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To my father… who is gone.

Acknowledgment

I would like to thank Konstantina Skolariki for the help she provided during the editorial process.

Contents

In Silico and In Vivo Studies on Quercetin as Potential Anti-Parkinson Agent

Hemanth Kumar Boyina, Sree Lakshmi Geethakhrishnan, Swetha Panuganti, Kiran Gangarapu, Krishna Prasad Devarakonda, Vasudha Bakshi, and Sandhya Rani Guggilla

1 Introduction

Parkinson's disease (PD) is the second most common age-related neurodegenerative disorder and is characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the accumulation of proteinaceous cytoplasmic inclusions known as Lewy bodies (LBs) (Michel et al. 2016; Chauhan and Jeans 2015).

Central nervous system dysfunction in PD patients results in symptoms such as bradykinesia, resting tremors, postural instability, and muscular rigidity (Jankovic 2008). Recent studies have provided insight into the major events involved in PD pathogenesis, including mitochondrial dysfunction and proteasome system dysfunction (Ghiglieri et al. 2018). Alpha-synuclein is the major protein constituent of LBs and Lewy neuritis aggregation. It is also linked with the accumulation of misfolded or damaged proteins and oxidative stress in the substantia nigra (Yan et al. 2018).

Rotenone, a neurotoxin that belongs to the family of isoflavones, naturally found in the roots and stems of several plants, is used as a broad-spectrum pesticide. It is highly lipophilic, thus easily crossing the BBB. Once in the cell, rotenone accumulates at mitochondrial complex I where it inhibits the transfer of electrons from iron-sulfur (Fe-S) centers to ubiquinone (Gowthami et al. 2018).

Increased reactive oxygen species production has been associated with complex I dysfunction induced by rotenone, which may produce oxidative damage to DNA

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and proteins of neural cells, hence leading to the death of DA neuron (Surmeier 2018; Gould et al. 2018). Currently, levodopa (L-dopa), although is considered a gold standard replacement therapy in PD, so far only alleviates the clinical symptoms. Furthermore, patients usually experience severe side effects several years after the L-dopa treatment. Therefore, efforts are made not only to improve the effect of L-dopa treatment for PD but also to investigate new drugs with both anti-parkinsonian and neuroprotective effects (Cenci and Crossman 2018).

Growing evidence suggests that abnormal redox active metal accumulation caused by dysregulation plays a central role in the neuropathology of PD. Redox active metals like Fe and Cu catalyze essential reactions for brain functions. However, these metals can also participate in the generation of highly toxic free radicals (*fenton reaction*) that can cause oxidative damage to cells and ultimately lead to the death of dopaminergic neurons (Aguilera et al. 2018). Flavonoids are naturally occurring plant molecules that are able to bind to free iron atoms and also offer powerful antioxidant protection. Quercetin, a polyphenolic compound, chelates iron atoms involved in fenton reaction in L-dopa metabolism hence acts as powerful iron-chelating agent. Quercetin's antioxidant effects are closely related to iron-chelating capacity which accounts for its ability to prevent neurodegeneration in Parkinson's disease (Castañeda-Arriaga et al. 2018). Recent studies suggest the neuropharmacological efficacy of polyphenolic quercetin against Parkinson's disease (Sarubbo et al. 2018). This study is designed to investigate the ameliorative role of quercetin with and without L-dopa in rotenone-induced Parkinson's disease (El-Horany et al. 2016; Kabel et al. 2018).

2 Materials and Methods

Forty-two adult male Wistar rats aged 7 weeks, weighing 150–250 g, were used. All animals were maintained under standard husbandry conditions. The rats were randomly assigned to six groups ($n = 6$) (Table. 1), and the experimental protocol was duly approved by the Institutional Animal Ethics Committee, and study design is

Table 1 Animal grouping

Groups	Treatment
I	Served as normal control group received vehicle (control)
II	Parkinsonism was induced by subcutaneous administration of rotenone for 28 days at a dose of 1.5 mg/kg (rotenone-R)
III	Co-treated with rotenone and L-dopa (R-L-dopa)
IV	Co-treated with rotenone and low dose of quercetin. (R + LD of quercetin)
V	Co-treated with rotenone and high dose of quercetin (R + HD of quercetin)
VI	Co-treated with rotenone and low dose of quercetin and L-dopa (R + LD of quercetin + L-dopa)
VII	Co-treated with rotenone and high dose of quercetin and L-dopa (R + HD of quercetin + L-dopa)

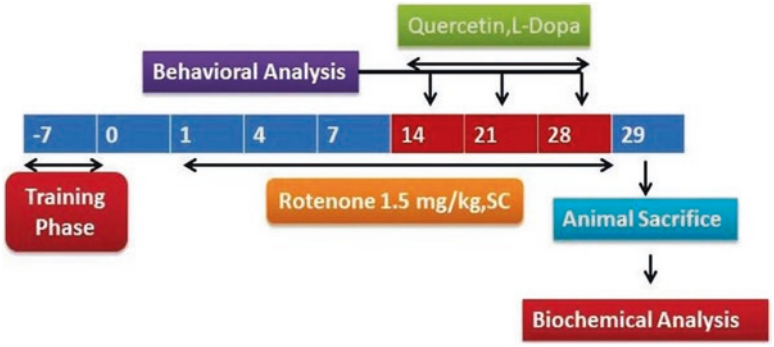

Fig. 1 Study design (duration of drug treatment and parameters assessment)

depicted in Fig. 1. Rotenone was dissolved in 1% DMSO solution and administered subcutaneously daily for 28 days at a dose of 1.5 mg/kg. Quercetin and L-dopa were dissolved in sterile water for injection, and quercetin was given at 15 mg/kg and 50 mg/kg doses. L-dopa and carbidopa were given in 1:10 ratio at doses of 20 mg/kg and 2 mg/kg i.p. All treatments were given 1 h prior to the rotenone administration from 15th to 28th day. The body weight of animals was measured prior to rotenone administration (first day) and on the last day of the study (28th day). The percentage change in body weight was calculated as follows: change in bodyweight = [(first day body weight−28th day body weight)/first day body weight] x100. Behavioral parameters like catalepsy, grip strength, and locomotor activity on rotarod were assessed on 14th, 21st, and 28th day of the study. Terminally on 29th day, the rats were sacrificed, and the striatum was separated. The 10% homogenate of striatum was made in 0.9% cold saline and used for biochemical assays (superoxide dismutase (SOD), catalase, reduced glutathione (GSH), and hydrogen peroxide (H_2O_2)). In addition to the iron-chelating activity of quercetin, serum iron assay was also determined. All antioxidant assays were carried out according to earlier described procedures (Hemanth Kumar et al. 2016; Hemanth Kumar et al. 2017; Boyina et al. 2018). Catalepsy test was carried out according to the method described by Costall 1974 (Costall and Naylor 1974). Rotarod was evaluated by the method as described by Kelly et al. (1998). Molecular docking studies were carried out using MOE in order to predict the possible binding interactions of quercetin with aromatic L-amino acid decarboxylase (AADC) and catechol-O-methyltransferase (hCOMT)(Ruddarraju et al. 2019). The crystal structure of the protein was retrieved from a protein data bank (http://www.rcsb.com).

Statistical Analysis: The data was statistically analyzed, using Graph Pad prism 5.0, and all values are mentioned as mean ± SEM. The behavioral data were analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test for multiple comparisons. For biochemical parameters, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test and then by Dunnett's multiple range tests was performed. The statistical significance of difference was taken as $P < 0.05$.

3 Results

Group II rats showed a significant decrease in body weight which was recorded at the end of the fourth week of rotenone exposure ($p < 0.001$) compared to Group I. In all other groups (Group III, Group IV, Group V, Group VI, and Group VII), significant restoration of the decrease in the body weight ($p < 0.05$; $p < 0.01$) was observed when compared to Group II (Fig. 2).

However, after 2 weeks of rotenone treatment, the increase in catalepsy was significantly higher as compared to control ($p < 0.001$), whereas Group III, Group IV, Group V, Group VI, and Group VII significantly ($P < 0.001$) decreased the catalepsy score as when compared to Group II (Fig. 3).

The grip strength was found to be significantly decreased ($p < 0.001$) in group II as compared to Group I, whereas Group III, Group V, Group VI, and Group VII significantly ($P < 0.001$) improved the grip strength as compared to Group II (Fig. 4a). The animals of all groups were trained on the rotarod prior to the start of the experiment till they learned. On the 14th, 21st, and 28th day of the study, all the animals of each group were tested on the rotarod for fall of latency. Group II animals showed a significant depletion in muscular coordination skill as compared to the control animals of Group I, and the groups treated with quercetin in combination with L-dopa (Group VI and Group VII) were found to be effectual in the recovery of muscular in-coordination when compared to Group II. There was a partial recovery of muscular coordination in animals treated with L-dopa, LD-quercetin (Group III), and high dose of quercetin (Group V) when compared to Group II (Fig. 4b).

The iron-chelating ability of quercetin showed that quercetin can chelate iron. Quercetin exhibited a potent iron-chelating ability. In order to better explore the extent of retention of Fe2+ by quercetin as a function of time, the absorbances

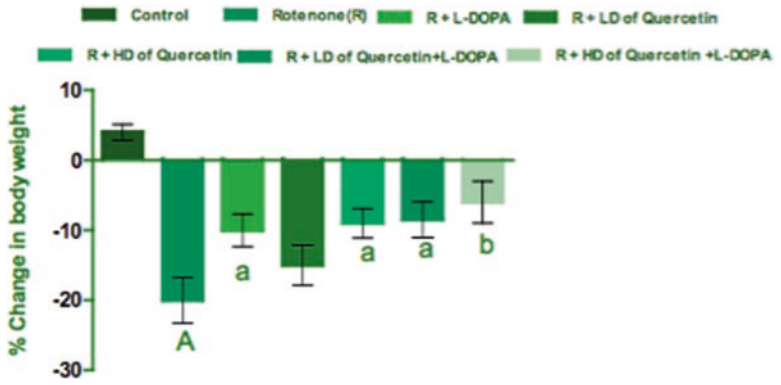

Fig. 2 Effect of quercetin, L-dopa, and quercetin + L-dopa treatment on body weights in rotenone-treated rats. Values are expressed as mean ± SEM ($n = 6$). Data was analyzed by one-way ANOVA followed by Tukey and Dunnett's post hoc tests. Significance is indicated as follows: A$P < 0.001$ when rotenone-treated group is compared to control and a$P < 0.05$, b$P < 0.01$, and c$P < 0.001$ when all treated groups were compared with rotenone-treated group

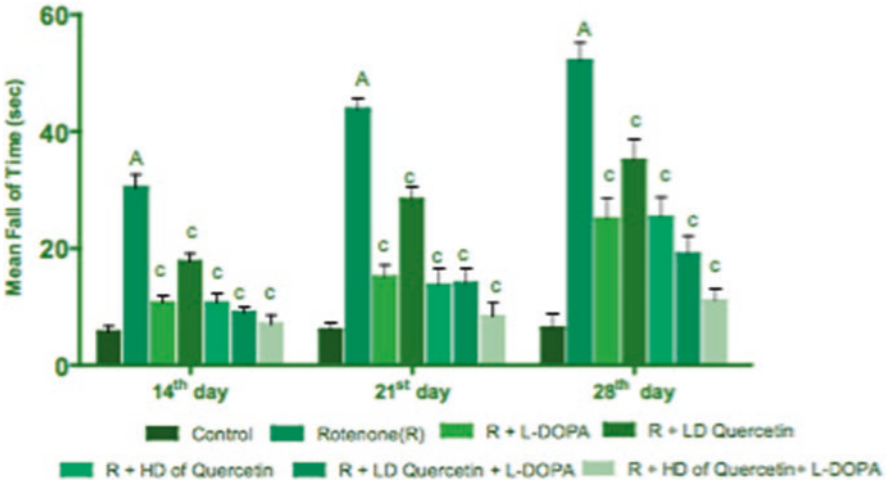

Fig. 3 Effect of quercetin, L-dopa, and quercetin + L-dopa treatment on catalepsy score in rotenone-treated rats. Values are expressed as mean ± SEM ($n = 6$). Data was analyzed by two-way ANOVA followed by Bonferroni post hoc test. Significance is indicated as follows: A$P < 0.001$ when rotenone-treated group is compared to control and c$P < 0.001$ when all treated groups were compared with rotenone-treated group

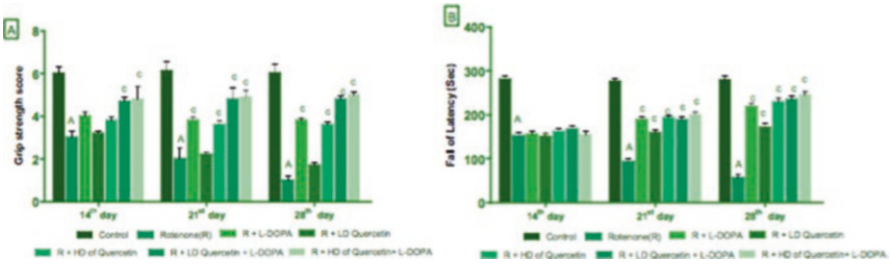

Fig. 4 Effect of quercetin, L-dopa, and quercetin + L-dopa treatment on (**a**) The grip strength activity and (**b**) locomotor activity on rotarod in rotenone-treated rats. Values are expressed as mean ± SEM ($n = 6$). Data was analyzed by two-way ANOVA followed by Bonferroni post hoc test. Significance is indicated as follows: A$P < 0.001$ when rotenone-treated group is compared to control and c$P < 0.001$ when all treated groups were compared with rotenone-treated group

of quercetin were also taken over a period of 60 min after the addition of bathophenanthroline. Results were obtained when 1, 2, 4, and 6 μ gm of quercetin were used (Fig. 5).

The brain GSH levels were significantly ($P < 0.001$) decreased in Group II when compared to Group I. There was a significant ($P < 0.01$, $P < 0.05$, $P < 0.01$, $P < 0.001$) and ($P < 0.001$) increase in GSH levels in Group III, Group IV, Group V, Group VI, and Group VII, respectively, when compared to Group II (Fig. 6a). The brain catalase levels were significantly ($P < 0.001$) decreased in Group II when compared to Group I. In addition, there was a significant ($P < 0.001$, $P < 0.05$,

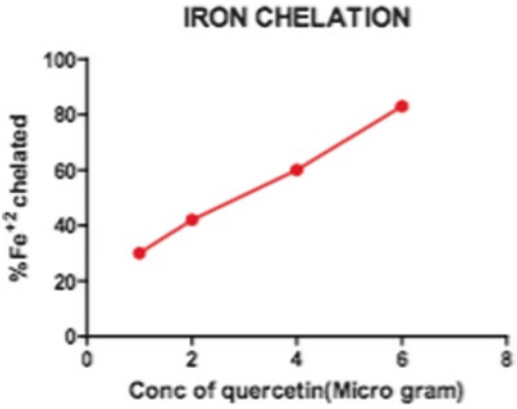

Fig. 5 Effect of quercetin on iron-chelating assay

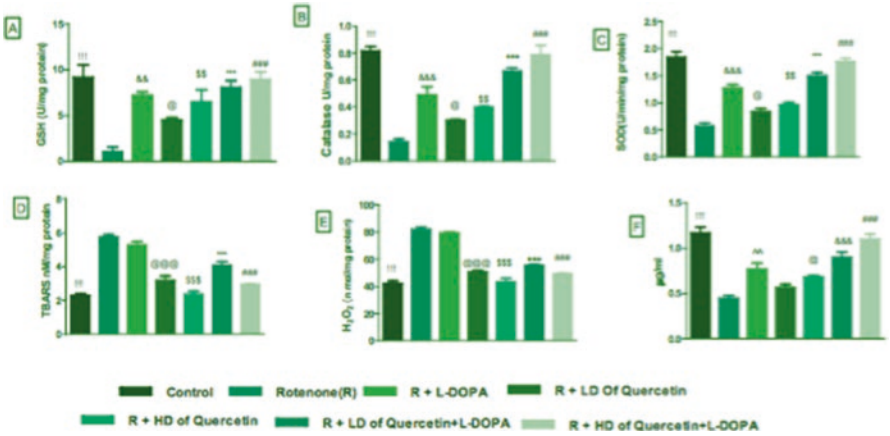

Fig. 6 Effect of quercetin, L-dopa, and quercetin + L-dopa treatment on (**a**) reduced glutathione (GSH), (**b**) catalase, (**c**) SOD, (**d**) TBARS, (**e**) H_2O_2, and (**f**) serum iron. All values are expressed as mean ± SEM. Significance is indicated as follows: !!!$P < 0.05$; \$\$$P < 0.01$; @$P < 0.05$; and ***$P < 0.001$ when treated groups were compared with control and negative control groups, respectively

$P < 0.001$, $P < 0.001$) and ($P < 0.001$) increase in catalase activity when Group III, Group IV, Group V, Group VI, and Group VII, respectively, were compared with Group II (Fig. 6b).The effect of quercetin and quercetin + L-dopa on antioxidant enzyme SOD is shown in Fig. 6c. The brain SOD levels were significantly ($P < 0.001$) decreased in Group II when compared to Group I. Also, there was a significant ($P < 0.001$, $P < 0.05$, $P < 0.001$, $P < 0.001$) and ($P < 0.001$) increase in enzyme levels in Group III, Group IV, Group V, Group VI, and Group VII, respectively, when compared with Group II. The effect of quercetin, quercetin + L-dopa, and L-dopa on brain TBARS is shown in Fig. 6d. The brain TBARS significantly ($P < 0.001$) increased in Group II when compared with Group I. Group IV ($P < 0.001$), Group V ($P < 0.001$), Group VI ($P < 0.001$), and Group VII ($P < 0.001$)

significantly decreased TBARS when compared with Group II. In Group III, treatment with L-dopa revealed no significant effects in TBARS levels when compared to Group II. The effect of L-dopa, quercetin, and quercetin +L-dopa on H2O2 is shown in Fig. 6e. The brain H_2O_2 significantly ($P < 0.001$) decreased in Group II when compared to Group I. There was a significant ($P < 0.05$, $P < 0.01$, $P < 0.001$, $P < 0.001$) and ($P < 0.001$) increase in H_2O_2 levels in Group III, Group IV, Group V, Group VI, and Group VII, respectively, when compared with Group II. The effect of L-dopa, quercetin, and quercetin + L-dopa on serum iron concentration is shown in Fig. 6f. The serum iron levels were significantly ($P < 0.001$) decreased in Group II when compared with Group I. Furthermore, there was a significant ($P < 0.01$, $P < 0.05$, $P < 0.001$) and ($P < 0.001$) increase in iron levels in Group III, Group V, Group VI, and Group VII, respectively, when compared with Group II. No significance was observed in Group IV when compared to Group II.

3.1 In Silico Studies on Quercetin Against Parkinson Targets

To investigate whether quercetin had the ability to bind on targets responsible for Parkinsonism, the docking calculations were performed on aromatic L-amino acid decarboxylase (AADC) and human catechol-O-methyltransferase (hCOMT). The PDB structures of AADC (1JS3) and hCOMT (3BWM) were retrieved from a protein data bank. The protein structure was optimized using structure preparation. Energy minimization was carried out using MOE default settings, and active sites were determined using the "site finder" tool of the program. The ligand-protein interactions on AADC of quercetin as well as crystal ligand carbidopa revealed that there could be potential inhibitory action against AADC. The binding free energy dG, ligand-protein interactions with distance and energy, were calculated and are shown in Table 2. The energy data showed that quercetin exhibited highest binding energy against 3BWM with dG value −6.11 kcal/mol, and these interactions where comparable with crystal ligand 3,5-Dinitrocatechol (DNC). All the interactions are shown in Table 2 and Fig. 7a–c.

4 Discussion

Parkinson's disease is a neurodegenerative disorder characterized by motor impairment in patients over the age of 65. The degeneration of dopaminergic neurons in the substantia nigra of the midbrain, frequently accompanied by the appearance of Lewy bodies (LBs), is considered to be the morphological hallmark of the disease. For understanding the molecular pathogenesis of neurodegeneration in Parkinson's disease (PD), many studies indicated that chronic exposure to rotenone causes highly selective nigrostriatal dopaminergic degeneration that is associated with neurochemical, behavioral, and neuropathological features of PD (Sharma and Nehru, 2013; Sharma et.al. 2018).

Table 2 Docking interactions of quercetin and crystal ligands on AADC and COMT

Compound (PDB ID)	Residues	Interaction	Distance	London dG (kcal/mol)
Carbidopa (1JS3)	Thr 82	H-donor	2.42	−6.3
	Lys303	H-acceptor	2.78	
	His192	H-acceptor	2.64	
	Thr246	H-acceptor	2.46	
Quercetin (1JS3)	Asp-271	H-donor	2.94	−5.43
	His-192	pi-pi	3.98	
DNC(3BWM)	MG-300	Metal	2.85	−6.44
3,5-Dinitrocatechol	Lys-144	H-donor	2.53	
	Glu-199	H-donor	2.32	
	Asn-170	H-donor	4.16	
Quercetin (3BWM)	MG-300	Metal	2.38	−6.11
	Trp-143	H-pi	3.77	
	His-142	pi-H	3.82	
	Lys-144	pi-H	3.93	

In the current study, rotenone was found to develop slow, progressive degeneration that warrants the use of this model in studying neuroprotective strategies (Sharma and Nehru, 2013; Sharma et al. 2018). It satisfied the basic findings in humans, where bradykinesia and rigidity were manifested as progressive increase in catalepsy score and decrease in grip strength. Moreover, it reproduced marked decrease in serum iron, SOD, catalase, and GSH levels with increase in TBARS levels. Furthermore, these neurochemical parameters exhibited a significant correlation with the catalepsy and grip strength as well as rotarod score, confirming the implication of mitochondrial dysfunction-oxidative stress-apoptosis axis in the pathogenesis of Parkinsonism. In silico molecular docking studies have also shown that quercetin could be an ideal drug target for aromatic L-amino acid decarboxylase and catechol-O-methyltransferase enzymes is from the present study we hypothesized that iron induced-oxidative stress-dependent apoptotic pathways play a critical role in degeneration of dopaminergic neurons in Parkinson's disease. An important insight into the mechanism of oxidative damage in the SNpc comes from the observation that iron is selectively elevated in the SNpc of individuals with PD. The cause of elevated iron levels in the SNpc of individuals with PD is currently unknown, although low SNpc ferritin levels and dopamine-mediated release of ferrous iron from ferritin have been hypothesized to lead to increased iron levels.

Quercetin – a flavonoid found in berries and other plants – chelates iron atoms as powerful as prescription drugs used in managing severe cases of iron overdose. The 4-keto-5-hydroxy region is a possible metal chelation site. The choice of quercetin's doses used in the present study (15 mg/kg and 50 mg/kg) was based on previous studies that confirmed its antioxidant and other pharmaological activities (Erboga et al. 2015; Shi et al. 2019; Jeon et al. 2017; Keddy et al. 2012). Our results showed that there was a marked symptomatic improvement in catalepsy, rotarod, and grip strength score along with neurochemical parameters

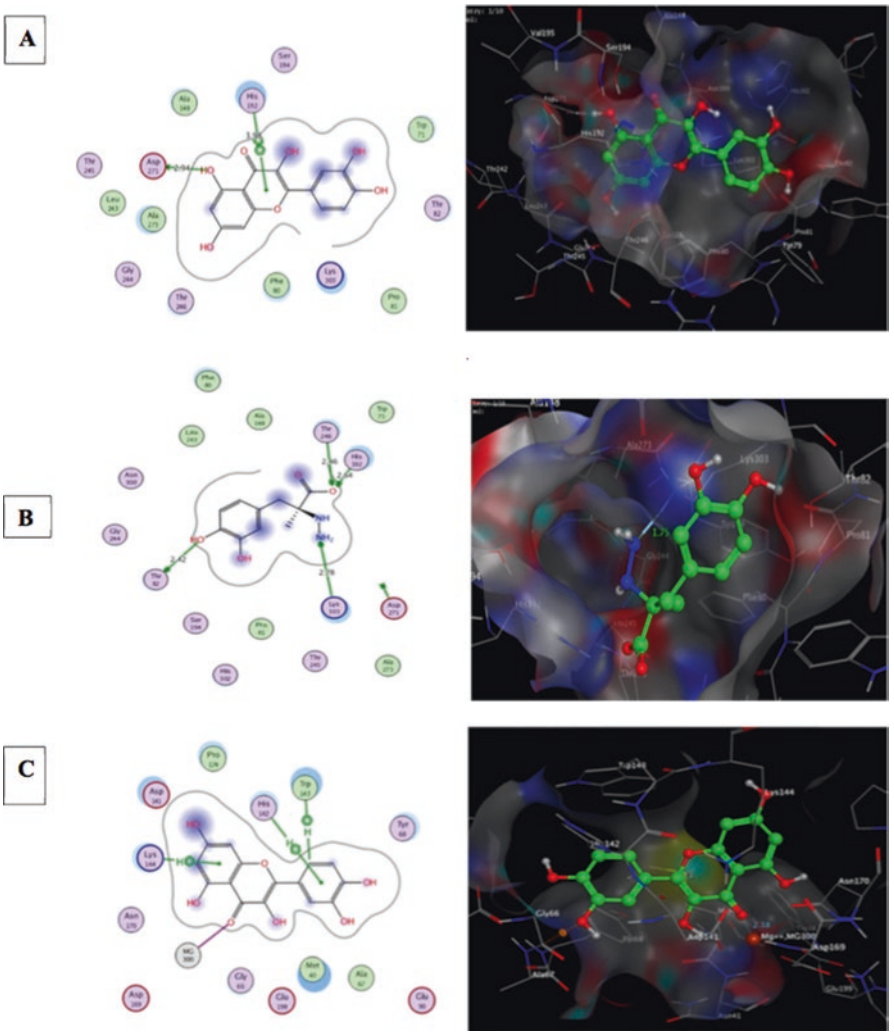

Fig. 7 Docking studies of quercetin and crystal ligands on AADC and COMT (**a**) 2D and 3D model of quercetin-AADC, (**b**) 2D and 3D model of carbidopa-AADC, and (**c**) 2D and 3D model of quercetin-COMT

when L-dopa was given in combination with quercetin. Quercetin reversed the increases in brain H_2O_2 and serum iron levels that were a consequence of the oxidative stress generated by rotenone administration. Hence, the present study provided clear evidence that quercetin possesses promising iron-chelating activity against rotenone-induced neurodegeneration. Thus, it may be useful against neurotoxicity, induced by environmental neurotoxins.

In the present study, the treatment of Parkinsonism with quercetin provided neuroprotection when given early in the course of the disease. This neuroprotective

role was proved as a result of the intervention of iron induced-oxidative stress-dependent apoptotic pathways. This was based on the concept that early detection and initiation of appropriate therapy present a high opportunity for slowing or even stopping the disease process earlier, before the condition becomes debilitated by both the primary disease process and secondary complications. The current study exhibited that quercetin efficiently halted the deleterious toxic effects of L-dopa on the underlying pathogenesis of PD. Combination of quercetin with L-dopa revealed normalization of catalepsy and rotarod score, in addition to amelioration of the measured neurochemical parameters indicating the benefit of both symptomatic and neuroprotective therapies.

5 Conclusion

In conclusion, the present study provided clear evidence that quercetin possesses promising iron-chelating activity against rotenone-induced neurodegeneration and could be recommended as a disease-modifying therapy when given with L-dopa early on in the course of Parkinson's disease.

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Conflict of Interest The authors declare no conflict of interest.

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Exhaled Breath Condensate (EBC): Is It a Viable Source of Biomarkers for Lung Diseases?

Stefanos Patsiris, Themis Exarchos, and Panayiotis Vlamos

1 Introduction

Advanced techniques and methods with the least invasive intervention to the respiratory system for diagnostic and monitoring purposes of respiratory diseases have always been in need. One bioelement that falls into this category (non-invasive) is the exhaled air and its derivatives such as the exhaled breath condensate. Both the exhaled air and the exhaled breath condensate are rich sources of markers which are emanated from the respiratory tract and reflect the normal and abnormal state of the lungs (airways and parenchyma) (Horváth et al. 2017). These markers cover a big range of volatile and non-volatile compounds and vary in type and concentration depending on the respiratory condition that is studied. It is believed that some of these markers can be set as biomarkers of various respiratory diseases (Ahmadzai et al. 2013; Bajaj and Ishmael 2013). This is supported by various studies. Compared to other sampling techniques, our proposed tools are found more advantageous regardless of the problems that exist in the total analytical process (pre-analytical, analytical and post-analytic phase) which can be minimized by the careful performance of the collection and analysis (Rosias 2012). This paper attempts to give a picture of the potential character of the exhaled breath condensate to provide biomarkers of respiratory diseases.

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2 Exhaled Breath Condensate

The exhaled breath condensate is a biological product. It is produced by cooling the exhaled air, a process which happens by exhaling through a cooling condenser system. Its analysis has shown that it is a mixture of substances, and although it consists mainly of water vapour (99%), there are several biomolecules in it, as well. The latter is a sum of water-soluble volatile organic compounds and aerosolized microparticles that contain non-volatile constituents. The group of non-volatile compounds consists of small inorganic ions, organic compounds and large molecular compounds (Kuban and Foret 2013). The size of the particles in the exhaled breath condensate provides a picture of their possible source. The submicron particles may come from the level of the pulmonary alveolus. Slightly larger particles originate from bronchial surfaces, and large particles are correlated with the mouth cavity (Haslbeck et al. 2010; Zamuruyev et al. 2017). All the parts of the exhaled breath condensate present a variety in their range and their concentration (Dodig and Cepelak 2013).

The exhaled breath condensate is a matrix of various potential biomarkers and not a single biomarker. This is based on the fact that its substances derive from the whole respiratory tract and they correspond to the composition of the respiratory tract lining fluid (airway lining fluid as it is also known). Their potential character relies on their production with changes in the consistency of the exhaled breath condensate leading to a presentation of differences between a healthy state and a pathological condition (Konstantinidi et al. 2015). There are three main groups that the biomarkers of the exhaled breath condensate can fall into after categorization and are shown in the following Table 1 (Davis et al. 2012).

Table 1 EBC biomarker groups and categorization

EBC biomarker groups and categorization	
Group 1	VOCs (volatile compounds) Non-VOCs (non-volatile compounds) Non-VOCs deriving from VOCs
Group 2	Molecular compounds of very low weight Molecular compounds of low weight Polypeptides Proteins Nucleic acids
Group 3	Organic and inorganic molecules Redox-relevant molecules pH-relevant molecules Lipid mediators Cytokines and chemokines

3 Reasons to Use Exhaled Breath Condensate as a Source for Biomarkers

The application of techniques and methods to detect and monitor respiratory diseases via the study of the exhaled air and especially the exhaled breath condensate has been used for some decades now (Rattray et al. 2014). Although the composition of the exhaled breath condensate contains many characteristic constituents regarding their function and concentration in pathological conditions, it is still a research tool, and there is a continuous attempt to make it useful and reliable for everyday clinical practice. There are many arguments and reasons to support its application in the field of respiratory medicine and to overcome the various inhibitions or doubts, since it seems to have more advantages compared to other traditional sampling methods.

The collection of the exhaled breath condensate is totally non-invasive (Harshman et al. 2014). This is very important because there is no intervention within the respiratory tract. The collection of the exhaled air and the formation of the exhaled breath condensate do not disturb or alter the ongoing metabolic processes in the respiratory system and especially those with pathological character. That means that the metabolomic contents of the exhaled breath condensate emanate from all the actions happening in the respiratory system and reflect pathophysiological changes. The other traditional means that are used to obtain samples from the airways influence its function and/or cause further inflammation by damaging the surrounding structures (Zimmerman 2012).

The exhaled breath condensate is a biofluid full of markers. Its wealth in markers and their origin, even from areas such as the "silent zone" of the lungs, makes exhaled breath condensate a precious option for diagnostic and monitoring purposes (Beck et al. 2016). Moreover, the similarity in content between the exhaled breath condensate and the airway lining fluid (different only in concentration) is valuable as it provides information on the insight of the functions and mechanisms of the respiratory system both in healthy and diseased status. This similarity relies on the reopening of the bronchioles and alveoli that were closed and can be captured in the exhaled breath condensate (bronchiole fluid film burst theory BFFB) (Horváth et al. 2017; Hayes et al. 2016) and the fact that particles are aerosolized because of the turbulence in the airways. The study of inflammation and oxidative stress which are the two main pathogenic processes of respiratory diseases via the collection and analysis of the exhaled breath condensate is sufficiently sensitive regarding the detection of their markers. This might be of great usefulness in the categorization of the respiratory diseases (Chhabra and Gupta 2012). Although, the exhaled breath condensate contains only chemical compounds and not cells, its gaseous and liquid constituents offer the opportunity to study products of the pathophysiology of respiratory diseases and receive a broader picture of their mechanisms. The volatile and non-volatile constituents of the exhaled breath condensate provide different details because of

their variety in chemical characterists (Liang et al. 2012). The metabolomic content of the exhaled breath condensate is more stable regarding its general handling process (Zamuruyev et al. 2017).

There are two very important advantages that accompany the collection of the exhaled breath condensate. First, it is the only sampling technique available for use in all ages, and second, it can be applied in the whole spectrum of a respiratory disease independently of their severity even in mechanically ventilated patients. The two benefits previously mentioned give the exhaled breath condensate the potential to measure the severity of respiratory diseases and contribute to its diagnostic character (Hunt 2011). It also seems that the gender and the age do not significantly affect the collected volume of exhaled breath condensate (Carter et al. 2012; Roca and Masclans 2012; Παναγιωτοπούλου – Γαρταγάνη Π. et al. 2009).

The collection of the exhaled air is a simple and easy task to perform. The participants exhale in a machine which consists of a mouth piece, a one- or two-way valve and a cooling condenser. Tidal breathing (quiet breathing) is the chosen pattern used for the collection process because it does not affect the lung function, a fact that makes it further easier and of course readily available. It has no adverse effects on the participants' status either. Its capability for repetitive performance makes it a perfect choice for serial longitudinal studies as well as epidemiological ones. Its short duration (10–15 minutes) allows time to collect sufficient volumes, it avoids causing tiredness to the participant, and it saves time for the staff responsible of performing the procedure (Dodig and Cepelak 2013; Hayes et al. 2016). It seems that lung function parameters do not affect the volume of the collected exhaled breath condensate. Furthermore, it does not require special personnel or distinct knowledge in order to collect it. So, based on all these factors, it is an easy, time-efficient, repeatable and without special requirement technique. One additional advantage is that the equipment used for its collection is very simple and relatively cheap compared with the ones used in other techniques (Rosias 2012; Loukides et al. 2011).

The area of the small airways is the location of the early start of airways' disease pathophysiology. The same area is also the target for the medication of many treatments. The fact that some of the products of the exhaled breath condensate and especially the aerosolized particles originate from this area and contain pathway mediators of respiratory disease pathogenesis can define it as the appropriate choice for disease phenotyping (Maniscalco and Motta 2017) as well as the suitable tool for monitoring the progress of respiratory diseases and the outcome of treatments (Esther et al. 2014; Lim and Thomas 2013). It is worth mentioning that the biomarkers of the exhaled breath condensate and those from other biological fluids are equivalent (Zamuruyev et al. 2017). A great number of substances that are detected in the exhaled breath condensate are also found in other sampling techniques such as the bronchoalveolar lavage and induced sputum (Balbi et al. 2007).

The analysis of the exhaled breath condensate must be highlighted as well because it contributes to its strengths as a sampling technique. Big variety of very sensitive methods such as mass spectrometry and liquid chromatography provides a wide range of qualitative and quantitative information about compounds that are

trapped in the exhaled breath condensate. Their usage helps to obtain a clearer picture of what is enclosed in the exhaled breath condensate offering opportunities to understand the pathways of the pathogenesis of respiratory diseases (Rahimpour et al. 2018).

4 Conclusion

Exhaled breath condensate is a sampling technique with many benefits. Although, there are many issues that have to be settled such as its standardization, the strengths that characterize it seem to give it a solid place in the field of diagnosis, monitoring and therapeutic assessment of respiratory diseases. Its non-invasiveness, its application in all ages and the fact that it can be independently applied despite the severity of the disease are some of its greatest advantages that make it the most suitable choice as sampling technique for respiratory diseases. The fact that it can be used in all the stages of respiratory diseases allows for the study of the whole spectrum of these diseases. Thus, contributing to the understanding of their pathological mechanisms and their reactions to therapeutic regimens. That is based on the study of its contents and its similarity with the respiratory tract lining fluid which covers the inner side of the airways. However, more research is required to develop and establish every step of its conduct because of the lack of standardization and the variety of methods used.

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Treatment Development for Alzheimer's Disease: How Are We Doing?

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Over 40 million people worldwide suffer from dementia. This number is projected to exceed 110 million by 2050 because of the aging of the worldwide population, especially in lower- and middle-income countries. The most common cause of dementia is believed to be Alzheimer's, a brain disease associated with deposition of beta-amyloid protein and hyperphosphorylation of intraneuronal tau protein leading to synaptic degradation, neuronal loss, brain circuit disruption, a range of symptoms, and eventually, if the person lives long enough, death. Over the last few decades, treatment development has focused on the deposition of beta-amyloid protein (A-beta 1-42) that is produced in the brain in huge quantities continuously and is thought to be toxic. Unfortunately, amyloid oriented therapies targeting individuals with dementia, or its prodrome mild cognitive impairment (MCI), have not been successful therapeutically even though they have been associated with reductions in amyloid. Currently, efforts are underway to deliver these therapies to individuals with very early symptoms or at risk for Alzheimer's dementia by virtue of genetics or a brain amyloid PET scan. Results from these studies are expected to begin to emerge by early 2020. In the meantime, since the amyloid hypothesis has been called into question, a number of different avenues are being pursued for treatment development. These are driven in part by new findings related to the polygenic nature of Alzheimer's as well as the interaction between this brain disease with factors such as brain vascular disease, insulin resistance, and/or brain inflammation. The expected future of AD treatment development is thought to be precision medicine.

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Microbiome Hijacking Towards an Integrative Pest Management Pipeline

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1 Introduction

Most agricultural crops have well-known insect pests, which can seriously affect crop quantity and quality (Metcalf 1996; Oerke 2005; Oerke and Dehne 2004). The chemical pesticides used to combat these pests are usually nonspecific and therefore toxic to other beneficial insects, such as bees, or even to animals (Aktar et al. 2009). Developing specific pesticides for each type of pest would mean less harm to the environment and a lower load of chemicals on crops, which would also benefit consumers. In this work we propose such a method, based on the study and

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exploitation of the insect microbiome to specifically combat each insect pest. Just like the human microbiome plays an important role in our health, the insect's microbiome often contains bacteria vital to its survival. Identifying these vital bacteria for each pest paves the way for the study of specific inhibitors which target specific bacterial proteins. Targeting the bacteria affects the health of the insect host and thus its ability to infect the plant as a pest. We demonstrate this approach with a study of the olive fruit fly *Bactrocera oleae*, which affects the olive tree (*Olea europaea* L.) and harbors the endosymbiotic bacterium *Candidatus Erwinia dacicola*.

