO synthesis).
m_6	t_{39}, t_{42}, t_{43}, t_{50}, t_{51}	Response of damaged endothelium in which secretion of macrophages is engaged. Macrophages uptake of oxLDL causes formation of foam cells and also destruction (if oxLDL level is high). When foam cell dies necrotic core is released and stimulates plaque creation (development of atherosclerosis).
m_7	t_{94}, t_{97}, t_{98}, t_{99}	Development of inflammation caused by cigarette smoke. It simulates macrophages to increase of cytokines secretion.
m_8	t_{25}, t_{28}, t_{108}	Inhibition of harmful mechanisms (platelet aggregation, proliferation of VSMC, and other), when NO synthesis is correct.
m_9	t_4, t_{112}	Endothelial dysfunction caused by high blood pressure.
m_{10}	t_5, t_{113}	Endothelial dysfunction caused by toxins.
m_{11}	t_6, t_{114}	Endothelial dysfunction caused by other factors.

MCT set	Transitions	Biological meaning
m_{12}	t_{10}, t_{11}	Inhibition of NO synthesis caused by ADMA, which is eNOS inhibitor.
m_{13}	t_{18}, t_{19}	Inhibition of NO synthesis caused by L-NMMA, which is L-arginine inhibitor.
m_{14}	t_{20}, t_{41}	Response of damaged endothelium and transformation to macrophages. Precisely, damaged endothelium secretes chemokines, what induces monocytes.
m_{15}	t_{24}, t_{27}	Regulation of blood pressure in case of proper NO synthesis.
m_{16}	t_{37}, t_{38}	The formation of VCAM-1 and monocyte complex, which leads to macrophages transformation.
m_{17}	t_{44}, t_{45}	Development of atherosclerosis is promoted by plaque rupture and formation of thrombus.
m_{18}	t_{73}, t_{83}	Proliferation of VSMC is stimulated by LDL oxidation.
m_{19}	t_{80}, t_{81}	Increase and activation of platelets (caused by cigarette smoke) promote atherosclerosis.
m_{20}	t_{87}, t_{88}	Increased quantity of LDL and triglycerides caused by modification of lipid profile.
m_{21}	t_{95}, t_{106}	Decreased quantity of plasminogen activator caused by cigarette smoke stimulates prothrombotic state and thrombus formation.

5.2. Clusters analysis

The analysis of the presented model is based mainly on t-invariants, which correspond to some subprocesses occurring in the modeled system. The number of t-invariants is dependent on the nature of the analyzed process. t-invariants can be calculated using many freely available tools, e.g., Charlie [14] or MonaLisa [6]. In our analysis t-invariants have been grouped into t-clusters and the biological meaning has been assigned to each such a cluster. Subprocesses corresponding to t-invariants being elements of the same t-cluster can be related in some way and influence each other. For this reason the analysis of clusters can be crucial for confirmation of some hypotheses and discovering of unknown properties of the modeled system. In Table 4 biological interpretation of 18 t-clusters has been shown. The clusters have been determined using average linkage method and Pearson similarity measure. The Mean Split Silhouette (MSS) is an index which has been used to identify the best clustering in the set of clusterings obtained using various methods (cf. [7, 8]). MSS evaluates a fit of each t-invariant to its cluster and an average quality of a given clustering [17, 26]. Calinski-Harabasz coefficient has been used to find the best number of clusters [4]. This coefficient has been calculated for clusterings whose number of clusters was in the range from 2 to 20 and the optimal number of clusters has been indicated by its highest value.

The obtained results confirm that oxidative stress (respiratory burst) and inflammatory process are the main paths that are related with endothelial dysfunction. These processes lead to increased secretion of ROS and free radicals, which are involved in excessive LDL oxidation. On the other hand, LDL oxidation influence on harmful mechanisms which are associated with decreasing NO bioavailability. These processes promote narrowing of the coronary blood vessels, stimulate increasing blood viscous and creation of

thrombus which leads to development of atherosclerosis. Cluster analysis showed also how big influence on the development of atherosclerosis has cigarette smoking. Both direct and indirect endothelial damage have influence on inflammation through increased quantity of macrophages and cytokines. Disorders in prothrombotic states have influence on creating thrombus and increasing blood viscosity which more directly stimulate atherosclerotic plaque formation.

Clusters analysis revealed how complex and wide is the interaction net of inflammation process and oxidative stress. Both of them are engaged in almost all mechanisms which are described in Table 4. These results reveal which of the processes are crucial for the modeled system.

Table 4: List of t-clusters. The column "t-cluster" contains names of t-clusters, while the column "Biological meaning" includes their biological interpretations.