The olive tree (*Olea europaea* L.) is a major agricultural crop in the Mediterranean basin playing an important economical role in countries of this region. Its contribution to agriculture is recognized since antiquity (Kaniewski et al. 2012). Olive domestication occurred in the Middle East about 6000 years ago (Zohary and Spiegel-Roy 1975) and then spread across the Mediterranean basin (Damania 1995; Lavee 2013). 98% of the world's olive groves are cultivated around the Mediterranean basin, and the European Union is the greatest consumer. Greece is among the leading world producers in olive oil, being third in production globally, after Spain and Italy (Food and Agriculture Organization of the United Nations 2018). Olive cultivation in Greece corresponds to about 80% of the total tree cultivation, with approximately 150 million trees, covering 21% of the total agricultural land and producing 400,000 tons of olive oil yearly (Hellenic Republic – Ministry of Rural Development and Food 2018). Over the last 25 years, world consumption of olive oil has increased by one million tons (International Olive Council 2018). Virgin olive oil is characterized by its high nutritional values and is an integral part of the Mediterranean diet, a diet with a high content of bioactive substances such as vitamins, flavonoids, and polyphenols. Polyphenols have been demonstrated to have a positive effect against cardiovascular diseases and certain cancers (Andrikopoulos et al. 2002; Hertog et al. 1995; Kris-Etherton et al. 2002). Phenolic compounds also play an important role in the organoleptic characteristics of olive oil. The phenolic profile of olive trees, as well as the olive oil quality and composition, depends on many factors, such as genotype, tissue type, developmental stage, geographical origin, fruit maturity stage, harvesting method, olive storage, and oil extraction technique (Mitsopoulos et al. 2016; Tuck and Hayball 2002; Vinha et al. 2005). Another important factor affecting olive oil quality and composition is fruit health.

The most important enemy of the olive tree is *Bactrocera oleae*, the olive fruit fly, which poses a severe economic threat for commercial olive growers, as it can cause up to 30% reduction in the production of olive fruit (Neuenschwander and Michelakis 1978). Infection by *B. oleae* affects not only the quantity but also the quality of the olive fruit and olive oil, due to the increase of oleic acid, which is caused by the larvae's feces as well as the entry of secondary bacteria and fungi through the spot created by the insect during larvae deposition. Various methods of pest control are used, only some of which are environmentally friendly. The main method used is chemical insect control. Chemical substances such as dimethoate, pyrethroid, or spinosad are used to reduce the population of the pest, but the insect has developed resistance to these pesticides (Hsu et al. 2004; Margaritopoulos et al. 2008; Pavlidi et al. 2017; Skouras et al. 2007; Vontas et al. 2001; Vontas et al. 2002;

Vontas et al. 2011). The McPhail trap, which contains pheromones, ammonium salts, or hydrolyzed proteins and attracts the insect, is used to observe and estimate the size of the population, in order to plan the right amount and the correct timing of pesticide application (Economopoulos 1977). The biological control method is based on the action of other insect populations that are parasites, predators, or pathogens for the target insect, but it is not so widely used (Manikas and Tsiroyannis 1982; Navrozidis et al. 2000; Neuenschwander and Michelakis 1978). Furthermore, the sterile insect technique aims at the rapid reduction of the population through the release of sterile insects into the environment (Knipling 1955; Ras et al. 2017).

The larvae of the olive fruit fly grow and feed on the mesocarp of ripe and unripe olive fruits, which allows them to carry out several generations until the fruits ripen (Ben-Yosef et al. 2014, 2015). In contrast to the ripe fruit, the unripe olive fruit is an inhospitable environment in which the insect cannot survive, as oleuropein inhibits the growth of the larva. The metabolite oleuropein accumulates at high levels during the early stages of olive fruit ripening and decreases gradually as the olive fruit ripens (Soler-Rivas et al. 2000). Oleuropein has the ability to inactivate enzymes and reduce lysine digestion, thus inhibiting larvae growth (Ben-Yosef et al. 2015). However the larvae manage to survive in the hostile environment of the unripe olive fruit due to the presence of the endosymbiotic bacterium, *Candidatus Erwinia dacicola*, in the gut of the insect (Capuzzo et al. 2005; Estes et al. 2012). The mechanism of action of this bacterium is difficult to determine as Ca. *E. dacicola* remains uncultivated, but it is assumed that the bacterium can provide a source of dietary proteins or amino acids for the larvae. This may be achieved by the secretion of chemical substances by the bacterium which may facilitate the dissociation of oleuropein-protein complexes in the insect's gut. This way, proteins and lysine can be easily assimilated by the insect, similar to mechanisms seen in other insect-associated symbiotic fungi and bacteria (Ben-Yosef et al. 2014, 2015; Pavlidi et al. 2017).

Ca. *E. dacicola* is transmitted vertically from the female fruit fly to her offspring (Estes et al. 2012), although horizontal transmission of the bacterium between larvae living and feeding on the same olive fruit has also been hypothesized (Bigiotti et al. 2018; Viale 2014). The olive fruit fly is closely linked to symbiotic bacteria throughout its life stages (Ben-Yosef et al. 2010). Both larvae and adult flies have been morphologically adapted to host bacteria in their gut. The abundance of Ca. *E. dacicola* changes during olive fruit fly development (Behar et al. 2008). The larvae present a higher relative abundance of Ca. *E. dacicola* than the egg and the pupa (Estes et al. 2012). Furthermore, the ovipositing female has the highest relative abundance of Ca. *E. dacicola*, compared to males that have mated, virgin females and adults aged less than 12 hours (Estes et al. 2012). Ca. *E. dacicola* is one of the few examples of endosymbiotic bacteria, which switch from an intracellular to an extracellular existence during host-insect development. The bacterium is intracellular at the larval midgut, but it is extracellular at the front gut in the adult insect (Estes et al. 2012). Phylogenetic analysis of the Ca. *E. dacicola* demonstrates that this bacterium is closely related to various phytopathogenic and free-living *Erwinia* species, such as *E. amylovora*, *E. pyrifoliae*, *E. tracheiphila*, and *Pantoea stewartii*,

pathogens which persist primarily in association with their plant hosts and insect vectors with limited survival in the soil (Estes et al. 2012).

The genome of Ca. *E. dacicola* was recently sequenced (Blow et al. 2016). We used this information to select three proteins of interest, which have been extensively studied in other organisms for their role and mode of action. We created homology models for these three proteins from Ca. *E. dacicola* and used these models to deduce pharmacophore structures for potential small molecule inhibitors. This approach will allow us to screen chemical libraries for potential new inhibitors of vital functions of Ca. *E. dacicola*. Specifically, targeting Ca. *E. dacicola* in wild populations of *B. oleae* is expected to result in a reduction in the fruit fly's ability to infect unripe olives and will thus greatly reduce their potential as pests.

2 Methods

2.1 Identification of Template Structures and Sequence Alignment

The amino acid sequences were retrieved from the conceptual translation of the NCBI database (http://www.ncbi.nlm.nih.gov/). The blastp algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify homologous structures by searching the Protein Data Bank (PDB). The multiple sequence alignment for homology modelling was performed using MOE (Dalkas et al. 2013; Vilar et al. 2008; Vlachakis et al. 2017).

2.2 Molecular Modelling

All calculations and visual constructions were performed using the Molecular Operating Environment (MOE) version 2013.08 software package developed by Chemical Computing Group (Montreal, Canada) (Vilar et al. 2008).

2.3 Homology Modelling

The homology modelling of the three proteins of interest was carried out using MOE (Vilar et al. 2008). The selection of template crystal structures for homology modelling was based on the primary sequence identity, similarity, and the crystal resolution (Nayeem et al. 2006; Papageorgiou et al. 2014; Papageorgiou et al. 2017; Papageorgiou et al. 2013). The MOE homology model method is separated into four main steps: (a) primary fragment geometry specification, (b) insertion and deletions task, (c) loop selection and side-chain packing, and (d) final model selection and refinement (Papageorgiou et al. 2014).

2.4 Molecular Electrostatic Potential

Molecular electrostatic potential surfaces were calculated by solving the nonlinear Poisson-Boltzmann equation (Vishnyakov et al. 2007), using the finite difference method as implemented in the MOE and PyMol (Mooers 2016; Vilar et al. 2008). The potential was calculated on solid points per side. Protein contact potential was calculated using Amber99 charges and default atomic radii (Chen and Pappu 2007).

2.5 Model Optimization and Molecular Dynamics

Energy minimization was done for all three models in MOE (Vilar et al. 2008), initially using the Amber99 force field implemented into the same package, up to a root mean square deviation (RMSd) gradient of 0.0001, in an effort to remove the geometrical strain (Sellis et al. 2009). The models were subsequently solvated with simple point charge (SPC) water using the truncated octahedron box extending to 7 Å from the model, and a set of molecular dynamic simulations was performed at 300 K and 1 atm with 2-second step size for a total of one hundred nanoseconds, using the NVT ensemble in a canonical environment (NVT stands for *n*umber of atoms, *v*olume, and *t*emperature that remain constant throughout the calculation). The results of the molecular dynamics simulation were collected into a database by MOE for further analysis (Loukatou et al. 2014; Vlachakis et al. 2014).

2.6 Model Evaluation

The models produced were initially evaluated within the MOE package by a residue packing quality function, which depends on the number of buried nonpolar side-chain groups and on hydrogen bonding. Moreover, the PROCHECK suite (Laskowski et al. 1996; Papageorgiou et al. 2017) was employed for further evaluation of the quality of the produced models. Finally, Verify3D (Von Grotthuss et al. 2003) was used to evaluate whether the three models are similar to known protein structures of their family.

2.7 Pharmacophore Elucidation

Computerized representations of hypothesized pharmacophores were analyzed in MOE, using the *pharmacophore query* feature (Vlachakis and Kossida 2013). A MOE pharmacophore query is a set of *query features* that are typically created from ligand *annotation points*. Annotation points are markers in space that show the

location and type of biologically important atoms and groups, such as hydrogen donors and acceptors, aromatic centers, projected positions of possible interaction partners or R-groups, charged groups, and bioisosteres (Vlachakis et al. 2013). The annotation points on a ligand are the potential locations of the features that will constitute the pharmacophore query. Annotation points relevant to the pharmacophore are converted into query features with the addition of an extra parameter: a nonzero radius that encodes the permissible variation in the pharmacophore query's geometry.

2.8 Conserved Motifs Exploration

For each protein of interest, homologs were identified by blastp, using default parameters. Organisms which gave good hits by blast for all three proteins were selected, and thus a dataset of 105 homologous sequences was downloaded for each of the three proteins. Multiple sequence alignments of the three datasets were performed using Matlab's progressive alignment methods (Papageorgiou et al. 2016). Conserved sequences motifs were identified from the multiple sequence alignments, and sequence logos were generated using Jalview software (Waterhouse et al. 2009).

3 Results and Discussion

Three proteins were chosen for analysis from Ca. *E. dacicola*, as they have been widely studied by our group and others previously: Helicase, Polymerase, and Protease-C (Vimal et al. 2018; Docherty et al. 2003). The blastp algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify homologous structures by searching the Protein Data Bank (PDB). Multiple sequence alignment for homology modelling was performed using MOE (Vilar et al. 2008), and from these, the most closely related, and with best resolution, available structures were chosen as templates for homology modelling (template-PDB IDs – Helicase 4CEI, Polymerase 4GZY, Protease-C 1K7G). The three homology-based models were stereochemically and energetically evaluated and superposed to their templates (Fig. 1). As expected, the models retained the fold of their templates, but, more importantly, they shared similar physicochemical and kinetic profiles with the X-ray structures they are derived from.

Based on the homology modelling of the Ca. *E. dacicola* proteins, pharmacophore models were generated using MOE for each of the three proteins of interest (Fig. 2). A pharmacophoric feature characterizes a particular property and is not tied to a specific chemical structure. Different chemical groups may share the same property and also be represented by the same feature (Vlachakis et al. 2015; Chatzikonstantinou et al. 2017; Marinou et al. 2018). It is thus a mistake to name as pharmacophoric features, chemical functionalities such as guanidines or sulfonamides, or typical structural skeletons such as flavones or steroids. Once generated, a pharmacophore

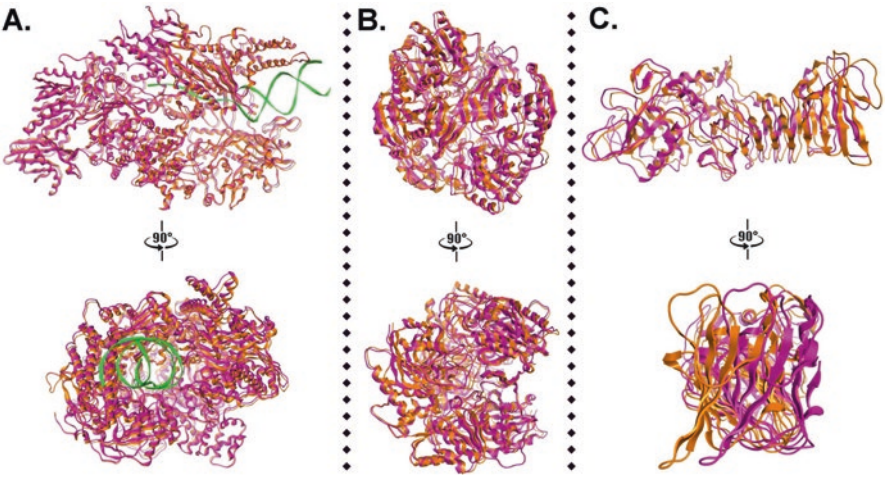

Fig. 1 Homology modelling. The homology models (orange colored) have been superposed to their templates (magenta colored) for each of the following targets: (**a**) Helicase, (**b**) Polymerase, and (**c**) Protease-C. The complexes are rotated by 90 degrees in the lower panel

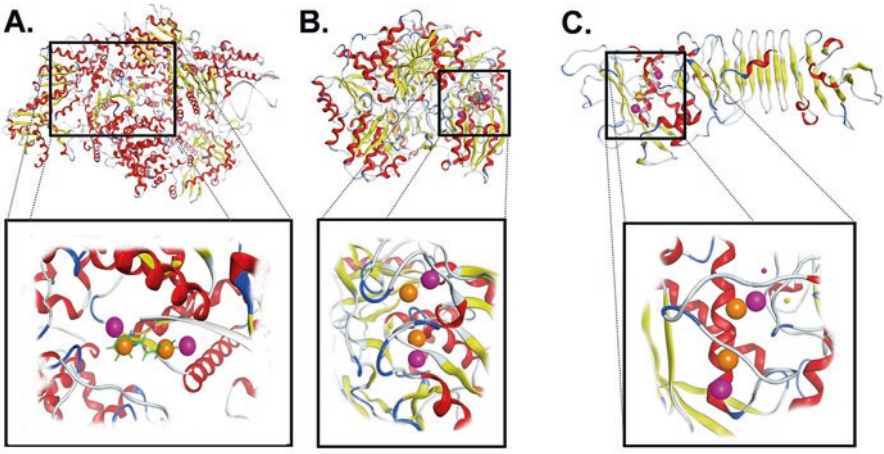

Fig. 2 Pharmacophore modelling. Pharmacophore models are given for each of the three target proteins: (**a**) Helicase, (**b**) Polymerase, and (**c**) Protease-C. The large magenta spheres represent electron accepting groups and the orange ones, aromatic moieties. The little spheres represent pharmacophoric features unique to the given protein. Yellow spheres represent sulfur groups capable of forming interactions of disulfide nature

query can be used to screen virtual compound libraries for novel ligands. Pharmacophore queries can also be used to filter conformer databases, e.g., output from molecular docking runs for biologically active conformations.

The main hindrance in in silico drug design and high-throughput virtual screening is the toxicity and nonspecific binding problem. This issue in the real world eventually escalates and results in resistance to the designed agents. Therefore, there is a dire

need for novel approaches and new strategies to be deployed. In this direction, the pharmacophore modelling refers to the generation of a pharmacophore hypothesis for the possible common binding interactions in a series of particular active sites (Vlachakis et al. 2015). In this study, the three different pharmacophore models were overlaid and reduced to their shared features so that common interactions were retained, in a so-called consensus pharmacophore. Such a *consensus pharmacophore* can be considered as the largest common denominator shared by a set of active molecules. As a result, we opt to eventually screen and discover compounds capable of interacting and inhibiting more than a single target. This novel stratagem is expected to act both in a parallel and serial mode. Blocking multiple targets cumulatively will be more destructive for the survival of the target organism (in this case, Ca. *E. dacicola*), and in case the bacterium establishes some form of full or partial resistance to one of our pharmacological targets, chances are that the other two targets will remain valid. This is based on the function of these three targets and the fact that they are involved in distinct and quite independent cellular pathways (i.e., inhibiting one of the three enzymes does not automatically block the other two).

The commonly reduced pharmacophoric features include two heavy aromatic regions (Fig. 2, orange color) and two hydroxy-like groups (Fig. 2, magenta color). The rest of the pharmacophore features shown in smaller spacefill spheres are unique for the given site and protein, so they were ignored given that they don't satisfy all three targets in this study. Electrostatic surfaces were drawn to be used as a filtering criterion for the screening process. It was found that all three sites share a mainly positively charged binding site (Fig. 3). Consequently, and based on both the electrostatic surface study and the pharmacophore modelling, the ideal compounds should be quite rigid and contain at least two aromatic rings as well as some -OH or -COOH groups. In addition, conserved motifs in each of the three proteins were analyzed by multiple sequence alignment to homologs identified by blast. Blastp was initially used to identify homologous sequences for the three Ca. *E. dacicola* proteins of interest in other organisms. The results were screened for organisms which gave significant hits to all three target proteins. Homologous sequences from 105 organisms were thus chosen and used in multiple sequence alignments. Conserved features were visualized with Jalview software (Supplementary Data 1, 2, and 3).

In conclusion, we present a novel approach for rational drug design against an important agricultural pest. On top of designing specific inhibitors for a certain protein of interest, based on the protein's structure, we combine the common characteristics of three protein active sites to design an inhibitor which targets all three. While this may mean we do not design the absolute best-fit inhibitor for each protein, the combined effect of a pesticide which has multiple targets means more efficient elimination of the pest and a huge reduction in the chance of developing resistance by chance mutations in three separate active sites. At the very least, this approach would greatly delay the development of any resistance and thus extend the effective life of the new compound. This approach will allow us to screen chemical libraries for potential new inhibitors of vital functions of Ca. *E. dacicola*. Specifically targeting Ca. *E. dacicola* in wild populations of *B. oleae* is expected to result in a reduction in the fruit fly's ability to infect unripe olives and will thus greatly reduce their potential

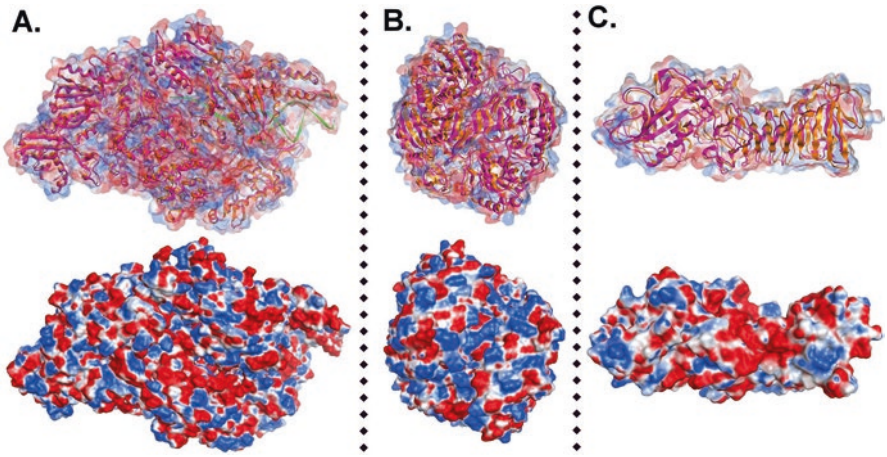

Fig. 3 Electrostatic surfaces. The electrostatic surface study for each of the following targets, (**a**) Helicase, (**b**) Polymerase, and (**c**) Protease-C, shows the area of positive charge in blue, area of negative charge in red, and neutral regions in white

as pests. Our methodology can be applied to other agricultural pests, reducing the chemical burden to the environment. With the recent availability of genomic data for ever-increasing numbers of pathogens and pests, as well as increasing knowledge of the role of the microbiome in the survival of an organism, this combinatorial approach to drug design is an important step forward.

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Regulation and Roles of Autophagy in the Brain

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Autophagy is crucial for neuronal integrity. Loss of key autophagic components leads to progressive neurodegeneration and structural defects in neuronal synapses. However, the molecular mechanisms regulating autophagy in the brain remain elusive. Similarly, while it is widely accepted that protein turnover is required for synaptic plasticity, the contribution of autophagy to the degradation of synaptic proteins is unknown. We find that BDNF signaling via the tropomyosin receptor kinase B (TrkB) and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway suppresses autophagy in vivo. Autophagy is differentially regulated by fasting, in different brain regions. Suppression of autophagy is required for BDNF-induced synaptic plasticity and for memory enhancement, under conditions of nutritional stress. BDNF signaling suppresses autophagy in the forebrain of adult mice. Indeed, BDNF ablation in the neural lineage causes uncontrolled increase in autophagy. In turn, increased autophagy mediates the synaptic defects caused by BDNF deficiency. Thus, fasting suppresses autophagy in regions of the mouse forebrain, thereby promoting synaptic remodeling and memory through a BDNF-regulated mechanism. We identify three key remodelers of postsynaptic densities as cargo of autophagy. Our results establish autophagy as a pivotal component of BDNF signaling, which is essential for BDNF-induced synaptic plasticity. This molecular mechanism underlies behavioral adaptations that increase fitness in times of scarcity.

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Three-Dimensional Models for Studying Neurodegenerative and Neurodevelopmental Diseases

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1 Introduction

In vitro systems and mouse models have been widely used in research aiming to elucidate brain development processes and neurodegenerative diseases. Various limitations that these systems have lead to the generation of in vitro 2D models, neural progenitors and neurons in adherent conditions, and more recently to more complex 3D models, brain organoids. Having mouse and human stem cells as a starting point, these systems can be highly accessible and allow various manipulations. Models expanded on three dimensions can more closely resemble some features of the human brain (Kelava and Lancaster 2016).

Initial efforts toward 3D in vitro models were mainly focusing on multicellular aggregates, the neural spheroids which could recapitulate the fundamental features of the human brain, like cell diversity, electrophysiology, extracellular matrix production, and mechanical stiffness (Camp et al. 2015; Zhuang et al. 2018). Organoids in definition are derived from stem cells or organ progenitors and contain several

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cell types with enormous self-organizing capacity, capable of forming whole tissues and structures similar to the organs in vivo, as well as recapitulating some specific function of the organs (e.g., excretion, filtration, neural activity, contraction) (Lancaster and Knoblich 2014a; Lancaster et al. 2013). Although cellular behavior is highly dependent on culture conditions, organoids can recapitulate in a faithful way the in vivo organization of cortical progenitors and neurons. This renders the organoid models a powerful tool to study molecular, cellular, and functional properties of the human brain and to follow up the progression of neurodevelopmental and neurodegenerative diseases, in an accessible, 3D structure resembling the human brain development (Buchsbaum and Cappello 2019; van den Ameele et al. 2014).

Organoids survive for long time periods, show enhanced maturity after a long period, and can be utilized for structural abnormalities, interactions between different cell types, and functional connectivity (Sun et al. 2018). Organoids can be used as more specific neurodegenerative disease models. In particular, the generation of organoids is possible by using induced pluripotent stem cells (iPSCs) from patients or iPSCs from healthy individuals in which specific mutations can be introduced through gene-editing technologies such as CRISPR (Kelava and Lancaster 2016). At the same time, human iPSCs-derived brain organoids could be used to elucidate the mechanisms of brain disorders caused by infections, as it has been shown for Zika virus-induced microcephaly (Qian et al. 2017). In addition, these organoids will contribute in drug testing and personalized therapy for neurodegenerative diseases, while at the same time, they could reduce the number of animals that are used in research (Kelava and Lancaster 2016).

Overall, brain organoids constitute one of the greatest achievements in research during the last years. These models of the human brain could possibly be used to unravel the molecular mechanisms that underlie the development of human brain and the neurodegenerative diseases related pathways (Kyrousi and Cappello 2019).

2 3D Brain Organoids in Disease Modelling: The Example of Neurodevelopmental and Neurodegenerative Diseases

2.1 Neurodevelopmental Diseases

So far, several neurodevelopmental diseases have been studied using organoids. Microcephaly, a clinical condition characterized by significantly reduced brain size (Woods et al. 2005), was the first example of organoid modelling performed by Lancaster et al. (2013), the same group that established iPSCs cerebral organoid protocol (Lancaster and Knoblich 2014b). In this study, cerebral organoids grown from patient-derived iPSCs have been used to investigate the implication of CDK5RAP2 mutation in the progression of microcephaly. Additional studies have also modelled microcephaly caused by different gene mutations, in the centrosomal-P4.1-associated protein (CPAP) (Gabriel et al. 2016) and the abnormal spindle-like (ASPM) (Li et al. 2017b). In the same context, brain organoid

systems were generated in order to unravel the involvement of Zika virus infection during pregnancy in the development of microcephaly to newborns (Cugola et al. 2016; Mlakar et al. 2016). Interestingly, Zika virus was shown to preferentially infect neural progenitors leading to delayed cell cycle progression, increased cell death, and reduced brain organoid size (Dang et al. 2016; Garcez et al. 2016; Qian et al. 2017).

Another application of cerebral organoids in disease modelling is the study of macrocephaly, a condition where patients are characterized by brain overgrowth. Mutations in phosphatase and tensin homolog PTEN gene, an important negative regulator of PI3K/AKT/mTOR signaling pathway (Georgescu 2011; Lee et al. 2012), have been associated with the development of macrocephaly (Butler 2005; Marchese et al. 2014). Indeed, Li et al. (2017a) demonstrated by employing a genetically modified by CRISPR/Cas9 cerebral organoid system that PTEN deletion leads to the upregulation of AKT activity, abnormal neurogenesis, and generally larger organoids with intense gyrification (Li et al. 2017a).

Autism spectrum disorder (ASD) is a phenotypically heterogeneous neurodevelopmental and psychiatric disorder with many different clinical subtypes based both on complex genetic basis and interactions between genetic and environmental factors (Ilieva et al. 2018; Park et al. 2016). Patient-derived or genetically modified brain organoids appear to be suitable for studying the development of such disorders. Indeed, cerebral organoids derived from idiopathic autism patients and from CRISPR/Cas9-mediated iPSCs exhibited a decrease in the cell cycle length of neural progenitors and an increase in the number of GABAergic inhibitory neurons disrupting the excitatory/inhibitory balance (Mariani et al. 2015) and revealed significant genes implicated in the etiology of ASD (Mariani et al. 2015; McCarthy et al. 2014). The aforementioned paradigms are some of the most interesting cases of organoid's applications. More studies have been performed investigating the progression of other diseases such as lissencephaly (Bershteyn et al. 2017; Li et al. 2017a), periventricular heterotopia (Klaus et al. 2019; O'Neill et al. 2018a, b), Rett syndrome (Mellios et al. 2018b, a), schizophrenia (Ye et al. 2017), Miller-Dieker syndrome (Bershteyn et al. 2017; Iefremova et al. 2017), Sandhoff disease (Allende et al. 2018), and Timothy syndrome (Birey et al. 2017).

2.2 Neurodegenerative Diseases

In contrast to neurodevelopmental diseases, modelling neurodegenerative diseases has been proved to be more challenging as symptoms developing in aged patients should be developed in the 3D systems. The majority of studies has focused on the most common type of dementia, Alzheimer disease (AD) which is characterized by aggregation of β-amyloid peptide (Aβ) plaques and neurofibrillary tangles, consisting of hyperphosphorylated Tau protein (Querfurth and LaFerla 2010). As 2D cultures and animal models cannot recapitulate efficiently the Alzheimer's phenotype, many efforts have been made in order to develop 3D culture systems suitable for

studying AD-like pathologies (Choi et al. 2014; Lee et al. 2016; Zhang et al. 2014). Most interestingly, Raja et al. (2016) first recapitulated Alzheimer's disease phenotype by generating brain organoids derived from early-onset familial Alzheimer's (fAD) patient's iPSCs. In this model, AD's characteristics were observed including β-amyloid (Aβ) aggregation, hyperphosphorylated Tau (pTau), and endosome abnormalities, and the organoids were further treated with different compounds aiming to attenuate the pathology (Raja et al. 2016). Cerebral organoids from frontotemporal dementia were generated from patient-derived-iPSCs carrying a Tau mutation to investigate the pathogenetic mechanisms leading to tauopathies by examining the role of p25/Cdk5 in tauopathy progression (Seo et al. 2017). The increase in expression levels of p25, a proteolytic fragment of the regulatory subunit p35, aberrantly activates cyclin-dependent kinase 5 (Cdk5) which has been linked to neurodegenerative disorders such as AD. Blockage of p25 by generating mutant iPSCs with CRISPR/Cas9 was capable of reducing Tau phosphorylation in organoids (Seo et al. 2017). Thus, early-onset fAD is the only neurodegenerative disease modelled using organoids and appears promising for studying in more details the etiology and use of these models for drug testing.

Apart from Alzheimer, creating brain organoids is indispensable to recapitulate many other neurodegenerative diseases as Parkinson and Huntington. Meanwhile, there is no fully functional organoid generated and used for studying the development of those diseases. However, a number of human midbrain-like organoids containing dopaminergic neurons have been developed from human iPSCs or neural stem cells (Edinson Lucumi Moreno 2015; Jo et al. 2016; Monzel et al. 2017). These models need optimization including differentiation and maturation in order to be efficient to mimic later stages of neurodevelopment and neurodegenerative diseases. The limitation of their growth potential occurs due to the lack of vascular and immune system leading to the necrosis of the organoid center and its smaller size. Further advancements are necessary in order for brain organoids to be an established and powerful tool in neurological studies and treatment approaches. It is believed that a key point would be the development of in vivo processes such as communication between different cell types of an organoid and the introduction of vasculature and immune cells, in order to better recapitulate the in vivo physiological microenvironment. The introduction of the microfluidic technology and more complex bioengineering approaches will greatly contribute toward this direction (El-Ali et al. 2006; Sontheimer-Phelps et al. 2019).

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Assessment of Factors Contributing to the Enhancement of Memory and Cognitive Abilities in the Context of Neurosciences

Spyridon Ktenas

1 Introduction

By 2050, the proportion of people aged over 60 is estimated to reach 22% of the world's population. Reduced cognitive abilities are one of the most important consequences of aging, which affect the individual's autonomy and the functioning of the body and generally aggravate the quality of life. From an economic point of view, reduced cognitive abilities increase healthcare costs, which are almost ten times higher for older people (Smith et al. 2009). At the same time, different cognitive issues are affecting younger populations from the infant age. The above facts make this area of interest not only a fascinating scientific topic but an existing challenge in need of solutions and improvements for millions of people of every age.

1.1 Looking into the Brain Imaging Techniques

Different imaging techniques are used in order to better understand the brain and identify possible problems. The most important ones include electroencephalography (EEG) (Klimesch 1998), magnetoencephalography (MEG), computed tomography (CT), magnetic resonance imaging (MRI), functional MRI (fMRI), positron emission tomography (PET), and single photon emission computed tomography (SPECT).

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2 Problems of Cognitive Ability and Memory

There are a number of known diseases and cognitive impairments due to various neurodegenerative conditions such as Alzheimer's disease, dementia with Lewy bodies, frontotemporal dementia, mild cognitive impairment, normal pressure hydrocephalus, and Parkinson's disease dementia. Besides these, there are also cases of learning difficulties in very young ages, such as dyslexia or attention deficit hyperactivity disorder (ADHD), which also affect cognitive ability.

Alzheimer's disease (AD) is a chronic neurodegenerative disease that usually starts slowly and worsens over time (World Health Organization 2015). It is the cause of 60% to 70% of cases of dementia. The most common early symptom is difficulty in remembering recent events (short-term memory loss).

Dementia with Lewy bodies (DLB) is a type of dementia that worsens over time (National Institute on Aging). Additional symptoms may include fluctuations in alertness, visual hallucinations, slowness of movement, trouble walking, and rigidity.

Frontotemporal dementia (FTD) is the clinical presentation of frontotemporal lobar degeneration, which is characterized by progressive neuronal loss predominantly involving the frontal or temporal lobes and typical loss of over 70% of spindle neurons, while other neurons remain intact (Chen 2009). Second only to Alzheimer's disease (AD) in prevalence, FTD accounts for 20% of young-onset dementia cases.

Mild cognitive impairment (MCI), also known as *incipient dementia and isolated memory impairment*, is a neurological disorder that occurs in older adults which involves cognitive impairments with minimal impairment in instrumental activities of daily living (Petersen et al. 2017).

Normal pressure hydrocephalus (NPH) (Smolinsky 2008), also termed as *Hakim's syndrome and symptomatic hydrocephalus*, is a type of brain malfunction caused by the expansion of the lateral cerebral ventricles. Typical symptoms are urinary incontinence, dementia, and gait disturbance.

Parkinson's disease begins by changes in the region of the brain that plays a key role in movement. In Parkinson's, the brain changes gradually spread, and they often begin to affect mental functions, including memory as well as the ability to pay attention, make sound judgments, and plan the steps needed to complete a task. Parkinson's disease is a fairly common neurological disorder in older adults, and it is estimated to affect nearly 2 percent of people older than age 65. It is estimated that 50–80 percent of those with Parkinson's disease eventually experience Parkinson's disease dementia.

Dyslexia appears in young ages as a difficulty in written and oral speech. In dyslexia, the temporoparietal and inferior frontal cortices show some alterations. Also, specific genes appear to be related to dyslexia.

ADHD (attention deficit hyperactivity disorder) is a common child disorder that affects focus and self-control. It is thought to be caused by developmental differences in the brain that affect the parts controlling attention, concentration, impulsivity, activity levels, and memory.

3 Enhancement of Cognitive Ability and Memory

Based on a variety of evidence, we can safely support that it is possible to improve cognitive function in a number of areas such as memory, attention, processing speed, and performance with the use of different methods. Neuroscientists have well established that the brain has a capacity to change in response to environmental stimuli (plasticity). This involves creating and strengthening some neuronal connections and weakening or eliminating others. The degree of modification depends on the type of learning that takes place, with long-term learning leading to more profound modifications. It also depends on the period of learning, with infants experiencing extraordinary growth of new synapses. But a key message is that plasticity is a core feature of the brain throughout life.

Studies have shown that learning can be an effective way to counteract the reduced functioning of the brain: the more the opportunities for elderly people to continue learning (whether through adult education, work, or social activities), the higher the chances of deferring the onset or delaying the acceleration of neurodegenerative diseases.

In terms of structured interventions, brain training can benefit the elderly. Such benefits can lead to improved functional outcomes even up to 5 years after training. Post-analysis of the effectiveness of traditional cognitive interventions and computer-based interventions in healthy elderly populations has shown similar positive results. While traditional cognitive interventions often involve extensive interpersonal contact with specialized trainers, computer-based interventions (neurofeedback, brain-computer interaction applications) are cost-effective and can be applied anywhere, which is important for the elderly who may have motor and financial problems. They are also often designed to be enjoyable, motivating users to follow the training program.

At the same time, the *pharmaceutical approach* (short-term benefits with side effects) and lifestyle changes can improve areas of cognition. Among the chemical cognitive enhancers, Modafinil (Morgenthaler et al. 2007) seems to be a promising one. Other substances like *caffeine* can improve "lower" cognitive elements like the reaction time to a stimulus.

Diet is also an important factor. For example, a diet rich in omega-3 fatty acids has proven to be beneficial for cognitive processes in humans (McCann and Ames 2005) and for the regulation of genes important for the maintenance of synaptic function and plasticity in rodents (Wu et al. 2007). In turn, a diet high in saturated fat is now widely known to negatively affect the molecular supports associated with cognitive processing and is also known to increase the risk of neurological dysfunction in both humans and experimental animals (Greenwood and Winocur 2005).

Physical exercise is probably the top cognitive booster. Many studies reach the same conclusion that exercise improves the brain's functionality.

Finally, *social interactions, sleep, and stress reduction* can also improve cognitive abilities.

4 Conclusions

The human brain has many abilities, of which very few and only in recent studies are explained in more detail. One of them is the brain's ability to adapt and self-improve its operation. In addition to this physiological function, we can improve our ability to learn and remember, but we currently have limited resources to do so. Every organ of the body can be influenced by our habits and activities, and it would be strange for our brain to be an exception.

With regard to differences in methods of improving cognition and memory, it seems that what we can do only little in order to positively intervene. Brain training does not always help without repetitive sessions. It also appears to improve the abilities that are linked to the nature and characteristics of the particular training session. While there are no particularly negative side effects from such activities, we must be very suspicious and skeptical of the messages from companies that promote different brain training applications and potentially promise multiple benefits.

Among the chemicals (drugs), it appears that Modafinil is promising, but some studies show that it offers nothing more than natural substances such as caffeine. In any case it is known that such substances have side effects and should never be used without sufficient justification by a specialist. In most cases, chemicals can offer immediate and short-lasting improvement without lasting results, and their continued use may have serious side effects and very unpleasant long-term implications.

After studying and evaluating the relevant research, it seems that a combination of physical exercise and nutrition has a positive effect on molecular systems associated with synaptic plasticity, while brain training with scientific techniques can improve performance in specific areas. Appropriate physical exercise and proper nutrition have additional benefits for the body. Further studies will be useful in specifying the benefits and the ways that both exercise and nutrition can be used in order to design therapeutic interventions.

In the future, scientists are sure to develop even more ways to improve brain capabilities, but the path is long, and it begins with the improvement of our understanding of the brain and the mapping of its functions. Even when we know how to intervene more effectively, we will continue to tackle moral dilemmas, especially in non-pathological situations, involving interventions and improvements, seeing as they are not available to all people, ultimately leading to a lack of equal opportunities.

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Buccal Mucosa Biomarkers in Alzheimer's Disease

Antigoni Avramouli and Panayiotis Vlamos

1 Introduction

Alzheimer's disease (AD), the most prevalent neurodegenerative disease, is characterized clinically by cognitive decline, memory loss, and visuospatial and language impairment. The greatest risk factor for contracting the disease is advancing age, and risk is significantly increased beyond the age of 70 years (Vieira et al. 2013). AD is an inevitable progressive dementia with long prodromal stages. To date, clinical diagnosis of AD is based on criteria of cognitive impairment and behavioral changes. Nevertheless, the diagnosis for having AD is not quite accurate when using these criteria and can only decisively be confirmed after histopathological investigation (Dickson 1997; Braak and Braak 1998). On the other hand, biomarkers that may identify individuals who are at an early stage of AD would allow timely preventative intervention. To narrow the gap between the increasing number of people facing AD and the established clinical diagnosis methodologies, a simple, inexpensive, noninvasive procedure is needed. This test should facilitate preclinical detection of the disease, in order to monitor disease progression and therapeutic efficacy.

To that end, the use of broadly researched neuroimaging and cerebrospinal fluid (CSF) biomarkers has received increasing attention in the last two decades. However, these techniques continue to face challenges associated with the invasiveness of sample collection, the cost, and interlaboratory variation. At the same time, their ability to distinguish AD from non-AD dementias or AD early diagnosis and application for widespread screening remains questionable. For this reason, a growing body of literature has reported that amyloid pathogenesis and tau metabolic pathophysiological changes are not restricted to the brain but are omnipresent in the

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human body and found in blood, saliva, skin, and other peripheral tissues, raising the possibility of detecting changes that could serve as biomarkers of AD. AD has been also associated with several systemic manifestations in peripheral cells, including dysfunction in metabolic, oxidative, inflammatory, and biochemical pathways.

Each of these cell types contains measurable components that differ between individuals with AD versus non-AD dementias or normal subjects and thus hold promise as candidate biomarkers for early diagnosis of AD. Therefore, diagnostic assays that measure biomarkers of AD pathology in these cells could be the best alternative to CSF-based assays for AD early screening.

Among others some of the best-known peripheral cells that are affected by AD pathology are buccal cells of the oral mucosa. Many studies have shown that individuals who had just been clinically diagnosed with AD and prior to any medication had a significantly different buccal cytome profile compared to normal controls (Francois et al. 2016).

This study summarizes the findings in buccal cells that raise the possibility of detecting changes that could be used for AD diagnostic assays and provides a brief description of a protocol that will be set up in Bioinformatics and Human Electrophysiology Laboratory of the Ionian University.

2 Buccal Cell-Based AD Biomarkers

Buccal cell-based AD biomarkers are a recent development. A large amount of data supports that there is an association between the biochemical changes in brain and peripheral tissues with ectodermal origin. Similar to skin cells, the buccal mucosa (BM) and the brain are derived from ectoderm during embryogenesis (Zoumakis et al. 2007; Schreml et al. 2010). Also, BM is an easily accessible tissue for sampling cells in a minimally invasive manner with an inexpensive procedure. Buccal cells share several pathological changes that are related to AD, such as higher expression of tau protein. Furthermore, they exhibit cytological and nuclear morphological changes; specifically, DNA content and damage, chromosome 17 and 21 aneuploidy, considerably shorter telomere length, neutral lipid content, and altered nuclear shape have been identified in buccal cells of AD patients.

Studies conducted up to now and confirm that the BM is a potential tissue for AD diagnostic biomarkers are described in Table 1.

These changes are potential diagnostic targets for achieving diagnosis at an even earlier stage. Therefore, further research must be undertaken in order to obtain a better understanding of the biology of buccal cells, to replicate such studies, and to investigate other potential markers of AD that might show differences along the progression of the disease.

Table 1 Summary of studies on buccal cell-based AD biomarkers

Samples	Method	Conclusion	Ref.
AD: 34 Vascular dementia: 29 Controls: 34 age-matched controls aged 65 years or older 33 young controls aged less than 65 years old	Western blot, ELISA, tau protein to total protein concentration	Levels of tau protein in oral epithelia showed a significant positive correlation with those in the CSF. AD patients had significantly higher levels of tau protein than patients with vascular dementia and controls. AD patients with a younger age at onset showed a higher level of the protein than the patients with later age at onset	Hattori et al. (2002)
AD: 54 (age 58–93 years) Controls: 26 (age 66–75 years)	Buccal cytome assay	Frequencies of basal cells, condensed chromatin cells, and karyorrhectic cells were found to be significantly lower in AD patients	Thomas et al. (2007)
AD: 54 (age 58–93 years) Controls: 26 old (age 64–75 years) 30 young (age 18–26 years)	Quantitative real-time PCR	Lower length of telomeres in white blood cells and buccal cells in AD patients relative to healthy age- matched controls (31.4% and 32.3%, respectively) Greater length of telomeres in hippocampus cells of Alzheimer's brains compared to control samples Telomere length in buccal cells was 52.2–74.2% shorter than that observed in white blood cells	Thomas et al. (2008)
AD: 54 (age 58–93 years) 21 individuals with Down's syndrome Controls: 26 old (age 64–75 years) 30 young (age 18–26 years)	Hybridization and detection for chromosome 21	1.5-fold increase in trisomy 21 and 1.2-fold increase in trisomy 17 in buccal cells of AD patients compared to age- and gender-matched controls Aneuploidy of chromosomes 17 and 21 in hippocampus tissue did not differ between AD cases and controls	Thomas and Fenech (2008)
AD: 10 Control 9 age-matched	Nucleator method	Reduction in intermediate cells and in the nuclear/cytoplasmic area ratio in AD patients, compared with the control patients	de Oliveira et al. (2008)
Mild AD: 21 Controls, mild AD 21 Moderate AD 10 Controls, moderate AD10 Severe AD: 10 Controls, severe AD 10	Three-dimensional quantitative fluorescent in situ hybridization (3D Q-FISH)	Matched controls have different 3D telomere profiles compared to mild, moderate, and severe AD patients Distinct profiles were also evident for each AD severity group	Mathur et al. (2014)

(continued)

Table 1 (continued)

Samples	Method	Conclusion	Ref.
Mild cognitively impaired: 13 AD: 13 Control: 26	Laser scanning cytometry measurement of nuclear DNA content, nuclear circularity, neutral lipids, and cell subtypes	DNA content was higher; abnormal nuclear shape (circularity) was significantly increased in cell types in both MCI and AD compared with controls Neutral lipid content of buccal cells was significantly lower in the MCI group compared with the control group The ratio of DNA content/ORO in buccal basal cells for both MCI and AD was significantly higher compared to the control group Negative correlation between buccal cell DNA content and ORO content in the AD group but not in MCI or controls	François et al. (2014)
MCI: 17 AD: 19 Control:18	Immunofluorescence	Combined biomarker panel (CK14 expression, plasma vitamin B12, and homocysteine) was significantly lower in the MCI ($p = 0.003$) and AD ($p = 0.0001$) groups compared with controls	Leifert et al. (2015)
MCI: 20 AD: 20 Control:20	Western blots Tau ELISA Laser scanning cytometry	Lower frequency of basal and karyorrhectic cells in the MCI group compared with controls. DNA content, aneuploidy, neutral lipids, and tau were similar in all groups Lower tau protein in both basal and karyolytic buccal cell types compared with differentiated buccal cells Aβ was significantly higher in the AD group compared with the control group. Buccal cell Aβ was correlated with mini-mental state examination (MMSE)	Francois et al. (2016)

(continued)

Table 1 (continued)

Samples	Method	Conclusion	Ref.
Mild AD: 19 Controls, mild AD 19 Moderate AD 14 Controls, moderate AD 14 Severe AD: 4 Controls, severe AD 4	3D structured illumination microscopy (3D-SIM)	Nuclear super-resolution DNA structure of individuals with AD significantly differs from that of their controls with an overall increase in the measured DNA-free/poor spaces Increase in the interchromatin compartment DNA structure of AD significantly differs in mild, moderate, and severe disease with respect to the DNA-containing and DNA-free/poor spaces	Garcia et al. (2017a)
Mild AD: 24 Controls, mild AD 24 Moderate AD 15 Controls, moderate AD15 Severe AD: 5 Controls, severe AD 5	Three-dimensional quantitative fluorescent in situ hybridization of telomeres (3D Q-FISH)	3D telomere profiles can differentiate between AD and non-AD and between AD severity groups 3D telomeric profiles allow for the distinction between AD and non-AD individuals	Garcia et al. (2017b)
Mild cognitive impairment: 16 Severe cognitive impairment: 34 Controls: 31	Immunofluorescence Immunocytochemistry Quantitative real-time polymerase chain reaction Flow cytometry	Presence of p-tau and tau transcript in the oral mucosa of cognitively impaired subjects when compared with healthy subjects Higher presence of p-tau and tau transcript	Arredondo et al. (2017)
AD: 29 Idiopathic PD: 30 Controls: 30	Light microscopy – Cytomorphometric analyses	No differences in nuclear (NV) or cytoplasmic (CV) volumes among groups No significant differences in the cytoplasmic and nuclear volumes of buccal cells among groups	Ozlece et al. (2018)

3 Buccal Cell Biomarkers Examination

Only a few studies have investigated changes in the oral mucosa in AD investigating cytological parameters, cell-type composition, and quantification of certain proteins. To that end we provide a brief outline of a protocol for early screening of the population according to biomarkers that reflect inflammation in buccal cells. Our objective is to identify biomarkers that may detect individuals who are at an early stage of AD, allowing thus timely preventative intervention.

3.1 Recruitment and Characteristics of Participants

The study will be conducted in accordance with the principles of Helsinki declaration and is already approved by the Institutional Review Board. All subjects will give informed consent, and the procedures followed will be in accordance with institutional guidelines. In this study will be enrolled persons with mild cognitive impairment (20–50) and AD (10–20). Age- and gender-matched controls will be enrolled as well. AD patients will be recruited from hospitals and associate and partner organizations. Diagnosis of AD will be made by clinicians according to well-recognized standards. The study will be conducted at the Bioinformatics and Human Electrophysiology Laboratory, at the Informatics Department of the Ionian University, and will be conducted in accordance with the principles of Helsinki declaration and will be approved by the Institutional Review Board.

3.2 Sample Collection

In our assay cells derived from the BM will be harvested from the inside of a patient's mouth after a brief information session outlining the purpose of the study. Cells will be collected using a modified version of the method used by Thomas et al. (2007).

3.3 Methods

Buccal cell smears will be fixed immediately in 95% alcohol and applied to a clean slide. Subsequently, the cytological smears will be stained by the standard Papanicolaou or May-Grunwald-Giemsa methods and routinely processed for microscopic examination. These stains allow both bright field and permanent fluorescence analysis that can be undertaken microscopically. Cells will be counted for each field of good quality, i.e., adequate stain and appropriate cell density to enable cell counts. The cytologic smears will be analyzed by immunocytochemistry and immunofluorescence assays. The selected proteins will be encoded by genes enabled in the pathogenesis of familial or sporadic AD. The selected proteins will also be all expressed in buccal mucosa and will be selected from the Human Protein Atlas platform available from www.proteinatlas.org (Uhlén et al. 2015; Thul et al. 2017). This human tissue proteome platform includes a basic description of a defined proteome and includes analyses of expression patterns, gene lists, and examples of protein expression on a cellular level. Buccal cells will be also prepared for electron microscopy, and possible changes in the ion concentration and localization will be observed by energy-dispersive X-ray spectroscopy analysis.

4 Concluding Remarks

Peripheral cell-based AD biomarkers hold considerable promise for detecting dysfunctional molecular signaling occurring in the early stages of AD. Yet most of the cell-based AD peripheral biomarkers are in discovery stages, and their development remains underfunded. Therefore, more validation studies are required in large investigations that involve minimally invasive nonneural tissue for cell-based AD biomarkers of MCI/AD risk. This study summarizes some of the knowledge gaps in buccal mucosa as a peripheral tissue for AD diagnostics. If our results will be combined with findings from other laboratories, new biomarker sets could emerge that may identify individuals who are at increased risk or are at an early stage of AD with much higher certainty.

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Conflict of Interest The authors confirm that this article content has no conflict of interest.

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Engaging Social Interest and Creating Awareness for the Behavioural and Psychological Symptoms of Dementia

Kristine Newman

Novel multimodal sensing study that has been installed and tested in a geriatric psychiatry inpatient unit is reviewed. Perspectives and experiences of behavioural and psychological symptoms of dementia are also examined. Further, the Spare a Thought for Dementia Through the Your Story My Story campaign, a project that seeks to amplify empowering stories from persons living with dementia and their friends and family carers in order to combat stigma and show carers that they are not alone in their journeys, is also discussed.

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Structural Study of the DNA: Clock/Bmal1 Complex Provides Insights for the Role of Cortisol, hGR, and HPA Axis in Stress Management and Sleep Disorders

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1 Introduction

The stress system is a highly conserved neuroendocrine system that helps organisms to cope with a variety of internal or external stimuli, *the stressors*. This system consists of two distinct components, the locus caeruleus/norepinephrine autonomic nervous systems and the hypothalamic-pituitary-adrenal (HPA) axis, which interact functionally with each other to effectively achieve the internal balance, known as

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homeostasis (Charmandari et al. 2005; Chrousos 2009; Chrousos and Gold 1992; Nicolaides et al. 2015b). The locus caeruleus/norepinephrine autonomic nervous system signals through catecholamines (epinephrine and norepinephrine) via well-identified and extensively studied G protein-coupled receptors (GPCRs), whereas the HPA axis provides the stress response via a ubiquitously expressed ligand-activated receptor, the glucocorticoid receptor (GR), which belongs to the steroid receptor subgroup of the nuclear receptor group of transcription factors (Chrousos and Kino 2005). Upon ligand binding, the GR undergoes substantial conformational changes that enable the protein to translocate to the nucleus and bind, as homo- or heterodimer, to well-recognized DNA domains, the glucocorticoid response elements (GREs), located in regulatory regions of glucocorticoid target genes (Chrousos and Kino 2005; Nicolaides et al. 2010).

In addition to facing stressors, organisms have to synchronize their daily activities to light/dark cycles. This synchronization is tightly achieved by another biologic system, the circadian clock system (from the Latin term "circa diem" that means "approximately a day"), which regulates a large number of physiologic functions in a circadian fashion. This system consists of a central component, the "master" clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus, and myriad peripheral clocks expressed in every tissue (Ko and Takahashi 2006; Takahashi et al. 2008). At the molecular level, the circadian oscillation of gene expression is mediated by transcriptional/translational loops created by the circadian locomotor output cycle kaput/brain-muscle-arnt-like protein 1 (CLOCK/BMAL1) heterodimer and important negative transcription factors, such as the Periods (PER1, PER2 and PER3) and Cryptochromes (CRY1 and CRY2), which form the principal or core transcriptional loop. Upon binding of the activated CLOCK/BMAL1 heterodimer to the E-box response elements, *Pers* and *Crys* are induced. The homonymous expressed proteins are then phosphorylated by casein kinase (CK) 1ε and δ. The phosphorylated isoforms repress the activity of the transcription factors CLOCK/BMAL1, forming a negative transcriptional/translational loop. In addition, several other transcription factors, including retinoic acid receptor-related orphan receptor α (RORα) and reverse viral erythroblastosis oncogene product (REV-ERBα), contribute to the auxiliary transcriptional loop that maintains the activity of the principal loop (Kiyohara et al. 2006; Kondratov et al. 2006; Nader et al. 2010; Padmanabhan et al. 2012).

Accumulating evidence suggests that the stress system and the circadian clock system communicate with each other at multiple neuroanatomical levels

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(Nicolaides et al. 2017). Glucocorticoids, the effector molecules of the HPA axis, contribute substantially to the synchronization of peripheral clocks by phase-shifting the expression of *Pers* in peripheral target tissues. In addition, these hormones through their cognate receptors cause transrepression of the *Rev-ERBα* and *RORα* genes altering directly the activity of the auxiliary transcriptional/translational loop. Furthermore, prolonged administration of prednisolone or adrenalectomy has been shown to alter the expression of clock-related genes. In addition, neuronal projections from the central clock in the SCN to the paraventricular nucleus (PVN) of the hypothalamus contribute to the daily oscillation of serum glucocorticoid concentrations, which are higher during the morning for diurnal species and during the evening for nocturnal species (Nicolaides et al. 2017).

Moreover, a recently identified circadian CLOCK component, termed as "Chrono" ("ChIP-derived repressor of network oscillator"), was found to interact with the GR, providing an association between the circadian clock system and the HPA axis (Anafi et al. 2014; Annayev et al. 2014; Goriki et al. 2014; Robinson 2014). Since the two systems interact with each other, any dysfunction could lead to many pathologic conditions, including inflammatory, metabolic, mood, malignant and sleep disorders (Nicolaides et al. 2017).

In normal conditions, deep sleep inhibits the HPA axis activity. Excessive or prolonged activation of the HPA axis can cause arousal and sleeplessness. Insomnia has been associated with increased activity of the HPA axis, as shown by increased plasma ACTH and serum cortisol concentrations. In excessive sleepiness and fatigue, the inflammatory cytokines interleukin 6 (IL-6) and tumor necrosis factor (TNF) are increased, suggesting that inflammation might contribute to the complex pathogenesis of these conditions (Chrousos et al. 2000). Sleep disorders are also associated with defects in molecules involved in circadian rhythms. Representative examples are familial advanced sleep phase (FASP), which has been attributed to the PER2-S662G mutation or genetic defects in *CRY2* and *PER3* genes, and delayed sleep phase disorder (DSPD), which was associated with polymorphisms in *PER3* and *AANAT* that encode an enzyme participating in melatonin synthesis (Chong et al. 2018).

2 Methods

2.1 Sequence Database Search

A combination of key terms and BLAST searches was employed in order to identify CLOCK and BMAL1 sequences. The names and/or accession numbers of all retrieved entries were used to recover their corresponding amino acid sequences from UniProtKB. Subsequently, these sequences were used as probes to search the nonredundant databases UniProtKB and GenBank by applying reciprocal BLASTp and tBLASTn. This process was reiterated until convergence (Vlachakis et al. 2017, 2014, 2013d).

2.2 Energy Minimization

Energy minimizations were used to remove any residual geometrical strain in each molecular system, using the Charmm27 force field as it is implemented into the Gromacs suite, version 4.5.5. All Gromacs-related simulations were performed through our previously developed graphical interface. An implicit Generalized Born (GB) solvation was chosen at this stage, in an attempt to speed up the energy minimization process (Papageorgiou et al. 2014, 2016; 2017; Schnerch et al. 2016; Sertedaki et al. 2016).

2.3 Molecular Docking Simulations

The experimentally identified structures and the constructed models were used for molecular docking with ZDOCK version 3.0, while RDOCK was utilized to minimize the ZDOCK molecular complex outputs and rank them according to their recalculated binding free energies. Principal component analysis was done using PyMOL (DeLano, W. L., the PyMOL Molecular Graphics System (2002) and the Ca atom root-mean-square function of deep view).

2.4 Molecular Dynamics Simulations

Molecular systems were subjected to unrestrained molecular dynamics simulations (MDs) using the Gromacs suite, version 4.5.5. MDS that took place in a SPC water-solvated, periodic environment (Balatsos et al. 2012; Boulaki et al. 2018; Chatzikonstantinou et al. 2017; Tagkalakis et al. 2017; Vlachakis et al. 2013e). Water molecules were added using the truncated octahedron box extending 7Å from each atom. Molecular systems were neutralized with counterions as required. For the purposes of this study, all MDS were performed using the NVT ensemble in a canonical environment, at 300 K, 1 atm and a step size equal to 2 femtoseconds for a total 100 nanoseconds simulation time. An NVT ensemble requires the number of atoms, volume, and temperature to remain constant throughout the simulation.

3 Results and Discussion

The DNA-bound Clock and Bmal1 structures have been co-crystalized in a single crystal molecular system (PDB id: 4H10) (Wang et al. 2013). Herein, the hypothetical role of cortisol in the DNA – Clock/Bmal1 has been determined using an in silico pipeline. The study begins by a thorough investigation of the crystal structure, molecular docking, energy minimizations, and molecular dynamics.

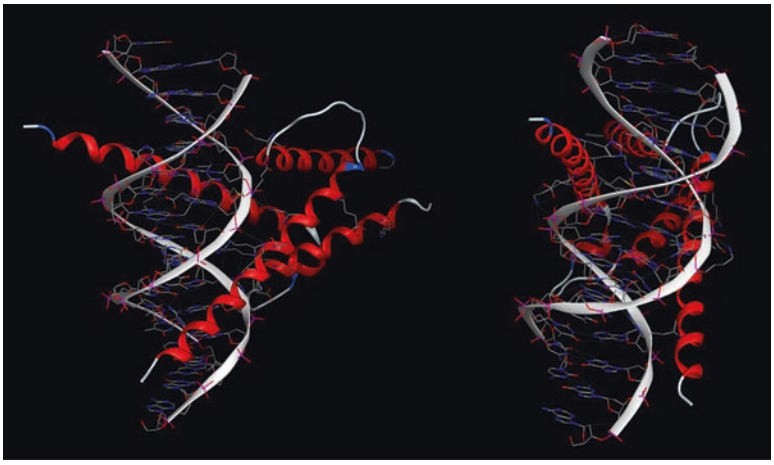

Fig. 1 The two alpha helices protrude into the dsDNA major grooves and establish strong interactions

The Clock/Bmal1 heterodimer protein complex is composed by two almost symmetrical alpha helices (Ko and Takahashi 2006). The longer alpha helix is the one that protrudes into the major groove of the double-stranded DNA molecule and secures the interaction (Fig. 1) (Kiyohara et al. 2006). In an effort to investigate the role of cortisol in the DNA/Clock/Bmal1 interaction, the molecular system was energetically minimized and subsequently used to dock a single molecule of cortisol (Brancale et al. 2008; Carvalho et al. 2013; Marinou et al. 2018; Vlachakis and Kossida 2013).

The docking algorithm explored numerous sites to accommodate the cortisol molecule. The by far most favorable one, energetically wise, was the minor groove of the DNA (Fig. 2). The Clock/Bmal1 proteins were deleted, and the docking was repeated in an attempt to eliminate the possibility of cortisol binding to the unoccupied major grooves of the DNA. It was concluded that not a single docking pose of cortisol was found in the major groove. On the contrary the most thermodynamically favorable pose was again the more well-defined minor groove of the DNA fragment.

The docking experiment was followed by exhaustive molecular dynamics simulations in a periodic system with explicitly solvated single point charge (SPC) water molecules (Amidi et al. 2016; Theoharaki et al. 2018; Vangelatos et al. 2009, 2013b). The molecular dynamics simulation was allowed to proceed until full equilibrium for the whole molecular system was reached, having allowed all degrees of freedom (Balatsos et al. 2009; Palaiomylitou et al. 2008; Sellis et al. 2012; Vlachakis 2009, 2013a).

The DNA molecule was removed, and the molecular dynamics simulation was continued. Interestingly the cortisol molecule kept its position as if the minor groove was still present (Fig. 3). However, it was clear that the Clock/Bmal1 complex was

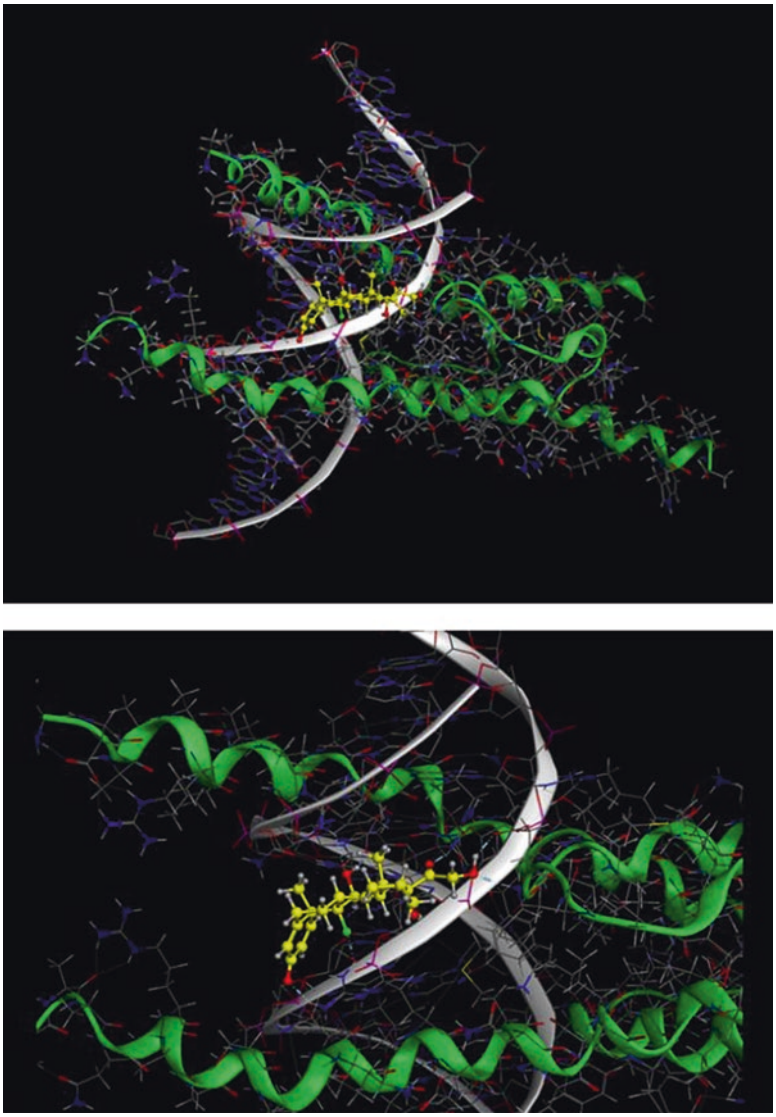

Fig. 2 The cortisol molecule docked in the minor groove of the dsDNA fragment

now bended inward. The presence of the cortisol compound was pulling the two alpha helices toward it.

Figure 4 shows the superposition of the original conformation of the Clock/Bmal1 complex without any cortisol as well as the Clock/Bmal1 complex in the presence of the docked cortisol and upon molecular dynamics (Amidi et al. 2017; Dalkas et al. 2013; Kandil et al. 2009; Vlachakis et al. 2012, 2013c). The original cortisol-free complex is showing in red ribbon representation and the cortisol-bound

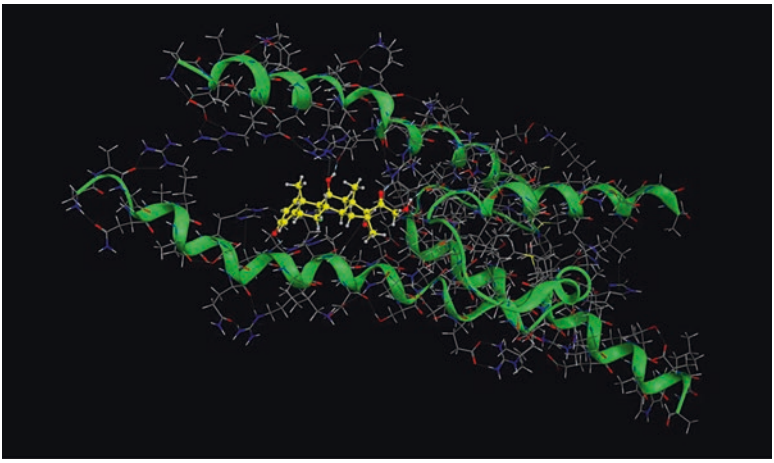

Fig. 3 The cortisol molecule stabilized in molecular dynamics simulations in the absence of the dsDNA fragment

Clock/Bmal1 complex is showing in green ribbon representation. The final Clock/Bmal1 complex (with the bound cortisol and upon molecular dynamics) is now bended inward the DNA molecule, and it forms much stronger interactions with it. The cortisol-bound Clock/Bmal1 complex has adopted an optimal anatomy in order to increase (times 4) the interaction of molecular surface with the major groove of the DNA, thus establishing many more hydrogen bonds and hydrophobic interactions (Fig. 4). The final superposed model is showing in Fig. 5, following the color conventions of the previous figures.

Our in silico study is showing a strong contribution of cortisol in the enhanced interaction of Clock/Bmal1 complex and dsDNA. Therefore, we suggest that in the presence of cortisol, the Clock/Bmal1 complex establishes stronger interactions with the dsDNA molecule, thus prolonging its DNA-bound conformation and its signaling effect. Consequently, this could account for changes and shifts in cellular circadian rhythms and biorhythms. When cortisol levels are too high, the chances of cortisol intercalating in the minor groove of the DNA are higher. That results in established interactions between the phosphate groups of the DNA backbone and the network of hydroxy groups of cortisol. Eventually in the presence of cortisol, the minor groove of the DNA reduces in size by 15%. This narrowing of the minor groove of the DNA results in a widening of the adjacent two major grooves. We speculate that the wider major grooves make it easier for the Clock/Bmal1 complex to accommodate itself on the dsDNA molecule.

The most important part of the brain involved in coordinating the sleep process is called an activating grid (RAS). It consists of a large number of nerve cells throughout the brain and is responsible for wakefulness adjustment. When RAS neurons stop functioning, alertness decreases. There is a continuous interaction between RAS and other brain regions that regulate sleep. These regions include the

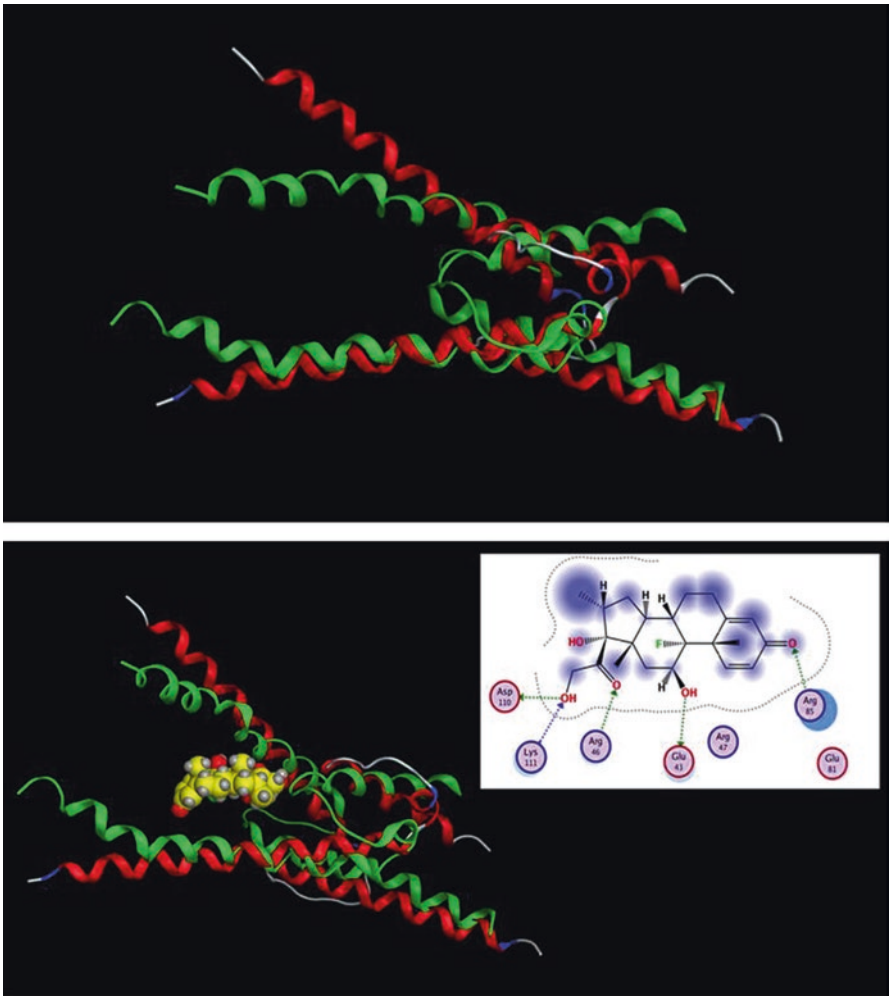

Fig. 4 Superposed the Clock/Bmal1 complex in the absence (red ribbon) and in the presence (green ribbon) of cortisol. The insert depicts the interactions that cortisol establishes with the two alpha helices of the Clock/Bmal1 complex

thalamus, the medulla, the hypothalamus, the bridges, the middle brain, the spinal cord, the pineal gland, the symmetrical suture nuclei, the basal forebrain, the hippocampus, and the suprachiasmatic nucleus.

The suprachiasmatic nucleus or nuclei (SCN) is a tiny region of the brain in the hypothalamus, situated directly above the optic chiasm. It is responsible for controlling circadian rhythms. The neuronal and hormonal activities it generates regulate many different body functions in a 24-hour cycle, using around 20,000 neurons. The SCN is situated in the anterior part of the hypothalamus, it is superior (hence supra) to the optic chiasm (CHO), and it is located bilateral to (on either side of)

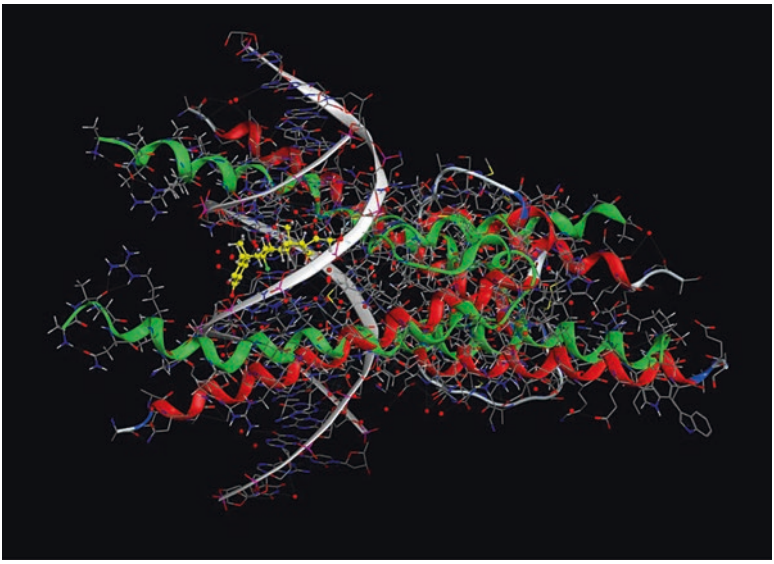

Fig. 5 Superposed the Clock/Bmal1 complex in the absence and in the presence of cortisol bound on the dsDNA fragment. Color conventions follow that of Fig. 4. Note that cortisol in yellow ball and stick is docked in the minor groove of the dsDNA fragment

the third ventricle. The SCN interacts with many other regions of the brain. It contains several cell types and numerous different peptides including vasopressin and vasoactive intestinal peptide and neurotransmitters. Through a network of connections, pineal projections lead to the production and release of melatonin in the circulation, where it modifies the activity of the brain stem networks which eventually control the sleep-wake cycle. It is significantly affected by light, and it promotes alertness, as evidenced by the high firing rate of neurons during the day and their low rhythm during the night. The light activates specific receptors in the retina, which in turn affect the suprachiasmatic nucleus. Exposure to light increases alertness, while darkness has the opposite effect. Therefore, light is important for the regulation of sleep and wakefulness, and it supports proper circadian function. The suprachiasmatic nucleus also controls functions that are coordinated with the sleep-wake cycle, such as temperature, hormone secretion (e.g., cortisol), blood pressure, and urea production (with antidiuretic hormone control not available in the elderly and children) (Nicolaides et al. 2015a, 2016).

4 Conclusions

All in all, our study shows that elevated concentrations of cortisol could result in higher intercalation levels of the latter in the DNA minor groove. This will consequently widen the nearby major grooves and will provide the Clock/Bmal1 complex

more space to dock and to interact with DNA. Moreover, on a second phase, the strong negatively charged hydroxyl groups of cortisol will attract a network of arginine and lysine amino acids on the Clock/Bmal1 complex and will bend it inward, thus securing a much stronger and prolonged interaction pattern. As a result, the Clock/Bmal1 complex in the presence of cortisol will remain in its DNA-bound form for longer periods and will inevitably affect circadian rhythms, stress response, and biorhythms. Our study paves the way for further investigation to be conducted regarding the role of elevated cortisol in stress, inflammation, and sleep disorders as a result of prolonged and stronger dsDNA – Clock/Bmal1 interactions.

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The Effects of Quantum Entanglement on Chromatin and Gene Expression

Michael Harney

1 Introduction

The quantum world has been traditionally constrained to nanometer scales with very little knowledge of the transition to the macroscopic scale. This is partly due to the incoherence associated with entropy and thermal variation in atomic scale entities at the macroscopic level which breaks the correlation between entangled states. However, quantum entanglement has recently been demonstrated at the macroscopic scale at room temperature. Lee et al. (2011) has demonstrated entanglement through nonclassical photon correlation in diamonds at the millimeter scale at room temperature, and Klimov et al. (2015) has demonstrated entanglement between electron-nuclear spin ensembles separated at the micron scale in a silicon carbide array, with distances that mimic cellular volumes (Lee et al. 2011; Klimov et al. 2015). Both experiments demonstrate the potential coherence between electron-nuclei pairs within carbon matrices and therefore open the door to considering coherence and entanglement in other, highly ordered carbon arrays, such as those that exist in biological molecules. As in the silicon carbide or diamond array, the requirements for coherence and stability in the biological carbon array are necessary, considering the high thermal energy, so carbon matrices with interacting mechanisms over a macroscopic scale are preferred to maintain stability. Although many long hydrocarbon chains may seem ideal for this situation, the lack of interlocking thermal stability from the beginning of the chain to its end represents a challenge for the coherence of the system. One example of particular significance where there is interlocking stability is through the coiled nature of chromatin, the storage structure of DNA. The basic unit of chromatin is a protein octamer containing histones H2A, H2B, H3, and H4.

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Coiled around the histone proteins is 147 bp of DNA, and extending from the histone proteins are amino-terminal tails that allow for covalent modification from external transcription factors (Allis et al. 2009). The supercoiled 30-nm fiber around the histones presents close proximity and electrostatic interaction to the previous windings and affects the larger scale structure of chromatin and of associated transcription factors (Ho et al. 2006). These closely interacting factors between 30-nm and 100-nm chromatin structures may facilitate the coherence and stability associated with quantum entanglement.

2 Gene Expression Through Coherence of Chromatin

Based on the experimental work of Klimov et al. (2015) demonstrating coherence in silicon carbide arrays and the compacted nature of chromatin, there is a reasonable expectation of coherence between some segments of chromatin in the approximately 3 billion base pairs of human DNA. At a very broad level, with the approximate coiling of 147 bp around the histone proteins and based on the millimeter scale coherence results of Klimov, we would expect many potential interactions between base pairs ($n = 3 \times 109$, $k = 147$) assuming no limit to range:

$$\binom{n}{k} = \frac{n!}{k!(n-k)!} \tag{1}$$

A more realistic interpretation may be to define k based on the median size of transcriptional factors and interacting locations ($k*$) and to modify the level of interaction based on range, such as a Yukawa coupling. There is evidence for the limited range of entanglement in DNA based on the model of the coulomb potential dipole in the electron cloud of the DNA base, where phonon interaction models show limited range (Rieper et al. 2011). Based on this model, we can modify (1) with a Yukawa scale factor based on a coulomb potential:

$$\binom{n}{k*} = \frac{n!}{k!(n-k)!} e^{-ur} \tag{2}$$

Where u is the scale factor and r is the range of interaction between transcriptional factors and gene promoters or inhibitors. It is well known that histone proteins have modification sites for covalent bonding through methylation, phosphorylation, acetylation, ubiquitylation, and sumoylation (Allis et al. 2009). These posttranslational modifications have effects on gene expression within the compacted chromatin and may also activate the Yukawa coupling that establishes coherence between transcriptional factors and gene promotion or inhibition sites. It is well known that remote activation of eukaryotic genes can be up to many kilobases in distance from the gene locus, and due to these limits of how far the remove

activation occurs, we propose the Yukawa potential as a way to model the quantum range limit (Ho et al. 2006). Although the explanation for these long-range activators is currently unknown, a possible explanation is the coherent Yukawa coupling of chromatin due to early interaction in the cellular environment through previous replication and transcription. One way to determine if this coherent interaction is occurring is to measure the Yukawa scale factor u based on gene expression outcomes from the same modifiers as a function of distance in coiled chromatin. A scale factor that remains consistent throughout many experiments of several different modifiers would provide evidence for quantum entanglement of these interactions.

Additional modeling by Rieper et al. (2011) of nucleotide bases as phonons (due to dipole interactions of the electron cloud associated with the base) over a long range of the helix shows quantum coherence at room temperature (Rieper et al. 2011). The effects of longer standing waves over the length of the helix result in phonon trapping, which has been demonstrated in silicon structures of a similar size at room temperature. The entanglement from the phonon interaction binds the DNA helix, as it would normally completely collapse from a classical standpoint. These models add additional evidence to the theory of gene expression by remote modifiers through quantum entanglement.