t-cluster	**Biological meaning**	**t-cluster**	**Biological meaning**
c_1	Inflammatory response of damaged endothelium: monocytes adhesion, which are engaged in the diapedesis (monocytes movement out of the vessel wall and transformation to macrophages).	c_{10}	Endothelial dysfunction caused by high LDL level. This LDL can be oxidized, then oxLDL stimulates harmful proliferation mechanism and adhesion of molecules. This cluster includes also inhibition of molecules adhesion and proliferation, when NO synthesis is correct.
c_2	Tissue remodeling caused by impairment of fibromuscular dysplasia (FMD) - it has direct influence on endothelial dysfunction.	t_{11}	Modification of lipid profile caused by cigarette smoke has an influence on increased quantity of LDL. This LDL can be oxidized, then oxLDL stimulate harmful proliferation mechanism. This cluster includes also inhibition of molecules adhesion and proliferation, when NO synthesis is correct.
c_3	The formation of VCAM-1 and monocyte complex, which leads to macrophage transformation. Macrophages secrete cytokines and growth factor, which lead to proliferation of VSMC.	c_{12}	Endothelial dysfunction caused by high LDL level. This LDL can be oxidized, then oxLDL stimulate harmful proliferation mechanism. This cluster includes also inhibition of proliferation and molecules adhesion, when NO synthesis is correct.
c_4	Cigarette smoke influence on development inflammation by stimulating macrophages. This cluster includes also harmful activity of macrophages, which leads to proliferation.	c_{13}	Endothelial dysfunction (caused by high LDL level) stimulates inflammatory response, which is related with LDL oxidation (oxLDL stimulate harmful mechanisms).
c_5	Endothelial dysfunction caused by high blood pressure induces inflammatory response: secretion of chemokines and adhesion of monocytes.	c_{14}	Endothelial dysfunction (caused by modification of lipid profile in smokers) stimulates inflammatory response, which is related with LDL oxidation (oxLDL stimulates harmful mechanisms).

t-cluster	Biological meaning	t-cluster	Biological meaning
c_6	Endothelial dysfunction caused by toxins induce inflammatory response: secretion of chemokines and adhesion of monocytes.	c_{15}	Endothelial dysfunction caused by various factors stimulates inflammatory response, this process is additionally stimulated by modification of lipid profile in smokers. These processes have influence on LDL oxidation and reduction of NO bioavailability (inhibition of NO synthesis caused by BH4 inhibition, eNOS inhibition by ADMA, L-arginine inhibition by L-NMMA). In other side this cluster includes NO that have vasoregulatory functions and can inhibit LDL oxidation.
c_7	Endothelial dysfunction caused by other factor (for example high glucose level in diabetes) induce inflammatory response: secretion of chemokines and adhesion of monocytes.	c_{16}	This cluster includes almost all mechanisms. Distinctive processes: respiratory burst and LDL oxidation. Both are additionally stimulated by cigarette smoke and aggravate atherosclerosis.
c_8	Endothelial dysfunction caused by impairment of fibromuscular dysplasia (FMD) - it has direct influence on endothelial dysfunction and induce inflammatory response: secretion of chemokines and adhesion of monocytes.	c_{17}	This cluster includes almost all mechanisms. Distinctive processes: NO synthesis and inflammation, which are additionally stimulated by cigarette smoke.
c_9	Endothelial dysfunction caused by modification of lipid profile induce inflammatory response: secretion of chemokines and adhesion of monocytes.	c_{18}	This cluster includes almost all mechanisms. Distinctive processes: NO synthesis and inflammation, which are additionally stimulated by cigarette smoke.

5.3. Conclusions

Systems approach is used for analysis of biological processes as complex systems. This approach forces the detailed knowledge of the modeling process but it can be difficult due to a lack of data and contradictory information. On the other hand, only in this way it is possible to know the nature of these processes, organize knowledge and even discover new dependencies.

Cluster analysis allows the discovery of new and interesting biological meanings, and also confirms the existing dependencies. Clusters are sets of t-invariants, therefore the number of t-invariants is important. When this number is small, all the interactions and dependencies can be considered and the biological interpretations can be determined. In the case of the presented model the number of t-invariants is very high (5344), and some clusters contain over 1000 and 2000 t-invariants, which can be problematic during the analysis. For this reason the descriptions of clusters 15-18 in Table 4 are more general, but they consist distinctive processes. On the other hand, by focusing on the smaller clusters dependencies can be determined in greater detail.

Clusters which contain t-invariants associated with endothelial dysfunctions (caused by high blood pressure, toxins, high glucose level in diabetes), always include a response of endothelial damage (stimulating chemokines and monocytes adhesion). These endothelial dysfunctions can be caused regardless of cigarette smoking. However, subsequent clusters reveal a direct influence of smoking on endothelial damage which are caused by impairment of fibromuscular dysplasia or modification of lipid profile. Clusters which include t-invariants associated with endothelial dysfunction caused by high LDL level (which can be additionally stimulated by cigarette smoke) beside inflammatory response also include oxidation process. If LDL is oxidized, it results in reduction of NO bioavailability. Clusters which contain t-invariants associated with NO synthesis show interesting interactions. NO can act positively and negatively, which is dependent on its concentration. Despite of mechanisms affecting the decrease of NO and promoting of LDL oxidation, NO can act positively and can inhibit harmful processes. The analysis of the clusters that include t-invariants associated with dual functions of NO showed that it can inhibit proliferation. In two of the three clusters containing NO it was revealed that it can inhibit adhesion of molecules and furthermore it can stimulate LDL oxidation.

Due to a lack of accurate data about the concentration of NO and a lack of information about the time of all of the studied reactions it is not possible to determine which mechanisms occur more frequently or faster. Despite this fact, this study confirmed that NO plays an important role in development of atherosclerosis.

The obtained results confirm that oxidative stress and inflammation are closely associated with endothelial dysfunction and development of atherosclerosis and both influence each other. These processes result in excessive production of ROS and free radicals, which are engaged in LDL oxidation and reduction of NO bioavailability. Additional source which stimulates these processes is cigarette smoking. The systems approach that has been used in the study allowed for a better understanding of the analyzed process and for distinguishing important signaling pathways. Participation of cigarette smoking in the atherosclerosis appears to be indisputable. These results are consistent with the literature.

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