3 Conclusions

Coherent, quantum entanglements have been considered as an explanation for the long-range interaction of gene modifiers and their locus targets. Additional experimental evidence that is focused on individual coherent interactions between specific modifiers and their gene locus targets is needed to validate if there are Bell states associated within chromatin. A Yukawa coupling model of electron potential is introduced to describe the entanglement interaction and limitations over distance. Similar models based on the interactions of the electron clouds of the base pairs as phonons show stability of the DNA helix only through quantum entanglement of the photons and instability if a classical model is used. The implications to science and biotechnology if quantum entanglement is shown to be active in gene expression would be substantial. From sequencing to gene therapy, there will likely be many improvements in the way medical care is delivered and the way new genes are discovered.

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Olive Oil Polyphenols in Neurodegenerative Pathologies

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1 Introduction

The aftermath of neuronal death as well as the progressive loss of structure or function of neurons in the brain or the spinal cord is specified as neurodegenerative disorders. The specific central nervous system region involved in the loss and decay of nerve cells characterizes the clinical features of the neurological disorder (Yacoubian 2017). The group of neurodegenerative disorders includes conditions such as amyotrophic lateral sclerosis (ALS) and Huntington's disease, but the most frequent are Alzheimer's disease and Parkinson's disease. The previously mentioned disorders are a primary health issue, predominately in the aging population

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(Antoniou et al. 2018a). In the near future, neurodegenerative disorders are anticipated to escalate, as life expectancy increases, leading to a financial and societal affliction (Gitler et al. 2017). Such diseases are most commonly caused by inflammation and oxidative stress (Barnham et al. 2004), and by the time the symptoms are obvious enough for a diagnosis to be made, the disorder has been developing for about 20 to 30 years prior. Alzheimer's disease and Parkinson's disease are related with high morbidity and mortality, while they leave very few alternatives for therapy (Antoniou et al. 2018b; Ritchie and Lovestone 2002). In the developed countries, a noticeable percentage of people suffer from the most common form of dementia, namely, Alzheimer's disease, which is considered a protein misfolding disease.

Alzheimer's disease is characterized by intracellular neurofibrillary tangles of hyperphosphorylated tau, or extracellular amyloid deposits (Aβ), located in defuse and senile plaques around cerebral vessels of dystrophic and degenerating neuritis of the brain (Citron 2010). Almost 30 million people are affected, nowadays, by Alzheimer's disease, with occurrence of the disease increasing by 1% in the age of 60–70 and 6–8% in the age of 85 years (Ferri et al. 2005; Kukull et al. 2002). These numbers are expected to grow to a great extent by 2050 (Citron 2010).

Parkinson's disease is located in substantia nigra, distinguished by the selective degeneration of dopamine-containing pigmented neurons (Cheng et al. 2010). Parkinson's disease is the most known neurodegenerative disease, and it influences more than 1% of the population above the age of 65 years (Farrer 2006; Harhangi et al. 2000).

Among the fatal brain disorders, and a form of dementia, is Creutzfeldt-Jakob disease (Sikorska et al. 2012), which is caused by misfolded proteins in neurons of the central nervous system. This disorder affects the signaling process and damages the neurons. Recent computational studies have attempted to address the 3D conformational arrangement of these proteins using advanced statistics and deep learning (Amidi et al. 2018, 2016, 2017; Bencurova et al. 2015). Also, novel approaches have been proposed in structural bioinformatics (Kontopoulos et al. 2016a, b; Carvalho et al. 2013).

2 The Setting

For more than 2500 years, olive tree has been the most prominent plant of the Mediterranean region. It has been used for social and religious purposes. Almost all of the ancient civilizations have used its derivatives, from ancient Greeks to ancient Egyptians. It has been mentioned in the bible and in the Koran. Since decades ago it has been linked with healing and longevity properties. Nowadays its correlation with longevity, lower incidence of coronary heart disease, cancer, and neurodegenerative disorders has been mentioned in a large number of studies (Visioli et al.

2018). Various studies have also termed olive derivatives as "medical foods." Medical foods are specific products, needed when a disease requires particular dietary management, that are established by medical and scientific evaluation (Lichtenstein 2017; Thaipisuttikul and Galvin 2012). Mediterranean diet seems to include a wide group of medical foods, the most prominent being extra virgin olive oil. Mediterranean populations are estimated to consume 25 to 50 ml of olive oil per day. Besides the dietary use of olive oil and olives, olive in the Mediterranean area is an asset of great importance, economically and culturally, while 90% of the world's olive oil is produced in that region. There are many olive cultivars that produce olive oils with different organoleptic characteristics. The beneficial role of extra virgin olive oil and its components are being revealed, and future research will show more about its benefits on human health (Nocella et al. 2018; Rigacci 2015). Olive oil is the principal source of fat in the Mediterranean diet and contains a great number of chemical compounds, such as several carotenes and phenolic compounds that act as antioxidants (Pandey and Rizvi 2009; Rigacci 2015; Vissers et al. 2004). Polyphenols are secondary plant metabolites that belong to a chemical group characterized by the presence of one or more aromatic rings with one or more hydroxyl substituent's group (Kennedy 2014). The olive tree synthesizes different and specific polyphenols, that are a part of the phytoalexin family, and their main role is to defend against microbial or fungal invasions or insect attacks (Cowan 1999). Polyphenols are located in the lipid and water components of olive oil. Primary polyphenols of olive oil are oleuropein aglycone, tyrosol, oleocanthal, oleacein, and hydroxytyrosol. The content of polyphenols in the olive depends on various circumstances such as the environment, ripening stage, storage, extraction, cultivar, etc. Said content can vary substantially, and it is known to reach levels as high as 60mg/100gr.

3 Is Olive Oil the Key?

At this moment, there are many limitations regarding the medication and treatment of neurodegenerative disorders since their main aim is to only address the symptoms. Therefore, there is no available therapy which can terminate the loss of neurons, especially for Alzheimer's and Parkinson's disease (Lanctot et al. 2009; Yacoubian 2017). Medications used for treatment of pathologies like Alzheimer's disease are restricted (Lanctot et al. 2009) and only delay the manifestation of the pathology or decrease the progression rate of symptoms, without blocking dopaminergic neuronal loss (Surmeier et al. 2010; Yacoubian 2017). Acetylcholinesterase inhibitors, (McGleenon et al. 1999) including donepezil, rivastigmine, galantamine, and glutamate receptor antagonist (Wang and Reddy 2017) such as memantamine, are currently the key therapeutic molecules used for treatment (Casey et al. 2010). Treatment of Alzheimer's disease has focused on the amyloid hypothesis, but this approach seems to have been unsuccessful so far

(Karran and Hardy 2014). Amyloid's product Aβ produces Aβ40 and Aβ42, and its accumulation seems to be connected to the pathological changes that Alzheimer's patients follow (Hardy 2009), but the most used acetylcholinesterase inhibitor, donepezil, has a moderate effect in decreasing Aβ pathology (Kim et al. 2014). Focusing on the detox mechanism is also very important (Axarli et al. 2016; Balatsos et al. 2012, 2009; Boulaki et al. 2018; Brancale et al. 2008; Chatzikonstantinou et al. 2017).

On the other hand, an extensive number of studies in the past few years have pointed out the beneficial effect of the Mediterranean diet against various pathologies, including neurodegenerative disorders (Alcalay et al. 2012). A decreased risk of dementia has been correlated with the consumption of olive oil, and by extension with its natural chemical products, "polyphenols" (Scarmeas et al. 2006). This results by enhancing cognitive function, reducing the risk of mild cognitive impairment and decreasing the progression rate of Alzheimer's disease (Scarmeas et al. 2009). The beneficial role of polyphenols, especially those found in extra virgin olive oil, is supported by a rising number of researchers. Extra virgin olive oil, as medical food, can benefit the majority of people, due to its wide availability and affordability (Table 1).

The cognitive behavior, of a group comprised of elderly people, presented a remarkable improvement when linked to a diet with extra virgin olive oil (Valls-Pedret et al. 2012), and similar results were obtained in a study with animal models after extra virgin olive oil consumption (Farr et al. 2012). The idea that the intake of polyphenols, as part of Mediterranean diet, contributes to a potent protection against neurodegenerative disorders has been reinforced by population studies and clinical trials (Rigacci 2015; Visioli et al. 2018). The existence of polyphenols has the ability to alleviate redox status and consequently reinstate optimal neuronal function, not only by their antioxidant ability but also by reinstitution of mitochondrial function, following activation of the nuclear factor pathway, which is involved in cell protection (Li et al. 2009). In addition, various beneficial effects have been revealed, including their potential to successfully impede protein aggregation, preventing self-assembly of misfolded proteins into toxic amyloid oligomers and fibrils (Daccache et al. 2011; Rigacci et al. 2011). When mice were fed with two different diets, one including extra virgin olive oil and one without, it was observed that the former showed a decrease in Alzheimer's disease (AD) symptomology (Lauretti et al. 2017). One of the most significant polyphenols which shows encouraging prospective in the determent of neurodegenerative diseases is hydroxytyrosol (Rodriguez-Morato et al. 2015). When tested to a mouse model related with Alzheimer's disease, it resulted in a remarkable development of the neurobehavioral dysfunction (Arunsundar et al. 2015). Moreover, plaque load in the hippocampus was significantly decreased when animals were administrated with Oleocanthal for 4 weeks, in a study performed on a human blood-brain barrier model and on the TgSwDI murine model of Alzheimer's disease. Furthermore, according to Batarseh et al., donepezil when combined with extra virgin olive oil nutrition significantly decreased Aβ load and its correspond-

Table 1 The major polyphenols of the extra virgin olive oil

A/A	Olive oil polyphenols	IUPAC name	Chemical formula
1	*Tyrosol*	4-(2-Hydroxyethyl)phenol p-Hydroxyphenethylalcohol 2-(4-Hydroxyphenyl)ethanol 4-Hydroxyphenylethanol	$C_RH_{10}O_2$
2	*Oleuropein aglycone*	4-[2-[2-(3,4-Dihydroxyphenyl) ethoxy]-2-oxoethyl]-5-ethylidene-6-[[(2S,3R,4S,5S,6R) 3,4,5-Trihydroxy-6-(hydroxymethyl)-2-tetrahydropyranyl]oxy]-4H-pyran-3-carboxylic acid, methyl ester	$C_{25}H_{32}O_{13}$
3	*Hydroxytyrosol*	4-(2-Hydroxyethyl)-1,2-benzenediol 3-Hydroxytyrosol 3,4-Dihydroxyphenylethanol (DOPET) Dihydroxyphenylethanol 2-(3,4-Di-hydroxyphenyl)-ethanol (DHPE) 3,4-Dihydroxyphenolethanol (3,4-DHPEA)	$C_8H_{10}O_3$
4	*Oleocanthal*	2-(4-Hydroxyphenyl)ethyl (3S,4E)-4-formyl 3-(2-Oxoethyl)hex-4-enoate	$C_{17}H_{20}O_5$
5	*Oleacein*	2-(3,4-Dihydroxyphenyl)ethyl (4Z)-4-formyl-3-(2-oxoethyl)hex-4-enoate; 2-(3,4-dihydroxyphenyl)ethyl 4-formyl-3-formylmethyl-4-hexenoate; (E)-3-(2-oxoethyl)-4-formyl-4-hexenoic acid 3,4-dihydroxyphenethyl ester; 3-(2-oxoethyl)-4-formyl-4-hexenoic acid	$C_{17}H_{20}O_6$
6	*Pinoresinol*	4-[(3S,3aR,6S,6aR)-6-(4-hydroxy-3-methoxyphenyl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-c] furan-3-yl]-2-methoxyphenol	$C_{20}H_{22}O_6$
7	*Vanillic acid*	4-Hydroxy-3-methoxybenzoic acid	$C_8H_8O_4$
8	*P-coumaric acid*	(E)-3-(4-hydroxyphenyl)prop-2-enoic acid	$C_9H_8O_3$
9	*Caffeic acid*	(E)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid	$C_9H_8O_4$
10	*Ferulic acid*	(E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoic acid	$C_{10}H_{10}O_4$

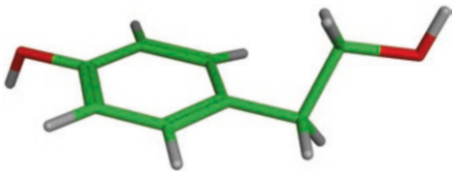

Fig. 1 Structural conformation of tyrosol phenolic compound in the 3D space

ing toxicity in a mouse model. The same researchers concluded that extra virgin olive oil integrated with donepezil upregulated synaptic proteins can elevate the blood-brain barrier tightness and minimize neuroinflammation (Batarseh and Kaddoumi 2018) (Fig. 1).

4 Polyphenol's Affiliation with Protein Related to Parkinson's Disease

One of the most important polyphenols present in the olive tree (*Olea europaea*), tyrosol, has been found to interact with a protein, which appears to be associated with neurodegenerative disorders (Table 2). In particular, the tyrosol polyphenol has been found to interact with the active site of tyrosinase, based on the crystal structure complex (PDB ID: 4P6T) (Goldfeder et al. 2014). Tyrosinase, a copper-containing protein, is a key molecule in the biosynthesis of melanin and other chemical compounds (Cass and Hill 1980). Hydroxylation of tyrosine to L-DOPA and oxidation of L-DOPA to dopaquine are both catalyzed by tyrosinase (Korner and Pawelek 1982; Sanchez-Ferrer et al. 1995). It is also possible that dopamine's oxidization to form melanin can be generated by tyrosinase (Miranda et al. 1984). Goldfeder et al. (Goldfeder et al. 2014), derived the following conclusions: (i)

Table 2 Tyrosol's related studies and function pathways in the PubChem database (Kim et al. 2016)

A/A	Used in drugs for human necessities under patent classification for	Biological test results were tested substance is active
1	Colorectal cancer	Inhibition of *Mycobacterium tuberculosis* recombinant Rv1284 beta-carbonic anhydrase
2	Prostate, kidneys, bladder	Inhibition of *Cryptococcus neoformans* recombinant Can2 beta-carbonic anhydrase
3	Genital or sexual disorders	Inhibition of *Candida albicans* recombinant Nce103 beta-carbonic anhydrase
4	Disorders of the urinary system	Inhibition of NADPH oxidase in human HUVEC cells
5	Antioxidant	Inhibition of human recombinant alpha-carbonic anhydrase 2
6	Disorders of the nervous system/ neurodegenerative disorders	Inhibition of *Mycobacterium tuberculosis* recombinant Rv3273 beta-carbonic anhydrase
7	Anti-Parkinson drugs	Toxicity against *Choristoneura fumiferana*
8	Mania or schizophrenia	Toxicity against *Saccharomyces cerevisiae*
9	Alcohol/opioid abuse	Inhibition of human recombinant carbonic anhydrase 2, 5A, 5B
10	Antibacterials/antivirals	Antioxidant activity assessed as oxygen radical scavenging activity
11	Leukemia	Activity at mushroom tyrosinase assessed as decrease in absorbance
12	HIV	
13	Anti-aging preparations	
14	Hypnotics	
15	Dermatological disorders	

albinism in human species is primarily generated by a mutated tyrosinase, (ii) tyrosinase is noticeably targeted by the pharmaceutical industry, and (iii) many organisms owe the production of melanin to tyrosinase. Moreover, as stated by Fairhead and Thony-Meyer (Fairhead and Thony-Meyer 2012), UV protection, detoxification of phenols, and wound healing are related to the action of tyrosinase. Olivares and Solano (Olivares and Solano 2009) supported the fact that melanoma can be affiliated with tyrosinase's mutations. There has been extensive research on the effects of such mutations (Dalkas et al. 2013; Filntisi et al. 2014; Inturi et al. 2014; Kandil et al. 2009; Kapasa et al. 2012; Maltezos et al. 2014; Marinou et al. 2018; Nicolaides et al. 2015, 2016). Dopamine levels in the brain are maintained through excessive oxidation of cytosolic dopamine and L-DOPA by tyrosinase, which is also preventing cell decay by dopamine auto-oxidation (Asanuma et al. 2003). Following the same study, in the absence of tyrosine hydroxylase, tyrosinase's ability for double-edge synthesizing and oxidizing in the dopaminergic system is indicated for the composition of dopamine in long-standing Parkinson's patients. Equally, tyrosinase's overexpression enlarged the amount of dopamine which accompanied by the development of melanin pigments that ultimately triggered apoptotic cell death (Hasegawa 2010). In closing, tyrosinase promoter is energetic during murine brain growth, and in accordance to Tief et al., it may be connected to neuromelanin's evolution in neurodegenerative diseases, like Parkinson's disease (Tief et al. 1996) (Fig. 2).

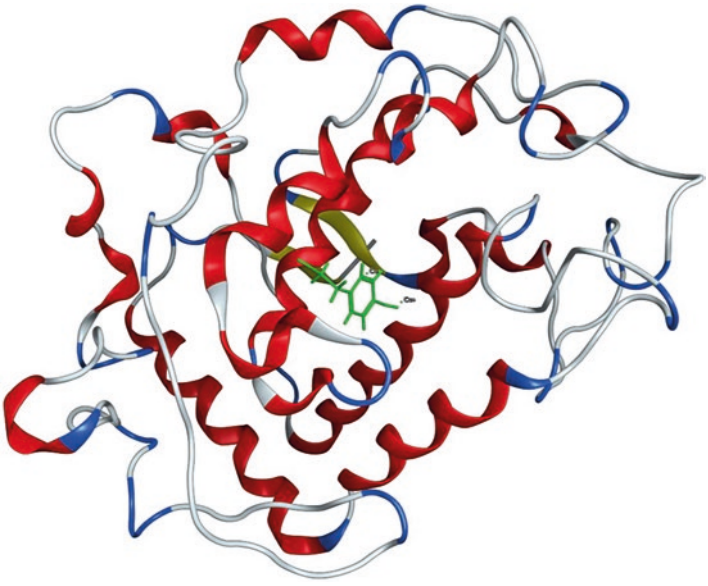

Fig. 2 Ribbon representation of the crystal structure (PDB ID: 4P6T) of tyrosinase protein bound with tyrosol (colored green) in its active site

5 Time to Change Viewpoint?

The discovery of exploitable molecules related to dietary regimens in order to decrease the risk of neurodegenerative disorders provides a great potential for effective treatment against such diseases (Scarmeas et al. 2009). The effects of Mediterranean diet, that contains polyphenols, in intercepting various age-related flaws, neurodegenerative disorders, as well as cancer have been well demonstrated by many researchers (Casamenti and Stefani 2017). More effective therapeutic approaches for neurodegenerative diseases, including treatments with the safe substitute of medical food therapies, need to be addressed. The level of contribution of olive polyphenols to neuroprotective effects and management of neurodegenerative disorders requires additional research (Rodriguez-Morato et al. 2015). A medical need in the coming years will be the discovery of molecules that are capable to differentiate protein and peptide aggregation by blocking the formation of the plaque accumulation (Vlachakis and Kossida 2013a, b; Vlachakis et al. 2013c, d). Moreover, functional portions of olive polyphenols, necessary to establish health tranquillity, will need to be examined. Future research and clinical trials on the role of Mediterranean diet and olive oil will provide further scientific knowledge, besides the beneficial effects on multiple neurodegenerative disorders as a general effect on the brain, and lead to a better understanding of the mechanisms interfering this association.

For better understanding of the olive oil benefits on health, many computational methods have been developed in the molecular and structural level (Palaiomylitou et al. 2008, 2016a, b, 2014, 2017). Comprehensive structural studies have helped in elucidating the role of key metabolizing enzymes (Pavlopoulou et al. 2013; Vlachakis et al. 2014a, b, 2017). All the information is handled by large computational systems (Theoharaki et al. 2018; Tsiliki et al. 2014; Vangelatos et al. 2009; Vlachakis et al. 2018, 2015; Vlachakis 2009; Polychronidou et al. 2015). Molecular modelling and structural bioinformatics are paving the way to a greater understanding of the role of olive oil ingredients in our health (Schnerch et al. 2016; Sellis et al. 2012; Sertedaki et al. 2016; Steinhauf et al. 2014; Tagkalakis et al. 2017; Vlachakis et al. 2013a, b). The metabolism of glucose, the level of steroids, and the nutrients that we intake from our diet are key to maintaining homeostasis (Vlachakis et al. 2014c, 2012, 2013e, f, g). In the long run, the maintenance of stable homeostatic parameters, like HPA axis, circadian rhythms, and hormone levels, will have a protective role against illness and disease.

6 Conclusions

Despite the great efforts that have been made so far in the fight against neurodegenerative disorders, there is no available treatment. Medications used for such pathologies only delay the progression of symptoms and have proven to be unsuc-

cessful thus far. Contrary, the consumption of extra virgin olive oil as a medical food and the olive polyphenols such as tyrosol, oleuropein aglycone, oleocanthal, and hydroxytyrosol appears to be potentially beneficial in the confrontation of neurodegenerative disorders, as stated by many researchers. Additionally, tyrosol interacts with the active site of tyrosinase, a protein responsible for albinism in mammals, which is a target excessively employed in the pharmaceutical industry, and it is also strongly associated with Parkinson's disease. In conclusion, there is a growing need to discover the potential beneficial effects of medical foods, like the extra virgin olive oil, against neurodegenerative disorders and unlock the connections of these pathologies in a molecular level. Current research has set the foundation for future studies and clinical trials for identifying the possible role of olive polyphenols on the human brain.FundingResearch was supported by a Microsoft Azure for Genomics research Grant (CRM:0740983) and by the FrailSafe Project (H2020-PHC-21-2015 – 690140) "Sensing and predictive treatment of frailty and associated comorbidities using advanced personalized models and advanced interventions," co-funded by the European Commission under the Horizon 2020 research and innovation program. EP was supported by the State Scholarships Foundation (IKY) – European Union (European Social Fund, ESF) and Greek national funds through the action entitled "Strengthening Human Resources Research Potential via Doctorate Research" in the framework of the operational program "Human Resources Development Program, Education, and Lifelong Learning" of the National Strategic Reference Framework (NSRF) 2014–2020.

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Human Pluripotent Stem Cells as In Vitro Models of Neurodegenerative Diseases

Vasiliki Machairaki

My main research focused in the last years has been the reprogramming of differentiated cell types, such as human fibroblasts, into pluripotent stem cells called induced pluripotent stem cells (iPSCs) and the application of this technology to studies of the nervous system and the diseases that affect it. We have been working on the generation of iPSC lines from Alzheimer's disease (AD) patients using recent developments in reprogramming strategies such as non-integrating episomal vectors to produce virus-free, clinical safe hiPSC. Our study shows that neurons differentiated from these cells display important disease properties and, thus, have the potential to serve as cellular models to explore various aspects of Alzheimer's pathogenesis. One of the lab's scientific goal is to use lines of familial Alzheimer's disease (FAD)-derived induced pluripotent stem cells (iPSCs) to generate brain-like structures ("organoids") mimicking native brains. Three-dimensional (3D) systems, called cerebral organoids, can recapitulate distinct architectures of the human brain, such as fluid-filled cavities resembling brain ventricles and tissues organized in layers including progenitor ventricular and subventricular zones present in the native brain. Recently, we have extended our research interests in the rapidly emerging field of exosomes and micro-vesicles (called as EMVs). Extracellular vesicles of either 50–200 nm in size (called exosomes) or 200 nm–1 μm in size (called micro-vesicles) are membrane-bounded vesicles that can carry RNAs, proteins, and other metabolites and are secreted from all cell types and are present in biological fluids such as serum and plasma. We have examined properties and functions of EMVs from human iPSCs that can be cultured infinitely under a chemically defined medium and compared them with the ones secreted by human mesenchymal stem cells (MSCs). Purified EVs produced by both stem cell types have similar sizes, but human iPSCs produced 16-fold more EVs than

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MSCs. When iPSC-EMVs were applied in culture to senescent MSCs, they reduced their elevated cellular ROS levels and alleviated aging phenotypes. We are currently exploring the potential application of EMVs in diagnostics, pathology, and therapeutics of AD. Extracellular vesicles secreted from AD patient derived neurons contain a relatively low amount of Aβ but have an increased Aβ42/ Aβ40 ratio; the majority of Aβ is located on the surface of the EVs. The results of our research can contribute substantially to the successful translation of stem cell biology into clinical therapy by improving our understanding of the pathogenesis and treatment of Alzheimer's disease.

Nutritional Lipidomics in Alzheimer's Disease

Efstathia Kalli

1 Introduction

Novel -omic approaches in medical and biological sciences including proteomics, metabolomics, nutrigenomics and lipidomics help researchers understand the molecular effect of diet/nutrients on human health. They also help them to identify metabolites implicated in human disease by gathering different set of data. Metabolomics is the measurement of endogenous metabolite concentrations in different cells and peripheral tissues as well as in biofluids, such as plasma, urine and CSF. In other words, metabolomics is defined as the study of the metabolome such as amino acids, lipids and fatty acids, signaling molecules, metabolic intermediates and secondary metabolites. Measuring these metabolites, molecular mechanisms and pathological pathways at the early stage of a disease can be identified.

Metabolomic research, using a nontargeted liquid chromatography/mass spectrometry conducted by Trushina et al. 2013, clearly demonstrated altered metabolic pathways in both plasma and CSF of AD and MCI subjects compared to cognitively normal subjects. Interestingly, the number of altered pathways increased with disease progression in both plasma and CSF. Both lipidomics and proteomics are two recently used metabolomic approaches to determine new AD biomarkers. The field of lipidomics is the analysis of lipid and lipid derivatives in healthy and diseased tissues and biofluids (blood plasma and serum). It can provide insights into complex metabolic networks and reveal biomarkers at an early stage of the disease. Using the appropriate technique analysis including desorption electrospray ionization mass spectrometric imaging (DESI–MSI) together with computational analysis workflows for omics data interpretation could help researchers to identify and in situ

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evaluate a tissue lipidome. The human lipidome has revealed a remarkable diversity of lipids (Shevchenko and Simons 2010).

Sphingolipids, glycerophospholipids and cholesterol play a crucial role in many cellular functions including cell membrane formation, energy storage and cellular signaling (Wong et al. 2017). The essential fatty acids, docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:4n-6, AA), account for about 8% and 6% of the dry weight of human brain, respectively (Muskiet et al. 2006).

2 Structural Membrane Lipids

Lipid molecules are important constituents of all living cells, whereas structural lipids as constituents of cell membranes contribute to cellular architecture. The three major types of membrane lipids are *phospholipids, glycolipids and cholesterol*. Phospholipids are mixed acid esters of glycerol with two fatty acids and one phosphoric acid moiety. Palmitoyl-CoA and serine condense to form dehydrosphingaganine which in turn converts to sphingosine. It then reacts with a long-chain acyl-CoA to form ceramide. The terminal hydroxyl group is substituted by phosphorylcholine to form sphingomyelin (Stryer 1988).

Phospholipids derived from glycerol are called *phosphoglycerides*. The commonest low-molecular-weight base that can be esterified with the phosphate is choline, and thus the phospholipid is known as *phosphatidylcholine* (lecithin). Other important glycerophospholipids are phosphatidylethanolamine and phosphatidylinositol.

Glycolipids or sugar-containing lipids and *cholesterol* are also found in many plasma membranes of eukaryotic cells. Most of the membrane's phospholipid and glycolipid molecules are arranged in a bilayer. The glycolipids are the most common membrane components of the central nervous system and peripheral nerve tissues. Cholesterol inserted between the fatty chains in the bilayer plays a crucial role in stabilizing the hydrophobic interactions within animal membranes allowing their proper functioning. It is abundant in cell membranes, up to one molecule for every phospholipid molecule (Stryer 1988).

3 Synthesis of Eicosanoids

Eicosanoids are bioactive oxygenated metabolites derived from DHA, AA, and EPA. The family of eicosanoids include prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), lipoxines (LXs), hydroperoxyeicosatetraenoic acids (HPETEs), and hydroxyeicosatetraenoic acids (HETEs) (Calder 1998). Eicosanoid dihomo-γ-linolenic acid is stored as phospholipid within cell membranes, where it serves as a precursor of prostaglandins designated E_1. Arachidonic acid is also stored within the phospholipid fraction of cell membranes, where it acts as a precursor to the family

of prostaglandins designated E_2. Considering the metabolism of the n-3 fatty acids, eicosapentaenoic acid is also stored within the phospholipid fraction of all membranes, where it may be converted to the family of prostaglandins designated E_3 and thromboxanes. In other words, PGE_1, PGE_2, and PGE_3 and other series are derived from dihomo-γ-linolenic acid, arachidonic acid, and eicosapentaenoic acids, respectively (Bergstrom et al. 1962).

4 Lipidomics in Alzheimer's Disease

4.1 Essential Fatty Acids and Prostaglandins in AD

The essential fatty acids are involved in the optimal membrane fluidity, intracellular signaling pathways, transcription factor activity, and gene expression (Logan 2004). According to Nys and Debruyne (2011), both EPA and DHA may increase neuronal and glial survival and in parallel may reduce the process of neuroinflammation.

Using animal studies and brain cells in culture, researchers found expression of cox-2 and an overactivation of PLA2 during chronic degenerative diseases such as AD. As indicated by Bazan et al. (2002), prostaglandin production and PLA2 activation initially trigger the neuropathology events in AD. It is well documented that the biosynthesis and metabolism of PGE2 in CSF may indicate the severity of Alzheimer's disease in an inverse manner. In particular, the levels of PGE2 were higher in MCI patients and lower in patients with advanced AD (Combrinck et al. 2006).

The role of n-6 PUFA and oxidized eicosanoid derivatives of n-6 PUFA have recently been reviewed as contributing to β-amyloid deposition, a hallmark of AD onset and progression (Whelan 2008). AA is located in several different cells in both the gray and white matter in the brain (Corsinovi et al. 2011). The role AA plays, in oxidative stress and lipid peroxidation, has already been discussed in relation to nonalcoholic fatty liver disease. However, oxidative stress and production of ROS has also been suggested to play a role in AD, thus suggesting a role of AA and lipid oxidation products (eicosanoids) in the onset and progression of the disease. Furthermore, the enhanced consumption of n-6 PUFA leads to an excessive production of the proinflammatory cytokines derived from AA through COX and LOX enzymatic activity which lead to brain damage. As an example, a study fed transgenic mice with memory impairment and β-amyloid deposition, a diet poor in n-3 PUFA but rich in n-6 PUFA. As a result, they had a significant decrease in the postsynaptic receptor complex in the brain which regulates memory and learning and a net potentiation of programmed cell death (Calon et al. 2005).

Both DHA and EPA have been shown to competitively counteract the production of proinflammatory eicosanoids derived from n-6 PUFA in the brain of AD patients. The neuroprotective role of EPA has been demonstrated since EPA competes with AA for incorporation into cell membrane phospholipids and for oxidation by the

COX enzyme, thus exerting anti-inflammatory actions. The resulting production of anti-inflammatory PGE_3 might result in decreased levels of proinflammatory PGE_2. The balance between the n-6 and n-3 PUFA ratio may therefore play a crucial role in the onset of AD. Such a dietary pattern consisting of a more balanced n-6:n-3 PUFA ratio may be a therapeutic tool in the pathogenesis of AD.

A novel transgenic model study with Tg2576 mouse expressing AD pathologies revealed that a diet could affect the fatty acid composition of different phospholipids in brain region (Bascoul-Colombo et al. 2016). More precisely, according to the results, the Tg2576 mouse corresponded to supplementary DHA diet regarding the phosphatidylethanolamine (PE) classes and ethanolamine plasmalogens rather than phospholipids (PL) classes. After undertaking a lipidomic approach, significant alterations in lipid composition have been observed in the cortex and hippocampus. Although further studies are needed to confirm a relation between low DHA levels in brain PE and AD, these findings indicate a protective role of dietary DHA in maintaining cognition by altering the brain lipid content.

Although interaction between peripheral lipid metabolism and the brain still remains unclear, Astarita and Piomelli (2011), using a multiorgan lipidomic approach, demonstrated that free DHA and DHA-containing glycerophospholipids were significantly lower in mid-frontal cortex of AD patients compared to control subjects. In addition to DHA molecules, a DHA-derived lipid mediator known as neuroprotectin D1 (NPD1) was reported to be lower in temporal lobes and in mid-frontal cortex of AD patients. That molecule is anti-inflammatory signal that contributes to survival of neuronal cells under uncompensated oxidative stress (De Roos et al. 2009; Belayev et al. 2017) through gene-expression programming that prevents the Aβ42-derived neurotoxicity (Lukiw et al. 2005). In addition, concentrations of liver DHA were reported to be reduced in AD patients compared to control subjects after using a linear regression analysis and taking into account confounding variables such as age, gender, and postmortem interval. The same study revealed that the apparent dysfunction in liver-mediated DHA biosynthesis was associated with visual and cognitive impairments in AD patients (Astarita and Piomelli 2011).

4.2 Dietary Cholesterol and AD Pathology

Cholesterol is the most widely distributed sterol in animal and human tissues. This simple lipid is a major constituent of the human brain since it accounts for about 20% of the body's total cholesterol and since it is present in the central and peripheral nervous system. Cholesterol is a crucial molecule for brain development, synaptogenesis, synaptic plasticity, dendrite formation, and proper neurotransmission.

Although a systematic review with meta-analysis failed to show any significant association between total serum cholesterol – at midlife – and cognitive impairment (Anstey et al. 2008), other researchers support the theory that elevated plasma cholesterol may contribute to the pathogenesis of AD, through different mechanisms

(Ledesma and Dotti 2006). As indicated in previous studies, the blood-brain barrier plays a crucial role in the homeostasis of the brain's microenvironment, and dysfunction of BBB precedes neurological diseases such as stroke and AD (Carvey et al. 2009).

Using the wild-type mice model, researchers reviewed the impact of high-cholesterol diet on the pathology of the disease. The results indicated that high dietary cholesterol increases cholesterol circulation in brain parenchyma and causes the aggregation of free cholesterol in neurons. This cholesterol buildup changes both the structure and the function of endolysosomes resulting in significant depositions of Aβ and phosphorylated tau in olfactory bulb neurons (Chen et al. 2010). Cholesterol-fed rabbits, 1% or 2% for 6 or 12 weeks, respectively, came up with increased levels of AB in the hippocampus and cortex, as demonstrated with immunohistochemistry and ELISA assays.

Numerous studies highlight the participation of dietary cholesterol in the Aβ deposition and thus in the AD pathology. In a transgenic-mouse model for AD amyloidosis, Refolo et al. (2000) examined the implications of a high-fat/high-cholesterol diet on the Aβ accumulation of the central nervous system (CNS). The authors, following a biochemical and a neuropathological analysis, concluded that hypercholesterolemia induces alterations in amyloid precursor protein processing and significantly increases the size and number of β-amyloid deposits.

4.3 Lipid Metabolites in AD and MCI Subjects

Lipidomic analyses suggest that altered lipid pathways in the brain and the peripheral tissues may affect the progression of the disease (Quehenberger and Dennis 2011; Astarita and Piomelli 2011). For instance, in an animal model study, APP/tau mice demonstrated increased levels of docosahexaenoyl (22:6), cholesterol ester (ChE), ethanolamine plasmalogens (pPEs), and sphingomyelins (SMs) compared to controls (Tajima et al. 2013).

Levels of isoprostane 8,12 were increased in both AD and MCI groups compared to age-matched controls ($P < 0.001$), suggesting an increased process of lipid peroxidation. Higher ceramide/sphingomyelin ratios (Han et al. 2011) and reduced ratio of desmosterol/cholesterol (Sato et al. 2012) have also been observed in the disease state. It has been noted that ceramides prolong the half-life of the enzyme beta-secretase or BACE 1 and through this mechanism facilitate the Aβ production.

Sphingolipids were altered in mild AD or during the predementia stage of mild cognitive impairment (MCI). In order to examine any relation between plasma ceramides and cognitive impairment, 100 women, aged 70–79, at baseline, in Women's Health and Aging Study II (WHAS II), were recruited and followed over 9 years. A high-performance liquid chromatography (HPLC) combined with electrospray ionization tandem mass spectrometry technique revealed that low levels of serum ceramides were associated with memory impairment in a cross-sectional way. However, high levels of ceramides predicted prolonged impairment, indicating

that these lipids could be biomarkers of how Alzheimer's disease progresses (Mielke et al. 2010).

A growing body of lipidomic studies supports that glycerophospholipids, reduced levels of desmosterol (Pratico et al. 2002) and sphingomyelin (SM [39:1]) (Olazarán et al. 2015), as well as lower levels of nonesterified fatty acids (22:6n-3, DHA) (Proitsi et al. 2015) are found in AD subjects compared to age-matched controls ($P < 0.01$). Moreover, higher levels of phosphatidylethanolamine (PE [36:4]) were found in AD groups versus age-matched controls (Olazarán et al. 2015). Desmosterol, sphingomyelins, and ceramides could show the presence of an abnormal lipid peroxidation and a dysfunction in integrity of cell membranes, and therefore, these specific lipids could act as biomarkers of AD. A disturbed cell membrane integrity at the preclinical stage of AD has also been demonstrated by Mapstone et al. (2014). In this observational study, researchers discovered an altered lipid profile in peripheral blood of community-dwelling participants with MCI and AD compared to matched controls, detecting the preclinical stage of the disease. Among metabolites, higher levels of dioleoylphosphatidic acid and lower levels of phosphatidylinositol and phosphatidylcholine (C38:4) were seen in the plasma of the AD group compared to the other two groups. In accordance to these findings, decreased phosphatidylinositol has been detected in the hippocampus (Prasad et al. 1998) and other cortical regions of patients with AD (Pettegrew et al. 2001). The data from the lipidomic analysis predicted progression from normal cognition to either amnestic mild cognitive impairment (aMCI) or mild AD over a 2–3-year period with 90% accuracy.

Furthermore, using combined gas chromatography/mass spectrometry methods, researchers (Lütjohann et al. 2000; Papassotiropoulos et al. 2002) determined increased plasma and CSF levels of 24S-hydroxycholesterol in AD and MCI patients. 24S-Hydroxycholesterol is a steroid derivative which crosses the blood-brain barrier and is converted enzymatically via cholesterol 24S-hydroxylase from CNS cholesterol (cholesterol + NADPH + oxygen → 24-hydroxycholesterol + NADP + water). According to Wishart et al. (2018), this enzyme is primarily located in the neuron region, and this pathway contributes to the brain cholesterol metabolism and the omission of brain cholesterol. There is speculation that changes in cholesterol metabolism may be involved in the pathogenesis of AD, and as a result the metabolite of 24S-OH-Chol could be used as a potential biomarker for the progression of the early stages of the disease. Apart from 24S-hydroxycholesterol, another oxidated product of cholesterol, known as 27-hydroxycholesterol, is also capable of crossing the blood-brain barrier. The ratio of 27-OH cholesterol to cholesterol, in patients with AD, with mild cognitive impairment and with vascular dementia appears to be lower compared to normal cognitive subjects (Kolsch et al. 2004).

Wisniewski et al. (2013) conducted a postmortem human study in order to investigate the metabolism of cholesterol in AD progression. Analysis of brain tissue samples by HPLC-MS combined with NMR showed that the concentration of desmosterol which is an intermediate in the synthesis of cholesterol was lower in AD patients compared to age-matched controls. In another study conducted by Sato et al. (2012), plasma desmosterol levels were also lower in AD and MCI patients,

compared with healthy elderly controls. Interestingly, significant changes in plasma desmosterol and in desmosterol/cholesterol ratio were noticeable in female AD patients and were also well correlated with the CSF desmosterol/cholesterol ratio.

Moreover, rats with diet-induced hyperlipidemia showed increased levels of galactosylceramide and sulfatide, in their hippocampus, leading to neural apoptosis (Stranahan et al. 2011). The proportion of sulfatide was found to be decreased in white matter and depleted up to 93% in gray matter in very early stages of Alzheimer's disease. Thus, sulfatide deficiency possibly indicates the earliest clinical stage of AD, according to Han et al. (2002). In addition, ceramides appeared to be at their highest concentration in white matter and at the stage of very mild dementia.

4.4 Saturated Fat and Brain Lipidome

High dietary intake of saturated fats enhances the brain's fatty acid uptake from plasma as indicated via positron emission tomography (PET) (Karmi et al. 2010). Dietary factors, in particular saturated fatty acids, have been suggested to enhance the production of the cytokines IL-1, IL-6, and TNF-α. All these cytokines possess proinflammatory functions and act as neuroinflammatory mediators in astrocytes and microglia causing neuronal death (Wang et al. 2012; Gupta et al. 2012). Contrary to the effects of DHA, a typical Western diet with 40% saturated fatty acids and 1% cholesterol increased the hippocampal Aβ deposition in transgenic APPswe/PS1dE9 mice, within 3–4 months (Oksman et al. 2006).

Going further in the field of lipidomics, Giles et al. (2016) conducted a study with male C57Bl/6 mice, in order to investigate whether plasma lipids derived from a diet rich in high saturated fatty acids are associated with modifications in cerebral lipid homeostasis. Male C57Bl/6 mice were fed either with a normal diet or with a SFA diet for 6 months. According to the analysis technique, lipids from the plasma, hippocampus, and cerebral cortex were analyzed by LC-ESI-MS/MS. The brain lipidome composition approach revealed significant differences in 50 lipid categories. The high-SFA diet-fed mice resulted in phosphatidylcholine (PC), phosphatidylethanolamine (PE), alkyl-PC, alkenyl-PC, alkyl-PE, alkenyl-PE, cholesterol ester (CE), diacylglycerol (DG), phosphatidylinositol (PI), and phosphatidylserine (PS) classes. These lipid species in the cerebral cortex were strongly relative to plasma lipid homeostasis.

5 Conclusion and Future Research

As stated in previous studies, diet can affect the lipid profile analysis in lipidomics. High intake of saturated fat and a dietary DHA deficiency are both capable of modifying the brain lipidome composition. Alterations in different lipid metabolites

including desmosterol, sphingomyelins, and ceramides are thought to take place in AD progression.

Patients presented significant reductions in sphingomyelin, desmosterol, and sulfatide as well as in DHA and in 27-OH cholesterol-to-cholesterol ratio. Furthermore, increased ceramide content and increased levels of isoprostane 8,12 were observed in MCI and AD patients, leading to neuronal cell death and lipid peroxidation, respectively.

Blood-based or CSF lipid biomarkers such as phospholipids, ceramides, and desmosterol should be investigated in the context of dietary patterns such as overall fat and especially saturated fat intake as well as DHA consumption and/or dietary (n-3)/(n-6) fatty acid ratio. Moreover, lipid biomarkers should be checked for correlation with other biomarkers of AD including PET imaging and concentration of Aβ, tau, and phosphorylated-tau protein. Confounding factors such as age, gender, and APOE genotype may all affect lipid metabolites in disease states. Validation and reproducibility from different laboratories are also two major concerns in the emerging field of lipidomics (Wong et al. 2017). A lipidomic approach combined with proteomics and genomics would allow early detection of lipid biomarkers, resulting in early AD diagnosis. This, in turn, would help researchers to design an individualized-based treatment and a personalized diet therapy at the early stages of the disease.

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Alzheimer's Disease Therapeutic Approaches

Maria Revi

1 Introduction

Alzheimer's disease (AD) is the most common cause of dementia, and with a new case occurring every seven seconds globally, the disease itself is becoming a slow pandemic (Ferri et al. 2005). One person for every 85 individuals can be expected to suffer from AD by the year 2050 (Brookmeyer et al. 2007). AD also imposes tremendous emotional and financial burden to the patient's family and community through the provision of care and loss of wages. The disease can be classified based on the age of onset, into early-onset AD and late-onset AD. Early-onset AD accounts for approximately 1%–6% of all cases and manifests roughly between 30 and 60 years of age. Late-onset form, accounting for around 90% of cases, has an age of onset later than 60 years of age. Etiology of AD is multifactorial with genetic, environmental, behavioral, and developmental components playing a role. The greatest risk factor is advancing age, others being a positive family history, head trauma, female gender, previous depression, diabetes mellitus, hyperlipidemia, and vascular factors (Kivipelto et al. 2001). The understanding of the pathophysiology of AD is constantly changing, for instance, the tangles, a well-known pathological hallmark of AD, earlier thought to be responsible for the disease, now rather seem to reflect the damage which the neurons have endured over a long period of time. The notion that amyloid beta peptide (Aβ) and phosphorylated tau are pathologic molecules is slowly changing, and it seems that they are present as a cellular adaptive strategy to oxidative stress. Apart from them, various deranged mechanisms such as chronic oxidative stress, mitochondrial dysfunction, Aβ production, neurofibrillary tangles accumulation, hormone imbalance, inflammation, mitotic dysfunction, calcium mishandling, and genetic components play a role in the disease process (Hippius and Neundorfer 2003;

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Dementia Fact Sheet No 362; Todd et al. 2013; Godyn et al. 2016; Graham et al. 2017; Dubois et al. 2014; Cummings et al. 2014). Although the mechanisms are diverse, the inevitable event of neuronal death occurs, resulting in AD.

2 Management of AD

In 1993, the release of tacrine, the first reversible acetylcholinesterase inhibitor (AChEI), reached the market for the treatment of AD but was withdrawn soon after because of reports of liver toxicity (Farlow et al. 1992; O'Brien et al. 1991). Three other cholinesterase inhibitors, donepezil, galantamine, and rivastigmine are currently used in the treatment of AD to reduce activity of acetylcholinesterase. These agents do not delay the progression of dementia but temporarily slow the loss of cognitive function.

The first representative of a new class of anti-Alzheimer molecules is memantine. Memantine works by blocking specific types of receptors called NMDA receptors to which transmitter glutamate usually adheres. A change in the way glutamate transmits signals within the brain is related to memory loss that accompanies the AD. Furthermore, hyper stimulation of NDMA receptors can result in damage or death of the cells. By suspending NDMA receptor, memantine improves the transmission of signals in the brain and reduces the symptoms of the disease.

The currently available treatment strategies include AChEIs and NMDA receptor antagonists (Silvestrelli et al. 2006). In order to modify the disease process, novel strategies have been developed. In this regard major developing is focused on the Aβ- and tau- based therapeutics, which could play a fundamental role in unlocking this disease in the near future (Anand et al. 2014; Kurz and Perneczky 2011). In this paper we highlight the currently approved pharmaceutical treatments.

Although AD is known for about a century (Ramirez-Bermudez 2012), four cholinesterase inhibitors and memantine are the only drugs approved by the US Food and Drug Administration for its treatment. These drugs provide symptomatic treatment but do not alter the course of the disease. Hence the modern therapeutic options that target the disease modification part are on a rise. The multiple mechanisms involved in the pathogenesis of AD create considerable difficulty in producing an effective treatment.

Unfortunately, none of the presented therapies can stop the progressive loss of neurons, and there is no treatment that can inhibit the progressive deterioration of cognitive abilities of patients. Consequently, the development of new drugs with strong properties against the disease is one of the greatest challenges of modern medicine.

According to recent studies, the synergistic action of acetylcholinesterase inhibitors (donepezil, galantamine, rivastigmine) and memantine seems to give better and promising results for the treatment of AD (Atri et al. 2013a). Combination therapies appear to have a better effect on cognitive and functional behaviors in patients with moderate to severe AD (Tariot et al. 2004; Porsteinsson et al. 2008a).

The clinical support for combination therapy is not complete, as it is based on the addition of memantine to existing AChEI treatment, with no data on the opposite order of initiation or the simultaneous treatment de novo with memantine and an AChEI.

3 Modulating Neurotransmission

The cholinergic group of neurons is the main neurotransmitter system involved in AD, and basal forebrain cholinergic loss is a well-recognized pathology. These neurons maintain cortical activity and cerebral blood flow and modulate cognition, learning, and task- and memory-related activities, development of the cerebral cortex, and regulation of sleep-awake cycle (Berger-Sweeney 2003; Schliebs and Arendt 2006). Considering the many functions of the cholinergic neurons, the symptom complex in AD can at least be partially understood. The dysfunction of the cholinergic system in AD occurs at various levels including a decrease in choline acetyltransferase activity and choline uptake, a decrease in acetylcholine synthesis (Slotkin et al. 1990), and altered levels of acetylcholine receptors (AChRs) (Xu et al. 2012). Glutamate is the primary excitatory neurotransmitter in the hippocampal and neocortical regions of the brain, and it plays a significant role in the cognition, learning, and memory process. The postsynaptic membrane has high density of the N-methyl-D-aspartate (NMDA) receptor. Studies have shown that there is an extracellular glutamate excess in AD, contributed both by an increased presynaptic glutamate release and decreased reuptake which, in turn, lead to a tonic activation of NMDA receptors (Revett et al. 2013a). The excitotoxicity is "slow" in contrast to the acute or rapid form that occurs with stroke or epilepsy. Impaired insulin signaling along with mitochondrial dysfunction and receptor abnormalities (Beal 1992) can be predisposing factors when glutamate is excitotoxic even at physiological concentrations (Novelli et al. 1988). Dysfunction in other neurotransmitter systems such as g-aminobutyric acid (GABA), histamine, and serotonin systems of neurons also leads to AD. The modulation of neurotransmission with drugs continues to remain the best approach in providing symptomatic improvement in patients. As of late, mechanistic insights into the disease modifying aspects have also been highlighted.

4 Cholinesterase System

The four acetylcholinesterase inhibitors (AChEIs) approved by the US Food and Drug Administration for the treatment of AD are tacrine, donepezil, rivastigmine, and galantamine. However, tacrineis now rarely used because of its hepatotoxicity (Watkins et al. 1994). AChEI enhance cholinergic neurotransmission through inhibition of acetylcholinesterase (AChE), thus decreasing the breakdown of acetylcholine.

It is clear that, when cholinergic transmission occurs, or their receptors become activated, there is an increase in long-term potentiation. The cholinergic AChRs are expressed on principal and inhibitory interneurons, both pre- and postsynaptically in most regions of hippocampus. Thus, the increase in acetylcholine levels in the synaptic cleft results in bidirectional influences (Drever et al. 2011). Galantamine also possesses agonist activity at the nicotinic a4β2 receptor subtype, and its clinical benefits are probably due to both mechanisms (Coyle and Kershaw 2001). More recently, it was shown that the muscarinic M1 AChRs are present in intracellular locations, especially in the hippocampal regions (Anisuzzaman et al. 2013). The cell surface M1 AChRs activate the phosphatidylinositol cascade, whereas intracellular M1 AChRs activate the extracellular regulated kinases 1/2 (Anisuzzaman et al. 2013). Both the pathways regulate long-term potentiation and synaptic plasticity. Cholinergic transmission also plays a role in modulating the mechanisms involved in adult neurogenesis (Bruel-Jungerman et al. 2011). In addition, studies suggest that AChEIs alleviate oxidative stress (Klugman et al. 2012). Various short-term trials with AChEI monotherapy have shown clinically apparent and encouraging improvement in cognitive function, reduction in the pace of functional decline or clinical worsening compared with placebo, as well as a decrease of behavioral symptoms in mild to moderate and moderate to severe AD patients. Data from meta-analyses also attest to the same fact (Birks 2006). Efforts to efficiently deliver standard drugs are another area of development. Transdermal delivery systems for all the three drugs are available but not currently approved. The oral dosing of AChEIs increases the plasma drug levels in a short time interval, which probably accounts for the observed gastrointestinal side effects. The transdermal patches can ensure "peak less" and prolonged delivery with minimal fluctuations in the plasma drug concentrations (Lefevre et al. 2008). Rivastigmine patches (9.5 mg/24 h) produced plasma drug concentrations and results that were comparable with oral capsules (12 mg/day). However, safety and tolerability profile of the patches were better. The subjects also experienced significantly decreased discomfort with patches (Winblad et al. 2007). Some novel AChEI molecules have also been developed. Memogain (GLN-1062; Galantos Pharma), the benzoyl ester of galantamine, is a pro-drug that is available as intranasal formulation. The drug has shown excellent efficacy and central nervous system (CNS) bioavailability in animal studies (Maelicke et al. 2010). Huperzine A is a natural alkaloid isolated from the Chinese moss shrub (*Huperzia serrata*) and possesses AChE-inhibiting action with modest effects on the amyloid precursor protein's (APP) metabolism and neuroprotection (Zhang et al. 2008). The drug showed promising safety profile in both phase I and phase II trials. At a dose of 400 mg twice a day, the drug was able to improve cognitive outcome by 2.27 points in patients with mild to moderate AD (Rafii et al. 2011). A pro-drug of huperzine, ZT-1, has shown admirable pharmacokinetic profile in a recent phase I study (Jia et al. 2013). Methanesulfonyl fluoride (SNX001) is an irreversible inhibitor of AChE, first reported in 1999 for its therapeutic value in AD (Moss et al. 1999). Preclinical studies demonstrating its benefit in cognition have recently revived the interest in the molecule. A phase I trial has studied the extent of AChE inhibition on healthy subjects and has shown promising results (Moss et al. 2013). Direct modulation of the cholinergic AChRs is also under considerable evaluation.

In AD, the levels of presynaptic M2 AChRs decrease, but those of postsynaptic M1 AChRs remain unchanged. A number of M1 partial agonists like AF102B, AF150(S), AF267B, and AF292 as well as allosteric agonists such as 77-LH-28-1, LY-593093, and Lu AE51090 are available. ML 169 is a recently reported M1 positive allosteric modulator (Reid et al. 2011). The strong side of the M1 agonists seems to be their role in APP processing and thus indirectly on other processes such as tau phosphorylation. Studies have shown that ablation of M1 AChRs leads to increased amyloid β(Aβ) generation (Medeiros et al. 2011). AF102B, an M1 partial agonist, significantly lowered CSF Aβ levels in AD patients (Nitsch et al. 2000), where AChEIs showed no effect (Parnetti et al. 2002). AF150(S) and AF267B have also shown promising results in the preclinical setup (Fisher 2008; Fisher et al. 2002). Another mixed muscarinic/s1 agonist, ANAVEX 2-73, is currently in phase I/IIa trials. This compound has partial agonistic activity at both muscarinic AChR and s1 protein (chaperone protein in endoplasmic reticulum activated by unfolded protein response) (Collina et al. 2013). In animal studies ANAVEX 2-73 attenuates Aβ-induced memory deficits and toxicity, decreases seeding of Aβ, and blocks the activation of glycogen synthase kinase-3 (GSK3b) and in turn the hyperphosphorylation of tau (Lahmy et al. 2013). While we slowly begin to understand the therapeutic potentials of muscarinic AChRs, the role of nicotinic AChRs in AD is debatable at best. Different neuronal systems express these receptors, and they play diverse functional roles in cognition, memory processes, trophism, and neuroprotection (Wallace and Bertrand 2013). Recent evidence has also uncovered their pathological side, and the possible mechanisms by which nicotinic AChRs may contribute to the pathophysiology of AD (Hernandez and Dineley 2012; Parri and Dineley 2010). Thus, their modulation is governed by a subtle balance. The levels of nicotinic receptors may remain unchanged or even upregulated, with progression of the AD process (Ikonomovic et al. 2009). Evidence shows that nicotinic AChR agonists produce both beneficial and damaging effects on neuronal function; hence, the net effect is not clear (Fisher 2012). Studies have shown that a7 nicotinic agonists attenuate Aβ-mediated toxicity (Kihara et al. 2001) but, on the other hand, modify the reactivity and increase the phosphorylation state of tau protein (Hellstrom-Lindahl et al. 2000). How modulating the same receptor decreases one pathology but increases the other is not clear. More surprisingly, antagonists of a7 nicotinic AChRs also cause similar effects (Mousavi and Hellstrom-Lindahl 2009). A few recent studies highlighted the cognitive enhancing potential of cotinine, a metabolite of nicotine, compound which is a positive allosteric modulator of a7 nicotinic AChRs (Echeverria and Zeitlin 2012). The properties which make cotinine a better ligand than nicotine would be its low toxicity profile, its nonaddictive nature and suitable blood-brain barrier clearance (Echeverria and Zeitlin 2012). Apart from its receptor modulation, the drug also inhibits Aβ aggregation (Echeverria et al. 2011). The pharmacokinetic and safety profile of cotinine has already been investigated in humans (Benowitz et al. 1983; Bowman and Mc 1962), but no documentation is available regarding its role in AD. Many novel ligands for a7 nicotinic AChR are also currently in development (Toyohara and Hashimoto 2010). EVP-6124 is a novel selective a7 partial agonist that improves memory performance in animals (Prickaerts et al. 2012). The compound has successfully completed phase II trials (NCT01073228). MT-4666 is another nicotinic

agonist that is currently in phase II trials (NCT01764243). Among other novel compounds, ABT-384 (NCT01137526) has completed phase II trials, while there is an ongoing trial with ABT-126 (NCT01527916). To summarize, the advantage of M1 agonists over AChEIs could be their potential role as disease modifying agents along with their symptomatic benefits. The role of nicotinic modulators, on the other hand, is not clear. From this group, only AChEIs are currently used in the clinical setting, the direct-acting ligands described would require more convincing evidence. Since neuronal dysfunction starts early in the course of the disease, the utility of AChEIs is to provide symptomatic relief during the transitional period by sustaining the function of the available neurons. However, with increasing neuronal damage, the therapeutic effectiveness of AChEI slowly diminishes. In regard to how long the drugs remain valid or how long the patients should be treated with AChEI, the answers vary. Reports indicate that the benefits may last up to 4 years (Rogers et al. 2000). The benefit of AChEIs in the behavioral symptoms of AD and their synergistic role in combination therapy with memantine are addressed in the following section.

5 N-Methyl-D-Aspartate Antagonism

Neuronal pathology in AD also extends to the glutamatergic system but at a later stage of the disease. Glutamatergic neurons regulate synaptic plasticity, neuronal growth, as well as differentiation, cognition, learning, and memory (Butterfield and Pocernich 2003). A "glutamate cycle" occurs between the pre- and postsynaptic neurons and astrocytes that determine the synaptic concentration of glutamate available for the receptors (Revett et al. 2013b). Cycle defects occur at different levels in AD, leading to a state of extracellular glutamate accumulation, increased NMDA receptor activation and excitotoxicity (Revett et al. 2013b). A large body of evidence shows mutual interaction between NMDA receptors and Aβ peptides. Studies suggest that NMDA receptors' activation leads to Aβ production and vice versa (Aβ oligomers binding and activating NMDA receptors), further substantiating the importance of the glutamatergic system in AD (Revett et al. 2013b; Dinamarca et al. 2012). Memantine is an uncompetitive NMDA antagonist; it has voltage dependency, rapid blocking kinetics, and moderate affinity and blocks the channel by trapping it in open conformation (Gilling et al. 2009). Mg^{+} ions block the NMDA channel under resting conditions, when glutamate is available the blockade is relieved, and the NMDA channel is now open for Ca^{+2} inflow. In pathological states such as AD, there is a low and persistent state of NMDA activation even at resting periods. In such states, Mg^{+2} ions are excluded from the channel, thereby allowing continuous Ca^{+2} flow across the membrane. The moderate affinity and voltage dependency property of memantine allows it to block the persistent NMDA activation and is, thus, beneficial in AD. Evidence also indicate that memantine-mediated blockade is relieved by high glutamate concentrations in the synaptic cleft. Hence, when a physiological

impulse arrives, the glutamate overrides the memantine blockade, and physiological transmission can continue without interference (Parsons et al. 2013). Experimental evidence shows that memantine treatment improves spatial learning in animal models of AD, protects neurons from Aβ induced toxicity, and decreases apoptosis, free radical-mediated damage, and restored synaptic degeneration (Miguel-Hidalgo et al. 2012). Reportedly, memantine also seems to have antagonizing effects on other receptors such as a7 and a4b2 nicotinic AChRs (Buisson and Bertrand 1998; Maskell et al. 2003), 5-HT3 (Rammes et al. 2001), a3b2 (Lee et al. 2012), 5-HT2A, dopamine D2 receptors, and histaminergic neurons (Motawaj et al. 2011; Nakaya et al. 2011). Hence, the therapeutic benefits of memantine may not be strictly due to its effect on NMDA alone. However, except of the memantine's effects on a7 nicotinic AChRs, there is no solid evidence to show that its impact on other receptors occurs at the therapeutically administered concentrations in AD (Rammes et al. 2008). Currently memantine is the only drug approved for clinical use in moderate to severe AD in the USA and Europe. Several studies show convincing evidence of memantine's value (Hellweg et al. 2012; McShane et al. 2006). Although the effect of memantine is evident in late stages, its role in early AD is unclear. The three main studies that have studied the role of memantine in mild to moderate AD show that it has some beneficial effects on cognitive and global functioning status, but it does not impede the progression of the disease (Bakchine and Loft 2008; Peskind et al. 2006; Porsteinsson et al. 2008b). A recent meta-analysis also indicates the same (Schneider et al. 2011). Memantine's non-beneficial role in the early stages of AD is not well understood yet. The involvement of cholinergic neurons probably occurs early on in the disease, but damage to glutamatergic system and excitotoxic degeneration occurs late in the course of the disease (Ni et al. 2013). The effect of memantine on other receptor channels might as well play a role here. Memantine also blocks a7 nicotinic AChRs more potently at therapeutic concentrations (Aracava et al. 2005). This blockade could affect neurotransmission during the early stages of the disease when functioning cholinergic neurons are still available. Hence as of now, the use of memantine is restricted to the later stages of the disease. A number of investigators have looked at the possible advantage of memantine combination with AChEI in AD (Tariot et al. 2004; Porsteinsson et al. 2008b; Atri et al. 2013b; Dantoine et al. 2006; Howard et al. 2012; Lopez et al. 2009; Riepe et al. 2007). Results from most studies indicate that addition of memantine to AChEI may add to the therapeutic value and improve clinical outcome in subjects. However, two recent systematic reviews have concluded that there may be a few significant favorable changes from the combination therapy, but it is not currently recommended (Farrimond et al. 2012; Muayqil and Camicioli 2012). There is a current ongoing trial of memantine and donepezil combination therapy in moderate to severe stages of AD (NCT00866060). A "once-daily" fixed dose combination of memantine and donepezil has also been developed (ADS-8704, Adamas pharmaceuticals). The above compound is currently in phase III trials.

6 Conclusion

The pathophysiology of AD involves disturbances and imbalances, occurring in a variety of mechanisms. In spite of the wealth of knowledge that exists regarding AD, only a handful of options are currently available for its management. The disease process is also complex in its own ways. At this time, symptomatic treatment is the best way of AD management. However, incredible leaps have taken place in developing disease-modifying approaches. Recent evidence indicates the disease modifying potential of the previously thought symptomatic drugs (AChR ligands and memantine), and it is believed that their proper usage will improve the clinical outcome of AD.

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Stress and Wellbeing of Psychiatry Trainees: A Literature Review

Rhoda Lai and Christos Plakiotis

1 Introduction

There has been a wave of recent media coverage in Australia of the stress levels of trainee doctors, brought to the nation's attention by multiple tragic suicides (Reynolds 2017; Ripp et al. 2015; Verghis 2018). For psychiatry trainees in the state of Victoria in particular, a range of recent changes to training and workload have been introduced, which bring issues about trainee stress and wellbeing into question.

The Royal Australian and New Zealand College of Psychiatrists (RANZCP) changed the structure of the Fellowship training programme in 2012 to a competency-based one (Jurd et al. 2015), introducing a host of new programmatic assessments to be completed across the training period. While this has reduced concentrated periods of high stress by taking some pressure off examinations, it has also resulted in previously informal supervision sessions becoming assessment-oriented and the blending of the roles of supervisor and assessor.

A new Mental Health Act was also introduced to Victoria in 2014 (State Government of Victoria 2014). While the new act is designed to better protect patient rights, this has come with the need for clinicians to prepare much more detailed Mental Health Tribunal reports for compulsory patients. In addition to the heavier administrative burden, clinicians, who are often trainees, must also attend more frequent tribunal hearings in person. The changes have been expected to be

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incorporated into the already-heavy on-site clinical and study commitments of psychiatry trainees, without an increase in staffing to compensate for the extra workload.

Anecdotally, media articles have cited "long and irregular work hours, social isolation, a lack of autonomy and job insecurity" as stressors that trainee doctors face in the workplace (Reynolds 2017). However, a review of whether evidence supports these factors is timely in the wake of these changes to Victorian trainee psychiatrists' workplaces. It is important to evaluate, based on current knowledge of the factors leading to trainee stress, whether these changes may exacerbate the problem of overstressed trainees. A current review of the literature will also contribute to evaluating possible solutions for trainee stress and burnout and the growing problem of trainees using tragic methods to end their suffering.

1.1 Objectives

This paper examines and reviews the current literature on the contributing factors towards stress and wellbeing in trainee psychiatrists and how the recent changes to their training and workload may impact on them. Suitable articles were identified using the search terms "trainee OR resident OR registrar AND psychiatry AND stress OR wellbeing" (and all related terms) in the PubMed database. Original research articles and reviews on contributors to psychiatry or medical trainee stress or wellbeing, especially those written from a Victorian or Australian perspective, were selected, and other relevant articles were found from their reference lists.

2 Factors Contributing to Stress in Trainee Psychiatrists

2.1 Working Hours and Workload

Psychiatry registrars, or trainees, in Australia undergo 5 years of specialist training in psychiatry that combines clinical supervision and workplace-based learning with academic learning (Jurd et al. 2015). However, while this combination of work and study was designed to fit into full-time hours, the reality for most trainees is that there is too much work required to complete within these hours (Rotstein and Jenkins 2017). In fact, Rotstein and Jenkins' (2017) recent survey of Australian and New Zealand psychiatry trainees found that "having too much work in too little time" was the main stressor for the majority of their respondents.

This appears to be a universal problem among trainees. In a survey of nearly 2000 postgraduate medical trainees in Canada, only 1% worked within normal full-time hours of 35 or less hours per week. A large proportion (39%) worked between 66 and 80 hours per week, and a staggering 22% worked more than 80 hours per week (Cohen et al. 2008). Consequently, time pressure was the most reported source

of stress (Cohen et al. 2008). Among psychiatry trainees in particular, a large study of over 7000 participants in countries across Europe, Asia and Africa demonstrated that in none of the 22 countries did average weekly working hours drop below 38 hours (Jovanović et al. 2016). The majority of participants (68.2%) worked over 48 hours per week, and 47.6% were not receiving at least 11 hours of rest every 24 hours, indicating that their working hours were likely to be irregular, combining day and night shifts (Jovanović et al. 2016).

Importantly, Jovanović et al. (2016) found that long working hours were significantly correlated with severe burnout. It is plausible that the longer their work hours, the less time trainees would have to spend on activities that contribute to their wellbeing and the decrease of stress. In a national report on doctors' and medical students' wellbeing, Beyond Blue (2013) revealed that the most common source of work stress was balancing work and personal responsibilities. This finding was supported by a study by Evans and Young (2017) on the work-life balance of psychiatrists and psychiatry trainees, which found that 11.98% of psychiatrists and psychiatry trainees never experienced appropriate work-life balance and 44.51% only experienced it to some extent. It was apparent that work was impeding on their personal lives, with several participants reporting poor balance to be a reason for their lack of having a partner (Evans and Young 2017). Since interacting with friends and family was overwhelmingly quoted as the most important means of relaxation for the participants (81%), those who lacked this social support were likely to have fewer opportunities to relieve their stress, contributing to a cycle of poor wellbeing (Evans and Young 2017).

The problem of overworked doctors is not a new discovery, and although regulations and guidelines have attempted to restrict their working hours, Morrow, Burford, Carter and Illing (2014) noted that a decrease in workload often does not accompany these changes. Trainee doctors in their study were still working unlogged hours, taking work home to stay on top of their workload and meet expectations they perceived their fellow staff had of them (Morrow et al. 2014).

Despite their long hours and high levels of stress, however, trainees in Australia and New Zealand have reported feeling satisfied with their work or that the work they were doing was appropriate for their level of training (Rotstein and Jenkins 2017). It is clear that among trainee doctors, there is a shifted perception of what normal levels of stress are, likely the result of a culture in the medical sector that normalises poor work-life balance. This culture is illustrated effectively in a survey by Evans and Young (2017). In their survey, 43.52% of the psychiatrists and psychiatry trainees who participated said that they had an appropriate work-life balance most of the time. However, when asked how their significant other would rate their work-life balance, only 15.47% said it would be normally good (Evans and Young 2017). In this way, the participants acknowledged that their perception of work-life balance was somewhat skewed compared to those outside of medicine. Clearly, more than just regulating trainees' working hours is needed to improve their wellbeing – an improvement would require measures to shift this culture in the sector.

2.2 Emotional Burden and Bullying

In a survey of postgraduate trainee doctors, Ogundipe et al. (2014) found that one of their most prevalent sources of stress was emotional distress from the workplace. In some cases, the stress can be so high that it leads to dropping out of trainee programs (Russell et al. 1975). In the survey by Ogundipe et al. (2014), 28.9% of trainees exhibited signs of emotional distress, measured objectively using the General Health Questionnaire (GHQ-12), which effectively measures mental distress and depressive symptoms (Romppel et al. 2013).

The literature points to several possible common sources of emotional distress for trainees. The high probability of psychiatry trainees experiencing patient suicides may be a common source of distress. A 2012 study found that 5% of psychiatry trainees in the United States experienced a patient suicide in a 1-year period and estimated that 20% would experience at least one over their traineeship (Tsai et al. 2012). However, in a Canadian study, Pilkinton and Etkin (2003), who had a much higher response rate and more representative sample, found that 61.4% of psychiatry trainees had experienced a patient suicide during their traineeship. This didn't include the suicides that some participants mentioned they had experienced during their pretraineeship years or uncompleted suicide attempts encountered (Pilkinton and Etkin 2003).

Alarmingly, 19.9% had encountered the suicide of a fellow trainee or other physician (Pilkinton and Etkin 2003) and, unfortunately, psychiatry trainees commit suicide at a high rate compared to other medical specialties (Russell et al. 1975). Pilkinton and Etkin (2003) found that these very personal experiences had significant impacts on the trainees' physical health and relationships both within and outside work. It is also possible that becoming aware of the suicides of peers would particularly change the way in which the trainees view psychiatry and their work. Common feelings reported by trainees were a loss of confidence in their clinical abilities and guilt about seeking help (Pilkinton and Etkin 2003). This guilt, mixed with deteriorating relationships in which support could be reached, could make for a toxic combination leading to poor wellbeing.

Emotional burden does not just come from experiencing suicide. In a qualitative study of factors leading to psychiatry trainees' stress and wellbeing, Benson et al. (2018) found that burden could arise from everyday care of patients. Witnessing patients suffering or deteriorating and feeling helpless in their ability to relieve their predicament was found to result in the feeling of being emotionally overwhelmed (Benson et al. 2018). Importantly, trainees' burden was exacerbated when they were unsupervised and felt underequipped (Benson et al. 2018). Having a lack of support could be categorised as a separate source of emotional burden – the feeling of needing to go through stressful events alone, without time to grieve or reflect (Benson et al. 2018).

This feeling of isolation is further worrying when it is considered that up to 80% of psychiatry trainees experience bullying in a year (Ahmer et al. 2009) and that incidences of mistreatment experienced are not isolated for 52% (Cohen and Patten 2005). There is a huge difference between the number of trainees who spontaneously cited bullying as a source of workplace stress (6.9%) in the open-ended

survey by Rotstein and Jenkins (2017) and those who affirmatively responded to experiencing bullying when questioned with specific examples of bullying behaviour in the survey by Ahmer et al. (2009). Much like the culture of poor work-life balance in the medical sector, it seems that experiences such as being humiliated or undermined in front of colleagues have infiltrated medicine. When presented with a vignette where a medical student was humiliated by a superior, 32.4% of medical students agreed that such situations were to be expected in medical school (Heru et al. 2009). Worryingly, it seems that trainees may not identify bullying as problematic but rather part of the job.

The fact that it is the medical sector that perpetrates this perception was illustrated when Heru et al. (2009) reported that medicine had met only 40% of medical students' expectations for professionalism. Tariq et al. (2016) also found that medical students were very commonly exposed to their superiors making derogatory comments – distinguished from constructive criticism – about patients or colleagues. The pervasiveness of this derogatory culture was illustrated when Tariq et al. (2016) further found that many medical students felt privileged to be included in their superiors' derogatory venting, as if it was part of being in the "club". However, this culture that normalises almost bully-like behaviour among superiors and makes students feel like they must earn their way into a club has resulted in a reluctance for trainees to complain when mistreatment happens. Medical students showed concern that making a complaint would affect grades (32.9%) and cause others to perceive them as weak (51.4%) or a troublemaker (20.3%), even though they agreed (68.6%) that it would be the right thing to do (Heru et al. 2009). In a field with such high professional stakes in trainee years, trainees' emotional wellbeing must be supported without consequences for their professional trajectories.

2.3 Burnout

Many of the symptoms detailed above can be interpreted through Maslach and Jackson's (1981) definition of burnout, a syndrome they describe as being most common in staff in human service industries. They characterised burnout by emotional and intellectual exhaustion in three domains: emotional exhaustion, depersonalisation and reduced personal accomplishment (Maslach and Jackson 1981). Emotional exhaustion is defined as feelings of being emotionally drained when overworked. Depersonalisation is the resulting feeling of emotional distance from or lack of feeling towards those in one's care at work. Reduced personal accomplishment is the tendency for workers with burnout to feel incompetent or dissatisfied in their ability to work with people (Maslach and Jackson 1981). Jovanović et al. (2016) reported that 36.7% of psychiatry trainees worldwide experience severe burnout.

Each of the dimensions of burnout can have a circular effect in intensifying the others. For example, feeling emotionally exhausted may take emotional resources away from caring for others, which in turn may result in reduced feelings of

accomplishment. There is some evidence that improvements in one dimension can buffer against declines in the others. Trainees for whom psychiatry was their first career preference have been found to experience significantly less burnout (Jovanović et al. 2016), perhaps owing to their sense of personal accomplishment. Trainees with burnout have been found to be less likely to open up to their supervisors about their symptoms (Kealy et al. 2016), perhaps increasing their sense of depersonalisation. However, it is possible that supervisors who do not foster a friendly dynamic in turn contribute to these symptoms. In this way, it is important to keep in mind that each of the factors contributing to stress or wellbeing is heavily intertwined and reduction of stress in one aspect may often lead into improvements in another.

3 Improving Trainee Wellbeing

As mentioned earlier, a change in the culture of the medical workplace is needed in order to realistically reduce the working hours of trainees without consequences like raising their anxiety over not meeting the workload and the supervisor's expectations. Promisingly, in Australia, working hours of junior doctors have gradually reduced over the last 15 years even without national regulations to restrict hours (Glasgow et al. 2014). Glasgow et al. (2014) attributed this partly to a cultural change, with more doctors recognising the importance of time away from work to improve work-life balance. Indeed, most psychiatry trainees consciously engage in activities that provide relaxation, such as being social, physical exercise and reading (Evans and Young 2017). It is important to note that these activities occur outside of the work environment and it is crucial that trainees are given time away from work to partake in them (Benson et al. 2018) – 7.86% of the large sample of trainees in the survey by Evans and Young (2017) responded that they never got opportunities to unwind.

However, even if the trainees themselves recognise the importance of work-life balance, true cultural change must come from the top down – supervisors and managers must promote balance for trainees to internalise this value. Currently, trainees who work overtime to get ahead academically and professionally are rewarded, as illustrated in the qualitative study by Morrow et al. (2014), where many trainees stated that their extra hours were done voluntarily despite hospitals attempting to restrict their hours. While some expressed this in terms of personal sacrifice to advance their learning, others felt pressure from superiors and colleagues, believing that they would be risking their professional reputation and chances for good references should they leave work on time (Morrow et al. 2014). This is worrying as it highlights the negative influence on trainee wellbeing that can come from superiors.

The trainee-supervisor relationship is a crucial one that can greatly shape trainees' values and wellbeing. A lack of supervision is another major contributor to stress and feelings of inadequacy that lead to burnout (Benson et al. 2018). Kealy et al. (2016) reported that trainees who felt unsupported also said their

supervisors were less available. Concerning is the fact that trainees with burnout who did not turn to their supervisors for support were more likely to use unhealthy coping strategies such as alcohol use, excessive spending or unhealthy eating (Kealy et al. 2016). On the flip side, trainees are highly likely to turn to supervisors as a first port of call, following the experience of a suicide (Pilkinton and Etkin 2003). That trainees highly value this relationship was also shown in a survey by Cohen and Patten (2005), where colleagues and program directors were the most commonly identified sources of support. Supervisor unavailability does not always stem from a lack of desire to spend time with their trainees. There were supervisors in the study by Rotstein and Jenkins (2017) who expressed concern about not being able to give enough time to their trainees, showing that they, too, recognise the importance of this relationship.

Problems in personal relationships can contribute to burnout (Ogundipe et al. 2014), and this may be where stable professional relationships play an even more important role in supporting trainees' wellbeing. However, this cannot occur without supervisors being able to support trainees without the threat of career-ending or career-interrupting consequences. When asked about the barriers to seeking mental health care, trainees most commonly cite concerns about confidentiality, stigma and professional ramifications rather than personal reasons such as not believing in its helpfulness (Aaronson et al. 2018; Beyond Blue 2013). However, if the literature is interpreted using the Maslach and Jackson (1981) model of burnout, reducing emotional exhaustion by working through problems with a supervisor could have run-on improvements, resulting in feeling less detached from their work and more successful in solving work-related problems. Though there will always be more extreme cases in which reporting and formal intervention is necessary, for many trainees, simply connecting with their supervisors who are available to mentor them through their stressful situations could result in the trainees providing better care for their patients.

4 How the Victorian Changes May Impact on Psychiatry Trainees

The changes to the RANZCP's Fellowship training programme have resulted in more in-training assessments, where supervisors assess psychiatry trainees' abilities (Jurd et al. 2015). However, with only 1 hour of individual supervision per week (Jurd et al. 2015) (2 hours per week for Stage 1 registrars since 2019) and about ten assessments per 6 months (The Royal Australian & New Zealand College of Psychiatrists n.d.), these assessments can encroach on a large proportion of time that could otherwise be spent mentoring trainees through difficult cases, situations or issues. This could create a barrier to trainee wellbeing that prioritises Fellowship assessments over mentorship. Further, since concerns about consequences for their career and achievements are already the most prominent barrier for trainees seeking help from supervisors, conflating the supervisor and assessor roles may only deter

trainees from confiding in their supervisors even more. The stress-reducing benefits of relieving some pressure from external examinations may be welcomed by trainees (Jurd et al. 2015). However, simply reducing stress while not also increasing chances for wellbeing to be improved may not be enough, and this move may simply be shifting stress from exams into the supervisor-trainee relationship instead.

Similarly, the new Victorian Mental Health Act 2014 has discernible benefits for patients, being designed to better protect their rights (State Government of Victoria 2014). This has come at the cost, however, of clinicians (often trainees) being required to write longer, more detailed reports on their patients and attend more frequent Mental Health Tribunal hearings. While there have not been any studies on the effects of the new Mental Health Act on psychiatry trainees' stress and wellbeing, it can be expected that this has increased their workload, with more administrative work to be done within the same number of hours than previously expected. As discussed earlier, this is problematic as workload appears to affect whether trainees work overtime more than rules and regulations around working hours. Trainees also have been shown to exhibit a strong sense of responsibility to patients and colleagues, choosing to work longer hours in order to avoid burdening others with their work and make sure their patients are handed over and cared for properly (Morrow et al. 2014). With this in mind, it is likely that trainees will take on the extra responsibilities that the Mental Health Act demands, even if they result in burnout, for the sake of their patients' care. Managers should certainly consider this and take measures for the extra administrative burden that the Act places on trainees, ensuring that patient workload is balanced between enough personnel to ensure that trainees can fit their expected workload within regular full-time hours.

5 Conclusions

It is clear from the literature that changing the culture of the medical workplace from one that values overwork to one that values work-life balance is crucial to improving the wellbeing of psychiatry trainees. The effects of improving the wellbeing of psychiatry trainees include improvements to patient care through reducing trainee burnout. A reduction in burnout would mean trainees would not feel drained from overwork, enabling them to have the mental capacity to care for their patients effectively and feel connected to them and their work, resulting in feelings of accomplishment. Supervisors are key to this cultural change, as it is clear that supervisors' expectations and values greatly affect trainees' own attitudes to their work. The trainee-supervisor relationship needs to be protected, since trainees will often think of supervisors as a first point of call in times of need, and while it is difficult to control problems that occur in private or personal relationships, stable working relationships can contribute a great deal to trainee wellbeing and support.

The changes that have recently occurred in the psychiatry Fellowship curriculum in Australia and New Zealand and the Mental Health Act in Victoria will have significant impacts on Victorian psychiatry trainees' wellbeing. The new RANZCP curriculum limits the capacity for supervisors to be effective mentors by conflating

the supervisor and assessor roles, which will worsen trainee fears about the professional consequences of asking their supervisors for help in dealing with stress. In addition, the Mental Health Act has increased trainee workloads, which could result in trainees feeling more obliged to work overtime in order to maintain their standards of patient care and professionalism.

Both of these changes certainly work to improve the system – the RANZCP curriculum was designed to implement effective programmatic assessments that do not rely on high-stakes examinations, and the Mental Health Act was changed in order to protect patient rights. However, changes that affect trainee psychiatrists must also consider the trainees' wellbeing in light of the literature available. Each factor contributing to stress or wellbeing is interlinked with the others. It will now be important for the RANZCP and supervisors to consider other avenues for supporting trainees that do not run the risk of affecting their assessments. In addition, managers will need to consider the heightened workload that trainees have experienced due to the new Mental Health Act when planning staffing levels and workload balance and allow for trainees to have enough time to partake in stress-relieving activities outside of work. It is unfortunate that those who choose to care for others' mental health are often mentally unhealthy themselves, and this culture must change from the top down.

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Brief Cognitive Tests in the Case of Dementia and Alzheimer's Disease Early Diagnosis

Maria Sagiadinou and Antonia Plerou

1 Introduction

Even though early features related with Alzheimer's disease pathogenesis are not clear at the moment, neuroanatomical networks of episodic memory, as well as language, attention, executive functioning, and visuospatial ability-associated networks, are likely to be linked with AD appearance (Weintraub et al. 2012). The research involved with autopsy samples suggests that memory and cognitive test scores were significantly lower in AD patients in comparison to the control subjects (Grossman et al. 2007; Wang et al. 2009). Low performance in cognitive tests, 10–12 years before formal diagnosis, is related to Alzheimer's disease pathogenesis (Aretouli et al. 2013; Schneider and Gottesman 2013; Twamley et al. 2006; Johnson et al. 2009). According to Rajan, this period is years before the formal diagnosis (Rajan et al. 2015). The psychometric and cognitive tests are proposed in order to define the difference between cognitive loss in normal aging and cognitive loss caused by AD (Fage et al. 2015). Moreover, brief cognitive tests (BCT) are also valuable in this screening process (Fichman-charchat et al. 2015). Possible negative aspects of early dementia screening, such as initiating anxiety and/or depression and stigma (Panegyres et al. 2016), should be taken into serious consideration. Early disclosure of dementia is accompanied with risks both for the patient and the family and friends, including a preoccupation with the diagnosis, restriction of activities, higher vigilance from the family, distress, and increased anxiety (Wilcock and Carroll 2009; Bamford et al. 2004). However, the benefits of early screening for the patient and the patient's environment should not be underestimated (Cordell et al. 2013).

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2 MCI and Alzheimer's Disease

Although mild cognitive impairment is linked with cognitive changes, these changes are not severe enough to interfere significantly with the patient's daily life or independent function. It is estimated that 15 to 20 percent of people around 65 are dealing with MCI. People with MCI, especially MCI-involved memory problems, are more likely to develop Alzheimer's disease or other types of dementia. However, MCI does not always lead to dementia. Screening for MCI is crucial, so as to motivate the patient at risk to seek an expert for diagnosis and for the planning of a therapeutic intervention.

Dementia implies a cognitive decline in a person's daily function. While diagnostic criteria vary depending upon dementia subtype, general features according to the Diagnostic and Statistical Manual – III R (DSM III-R) are fundamental (American Psychiatric Association 1987). Dementia cases are associated with memory impairment that is not linked with normal aging, as well as with impairments in other cognitive domains such as attention, language, visuospatial skills, or problem-solving. These deficits are not severe enough to affect daily functional activities. In elderly people, dealing with a gradually progressive amnestic disorder (non-memory cognitive domains), Alzheimer's disease is the most common diagnosis.

Alzheimer's disease is related to prominent memory disturbance appearing early in clinical evaluation (Fleming et al. 1995). During AD's progression, cognitive and behavioral variations arise. Alzheimer's disease is a neurodegenerative disorder and is diagnosed under a postmortem brain evaluation. Typical neuropathological deficits that are noticed in AD cases are amyloid plaques and neurofibrillary tangles (Burns et al. 1990).

3 Brief Cognitive Test Evaluation Criteria

For the frame of this review study, validated cognitive tests were selected, which are used in studies for Alzheimer's Disease and Mild Cognitive Impairment Evaluation (English version). In addition, these tests are performed directly to patients and not to an informant. Moreover, these tests can last from 1–2 minutes to 20 minutes. Finally, self-reports, neuropathological tests, as well as tests that examine everyday function abilities were excluded. Sensitivity and specificity are basic aspects in the case of BCT. Low sensitivity is related to false negatives, therefore false diagnoses, whereas low specificity leads to false positives (Solomon and Murphy 2005). It is worth mentioning that the level of sensitivity and specificity of brief cognitive tests varies. However, almost 80% of these tests are generally accepted and are also applied to several scientific fields like biology and medicine (Mitchell et al. 2010b).

4 Brief Cognitive Test

4.1 Brief Cognitive Test: Administration Time 10–20'

Mini-Mental State Evaluation (MMSE) is considered to be the most commonly used psychometric test, both for clinicians and mental health experts. It includes 11 sections associated with recent memory, calculation, language, and visuospatial skills. The test duration is approximately 15 minutes and it is rated in a scale of 30 grades. Cutoffs for mild, moderate, and severe dementia are also provided. MMSE limitations refer to age, language, and educational variations (Folstein et al. 1975). MMSE sensitivity is not considered to be relatively high in the case of early dementia screening. At the cutoff for 24/30 grades, MMSE sensitivity reaches 87%, while specificity is measured to be approximately 82% (Tombaugh and McIntyre 1992).

Mini-Cog has been validated in population-based studies and in older community-dwelling adults, heterogeneous with respect to language, culture, and education. Mini-Cog test duration is almost 10 minutes, while a clock-drawing test and a verbal memory task are also included (Cordell et al. 2013). Mini-Cog sensitivity and specificity are suggested to be 76% and 89%, respectively (Borson et al. 2003; Borson et al. 1999).

Addenbrooke's Cognitive Examination Revised (ACE-R) test assesses five cognitive domains, namely, attention and orientation abilities, memory skills, verbal fluency, language ability, and visuospatial function level. Improved sections of MMSE are also incorporated. ACE-R test duration is approximately 20 minutes, quite long for the cases of brief cognitive tests; however, the specificity can reach 94% while the specificity almost 89% (Mathuranath et al. 2000; Reyes et al. 2009). Namely, Mini-Addenbrooke's Cognitive Examination (M-ACE) is a revised and shortened version of the ACE-R, which combines five elements, in order to evaluate orientation, memory, and visuospatial skills. Test duration is less than 5 minutes, while two cutoffs are proposed at 21 degrees with sensitivity of 61% and specificity of 100% as well as at 25 degrees with sensitivity of 85% and specificity of 87% (Hsieh et al. 2015).

Saint Louis University Mental Status (SLUMS) test is composed of 11 tasks in order to evaluate memory, orientation, attention, and executive functions. The screening process is about 10 minutes in order to diagnose dementia deficits. The cutoff point is 20, whereas sensitivity is around 82.86%, and specificity reaches almost 86.96% (Szcześniak and Rymaszewska 2015).

Montreal Cognitive Assessment (MoCA) test is utilized for mild cognitive impairment screening. MoCA test evaluates skills like attention, memory, orientation, language, visuospatial abilities, and executive functions. MoCA test duration is approximately 10 minutes. In case of the cutoff at 26 grades, MoCA test sensitivity is 81%, while its specificity reaches 95% (Vogel et al. 2015).

4.2 Brief Cognitive Test: Administration Time 1–5'

Memory Impairment Screen (MIS) is a verbal memory test, comprised of four modules which last only 4 minutes. Participants involved with MIS test are asked to loudly read four words that are written on a piece of paper. In addition, four general categories are involved, where four words need to be incorporated. After the completion of a "distraction task" like counting down from 20, the participants are asked to recall the words provided earlier. On the threshold of four points, the sensitivity of the MIS test is not considered to be high, almost around 80%; nevertheless, specificity reaches 96%. MIS test is not involved with age, gender, and educational level limitations (Alsaffar et al. 2016; Barral et al. 2016).

Clock-drawing test (CDT) is considered to be a commonly used test for dementia and cognitive impairment screening. This test lasts 1–2 minutes and is applied easily. CDT sensitivity and specificity are not very high, namely, 76% and 81%, respectively; nevertheless, these features could be improved depending on the way the clock-drawing test is implied. CDT provides both significant quantitative and qualitative information; therefore, it is embedded in others' test (Yoo and Lee 2016; Kim et al. 2016; de Paula et al. 2013). It is considered to be extremely sensitive of early dementia screening and identification (Beber et al. 2016; Vyhnálek et al. 2016; Ricci et al. 2016).

Abbreviated Mental Test (AMT) is used to examine mental states by assessing memory and orientation in reference to time and space. AMT's duration is relatively short, namely, 3 minutes, and in the case of cutoff in 8 grades, the sensitivity is high reaching 91%, whereas the specificity is considered to be quite low, namely, 75% (Endlebury et al. 2015).

*Six-Item Cognitive Impairment Test (*6-CIT) is a brief cognitive test that lasts less than 5 minutes and includes three orientation items: reverse counting from 20, reverse recall of the months, and recall of an address. For a cutoff at 7 grades, sensitivity is measured at 78.57%, whereas specificity at 100% (Callahan 2002).

Short Test of Mental Status (STMS) is used for the evaluation of orientation, attention, immediate recall, delayed recall, abstract thinking, executive function, and calculation abilities. STMS test's duration is almost 5 minutes. STMS's sensitivity and specificity are quite low; therefore, STMS's reliability is limited (Ivnik et al. 2003).

Animal naming is a short verbal term test commonly utilized for assessing cognitive impairment using measurements of category fluency measurement. In only 1 minute, the participants are asked to name as many animals as they can. It is used as a brief and primary screening test for dementia and milder cognitive impairment cases (Sager et al. 2006).

Rowland Universal Dementia Assessment (RUDAS) is used within the frame of multicultural settings and in illiteracy cases. RUDAS lasts for about 1 minute and is used to identify the ability of body part recognition, visuospatial function, reasoning, and memory. For a cutoff of 21 scores, the sensitivity and specificity of the RUDAS test are 83% and 86%, respectively (Naqvi et al. 2015).

Brief Alzheimer Screen (BAS) is involved with orientation modules in time, recalling ability (three words), distraction task (backward word), and categorical fluency (animal naming). 3 minutes is only required in the case of BAS's screening procedure. For a cutoff of 26 degrees, sensitivity and specificity are calculated to be 99% and 87%, respectively (Mendiondo et al. 2003).

General Practitioner Assessment of Cognition (GPCOG) evaluates time orientation skills, word recall ability, and recent event recall capacity while a clock-drawing test is included. GPCOG test provides additional six-question set addressing the informer examining changes that have been noticed lately. The score provided from the combination of both scales is considered to have higher sensitivity (85%) and specificity (86%) with respect to each section score independently. GPCOG duration is less than 4 minutes (Aprahamian et al. 2011).

Short Orientation-Memory-Concentration Test lasts about 5 minutes and includes six brief evaluation tasks. While following a cutoff score of 10/11 grades, the sensitivity of this test rises to 88% and the specificity to 94% (Davous et al. 1987).

Short Portable Mental Status Questionnaire (SPMSQ) is compromised from ten screening items, in order to evaluate orientation, memory, current event information, and calculation ability. Its sensitivity and specificity vary, with the specificity mainly being quite low (Malhotra et al. 2013).

Trail Making Test (TMT) evaluates visual search, processing speed, mental flexibility, and executive ability in less than 5 minutes (Tombaugh 2004; Salthouse 2012).

Five-word test is a simple and quick screening test, which lasts only 2 minutes and assesses episodic memory by evaluating the recalling ability of a short list. With a cutoff at 10 scores, sensitivity and specificity of the five-word test are 91% and 87%, respectively (Dubois et al. 1983).

5 Brief Cognitive Test Overview

Brief cognitive tests are used for early dementia, mild cognitive impairment, and Alzheimer's disease. According to the International Psychogeriatric Association (IPA) after reviewing 20 cognitive tests, the six most commonly applied are the Mini-Mental State Examination, clock-drawing test, Delayed Word Recall, Verbal Fluency Test, Similarities, and Trail Making Test (Shulman et al. 2006). In addition, researchers suggested that General Practitioner Assessment of Cognition (GPCOG), Memory Impairment Screen (MIS), and the Mini-Cognitive Assessment Instrument (Mini-Cog) are quick, effective, and clinically accepted with minimum limitations referring to age, education, gender, and nationality bias. According to Holsinger et al. (2013) the Memory Impairment Screen, Brief Alzheimer Screen, Mini-Cog, and Six-Item Screener are suggested for the effective screening of dementia.

In reference to the BCT effectiveness according to community, primary or secondary care bias, MIS is suggested to be suitable for community screening. MMSE and AMTS are more applicable in primary care settings, while 6-Items and Mini-Cog

in specialized settings (Mitchell et al. 2010a; b). According to Villars et al. (2010) MMSE, GPCOG, and the clock-drawing test are proposed as easily implemented, validated, and effective in order to identify dementia in primary care (Villars et al. 2010).

According to Kaiser Permanente Research Affiliates Evidence-Based Practice Center's (EPC) research, the MMSE test was the most frequently used in 55 studies based on brief cognitive tests. Additionally, several BCT tests, like MMSE, Clock Draw Test (CDT), Mini-Cog, Saint Louis University Mental Status (SLUMS) Exam, Abbreviated Mental Test (AMT), Blessed Orientation Memory Test, General Practitioner Assessment of Cognition (GPCOG), Short Portable Mental Status Questionnaire (SPMSQ), and Montreal Cognitive Assessment (MoCA), are suggested for the case of dementia screening in primary care settings (Lin et al. 2013). Moreover, in the review study of Velayudha, where 22 cognitive tools were compared, the Addenbrooke's Cognitive Examination Revised (ACE-R) is suggested as an excellent tool in the case of early dementia screening compared to MMSE (Velayudhan et al. 2014a; b). According to recent research within the frame Spain national surveys, MIS and MMSE are suggested for primary care, while CDT and Verbal Fluency Tests are proposed in cases where thoroughly screening is needed. In addition, in the case that specialized settings are necessary, MoCA, MMSE, ACE-R, CDT, MIS, and TVF are suggested (Olazarán et al. 2016). Finally, a clinical guideline published from the Mental Health Department of Greece Health Ministry, in the case of dementia evaluation, proposes MMSE, MoCA, and the "five-word test" for efficient dementia screening.

On the other hand, in the case of Alzheimer's disease, Galvin and Sadowsky (2012) suggested a stepwise algorithm in order to assess primary care. In particular, the first step is referred to be a pre-diagnostic test, while the second step incorporates cognitive assessment with the use of MMSE, MoCA, and Mini-Cog screening test. According to Alzheimer's Association, an algorithm for the Annual Wellness Visit (Cordell et al. 2013) is suggested, which proposes that MIS, GPCOG, and Mini-Cog are the most reliable for assessing AD in primary care.

6 Discussion and Future Work

Brief cognitive tests are essential as they provide a validated but not time-consuming assessment in order to indicate the risk of dementia. A variety of brief cognitive tests have been developed; nevertheless, BCT that are efficient, are patient-acceptable, and are suitable for the relevant care setting need to be identified. In many cases, cognitive impairment is linked with independence loss, decreased quality of life, and increased health-care costs. Considering cognitive impairment is crucial for public health impact, a timelier diagnosis of dementia is proposed. Authors' future work will focus on establishing a simple, rapid general cognitive assessment tool in order to be used in both clinical and research issues.

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Synthesis, Molecular Docking Studies and Biological Evaluation of N-Acylarylhydrazones as Anti-Inflammatory Agents

Tangirala Sarala Devi, Galla Rajitha, Konda Swathi, Katari Sudheer Kumar, and Amineni Umamaheswari

1 Introduction

Neurodegenerative diseases are a group of chronic, progressive disorders characterized by the gradual loss of neurons in discrete areas of the central nervous system (CNS). The mechanisms underlying their progressive nature prevent inflammatory reactions, which are essential for the integrity and proper function of the CNS. Substantial evidence acknowledges a common inflammatory mechanism in various neurodegenerative diseases (Gao and Hong 2008). Acylhydrazone moieties which possess the structure R1R2C=NNHCOR are the most important pharmacophoric cores of several anti-inflammatory (dos Santos et al. 2014; Soujanya et al. 2017) antinociceptive (Barreiro et al. 1998), antiparkinson's (Turan-Zitouni et al. 2018), antimicrobial (Jayaveera 2012), antitubercular (Küçükgüzel et al. 1999), antitumor (Mohareb et al. 2011), antioxidant (Sibelsuzen et al. 2009; SaralaDevi et al. 2010), analgesic (Alexandre et al. 2014) and antimalarial (Walcourt et al. 2004) activities. Studies have revealed that various substitutions on the acyl carbon and imine carbon significantly affect the reactivity and biological activity of hydrazone moiety. Curcumin, a natural constituent of *Curcuma longa*, has a styryl carbonyl moiety in its structure and displays anti-inflammatory activity (Sreejayan Rao 1994). Curcumin and dehydrozingerone were reported to be potent scavengers of

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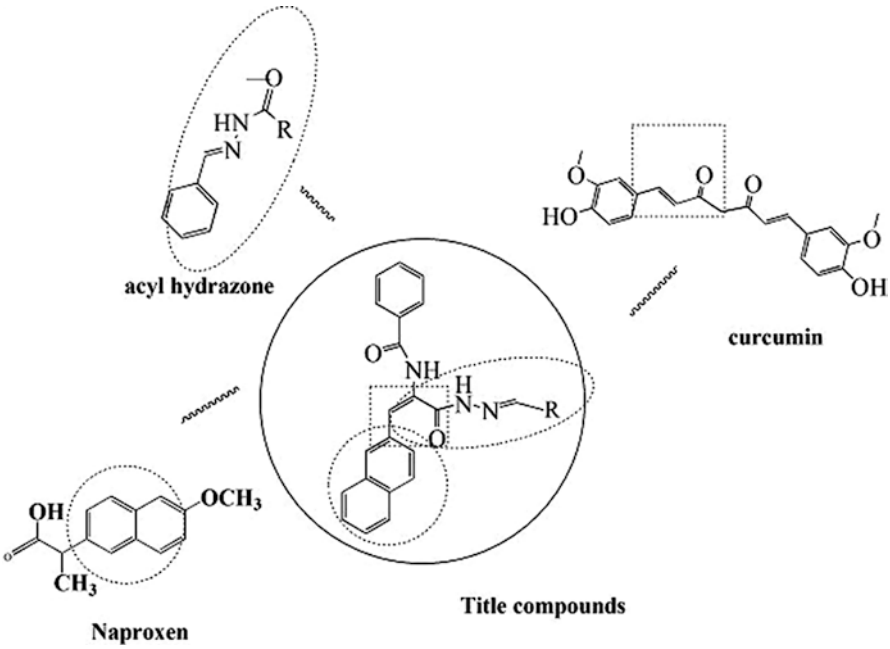


Fig. 1 Design strategy adopted for designing of title compounds **3a–3o**

oxygen free radicals and also possess good anti-inflammatory activity. Both are styryl ketones with similar substitution on the phenyl ring.

Naphthalene has occupied a central place among medicinally important compounds due to their diverse and interesting antibiotic properties with minimum toxicity (Rokade et al. 2009). In particular some of naphthalene derivatives were investigated in depth, as nonsteroidal anti-inflammatory drugs (NSAIDs), e.g. naproxen. Prompted by the above observations, we aimed to synthesize the various new series of N-acylarylhydrazones by incorporating napthy-2-lidene on acyl moiety and various aryl and heteroaryl substitutions on imine carbon and to study the possible contribution of both functionalized units by assessing for anti-inflammatory activity and their docking studies against human COX-2 enzyme (Fig. **1**).

2 Experimental Design

2.1 Material

All the chemicals and solvents used in the present study were purchased from Merck, HiMedia, S.D. Fine Chemicals Limited, Mumbai, and Sigma-Aldrich, USA. Melting points were determined in an open capillary tube in Thermonik

precision melting point-cum-boiling point (C-PMB) apparatus and are uncorrected. The purity of the compounds was ascertained by TLC on silica gel G plates (Merck). IR spectra (KBr discs) were recorded by Shimadzu FT-IR spectrophotometer using KBr-d6 pellets technique; 1H NMR spectra were recorded on Bruker 400-MHz NMR spectrometer using DMSO as solvent. Mass spectra were recorded on Apex mass spectrophotometer, and elemental analysis (C, H and N) was performed using Perkin–Elmer model 240C analyser.

2.2 Chemistry

2.2.1 Synthesis of 4-((Naphthalen-2-yl)methylene)-2-phenyloxazol-5 (4H)-one (1)

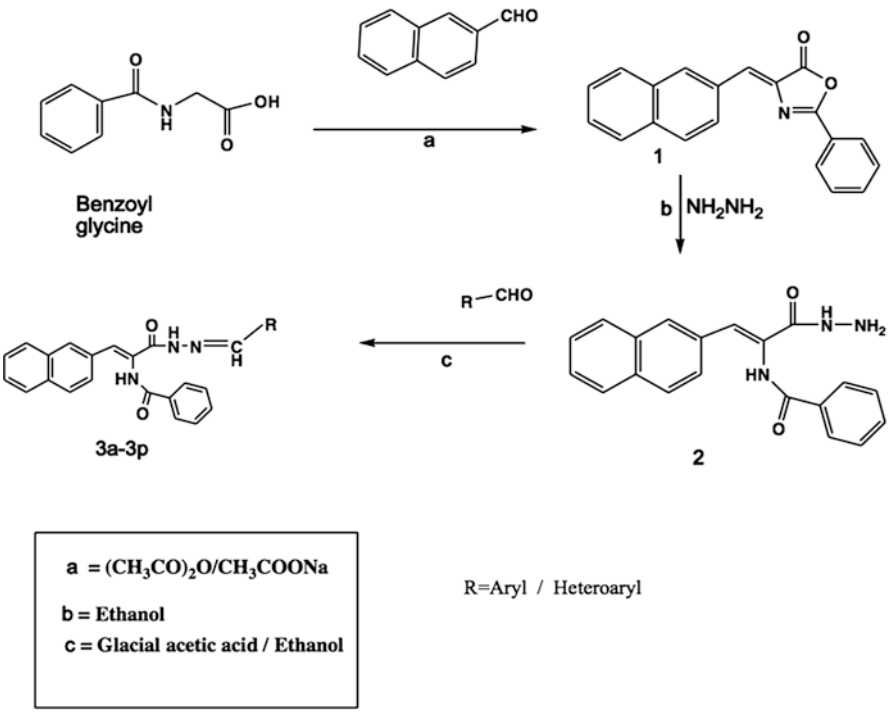
Synthesis of 4-((naphthalen-2-yl)methylene)-2-phenyloxazol-5(4H)-one was done in accordance with the previously reported method (Rajitha G. et al., 2011).

2.2.2 Synthesis of 2-(Benzamido)-3-(naphthalen-2-yl)acrylohydrazide (2)

For the synthesis of 2-(benzamido)-3-(naphthalen-2-yl)acrylohydrazide, 4-((naphthalen-2-yl)methylene)-2-phenyloxazol-5(4H)-one (1) (0.03 mmol) was stirred with a solution of hydrazine hydrate (0.06 mmol) in ethanol (20 ml) for 30 minutes. The deep yellow colour of oxazolone immediately changed to a light yellow coloured product, which was filtered, washed and recrystallized from methanol. Yield: 84%; M.P: 170–1720 °C; IR (KBr) cm^{-1}: 3228, 3163 (N–H_2), 3020 (N–H). ^{1}H NMR (400 MHz, DMSO-d_6); 4.47 (s, 2H, NH2), 7.41–7.95 (m, 12H, Ar-H), 8.07 (s, IH, C=CH), 9.67 (s, 1H, -NHCO), 9.74 (s, 1H, -CONHN=); Mass: m/z (M) 331, (M-H)—330.

2.2.3 Synthesis of N′-(Substituted)-2-(benzamido)-3-(naphthalen-2-yl) acrylohydrazide (3a–3o)

For the synthesis of N′-(substituted)-2-(benzamido)-3-(naphthalen-2-yl)acrylohydrazide (Scheme **1**), equimolar amounts of 2-(benzamido)-3-(naphthalen-2-yl)acrylohydrazide and various substituted aromatic aldehydes were heated at 60 °C with few drops of acetic acid in ethanol for 1 hour. The mixture was allowed to cool to room temperature. A solid product was formed, which was then filtered and recrystallized from ethanol. The various substituted 2-(benzamido)-N′-(substituted)-3-(naphthalen-2-yl)acrylohydrazides were prepared by a similar procedure.



Scheme 1 It represents the synthesis of N′-(substituted)-2-(benzamido)-3-(naphthalen-2-yl)acrylohydrazide (**3a**–**3o**).

2.3 Physico-Chemical and Spectral Data of Title Compounds

3a: 2-(benzamido)-*N*′-benzylidene-3-(naphthalen-2-yl)acrylohydrazide: Yield: 75%; M.P: 160–1620 °C; IR (KBr) cm^{-1}: 3437 (N-H), 3024 (Ar-H), 1771&1656 (C=O), 1547 (C=C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.47–8.13 (m, 17H, Ar-H), 8.14 (s, IH, C=CH), 8.51 (s, IH, N=CH), 10.03 (s, 1H, -NHCO), 11.80 (s, 1H, -CONHN=); 13 C NMR (100 MHz, DMSO-d_6): δ 124.43, 125.39, 125.74, 125.80, 126.03, 126.37, 126.47, 127.03, 127.82, 128.15, 124.43, 128.53, 128.63, 128.67, 128.74, 128.80, 129.04, 131.11, 131.27, 131.58,133.61,161.80; Mass: m/z (M) 419, (M-H)- -418, (M + H) + −420; Elemental analysis: Calculated: C, 77.31; H, 5.05; N, 10.02 Found: C,77.32; H,5.15; N, 10.12.

3c: N′-(3,4-dimethoxy benzylidene)-2-benzamido-3- (naphthalen-2-yl)acrylohydrazide (3c): Yield: 80%; M.P: 216–2180 °C; IR (KBr) cm^{-1}: 3437 (N-H), 3058 (Ar-H), 1753&1656 (C=O), 1597 (C=C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.82 (s, 6H, OCH3), 7.03–8.12 (m, 15H, Ar-H), 8.13 (s, IH, C=CH), 8.39 (s, IH, N=CH), 9.95 (s, 1H, -NHCO), 11.69 (s, 1H, -CONHN=); Mass: m/z (M) 479, (M-H)—478; Elemental analysis: Calculated: C, 72.64; H, 5.25; N, 8.76, Found: C, 72.54; H, 5.21; N, 8.74.

3d: N′-(3,4,5-trimethoxybenzylidene)-2-benzamido-3-(naphthalen-2-yl)acrylohydrazide: Yield: 80%; M.P: 204–2050 °C; IR (KBr) cm^{-1}: 3443 (N-H), 3056

(Ar-H), 1710&1676 (C=O), 1578 (C=C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.72 (s, 3H, OCH3) 3, 3.85 (s, 6H, OCH3), 7.04–8.00 (m, 14H, Ar-H), 8.11 (s, IH, C=CH), 8.40 (s, IH, N=CH), 10.11 (s, 1H, -NHCO); 11.90 (s, 1H, -CONHN=); Mass: m/z (M ± 1) 509, (M-H)- -508; Elemental analysis: Calculated: C, 70.71; H, 5.34; N, 8.25, Found: C, 70.72; H, 5.31; N, 8.27.

3e: N′-(4-hydroxybenzylidene)-2-benzamido-3-(naphthalen-2-yl)acrylohydrazide: Yield: 80%; M.P: 187–1880 °C; IR (KBr) cm − ^{1}: 3334 (N-H), 2923 (Ar-H), 1715&1691 (C=O), 1514 (C=C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.41–7.91 (m, 16H, Ar-H), 8.11(s, IH, C=CH), 9.63 (s, IH, N=CH), 9.92 (s, 1H, -NHCO), 11.59 (s, 1H, -CONHN=); Mass: m/z (M) 435, (M-H)-434, (M + H) + −436; Elemental analysis: Calculated: C, 74.47; H, 4.86; N, 9.65 Found: C, 74.42: H, 4.36: N, 9.66.

3f: N′-(4-hydroxy-3-methoxy-benzylidene)-2-benzamido-3-(naphthalen-2-yl) acrylohydrazide: Yield: 80%; M.P: 208–2100 °C; IR (KBr) cm^{-1}: 3385 (N-H), 3030 (Ar-H), 1760&1664 (C=O), 1510 (C=C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.85 (s, 3H, OCH3), 7.10–8.12 (m, 15H, Ar-H), 8.13 (s, IH, C=CH), 8.37 (s, IH, N=CH), 9.57 (s, 1H, OH), 9.96 (s, 1H, -NHCO); 11.67 (s, 1H, -CONHN=); Mass: m/z (M ± 1) 465, (M-H)- 464; Elemental analysis: Calculated: C, 72.24; H, 4.98; N, 9.03, Found: C, 72.25; H, 4.71; N, 9.05.

3g: N′-(N,N-dimethylamino benzylidene)-2-benzamido-3-(naphthalen-2-yl) acrylohydrazide: Yield: 70%; M.P: 166–1680 °C; IR (KBr) cm^{-1}: 3325 (N-H), 2726 (Ar-H), 1737&1694 (C=O), 1565 (C=C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.98 (s, 6H, N(CH3)2), 7.44–8.04 (m, 16H, Ar-H), 8.11 (s, IH, C=CH), 8.35 (s, IH, N=CH), 10.07 (s, 1H, -NHCO); 11.63 (s, 1H, -CONHN=); Mass: m/z (M) 462, (M-H)- -461, (M + H) + −463; Elemental analysis: Calculated: C, 75.30; H, 5.67; N, 12.11, Found: C, 75.26; H, 5.71; N, 12.14.

3h: N′-(4-methyl benzylidene)-2-benzamido-3-(naphthalen-2-yl)acrylohydrazide: Yield: 80%; M.P: 132–1340 °C; IR (KBr) cm^{-1}: 3433 (N-H), 3024 (Ar-H), 1737&1656 (C=O), 1502 (C=C) ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.33 (s, 3H, CH3), 7.28–7.90 (m, 16H, Ar-H), 8.12 (s, IH, C=CH), 8.46 (s, IH, N=CH), 10.11 (s, 1H, -NHCO), 11.87 (s, 1H, -CONHN=); Mass: m/z (M) 433, (M-H)- -432, (M + H) + −434; Elemental analysis: Calculated: C, 77.58; H, 5.35; N, 9.69, Found: C, 77.56; H, 5.31; N, 9.65.

3l: 2-benzamido-3-(naphthalen-2-yl)-*N*′-((E)-3-phenylallylidene)acrylohydrazide(3 l): Yield: 80%; M.P: 155–1620 °C; IR (KBr)cm^{-1}: 3432 (N-H), 3309 (Ar-H), 1708&1691 (C=O), 1505 (C=C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.05 (s, 2H, HC=CH), 7.34–7.91 (m, 17H, Ar-H), 8.11 (s, IH, C=CH), 8.26 (s, IH, N=CH), 9.93 (s, 1H, -NHCO); 11.69 (s, 1H, -CONHN=); Mass: m/z (M) 445, (M-H)- 444; Elemental analysis: Calculated: C, 78.18; H, 5.20; N, 9.43, Found: C, 78.14; H, 5.23; N, 9.47.

3m: 2-benzamido-3-(naphthalen-2-yl)-*N*′-((naphthalene-2yl)methylene) acrylohydrazide:Yield: 85%; M.P: 146–147 °C; IR (KBr) cm^{-1}: 3401 (N-H), 3305 (Ar-H), 1764&1681 (C=O), 1565 (C=C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.48–7.91 (m, 19H, Ar-H), 8.19 (s, IH, C=CH), 8.90 (s, 1H, N=CH), 10.42 (s, 1H, -NHCO); 11.91 (s, 1H, -CONHN=); Mass: m/z (M) 469, (M-H)- -468, (M + H) + −470; Elemental analysis: Calculated: C, 79.30; H, 4.94; N, 8.95, Found: C, 79.33; H, 4.91; N, 8.96.

3o: 2-benzamido-3-(naphthalen-2-yl)-*N′*-((4-pyridyl)methylene)acrylohydrazide: Yield: 75%; M.P:180–1820 °C; IR (KBr) cm^{-1}: 3330 (N-H), 3064 (Ar-H), 1751&1661 (C=O), 1565 (C=C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.46–8.12 (m, 16H, Ar-H), 8.46 (s, IH, C=CH), 8.67 (s, IH, N=CH), 10.00 (s, 1H, -NHCO); 12.09 (s, 1H, -CONHN=); Mass: m/z (M) 420, (M-H)- -419, (M + H) + −421; Elemental analysis: Calculated: C, 74.27; H, 4.79; N, 13.33, Found: C, 74.27: H, 4.69: N, 13.10.

3 In Vivo Experiments

3.1 Acute Toxicity Studies

The study was conducted as per OECD-423 guidelines and determined in healthy adult male albino Swiss mice. Animals were fasted for 24 hours and divided into groups of six animals. The test compounds suspended in normal saline were given intraperitoneally, in doses of 10 mg to 1000 mg per kg body weight, and the control group of animals received only the vehicle (normal saline). The animals were observed for 48 hours from the time of administration of the test compound and showed mortality in a dose of 1000 mg/kg (LD50). Subsequently, a tenth of the LD50 (100 mg/kg-ED50) was selected as a dose for screening of anti-inflammatory activity (Dixon 1991).

3.2 In Vivo Anti-Inflammatory Activity

The in vivo anti-inflammatory activity of all the title compounds (**3a–3o**) was evaluated using carrageenan-induced hind paw oedema test in male albino rats (150–180 g) of Wistar strain at 100 mg/kg body weight. The rats were divided into different groups. One group consisting of six animals served as control, while the other groups of six animals each received the test compounds and standard drug. The rats were administered orally with test compounds (100 mg/kg), 100 mg/kg phenylbutazone (positive control) or 10 ml/kg 0.5% sodium carboxymethyl cellulose (vehicle control) 1 hour before injection of 0.05 ml of 1% suspension of carrageenan into the sub-plantar region of the rat hind paw. A mark was made at the lateral malleolus of the right paw, and the foot was dipped to the same distance of the mark into the arm of plethysmograph. The volume of the injected paw was measured by water displacement in a plethysmograph immediately after carrageenan injection. The paw volume was again measured after 180 minutes. Average oedema volumes for test compound treated and positive control rats were compared statistically with those of the vehicle control animals and expressed as percent oedema inhibition, which is calculated using the formula (Winter et al. 1962). Percentage oedema inhibition = 100 (1 − Vt/Vc) where Vt = volume of oedema in treated group and Vc = volume of the oedema in the control group. Statistical significance of the results was tested by Anova followed by Dunnett's *t* test.

3.3 Molecular Docking Studies against COX-2

Molecular modelling and docking calculations were performed in a SGI workstation with 3.0 GHz processor, 4 GB RAM, 300 GB hard disc and an NVIDIA FX 1700 graphics card running in Linux operating system installed with Schrodinger software suite 2015–2 (Maestro v 10.2, Schrodinger, LLC: New York). Molecular docking studies of the title compounds (**3a–3o**) were performed by Glide v 6.7 against crystal structure of aspirin-acetylated human cyclooxygenase-2 (5F19) to observe the binding mode of new analogues at the active site. The drug likeness scores for all ligands were evaluated with the help of Lipinski rule of five and the ligands, standard drug were embedded in to the generated grid of human COX-2 protein to access their binding affinities. Ten thousand poses per each ligand was generated, clustered and discarded if RMS deviation is less than 0.5 A° and atomic displacement is less than 1.3 A° (Rajitha et al. 2014).

3.4 In Vitro COX-2 Inhibition Assay

Among the title compounds, selective active compounds which exhibited good activity in both in vivo anti-inflammatory activity and binding affinity towards COX-2 in docking were evaluated for their ability to inhibit COX-2 using an ovine COX-1/COX-2 assay kit (catalogue No. 560101, Cayman Chemicals Inc., Ann Arbor, MI, USA) according to protocol recommended by the supplier, and their IC50 (μM) values were determined.

4 Results and Discussion

4.1 Chemistry

Fifteen new compounds of N′-(substituted)-2-(benzamido)-3-(naphthalen-2-yl) acrylohydrazide (**3a–3p**) were synthesized, and the reaction sequence for the synthesis is outlined in Scheme. The intermediate 2-(benzamido)-3-(naphthalen-2-yl) acrylohydrazide **(2)** upon nucleophilic addition with functionalized aromatic/heteroaromatic aldehydes in ethanol and a few drops of acetic acid yielded the title compounds **(3a–3p)**, and the intermediate **(2**) was obtained by stirring 4-((naphthalen-2-yl)methylene)-2-phenyloxazol-5(4*H*)-(1) with hydrazine hydrate (99%) at room temperature in the presence of ethanol. 4-((naphthalen-2-yl) methylene)-2-phenyloxazol-5(4H)-one was synthesized by condensation of hippuric acid with 2-naphthaldehyde in presence of acetic anhydride and sodium acetate. Spectral data of all the newly synthesized compounds were in full agreement with the proposed structures.

4.2 In Vivo Anti-Inflammatory Activity

All the synthesized compounds were evaluated for their in vivo anti-inflammatory activity. Among these compounds the phenolic derivatives exhibited good anti-inflammatory activity. It has been found that compound **3e** (75%) exhibited remarkable anti-inflammatory activity, followed by compounds **3c** (74%) and **3f** (74%). The compound **3d** (71%) was found to be equipotent with the standard phenylbutazone. It is in agreement with previous reports that the presence of methoxy group at ortho position to the phenolic hydroxyl has shown the good effect on anti-inflammatory activity profile (Adriane et al., 1998; Cunha et al., 2003).

4.3 Molecular Docking Studies

The docking study may offer more insight into the understanding of the protein–ligand interactions and the structural features of the active site. The binding affinities of the synthesized compounds with the receptor were analysed. These results not only indicate about the activity but also explain the fragments (benzamido group, substituted aromatic ring) which are responsible for the interaction with hydrophobic cavity of target protein and also π–π staking interactions. All the compounds showed good binding affinity with COX-2; compound **3 m** showed good binding affinity with an XP-G score of 9.328 kcal/mol when compared to reference celecoxib (−5.107 kcal/mol).

4.4 In Vitro COX-2 Assay

The study was extended to in vitro COX-2 assay. Among the title compounds selective–active compounds that exhibited good activity in both in vivo anti-inflammatory activity and binding affinity towards COX-2 in docking were evaluated for their ability to inhibit COX-2. Unsubstituted **(3a)**, 3,4-dimethoxy phenyl **(3c)**, 4-hydroxyphenyl **(3e)**, 4-chlorophenyl **(3 k)** and pyridin-4-yl **(3o)** derivatives showed remarkable COX-2 inhibition. Among the evaluated compounds **3c** showed potent activity with IC50 value 0.5 μM which was comparable to celecoxib (0.8 μM). All the other compounds also showed IC50 values.

5 Conclusion (Figs. 2 and 3)

Compounds **3c, 3e** and **3f** exhibited potent in vivo anti-inflammatory activity in comparison to phenylbutazone as the reference drug. **3a, 3c, 3 k** and **3o** exhibited potent in vitro anti-inflammatory activity. Molecular docking studies further supported the activity of **3e** and **3c** and further helped in understanding the various interactions between ligands and enzyme active sites in detail and thereby helped to design novel potent inhibitors (Fig. 4, Table 1).

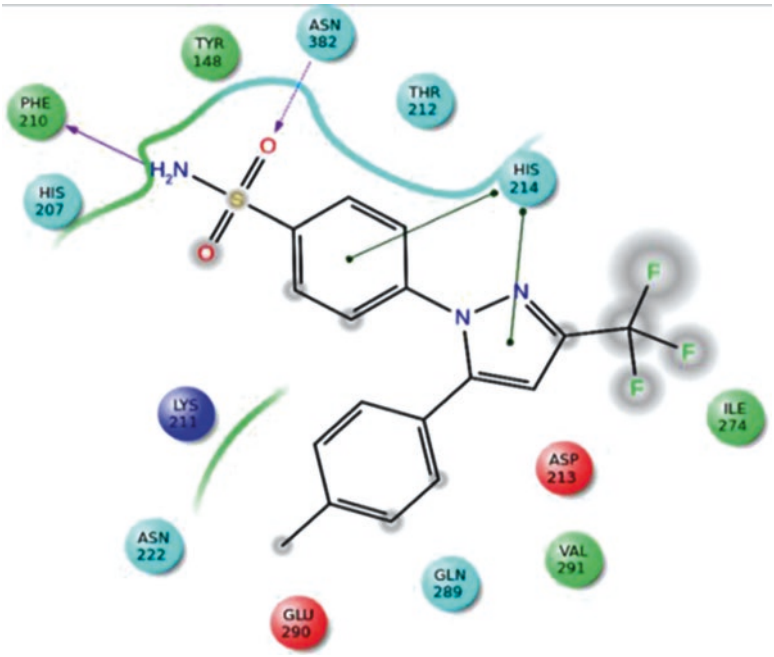


Fig. 2 2D ligand interaction of celecoxib with COX-2

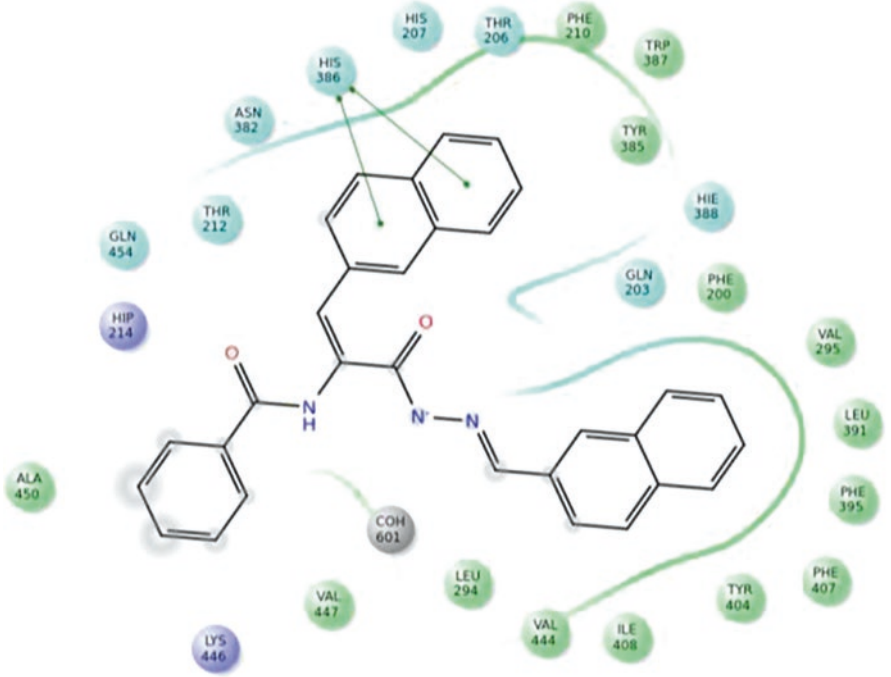


Fig. 3 2D ligand interaction of compound **3 m** with COX-2

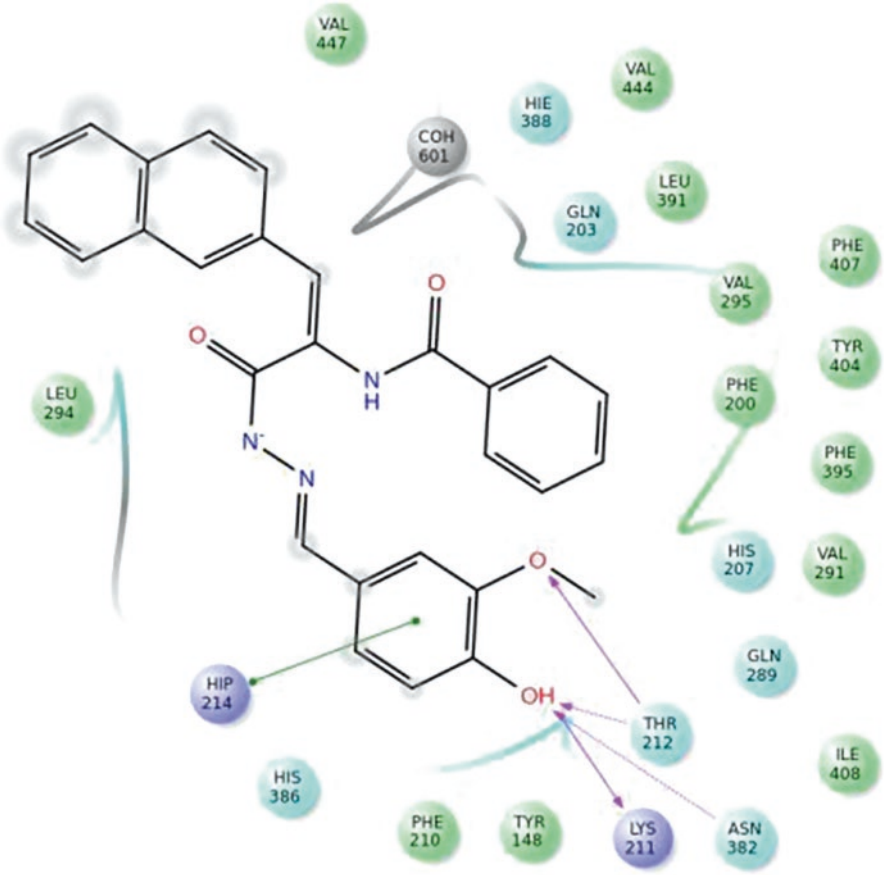


Fig. 4 2D ligand interaction of compound **3f** with COX-2

Table 1 Anti-inflammatory activity of N′-benzylidene-2-benzamido-3-(naphthalen-2-yl) acrylohydrazides (**3a**–**3o**)

Compound code	R	Carrageenan-induced rat paw edema method[a]		*In vitro* [d] COX-2 inhibition assay	Molecular docking studies against COX-2
		Edema volume after 3h (ml ± SEM)	% Inhibition of inflammation	IC_{50}(μM)	XP – G score (Kcal/mol)
3a	C_6H_5-	(0.58)[b] ± 0.037	58	0.7	−8.05
3b	(4-OCH_3)C_6H_4-	NT	NT	NT	−7.55
3c	3,4-(OCH_3)$_2C_6H_3$-	(0.36)[b] ± 0.034	74	0.5	−6.275
3d	3,4,5-(OCH_3)$_3C_6H_2$-	(0.40)[b] ± 0.003	71**	0.9	−8.05

(continued)

3e	4-OH C_6H_{4-}	$(0.34)^b \pm 0.001$	75^{**}	0.8	−8.268
3f	4-OH,3-$OCH_3C_6H_{3-}$	$(0.36)^b \pm 0.042$	74	0.9	−8.391
3g	4-$N(CH_3)_2C_6H_{4-}$	$(0.49)^b \pm 0.033$	65	NT	−6.952
3h	4-CH_3 C_6H_{4-}	$(0.91)^b \pm 0.034$	34	NT	−6.136
3i	3-NO_2 C_6H_{4-}	NT	NT	NT	−8.889
3j	4-CN C_6H_{4-}	NT	NT	NT	−6.148
3k	4-Cl C_6H_{4-}	NT	NT	NT	−7.622
3l	Styryl	$(0.54)^c \pm 0.037$	60	1.5	−6.791
3m	Naphthalene-2yl-	$(0.49)^c \pm 0.041$	64	2.4	−9.328
3n	Thiophen-2-yl-	NT	NT	NT	−5.776
3o	Pyridin-4-yl-	$(0.41)^c \pm 0.033$	70	0.7	−7.965
Standard	Phenylbutazone	$(0.40)^b \pm 0.040$	71	–	–
Standard	Celecoxib			0.8	−5.107

NT not tested

∗Significance levels $*p < 0.5$, $**p < 0.01$ and $***p < 0.001$ by Dunnett's *t* test

[a]At 100 mg/kg (p.o.), oedema volume was measured 3 h after carrageenan injection, and each value represents as the mean ± SEM ($n = 6$), activity presented as % inhibition of inflammation

[b]Control oedema volume = 1.39 (0.005)

[c]Control oedema volume = 1.37 (0.001)

[d]The in vitro test compound concentration required to produce 50% inhibition of human recombinant COX-2. The IC50 values were determined using the enzyme immunoassay kit (catalogue no. 560101, Cayman Chemicals, Inc., Ann Arbor MI, USA)

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Exosome Biomarkers Revolutionize Preclinical Diagnosis of Neurodegenerative Diseases and Assessment of Treatment Responses in Clinical Trials

Dimitrios Kapogiannis

Alzheimer's disease and other neurodegenerative diseases have long preclinical phases with active and progressively irreversible pathology. Therefore, biomarkers are essential for identifying patients early in the course of these diseases, when they may benefit the most from disease-modifying interventions. A limitation of biomarkers measured in the soluble phase of blood is their tenuous link to brain pathology. A new approach to biomarker discovery that addresses this limitation is deriving extracellular vesicles (EVs) enriched for neuronal and astrocytic origin from peripheral blood. EVs are membranous particles (subdivided into smaller exosomes and larger microvesicles) that are shed by all cells and found in all biofluids. Neuronal and astrocytic EVs have been implicated in the pathogenesis of several neurodegenerative diseases. Given their origin, neuronal and astrocytic enriched EVs harvested from blood can be used to interrogate brain pathologic processes previously inaccessible in vivo. In a long series of case control studies based on these EV subpopulations, we have identified candidate protein biomarkers for Alzheimer's disease and other neurodegenerative diseases. In GeNeDis 2018, an update of these studies and results from a validation study of these biomarkers in preclinical Alzheimer's disease will be presented. In addition, we will present results from studies demonstrating EV biomarker responses to experimental interventions. EV-based biomarkers are a valuable new tool that will enable researchers to test hypotheses in proof of concept studies with carefully selected participants at the preclinical phase, spearheading therapeutic discovery in neurodegenerative disease.

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Biomarkers as a Different Approach in Prevention and Treatment of Drug Addiction (Preliminary Study)

Maria Gonidi, Anna Tselenti, and Antonia Plerou

1 Introduction

Drug addiction is a chronic relapsing disorder and a burden on society and individuals with harmful and long-term consequences (Nora and Li 2004). Although imaging studies of brain function, neurobiological processes, and environmental factors show abnormalities among the addicted individuals, none of these dysfunctions have predictive validity. The understanding of complex interactions of genetic, neurobiological, and environmental factors could lead to specific criteria, including biomarkers, to identify or classify addicted individuals into categories and either assess or predict treatment response (Nora et al. 2015). Traditional biomarkers such as quantification of drugs in either body fluids like urine or blood or in hair are diagnostic biomarkers valuable for legal purposes. They can also be used to evaluate therapeutic benefits in different medication developments (Wada et al. 2010). However, all these traditional biomarkers have no predictable value for clinical outcomes and provide no additional insights regarding the process of the disease. A dimensional approach of drug addiction offers the opportunity for developing potential tools critical in treating them. Brain imaging and neurochemical imaging

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PET (positron emission tomography) provided information about neuronal circuits that are implicated in drug addiction and could be very helpful in identifying potential biomarkers associated with higher addiction risk as, for example, Dopamine D2Rs.

As for the diagnostic accuracy of D2Rs, although high levels of D2Rs in striatum are associated with resilience and low levels with rewarding effects, this method lacks specificity. On the other hand, genetic studies (Jonson et al. 2011) have shown that genes or genomic regions appear to contribute or modulate addiction risk. The heterogeneity of addictive diseases could be explained by an integrated procedure and measurement of phenotype. Recently, the activity of NFKβ and cytokines in the brain of drug addicted individuals was studied. NFKβ seems to play a crucial role as a key activator of inflammation and toxicity. However, NFKβ, through poorly defined mechanisms, has an anti-inflammatory activity (Nenning and Schank 2017; Zhong et al. 2016) in order to maintain homeostasis and tissue repair. Recently, minimally invasive methods of using biomarkers in squamous cells of the buccal mucosa have gained attention (Francois et al. 2016). Central nervous system may reflect changes in tissue of the same ectodermal origin as buccal cells. Oral exfoliative cytology is a simple, cost-effective, noninvasive, and painless procedure for microscopic and biochemical analyses. In the present study, we evaluate the expression of biomarkers in buccal cells of addicted individuals.

2 Material and Methods

Buccal smears of 35 individuals with addictive disorders (20) or substance use disorders (15) for more than 3 years were collected by the gentle brushing of the buccal mucosa. They were immediately fixed in 95% ethanol for Papanicolaou staining or in 5% formaldehyde for immunocytochemical staining. Immunocytochemical staining for the expression of NFKβ, IL-1β, IL-6, TNF-α, bcl-2, and ucp4 antibodies was performed on the epithelial cells, for the study of oxidative stress, toxicity, and inflammation. Papanicolaou staining was performed for the potential structural disorders. The study group age range was 20–35 years. There was a correlation with the clinical profile of each individual. Individuals with HIV or Tbc infection were excluded from this study.

3 Results

Cytomorphology and immunoprofile of the smears of chronic relapsers and substance users for more than 3 years revealed in 35 out of 35 individuals (100%) karyolitic changes undergoing necrosis or increased nuclear/cytoplasmic ratio in the squamous isolated cells. Increased cytoplasmic expression of the markers IL-1β, IL-6, and TNF-α and nuclear expression of NFKβ have been found in the smears of the individuals (15 of 15) with substance use for more than 3 years (100%). In the

group of addicted individuals without substance use (group of 20), there was a total increase of cytoplasmic expression of IL-1β, IL-6, and TNF-α. NFKβ was positive in 15 out of 20 individuals. On the other hand, decreased expression of bcl2 was correlated with a weak increased expression of ucp4 related with oxidative stress in 3 out of the 35 smears.

4 Discussion

Oral cavity cells may reflect changes in other tissues of ectodermal origin such as in the central nervous system. In recent studies, a spectrum of biomarkers and cytomorphologic changes of buccal cells were evaluated for patients with AD compared to normal individuals and as expected, significant differences were shown (Francois et al. 2016) The cytomorphometric analysis showed structural changes in nuclear/cytoplasmic ratio as well as in nuclear density and texture, findings that should reflect consequences of inflammation. In our preliminary study, cytomorphologic changes have been found in all the smears. Long-term use of drugs can modify brain function and alter metabolites and proteins, inducing inflammation and oxidative stress response that could be a permanent effect (Potenza et al. 2011). The standard goal of all the studies on drug addiction is to provide information for better understanding of the mechanisms and pathways underlying addiction as well as to introduce diagnostic and predictive biomarkers. The purpose of the study was to investigate the expression of inflammatory and oxidative stress markers, in buccal cells, between drug-treated and untreated groups. Studies in drug-treated animals, compared with healthy controls, revealed differences in pathways of energy-related biological processes and oxidative stress response neurotransmission (Lichti et al. 2011). In human plasma, serum, and recently saliva samples, altered proteins have been found (Kalivas and Volkov 2005) that could be potential biomarkers. Although protein profile of peripheral tissues could become a potential prognostic and diagnostic tool for addiction, changes in central nervous tissue may differ from changes of peripheral regions. In that way, the findings from our preliminary study are still inconclusive and require further investigation (Wang et al. 2016). The metabolic changes of drug addiction or after drug treatment stimulate the whole-body system, and further studies are needed to illuminate the correlation between central nervous system and peripheral metabolite changes.

5 Conclusion

Future holistic research in peripheral tissues including buccal epithelial cells could enhance the understanding of underlying mechanisms of drug addiction and provide potential diagnostic and prevention biomarkers.

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Sleep Disorders and Restless Legs Syndrome in Hemodialysis Patients in Greece: A Cross-Sectional Study

Pantelis Stergiannis, Maria Govari, Edison Jahaj, Christina Marvaki, Georgia Toulia, Katerina Marvaki, Georgia Chasioti, and George Intas

1 Introduction

Dialysis is defined as the use of an artificial kidney to purify urea, waste products of metabolism, toxins, and excess fluid from the blood. This procedure is selected as treatment of end-stage renal failure, transient renal failure, and specific cases of drug intoxication or overdose. The main purpose of hemodialysis is to treat kidney failure, where fluids, acids, electrolytes, and drugs are not effectively excreted in the urine. Hyperkalemia, uremia, fluid overload, acidosis, and uremic pericarditis are indicative cases requiring hemodialysis (TOH 2008).

Sleep is a behavior characterized by changes in body posture and eye condition. Sleep status is assessed in several dimensions either by self-assessment or behavioral analysis, physiological functions, and genetic information. While such analyses can be used to differentiate arousal states from full awakening to deep sleep, scientists internationally point out that the EEG or the sleep study is the gold standard for the objective assessment of sleep (Buysse 2014).

Over the last decade, there have been an increasing number of studies into the role of sleep in health, with undeniable evidence showing that sleep disorders,

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including insomnia and extreme sleep times, adversely affect the risk of developing illnesses and contribute to increased mortality rates regardless of the cause (Vgontzas et al. 2013). These findings have a significant impact on public health, as about 25% of the population report insomnia problems despite their health status (LeBlanc et al. 2009), while 10% meet the criteria for insomnia (Morin et al. 2006). As expected, the literature does not show the abundance of studies in Greece that examine the correlation between sleep disorders in patients undergoing hemodialysis.

This study will attempt to investigate sleep disorders experienced by hemodialysis patients in an attempt to draw useful conclusions and to propose solutions to address this serious problem.

2 Materials and Methods

This is a cross-sectional study, and the study sample, which was a sample of convenience, included 120 patients undergoing hemodialysis from February to April 2017 at the General Hospital "Agia Olga" in Greece.

2.1 Tools

The main tools of the study were the Athens Insomnia Scale, the Berlin Questionnaire, and the RLS (Restless Legs Syndrome) Questionnaire. Furthermore, specific patient demographics were recorded.

The Athens Insomnia Scale was developed in the Unit of Sleep Study of the Psychiatric Clinic of the University Hospital of Athens, by the Professor of Psychiatry, Mr. Konstantinos Soldatos, and his research team. It is a self-completed psychometric instrument consisting of eight questions. The first five questions on the scale (assessment of sleep difficulty, night awakenings, early morning wake, total sleep time, and overall sleep quality) correspond to criterion A for diagnosis of insomnia according to ICD-10, while the requirements of a minimum frequency (at least three times a week) and a duration (1 month) of any patient's complaint correspond to criterion B of ICD-10 (WHO 1992). The ICD-10 conditions for severe discomfort caused by sleeping and/or interference in daily routine activities (criterion C) are covered by the strictly subjective nature of the response options for each question on the scale as well as the content of the last three questions about the consequences of daydreaming insomnia (problems with a sense of well-being, daytime running, and daytime sleepiness). Each query on the scale can be rated from 0 to 3 (with 0 corresponding to "no problem" and 3 for "very serious problem"). The users are asked to respond positively if they have experienced the difficulty of sleeping described in each question at least three times a week in the last month or other time period, the duration of which depends on the purpose of the study for which the scale is used for (Soldatos et al. 2000).

The Berlin Questionnaire was developed by Netzer et al. (1999) for the assessment of sleep disorders in patients with sleep apnea syndrome. It was developed by researchers who wanted to determine whether patients who have completed a sleep apnea survey would help primary care physicians, by completing this tool, to identify which patients should perform further diagnostic tests for this condition. The golden rule for the diagnosis of sleep apnea is to carry out a sleep study. However, the results of the Berlin Questionnaire provide a clear indication for patients with sleep apnea syndrome who are promoted as a sleep study priority to confirm (most likely) or discard (less likely) the particular syndrome in the patient under study. The Berlin Questionnaire consists of ten questions organized in three categories. It incorporates questions about sleep apnea risk factors such as snoring behavior, sleepiness or fatigue, and the presence of obesity or hypertension. It has been weighted and validated in Greek by Bouloukaki et al. (2013).

The restless legs syndrome is directly related to unintentional semi-rigid leg movements during sleep, which are referred to as periodic movements of the limbs during sleep (PLMS) and which can often awaken the patient. RLS is associated with a variety of symptoms both day and night. The syndrome presents unpleasant and often painful sensations in the lower limbs that start or worsen during rest phases or periods of inactivity. Symptoms become more noticeable during the evening hours or occur only at night, causing the patient to have difficulty in sleeping. Sleep disorders often lead to excessive daily fatigue, difficulty in concentrating, and even depression. Symptoms of the syndrome may also adversely affect the patient's social activities that require immobility, such as long trips or watching a show. The plethora of symptoms associated with RLS demonstrate a significant reduction in the quality of life of patients due to the condition. For RLS assessment, the RLS Questionnaire was created, which is a reliable tool for diagnosing the syndrome through ten questions (The International Restless Legs Syndrome Study Group 2003).

2.2 Statistical Analysis

We conducted multiple Poisson regression analyses with dependent variable insomnia (yes vs no) and covariates the sociodemographic variables, sleep apnea and RLS. Poisson regression analysis is used instead of logistic regression because it calculates relative risks. So, it is easier to compare odds ratios (Zou 2004). Also, we made correlations by Pearson. All analyses were performed using SPSS IBM Statistics v.22. All tests were two-sided, with a significance level of 5%.

3 Results

Table 1 presents the sociodemographic and medical characteristics of the responders. Most of them were males (68.3%), aged 68.1 ± 14.1 years. 45% received less than 6 years of education. The BMI of responders was 24.2 ± 3.9, with

Table 1 Sociodemographic and medical data (n = 120)

Variables	N	%	Variables	N	%
Gender			*Morbidity*		
Male	82	68.3	Diabetes mellitus	83	69.2
Female	38	31.7	Hypertension	37	30.8
Education			*Medication*		
< 6 years	54	45	Analgesics	35	29.2
6–9 years	19	15.8	Hypnotics	21	17.5
10–12 years	25	29.2	Antidepressants	19	16.1
> 12 years	12	10	Anticoagulants	8	6.7
BMI, kg/m²			Antihypertensives	37	30.8
Underweight	5	4.2	Insulin	32	26.7
Normal	61	50.8			
Overweight	46	38.3			
Obese	8	6.7			

Table 2 Sleep characteristics

Variable	N	%	Variable	N	%
Insomnia (AIS)			*Restless Legs Syndrome (RLS)*		
Yes	61	50.8	No	45	37.5
No	59	49.2	Mild	23	19.2
Sleep apnea			Moderate	35	29.2
Low risk	81	67.5	Severe	12	10
High risk	39	32.5	Very severe	5	4.2

Table 3 Poisson regression model estimating factors affecting insomnia

Variable	RR	95% CI	P
BMI (normal vs overweight and obese)	0.180	0.032–1.003	0.05
Analgesics (yes vs no)	0.125	0.031–0.513	0.004
Hypnotics (yes vs no)	0.072	0.010–0.533	0.01
Restless legs syndrome (yes vs. no)	2.281	1.179–4.413	0.014

minimum value of 16.6 and maximum 40.8. About one third received antihypertensives, analgesics, and insulin. The mean hemodialysis time of the responders was 5.3 ± 5.7 years.

As is described in Table 2, half of the responders suffered from insomnia. About two thirds were at low risk for sleep apnea. Most of the participants suffered from restless legs syndrome (62.5%).

Table 3 reports results from the binary regression model estimating insomnia (yes vs no) according to the Athens Insomnia Scale. Insomnia was significantly associated with higher BMI (RR: 0.180; 95% CI [0.032, 1.003]), analgesics receiving (RR: 0.125; 95% CI [0.031, 0.513]), hypnotics receiving (RR:0.072; 95% CI

[0.010, 0.533]), and restless legs syndrome (RR: 2.281; 95% CI [1.179, 4.413]) after adjusting for sociodemographic variables.

Sleep apnea was significantly linked to analgesics (RR: 0.309; 95% CI [0.093, 1.027]; $p = 0.045$). RLS was significantly tied to hypnotics (RR: 0.077; 95% CI [0.008, 0.745]; $p = 0.027$) and insulin (RR: 0.019; 95% CI [0.001, 0.542]; $p = 0.020$).

Insomnia was found to be correlated with age ($r = 0.185$; $p < 0.001$) and analgesics ($r = -0.338$; $p < 0.001$) and hypnotics ($r = -0.365$; $p < 0.001$) receiving. Restless legs syndrome was associated with comorbidity ($r = 0.415$; $p < 0.001$), anticoagulants ($r = -0.258$; $p < 0.001$) and antihypertensives ($r = 0.597$; $p < 0.001$) receiving, as well as duration of hemodialysis ($r = 0.200$; $p < 0.001$) and sleep apnea ($r = -0.266$; $p < 0.001$).

4 Discussion

In this study, sleep disorders in 120 hemodialysis patients were investigated. The main observation of the study was that most patients undergoing hemodialysis experienced significant rates of sleep disturbances. Several of them were receiving medication. The results of our study also show that insomnia was significantly associated with higher BMI, analgesics and hypnotics receiving, and restless legs syndrome after adjusting for sociodemographic variables. Sleep apnea was significantly linked to analgesics. RLS was significantly tied to hypnotics and insulin. Insomnia was found to be correlated with age as well as analgesics and hypnotics receiving. Restless legs syndrome was correlated with comorbidity, anticoagulants and antihypertensives receiving, duration of hemodialysis, and sleep apnea.

Abassi et al. (2016) conducted a study in Iran to investigate the association between chronic end-stage renal failure and sleep disorders. Patients with chronic end-stage renal failure who took three dialysis sessions a week were enrolled in their study. According to the results of their study, the majority of the patients in the sample (95%) experienced sleep disturbances. Sleep disturbances were associated with older age, blood creatinine levels, upper airway obstruction, hepatomegaly, hepatic failure, higher blood TSH levels, hypothyroidism history, antihypertensive treatment, levodopa taking, and erythropoietin. The results of this study are consistent with the results of our study. In addition, Abassi et al. pointed out that although sleep disorders are a common symptom among patients with renal insufficiency, they are a complex issue and cannot simply be correlated with the elimination of substances from the body.

Losso, , Minhoto, and Riella (2014) in a survey conducted in Brazil found that hemodialysis patients had significant sleep disorders, agreeing with the results of our study, although they appeared in a reduced magnitude in the patients undergoing in automated peritoneal dialysis compared to the patients undergoing continuous portable peritoneal dialysis. This difference is attributable to the lowest average body mass index of hemodialysis patients.

Ezzat and Mohab (2015) performed a study aiming to assess sleep disorders in patients with end-stage renal failure. Their results showed high rates of sleep disturbances, with insomnia being the main symptom and restless legs syndrome occurring in a significant proportion of patients, in consistence to the results of our study.

The results from a study carried out by Brekke et al. (2014) showed that most of their renal end-stage patients had significant sleep problems, similar to our study. These patients had a lower age and higher rates of depression, also agreeing with the results of our study.

Trbojević-Stanković et al. (2014) tried to determine the prevalence of depression and poor-quality sleep and to investigate the correlation between these disorders and the demographic, clinical, and therapeutic characteristics of patients undergoing hemodialysis. Researchers observed that renal patients with depression were significantly older and had significantly lower dissolution capacity and significantly worse sleep quality, while there were no significant differences in sex, employment, and laboratory parameters. Other parameters studied were also not found to be related to the quality of sleep. In general, they concluded that depression and sleep disorders are often problems in patients with chronic renal insufficiency undergoing hemodialysis, in agreement with the results of our study.

DeFerio et al. (2017) conducted a research in the United States by studying patients with end-stage chronic renal insufficiency who underwent hemodialysis. According to the results of their study, patients exhibit symptoms of restless legs syndrome, which is positively related to the hemodialysis process and to the feelings associated with it (anxiety, depression, dysthymia, and other psychological disorders).

Al-Jahdali (2011), in a survey done in Saudi Arabia, studied sleep disorders and sleep apnea in renal patients. The results of his study showed high rates of insomnia among these patients. At the same time, he found that the nephropathy patients suffered from restless legs syndrome, agreeing with the results of our study, but the syndrome was more common in nephropathy patients undergoing peritoneal dialysis compared to patients undergoing hemodialysis. Overall, the investigator points out that sleep disorders are a common problem among renal patients, focusing on high insomnia rates and high rates of poor sleep quality, as in our research.

Mao et al. (2014) conducted a meta-analysis in order to investigate the relationship between demographic characteristics and comorbidity (age, gender, diabetes, etc.) and RLS in patients undergoing hemodialysis. The results of meta-analysis showed that risk factors for patients suffering from nephropathies to develop restless legs syndrome were lower hemoglobin and iron values. Also, Caucasians were more high-risk patients.

Wang et al. (2013) conducted a study in Taiwan investigating sleep disorders in patients undergoing dialysis. The aim of their study was to investigate the correlation between hemodialysis (shift) and subjective sleep quality in patients with chronic hemodialysis. The main result of their study was that dialysis during the morning shift is significantly associated with better subjective sleep quality. This parameter was not included in our study.

4.1 Limitations

The main limitations of the present study are the relatively small sample size and the fact that the study was conducted in only one hospital. These limitations prevent the investigation of correlations between various research parameters and especially demographic and personal data referring to similar studies.

5 Conclusions

Sleep disturbances are common symptoms among hemodialysis patients. The restless legs syndrome occurs at a lower frequency among hemodialysis patients but is a major problem for patients. As expected, the literature does not show the abundance of studies in Greece that examine the correlation between sleep disorders and hemodialysis. Doctors should assess sleep disorders in hemodialysis patients using questionnaires and to encourage them to partake in a sleep study, after confirming the existence of the disorder. Physicians, if deemed necessary, should also refer patients to a specialized psychiatrist or psychologist depending on the severity of the problem.

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Craniofacial and Neurological Phenotype in a Patient with De Novo 18q Microdeletion and 18p Microduplication

Christos Yapijakis, Antonia Angelopoulou, Emmanuel Manolakos, and Costas Voumvourakis

1 Introduction

Chromosome 18q deletion syndrome (18q-) is a rare chromosomal disorder with phenotypic variability, first described in 1964 (De Grouchy et al. 1964). The most common features of 18q- include small stature, mental deficiency with hypotonia, poor coordination, nystagmus, conductive deafness, seizures, microcephaly, midfacial hypoplasia with deep-set eyes, carp-shaped mouth, narrow palate, prominent antihelix, prominent antitragus, narrow or atretic external canal, long hands, tapering fingers and short first metacarpal with proximal thumb, vertical talus with or without talipes equinovarus, hypoplastic labia minora in females, cryptorchidism with or without small scrotum and penis in males, skin dimples over acromion, and cardiac defect (Wertelecki and Gerald 1971; Miller et al. 1990; Kline et al. 1993; Gondré-Lewis et al. 2015; Xiang 2010).

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Although several cases of chromosome 18q deletion syndrome have been observed before, there is only one recorded report of partial trisomy 18p and partial monosomy 18q (Bartels et al. 2011). Here we present a second case with features of 18q- syndrome who had combined 18q partial monosomy and 18p partial trisomy.

2 Methods and Results

The 50-year-old female patient was examined during genetic counseling of her healthy brother. She had a history of congenital cleft palate and developmental deficiency with hypotonia, hearing loss, and epilepsy until adulthood. The patient's family history was free of related cases (Fig. 1).

The clinical genetic examination of the patient noticed short height, hypotonia, macrodactyly, tremor, microcephaly, midfacial hypoplasia, strabismus, epicanthus, broad nasal base, repaired cleft palate, elevated upper lip, low-set ears with prominent antihelix, and narrow acoustic pore (Fig. 2). The neurological examination of the patient revealed cerebellar dysfunction with static and motor ataxia, gait difficulty, and imbalance. Mental deficiency was revealed with Mini-Mental State Examination score 19 ("moderate cognitive disorder") and instrumental activities of daily living score 18 ("slight dysfunction").

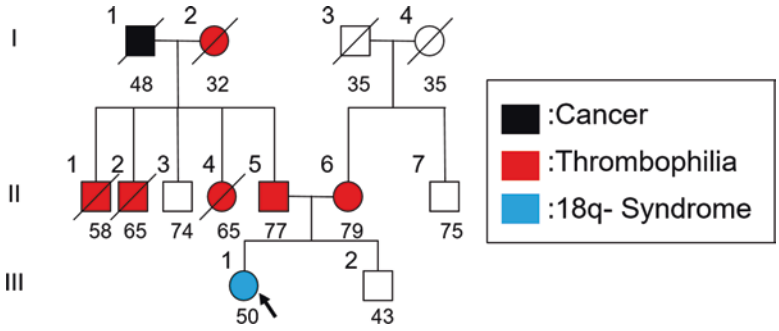

Fig. 1 Pedigree of the studied family. The patient (III-1) is indicated with an arrow

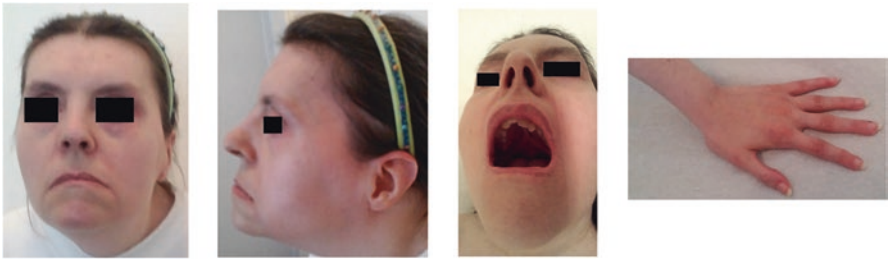

Fig. 2 Phenotype of the patient

The genetic investigation performed in the patient's blood samples included karyotype analysis and comparative genomic hybridization array (aCGH). The patient's karyotype was normal (Fig. 3). The aCGH analysis (Fig. 4) revealed an 8 Mb deletion (del18q22.3q23) and a 7.2 Mb duplication (dup18p11.32p11.23).

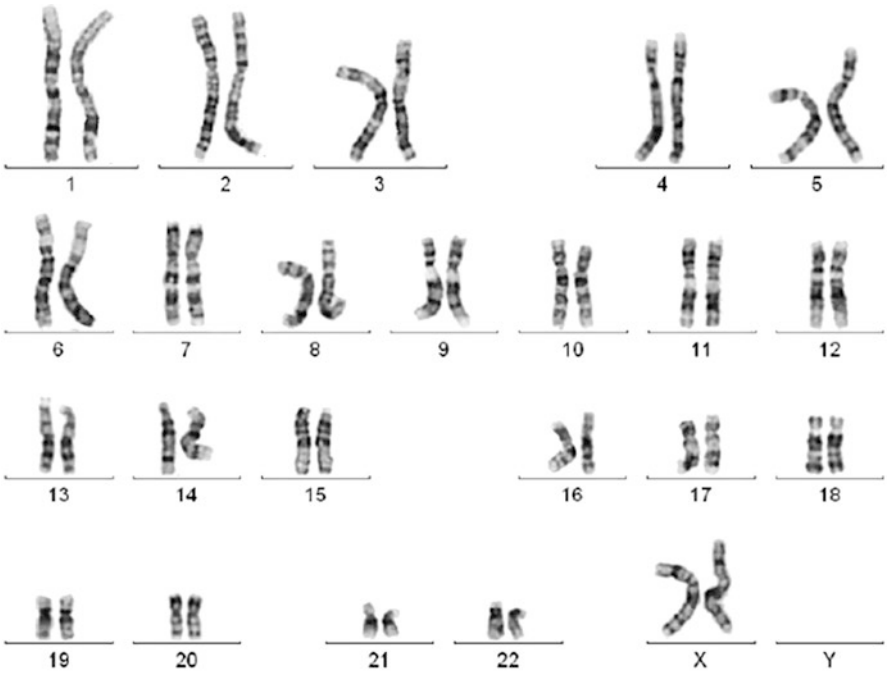

Fig. 3 Normal karyotype of the patient

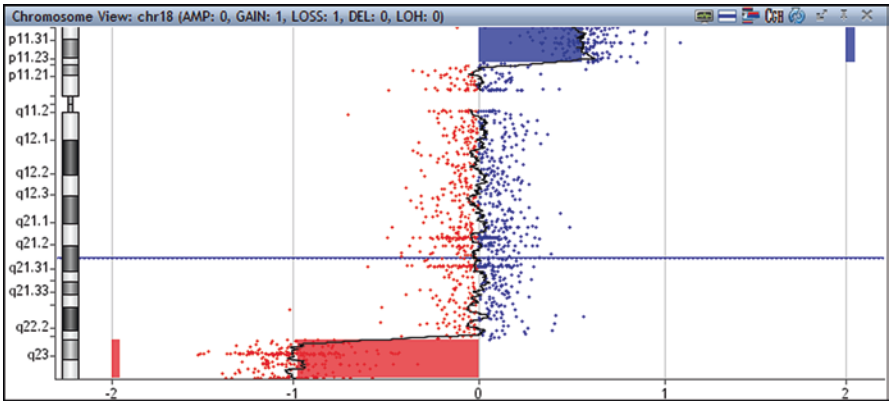

Fig. 4 Results of the aCGH analysis revealing del18q22.3q23 and dup18p11.32p11.23

3 Discussion

The patient had simultaneously a microdeletion in the long arm of chromosome 18 and a microduplication in the short arm of the same chromosome. This is the first report of these exact micro-rearrangements of chromosome 18, as far as we know. There are rare reports of cases with similar but not identical genotypes (Bartels et al. 2011). The combination of a partial duplication of the short arm and a partial deletion of the long arm of chromosome 18 is possibly caused by a de novo unequal recombination mechanism.

Depending on the involved chromosomal regions, the combined partial duplication of 18p and partial deletion of 18q may result in different phenotypes (Bartels et al. 2011). In our patient, almost all her clinical features are associated with 18q- syndrome.

Conflict of Interest Authors declare that they have no conflict of interest.

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What Do Recent Clinical Trials Teach Us About the Etiology of AD

Nikolaos K. Robakis

Alzheimer's disease (AD) is the most common type of dementia caused by severe neurodegeneration in the hippocampus and neocortical regions of the brain. In addition to neurodegeneration, AD brains contain high levels of amyloid plaques (APs) and neurofibrillary tangles (NFTs) which are used as neuropathological hallmarks of the disorder. Despite intense research efforts, the mechanism(s) of the AD neurodegeneration are imperfectly understood, hampering efforts for the development of efficient therapeutics. Furthermore, failure of clinical trials to benefit AD patients suggests that AD hallmarks are poor therapeutic targets and supports the suggestion that these hallmarks are sequelae of neurodegeneration. Although genetic evidence seem to support the amyloid theory of AD, additional empirical observations and experimental data are inconsistent with the amyloid/Aβ theories of AD [Robakis and Neve (1998), TINS vol. 21 pp.15–19; Robakis (2011) NBA vol. 32, pp 372–379]. This possibility is further supported by data that amyloid plaques and neurofibrillary tangles are found in a number of distinct neurodegenerative disorders and that animal models expressing high levels of AD pathological structures show little neuronal loss. Furthermore, genetic evidence linking genetic loci to disease reveal little about the molecular mechanisms involved. Mutants of APP, PS1, and PS2 cause familial AD (FAD) suggesting these mutants can be used as models to study mechanisms of neurodegeneration. Recent reports show that the ability of efnB1 and BDNF (factors) to rescue neurons from excitotoxicity depends on PS1 but is independent of γ-secretase. Interestingly, PS1 FAD mutations block the ability of factors to protect neurons from toxicity suggesting that FAD mutants may increase neuronal death by blocking neuroprotective activities of brain neurotrophins. Other reports also suggest that proteins involved in FAD have Aβ-/γ-secretase-independent functions that can play important roles in AD. Furthermore, non-neuronal brain cells like microglia are implicated in AD pathology.

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Developmental Biology and Transgenic Avian Embryology: Body Alterity Bioart Wet Lab

Adam Zaretsky

1 Introduction

Public experimental embryology opens a relationship between an embryo and an amateur transgenic designer. Artists produce real-world effects by forcing hereditary aesthetics on developing bodies. Through naming and funeral rites, we assign the embryos an uncertain amount of clout or cultural worth.

1.1 Goals

The goals of this research include to aid in public understanding of the relationship between transgenics and aesthetics; to immerse students in an experimental embryology while grounding the experience in real-world effects on developing bodies; to take an active and hands-on tactical stance on the role of hereditary designer and help in public analysis of the bioethics of genetic engineering; to understand the relationship between institutional oversight in pre-animal experimentation, embryonic dignity, and the problem of humane sacrifice; and to comprehend the politics and responsibilities involved in play at the level of heredity.(Evolution Haute Couture 2009; Imagining Science 2008; Transient Creatures (Catalogue) 2008; "Birdland", Nada Journal, Novembro 2007; Dangerous Liaisons and other stories of transgenic pheasant embryology 2009)

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2 Materials and Methods

1. Incubator 37 degrees Celsius 75% humidity
2. Fertile eggs (quail chicken and pheasant) 2–4 dozens
3. Labels with names for each fertile egg printed on them
4. Sterile tweezers and other standard dissection tools
5. Dissection microscope f. penicillin/streptomycin
6. Clear yellowish scotch tape
7. Pencils
8. Rubber gloves
9. Retinoic acid
10. Non-Pyrex disposable glass pipettes with rubber bulbs for DIY microdissection tools and microinjection needles
11. Plasmid, in this case, pDSRed-Dcl: The vector (transgene infectious agent) included in the plasmid is CMV (*Cytomegalovirus*.) Red stands for RFP+ (red fluorescent protein positive.) The chimeric proteins (pDS and Dcl) fused in the genomes of the embryonic somatic cell genomes. Together, pDS and Dcl express for a microtubule protein that is quite disruptive.
12. A wide variety of benzodiazepine powder drug family of compounds (valerian root derivatives) for avian embryonic euthanasia and researcher stressor tranquilization.

2.1 Methods (Imagining Science 2008)

1. Fertile eggs are stored at 12 degrees Celsius.
2. Fertile eggs are added to a 37 °C incubator, five a day, for a week before the lab (be sure to stabilize the incubator for a few days before beginning the experiment).
3. This leads to a timed start to development, and after 1 week, a 7-day spread of development should be experientially available.
4. On day 3 and day 4 before the lab, extra eggs are added to the 37 °C incubator (at least three eggs per student).
5. Labels with names for each fertile egg printed on them are stuck to these eggs. This is a list of the names used in this experiment:

   Health, Beauty, Longevity, Public Good, Knowledge, Profit, Erotism, Utility, Novelty, Animal, Poor, Queer, Captive, Slave, Fat, Ugly, Short, Stupid, Primitive, Unborn, Deformed, Poor in the World, Raw Material, Extinguish Humanely, Expendable Being, Educational Embryo, Pity Party, Defect, Murder Me, Loss and Lack, Death Row, Dead End, Destiny, Non-being, Non-human, Sub-human, Pre-human, Ancestor Rape, Use Me, Material, Fratricide, Torture Mirror, Somatic Machine, Responsive Behavior, Model Organism, Reflex Entity, Workhorse, and Factory.

6. At the inception of the lab, the eggs are removed from the incubator.
7. Class examination of normal embryological development: 1- through 7-day stages. The eggs are simply cracked into petri dishes and then put on display. Observation under the microscope is optional, but it often makes the heart beat easier to perceive.
8. Swab the egg with gauze soaked in 70% alcohol.

The large end of the egg is punctured with a needle to unseal the egg's lung (the air cell) and relieve pressure. Then students are instructed to put a knee up, rest one of their feet on a chair, and roll the egg back and forth five or ten times. This loosens stabilization in the amnion and lets the embryo float to the top of the egg where the window is to be made. It also makes the embryo veritably one sided in respect to gravity and, for the most part, unviable. A small window is made by the students in each 3- and 4-day incubated fertile egg. Windows are opened in the 3- and 4-day incubated egg's shells. This is so we can practice microsurgery, transplantation, teratology (with retinoic acid), and transgenics:

Microsurgery The non-Pyrex disposable glass pipettes are pulled with metal tweezers over a flame to make microsurgical scalpels. This is a standard lab practice, and a variety of shapes and intentions can be made with molten glass. The non-intuitive effects of incision on developing organisms are a gateway lesson in morphology, intercellular communication, differentiating body fields (protein crystals), cleavages (folds), and membranes (fabrics).

Transplantation Using newfound skills in microsurgery, explantation or transplantation is encouraged. A section, glob, or particular formative area (i.e., the limb bud, eye, heart, etc.) can simply be cut out of a 4- or 5-day embryo and pasted into a 3- or 4-day embryo (incised.) Due to timed developmental stages, the state of the 3-day incubated embryonic immune system, and the amount of differentiation which has occurred in both preemies, explants from one embryo to another at these stages are generally not rejected. Tungsten or platinum micropins are an unexplored option for this lab. They do help keep a transplant in place until it has grafted into *place*.

Teratology In this case retinoic acid was used as a teratogen. This is a standard teaching toxin because of the massive and obvious defects it causes. Simple application with pipette was offered. The point of this part of the research is to tout environmental toxicology as a way to prevent birth defects and screen environmental risks of pollution. At the same time, it is suggested that the relation between both the surgical methods and the chemical methods of causing morphological dissidence is conjoined in transgenic infection. Besides retinoic acid, alternative teratogens which can be used include ETA, vital dyes, colchicine-soaked microbeads, and concentrated parsnip juice.

Transgenic Infection The non-Pyrex disposable glass pipettes are pulled with metal tweezers over a flame. The transgenic bird embryos were made using a plasmid (which is an infective gene) called pDSRed-Dcl. This gene causes abnormalities in

embryo development and shape. Four-day-old manipulated embryos were microinjected by hand with a small amount of plasmid.

By comparing microinjection of a new gene into an embryo's somatic tissues to surgery, transplantation, and toxicological methods, the intention is to show the commonalities between the brute use of a micro scalpel and a poison to the process of gene insertion or transgene infection.

After manipulation, the bodies in the windowed eggs will need antibiotics (pen/strep) and a piece of clear yellowish scotch tape over the window for sterility. Add a few drops of penicillin/streptomycin and scotch tape over the window to prevent infection. (Remember to use the yellowish old school scotch tape and not the more expensive frosted "magic" tape which does not adhere in damp, yolky, incubator environs).

The incubated eggs are put back in the incubator eggs for at least another week of altered development.

The results analysis:

- Artist's reading of the resultant bodies.
- Come up with an impromptu group sculptural critique.
- Express an alternative version of process based on limits of self-expression.

The embryos are then culled humanely and disposed of or fixed properly for display. Alternatives are also offered. The methods of sacrifice commonly applied to avian embryos are death by autoclaving, refrigeration, putting down on ice, or simply pouring down the drain. I added two other options for my students: valium overdose and ritual sacrifice. In any case, the embryos are not hatched. The valium overdose seemed to be the most humane sacrifice for an embryo. It may have been the first time that an embryo was given such a respectful euthanasia.

"The selection of specific agents and methods for euthanasia will depend on the species involved and the objectives of the protocol. Generally, inhalant or noninhalant chemical agents (such as barbiturates, nonexplosive inhalant anesthetics, and CO2) are preferable to physical methods (such as cervical dislocation, decapitation, and use of a penetrating captive bolt). However, scientific considerations might preclude the use of chemical agents for some protocols. All methods of euthanasia should be reviewed and approved by the IACUC."(Guide for the Care and Use of Laboratory Animals n.d.).

2.2 DIY Plasmid Microinjection

Transgenic Avian Embryology: a note on the efficacy of manual application handmade microinjection needles to somatically introduce Plasmid into avian embryos as a Morphological Sculpting protocol. A lay summary of research:

Without expensive equipment we tested whether manual, intramuscular plasmid injection with homemade microinjectors could be a technique of pheasant, quail, and chick genetic modification. A plasmid is a circular ring of DNA that can unfurl

and insert its gene load into a living organism's cells. Secure in the knowledge that the hepatitis B vaccine is a working example of raw plasmid injection(Hui et al. 1999); the success of our research echoed the fact that plasmids need not be forced to invade their nuclear targets. Plasmids can find their own way through pores in cellular membranes and incorporate into genomes without the more brutish and costlier transgenesis skill sets (i.e., intranuclear microinjection, biolistics, viral vectors, heat shock, electroporation, etc.). When it comes to infectious genes, proximity is sometimes all that is needed to alter heredity.

3 Ethics and Considerations

3.1 Timing Death: Embryo Research Is Not Animal Research

Held at the University of Leiden, this lab cleared the Animal Experimentation Committee and Recombinant Safety. Although a part of AOL (all organisms living), it appears that avian embryos are not considered animals by definition in the EU as they are not "free-living beings." They are also not considered a recombinant safety hazard because they are incapable of reproducing. When a chicken embryo grows large enough to leave the egg and hatch, it changes rank and begins to be considered a free-living vertebrate animal. When a zebrafish embryo exits his eggshell, it leaves with a large chunk of egg yolk as a part of his abdomen. Research can continue on the non-animal until she firsts eats a meal from the external world, becoming a fish animal but with some rights reserved. These are all bioethical lines drawn in the sand, which change from nation to nation and are judged differently according to potential utilization premonitions. The focus on embryonic development is generally codified as pre-animal research or research with/on non-organisms, even in the arts.

Even our right to work with plasmid was in some way determined by the status of the embryos. Due to the fact that they we deemed not to be free-living, and that the end-of-life issues would be taken care of before an age of reproductive potential (i.e., in huevo), it was also rightfully assumed that they (the glob of not yet animal) would not represent a viable genetically modified danger. Simply put, embryos cannot get pregnant. In and out of the lab, transgenic embryo research is not animal research. The embryos just have to be culled before they become free-living.

3.2 Humane Sacrifice: Sacrificial Art and Sacrificial Science

"Euthanasia might be necessary at the end of a protocol or as a means to relieve pain or distress that cannot be alleviated by analgesics, sedatives, or other treatments. Protocols should include criteria for initiating euthanasia, such as degree of a physical or behavioral deficit or tumor size that will enable a prompt decision

to be made by the veterinarian and the investigator to ensure that the end point is humane and the objective of the protocol is achieved."(National Research Council 1996)

When to euthanize is determined by nation-state and cultural conceptions of feeling (nervous system development) and the fact that non-free-living beings cannot reproduce. The full cycle from conception to hatching is 21 days (assuming proper temperature and humidity.) Different countries have different protocols, but for embryo incubation to not be considered animal research, an end to observation should be planned for the seventh, 10th, 14th, or 20th day[1]. For this research, we did not study embryos older than 14 days.

3.3 Artists and Experiential Embryology

Mutagenesis impedes or coerces the imaginary into the lifeworld by pushing ideology into flesh. So, transgenic protocol is a ritual for the cultural production of liminal monsters. Just like artists, scientists have their methodologies of creative flourish and sacrifice. But, scientific and artistic play is often based on different paradigms of what the act of experimentation is. With that in mind, we have to open up the embryo to the philosophical discussion.

For its minute size, the embryo plays a big part in transgenic process arts. Developmental biological toolage entails processes that generally stem from the laboratory bench and fruit out into clinical/medical applications, speeding up food production and repopulating the earth with novel livestock, and "useful" industry plants. The appreciation for the organism in developmental biology is generally conceptualized as a platform for knowledge acquisition alone. The mutagenic developmental biology arts tend to study the same bodies (or bodies in progress) as embryologists and even wield the standard protocols lifted from developmental biology, high-tech animal husbandry, and human-assisted reproductive technology.

4 Conclusions (Fig. 1)

This hands-on research was documented in order to stimulate debate about the use of new biological methods for permanent alteration of morphological as well as hereditary genetic inheritance. The process involves an introduction to embryology and developmental biology as an art form. The protocol involves physical (surgical), chemical (teratogenic), and transgenic alteration of growing quail, chicken,

[1] The nerves develop around the third day but cold is considered an anesthetic (cryanesthesia) so it is suggested that simple removal from the incubator is all that is required to enervate and temporarily halt development.

Fig. 1 Collage, Transgenic Embryology Lab, with the Participating Students of Leiden University VivoArts Honors Class, The Arts and Genomics Centre, University of Leiden, NL, May 11th 2007 to May 22nd 2007, Video direction, Camera and Edit: Jeanette Groenendaal and Zoot Derks, g-netwerk.nl 2007. https://we-make-money-not-art.com/_yes_its_true_im/

and pheasant embryos. The manipulation of the embryos was the central focus of the research.

Transgenic sculpture laboratories give humanities students the tools and skills they need to in a real biotechnical relationship with their 4-day incubated and windowed eggs. This type of research is a furtherance of qualitative knowledge through hands-on artistic avian embryology and mutagenesis research. The documents mentioned are the embodied protocol and the results of imaging somatic difference. The habit of inserting an engineered plasmid into the genome of a cell line or organism is a physical artifact stemming from the mortal desire for lasting signature.

These stillborn sculptures, in accord with the libidinal economy of multigenerational directionality, have been impressed upon for the record alone. Consider their mutations to be a sort of genetic graffiti: signing, marking, branding, and tagging.

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Gene Editing, Sexual Reproduction, and the Arts: The Present, the Future, and the Imagined

Roberta Buiani

In recent years, popular culture has been graced with countless news announcing novel developments in genome editing. While many experiments are still in their early stages, genome editing seems very promising. Often betraying a sensationalist and triumphant tone, news coverage focuses on the potentials that these developments will have for the advancement of the human species, i.e., the eradication of disease, the extension of life, the improvement of the body and its appearance, etc. The future looks hopeful and unproblematic according to these accounts. On the opposite end of the spectrum, some may wonder whether these developments pose a potential worsening of the human condition: Are these developments safe? What are the ethical implications? Who will benefit from these developments? Given today's social divisions and cultural conflicts, these voices predict a rather unpromising future and warn against the pursue of innovation at any cost.

What these radically opposed positions share is that they all tend to make predictions and look at the future: a promising, improved future on the one hand, and a discouraging, problematic future on the other hand.

We propose before we look at what genome editing will bring us in the future to look how it affects the present. In particular, we ask: What are the unresolved issues in the present that we should address before launching ourselves into the future? Can we find interesting ways to draw attention to them and redress how they are currently treated through creative interventions? More specifically, We would like to explore issues regarding sexual reproduction, fertility, and sexual technologies. Artistic interventions pertaining to these topics, in addition to raising awareness about sexism, sexual rigidity, and the medicalization of the body, may also be suggestive of ways in which we might rethink the role of human enhancement and genome editing in sciences as well as in everyday life. We believe that present issues in gynecology, hormonal management, human enhancement, and sexual and cultural identity may be addressed, redressed, hacked, and reimagined through the arts.

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Amyotrophic Lateral Sclerosis: Current Status in Diagnostic Biomarkers

Katerina Kadena and Panayiotis Vlamos

1 Introduction

Amyotrophic lateral sclerosis (ALS) is a rare, neurodegenerative disease that affects the human motor system. ALS is a highly heterogeneous disease, depending on several causative factors. The heterogeneity of the disease is also reflected in the variation of the symptoms in ALS patients. The worldwide annual incidence of ALS is about 2.08 per 100,000 with uniform rates in Caucasian populations and lower rates in African, Asian, and Hispanic populations, while the number of individuals with ALS is expected to grow significantly between 2015 and 2040 with an estimated increase of 69% (Chio et al. 2013a; Arthur et al. 2016).

Taking into consideration its heterogeneity, it is of vital importance to design a precise diagnostic system which will allow researchers, clinicians, and policymakers to approach ALS and design effective treatments and efficient care structures.

Biomarkers are measurable indicators of an organism's state. Their measurements can be related and indicative of normal or pathological conditions. Biomarkers have been proved to be a trusted and validated component towards neurodegenerative diseases' diagnosis and personalized treatment. Biomarkers can also help monitor ALS progression or predict a person's outlook (Xu et al. 2016a).

Despite the fact that a lot of discussion and research has been made, there is a clear need to associate the neurobiology of ALS and its neurochemical abnormalities with cognitive deterioration which will lead to a set of validated indicators used to diagnose the disease (Tan et al. 2016). Moreover, since ALS has a recognized multifactor character, this leads to the need for designing a diagnostic and therapeutic strategy based on a systematic personalized approach. Taking into consideration that human biomarkers have extensively been used in other dementias as

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a diagnosis and prevention tool, a biomarker-guided diagnosis system is needed for the comprehensive dissection and classification of interacting and converging disease mechanisms, description of genomic and epigenetic parameters, natural trends of the patient, surrogate biomarkers, and indicators of risk and progression (Benatar et al. 2016).

Although ALS is the most predominant progressive neuromuscular disease worldwide, it seems that research and data related to its cause are insufficient compared to the progress made towards the diagnosis and treatment of other neurodegenerative diseases.

Having an unknown cause, research has shown that molecular alterations, including processing of RNA molecules, water channels, and calcium levels in different cells are some of the main factors involved in the progressive weakness of the motor system (Recabarren-Leiva and Alarcon 2018). According to NINDS ALS Fact Sheet of NIH, ALS is mostly sporadic and it occurs spontaneously without a known cause, while only 10% of incidents are considered familial due to inherited faulty genes.

Except for motor system impairment, ALS patients demonstrate mild to moderate cognitive disorders with numbers showing that up to 20% of ALS patients meet the full-blown dementia syndrome criteria, resembling the symptoms of Frontal Temporal Dementia (FTD) (Ringholz et al. 2005; Strong et al. 2009).

Up to now, clinical examination is critical for the diagnosis of ALS, and no biomarkers that can be solely used to diagnose and evaluate ALS progression have been identified. So, in this review, we will briefly discuss the main and most important molecules which could be biomarker candidates for the diagnosis of ALS and the prediction of disease progression which may lead to a set of indicators that can be used for ALS treatment approach in the future.

2 Biomarkers

Up to now, several biological and clinical studies have pointed out a number of diverse biomarkers that could be used to identify the ALS clinical phenotype. In the following paragraphs, we aim to assemble and briefly discuss the current status in this field.

2.1 Biological Biomarkers

2.1.1 Genes

A variety of studies have revealed many mutated genes responsible for familial inherited ALS, with the most known mutated gene being the superoxide dismutase 1 (SOD1) (Rosen 1993). More than 170 different mutations have been found in this gene and are associated with the pathological phenotype of ALS (Munch and

Bertolotti 2010). Apart from SOD1, mutations in genes related to nucleic acid metabolism, have been identified as causative factors for ALS (Blasco et al. 2016a).

In addition, mutations in TARDBP gene, which produces TDP43 protein and mutations in FUS gene, have been also implicated to ALS (Kamelgarn et al. 2016; Lee et al. 2011; Deng et al. 2014). At the same time, abnormal repetitions and differences in the length of specific genes have been correlated with the development of the disease. The GGGGGG hexanucleotide repeat expansion in the C9ORF72 gene is responsible for the majority of ALS cases, both familial and sporadic (Rohrer et al. 2015; Smith et al. 2013). Mutations have also been discovered at the vesicle-associated membrane protein-associated protein B (VAPB), which regulates the transportation and the elimination of abnormal proteins (Nishimura et al. 2004; Chen et al. 2010). The mutated VAPB cannot function properly and that leads to aggregation, and subsequently to motor neurons death (Suzuki et al. 2009).

Discoveries made thanks to the Genome Wide Association Studies (GWAS), which are able to identify associations between variants and characteristics of the ALS, have shown a number of new genes. In sporadic ALS, the gene UNC13A, which produces a protein responsible for the regulation of neurotransmitter release (van Es et al. 2009), is identified as a genetic factor in different cohort studies, made to Italian and Spanish ALS patients (Chio et al. 2013b; Vidal-Taboada et al. 2015).

Of course, the previous examples are some and the most prevalent causative factors of ALS. There are also other gene alterations identified in ALS patients, both in familial and sporadic subtypes, which can cause the disease. Regarding sporadic ALS, a study performed by Recabarren-Leiva et al. selected ten genes, playing an important role in the SALS, and tried to clarify the association between them and the four most common genes causing ALS (C9orf72, SOD1, FUS, and TDP-43). Surprisingly, there was no interaction between the proteins of the two groups (Recabarren-Leiva and Alarcon 2018).

Remarkably, the functional differences of the proteins produced by these genes, as well as the different clinical phenotypes of the patients, state once more the heterogeneity of ALS.

2.1.2 miRNAs

miRNAs are one of the three types of small non-coding, evolutionary conserved RNAs, which are responsible for the gene regulation (Hawley et al. 2017). miRNAs' length is approximately 22 nucleotides, and their function concerns the regulation of protein-coding genes, achieved through the repression of translation and the degradation of mRNA (Ha and Kim 2014).

To begin with, there are several mice studies that implicate the alteration of miRNAs' expression with the ALS (Rinchetti et al. 2018). In more details, mice bearing a mutation in the sod1 gene have elevated levels of miR-9 and miR-206 (Williams et al. 2009; Dobrowolny et al. 2015; Toivonen et al. 2014; Zhou et al. 2013). miR-155 shows increased levels in ALS, and miR-29 shows increased levels in ALS

in both the brain and spinal cord. Differential expression is observed for the miR-22, miR-155, miR-125b, and miR-146b (Cloutier et al. 2015; Parisi et al. 2013).

In human ALS patients, it was found that the expression of miRNAs was altered. Five different miRNAs (miR-524-5p, miR-582-3p, miR146a∗, miR-b1336, and miR-b2403) were found to interact with the neurofilament mRNA, which as we will discuss in the following paragraphs has a crucial role in ALS (Campos-Melo et al. 2013; Ishtiaq et al. 2014).

miRNAs are promising biomarker candidates, as they can be secreted by the cells in the extracellular space, remain stable in body fluids, and be isolated from them. Aiming to find the best potential miRNA biomarker, many researchers have published studies where they have analyzed patient samples, obtained from blood, serum, spinal cord, frontal cortex, leukocytes, and CSF, and have found alterations in the expressions of numerous miRNAs. This points out that a panel of miRNAs is a better biomarker than one specific miRNA, and it also can more accurately identify ALS subforms (Rinchetti et al. 2018; Cloutier et al. 2015).

2.1.3 Proteins

Proteins can be isolated from different sample types of ALS patients. The main types are plasma, serum, cerebrospinal fluid (CSF), urine, saliva, and brain tissue (Ryberg and Bowser 2008). Some of the most important proteins are described below.

2.1.4 Filaments

Filament proteins are one of the major categories that can be used as biomarkers for the diagnosis of ALS (Xu et al. 2016b). Neurofilaments (NFL) are intermediate filaments, parts of the cytoskeleton, mainly abundant in the axons, where under pathological conditions accumulate in cells and proximal axons of affected neurons. After an axonal injury, neurofilaments are released in the cerebrospinal fluid (CSF) and subsequently in the blood. As a result, NFL levels are increased in the blood of ALS patients. Furthermore, patients with ALS exhibit increased levels of phosphorylation of the neurofilament heavy chain (pNFH) in their CSF (Reijn et al. 2009; Brettschneider et al. 2006). This observation can be used as a prognostic factor, but also as a factor of disease progression and survival.

2.1.5 Inflammatory Mediators

Inflammation is one of the main characteristics of neurodegenerative diseases. Proteomic studies of the CSF of ALS patients, compared to healthy donors, revealed 248 proteins with differential expression related to inflammation and the complement cascade (Ransohoff 2016; McGeer and McGeer 2002). Alteration levels of

cytokines can be used as biomarkers. Similarly, in the blood, increased levels of pro-inflammatory cytokines such as IL-6 and IL-8 were found. Also, IL-5 and IL-2 levels were decreased, while TGF-β levels were increased and were correlated with ALS duration. Apart from the blood, increased levels of cytokines, such as TNFα, IL-1β, and IL-2, and decreased levels of IFN-γ were also observed in the plasma (Vu and Tsukahara 2017).

2.1.6 TDP-43

Although previously we have discussed the mutations that occur in the gene that produces the TAR DNA-binding protein of 43 kDa, now we will address the fact that the identification of these proteins in the CSF, the blood, or the plasma of ALS patients could be a critical biomarker since TDP-43 levels of ALS patients were found elevated compared to healthy donors or donors with other types of neurological diseases (Junttila et al. 2016; Kasai et al. 2009). However, several problems, including the different isoforms of the protein produced by the alternative splicing of the transcript and the self-aggregation capacity of this protein, have risen, and more sensitive techniques are required in order to establish TDP-43 as a biomarker. TDP43 positive ubiquitinated cytoplasmic inclusions have been identified not only in ALS patients but also in a great percentage of patients with frontotemporal dementia (FTD) (Steinacker et al. 2008; Xiao et al. 2015).

2.1.7 Cystatin C

Cystatin C is another biomarker for ALS diagnosis, as studies from different groups have shown. In general, the cystatin C is a cysteine proteinase inhibitor involved in the regulation of the extracellular matrix and the nervous system repairment (Wilson et al. 2010). Its levels in ALS patients have been found decreased in CSF, while in the plasma its levels are increased. Its reduced levels in CSF are positively correlated with the survival, while the opposite applies to the plasma levels (Chen et al. 2016a; Ranganathan et al. 2005).

2.1.8 New Insights by Omic Studies

During the last years, the omic studies, which are studies related to a specific field, have been a useful tool in examining the flow of information that underlies ALS. Proteomics study the proteome of an organism. In a similar way, metabolomics' studies concern the metabolome, which is the set of metabolites present in biological samples and produced due to the metabolism. In this paragraph, we will discuss the advancements that proteomics and metabolomics have offered in the research field of ALS biomarkers.

To begin with, proteomic analysis in the CSF of ALS patients has revealed several proteins that could be significant biomarkers. For example, Chen et al. have concluded that insulin-like growth factor II (IGF-2) could be an effective biomarker to monitor ALS progression. IGF-2 is downregulated in ALS patients, and simultaneously when ALS patients were compared with OND groups, IGF-2 showed a further decrease (Chen et al. 2016b). Another study performed by Collins et al. pointed out WDR63 protein as a potential biomarker. Levels of WDR63 were increased in ALS patients compared to healthy donors or donors with other neurodegenerative diseases, but still, a further investigation of this protein and its role is required. Moreover, a meta-analysis review has gathered the proteomic-concerning publications and evaluated their results. It concluded that there was low overlap among the studies, and a variety of reasons could be responsible for this. Sensitivity and reproducibility need to be increased so that solid data could be produced (Barschke et al. 2017).

Metabolomic studies in CSF, plasma, and serum of ALS patients have been conducted by several research groups. These studies revealed alterations in metabolic pathways of ALS patients, which at the same time were correlated with the progression of the disease. Furthermore, the comparison of the metabolome of sporadic ALS patients and of patients with SOD1 mutation demonstrated different metabolic signatures in the two groups (Blasco et al. 2016b, 2016c).

Proteomic and metabolomic studies are researching approaches which could reveal several new and interesting potential biomarkers for the diagnosis of ALS, especially if they are combined with other analytical techniques and clinical findings.

2.2 Clinical Biomarkers

2.2.1 Neuroimaging Biomarkers

As previously mentioned, there is not a specific diagnostic test able to identify ALS. For this reason, the clinicians rely on the upper and lower motor neuron signs of a region. It is a common that definitive diagnosis is delayed. The most important advance of neuroimaging biomarkers is that they could be applied in the clinical practice and may contribute to an early diagnosis (Grolez et al. 2016).

The candidate neuroimaging biomarkers are divided in two groups, the ones that belong to the radionuclide imaging and the ones belonging to the magnetic resonance imaging (MRI). The first category includes the single photon emission computed tomography (SPECT) and the positron emission tomography (PET), while the second one comprises of the voxel- and surface-based MRI morphometry (VBM and SBM), the diffusion tensor imaging (DTI), the functional MRI (fMRI), the magnetic resonance spectroscopy (MRS), and the spinal cord MRI (Chen and Shang 2015).

The MRI can track the hyperintensity of the corticospinal tracts and also the cerebral atrophy in ALS patients. Magnetic resonance spectroscopy can detect proton-containing metabolites, which serve as markers for the detection of upper

motor neuron dysfunction. On the other hand, diffusion tensor imaging can point out decreased fractional anisotropy in the corticospinal tract. PET is able not only to clarify specific changes in the neuronal receptors of the extra-motor cerebral in ALS patients but also to provide information concerning the inflammatory mechanisms that govern ALS (Grolez et al. 2016).

The main results of the previously mentioned neuroimaging biomarkers are the discovery of the hyperintensity of the corticospinal tract in FLAIR/T2 and the hypointensity of the motor cortex on SWI. Simultaneously, structural magnetic resonance imaging demonstrates the atrophy of the precentral gyri (VMB) or of the extra-motor regions (VBM). Also, it shows the cortical thinning of the primary motor cortex and extra-motor regions (SBM). The diffusion tensor imaging shows the decreased FA of the corticospinal tract, the corpus callosum, and the extra-motor regions, as well as the decreased ADC of the corticospinal tract. The magnetic resonance spectroscopy reveals the decrease of the NAA in the motor cortex and the different metabolites in the CST and the extra-motor regions. Quantitative iron imaging depicts the deposition of iron in the motor cortex, CST, and extra-motor regions. Finally, the functional magnetic resonance imaging is able to detect alterations of the cerebral activation during motor and other tasks, the abnormalities in the functional connectivity in the sensorimotor and other networks, and the coherence of the somatosensory and extra-motor areas (Mazon et al. 2018).

3 Conclusion

It is clear that although technological advancements and highly novel scientific studies have drawn attention to clinical observations and new molecules have provided insights regarding ALS pathogenesis, still a specific biomarker for the disease, prognostic or diagnostic, has not been found. Even though some promising candidates have been described, each one of them requires further development and study, in order to increase the accuracy and sensitivity of each method. Especially, due to the heterogeneity of the disease, the combination of different biomarkers seems more prominent for a better diagnosis. In other words, technological advances must come together with clinical measurements to form a complete understanding of the disease. This would give a remarkable opportunity to comprehend the disease, its causative factors, and its progression.

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Synthesis and Characterization of Biologically Significant 5-[N,N-dialkylamino alkoxy] azaindole 2-one, 3-thiosemicarbazones and 5-[N,N-dialkylamino alkoxy] azaindole 3-hydrazone, 2-ones

Konda Swathi, Galla Rajitha, and Manda Sarangapani

1 Introduction

Isatin is an endogenous compound first isolated in 1998 and reported (Pandeya and Raja 2002) to possess a wide range of central nervous system activities. Surendranath pandya (Pandeya et al. 2000) et al. reported the synthesis and anticonvulsant activity of some novel N-methyl/acetyl, 5-(un)-substituted isatin-3-semicarbazones. In the last few years, isatin derivatives have been discovered which show potential hypnotic (Padhy et al. 2004), antibacterial (Raviraj et al. 2004; Gupta et al. 2004; Ajitha et al. 2002), and MAO inhibitory (Krall et al. 1978) activity.

It is evident from the literature survey that isatin derivatives, dialkylaminoalkyl derivatives, show more promising central nervous system and anticonvulsant activities. Keeping in view these two molecular moieties, viz., 5-hydroxy azaisatin (resembles serotonin) and dialkylamino alkyl (resembles NT), it is our endeavor to bring such important moieties into a single molecular frame as a model for molecular conjunction by appropriate synthetic routes and to screen them for anticonvulsant activity and neurotoxicity.

We are reporting in the present study the synthesis and characterization of some new compounds: 5-[N,N-dialkylaminoalkoxy] Azaindole 2-one,3- thiosemicarbazones, 5-[N,N- dialkyaminoalkoxy] Azaindole,3- hydrazone, 2-ones.

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In the present work, some new 5-[N, N-dialkylaminoalkoxy] Azaindole 2, 3-dione derivatives were prepared from 5-hydroxy aza isatin and its thiosemicarbazones. All the compounds were evaluated for anticonvulsant activity by the maximal electroshock-induced convulsion method. These compounds were also evaluated for their neurotoxicity study by the skeletal muscle relaxant activity method. 5-[2- dimethylamino ethoxy] Azaindole 3-hydrazone, 2-one (Va) and 5-[2-dimethyl aminoethoxy] Azaindole 2- one,3-thiosemicarbazone(IIIa) showed good anticonvulsant activity when compared with standard drug phenytoin, and all the compounds showed reduced neurotoxicity when compared with standard drug diazepam. The antiepileptic effect of compound (IIIa) and compound (Va) on the brain has bit been experimentally confirmed. Therefore, the aim of the present investigation was to evaluate the effect of compound (IIIa) and compound (Va) in rat brain after induction of epilepsy by MES in albino Wistar rats.

2 Experimental

2.1 Materials and Methods

The compounds were mostly synthesized by conventional methods and described in experimental selection and also by the methods established in our laboratory.

2.1.1 Preparation of 5-Hydroxyazaindole 2-one, 3-thiosemicarbazone (II) and 5-Hydroxyazaindole 3- hydrazone-2-one(IV) Scheme 1

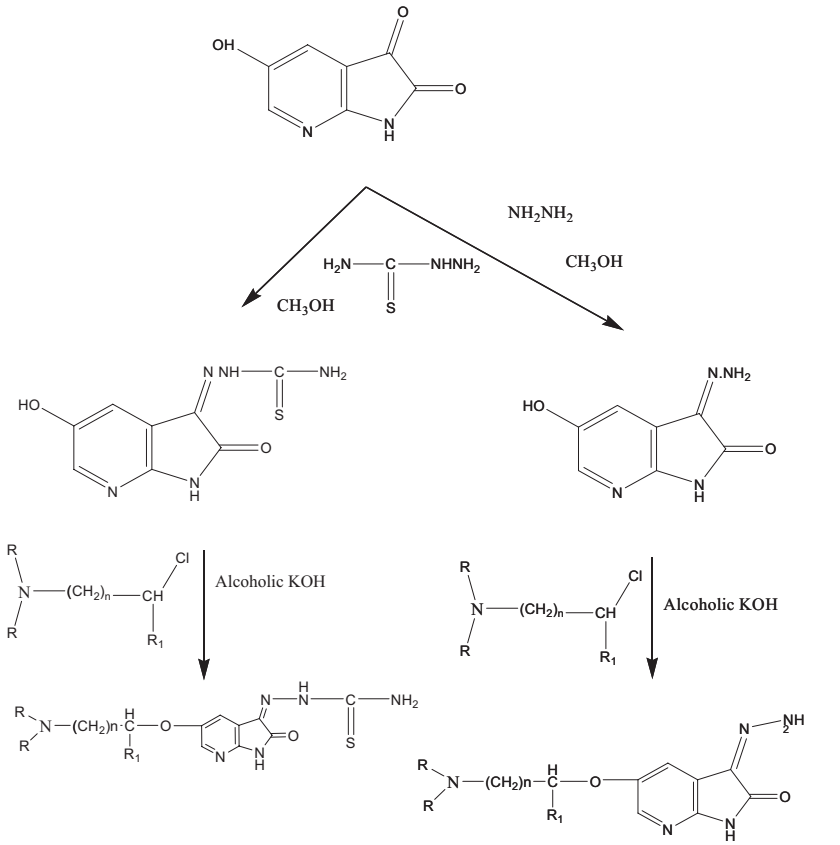
5-Hydroxyazaisatin was heated under reflux in methanol containing two or three drops of acetic acid with thiosemicarbazide hydrochloride/hydrazine hydrate for half an hour. The product thus separated was filtered and purified by recrystalization from suitable solvent (yield 89%, m.p.270 ºC (II); yield 90%, m.p.284 ºC (IV)). *2.1.2 Preparation of 5-[2(3)-dialkyl amino alkoxy] Azaindole 3-thiosemicarbazone-2- one (III) and 5-[2(3)-dialkyl amino alkoxy] Azaindole 3-hydrazone-2-one(V)* Scheme 1**.**

A mixture of 5-Hydroxyazaindole 2-one,3-thiosemicarbazone (II)/5-hydroxy azaindole 3- hydrazone 2-one(IV) (0.01 moles) and dialkylamino alkylhalide (0.01 moles) was placed in 10% alcoholic potassium hydroxide, and this mixture was stirred at room temperature for 6 hours. The alcohol was reduced to half of its volume and cooled. The product separated was filtered, washed with small portions of cold alcohol repeatedly, and dried. The physical data of the title compounds were presented in Table 1. The compounds were characterized by spectral data.

2.2 Spectral Data

The compounds have been characterized by the spectral data IR, PMR, and mass.

IR spectrum (KBr) of compound (I) exhibited absorption bands (cm-1) 3421.47(OH), 1630.08(C=O), 1548(Ar, C=C), 1282(C-O-C), and 883.85-579.8



5-Hydroxy-asaisatin-3-thiosemicarbazone derivative (III)

I I Ia: R=CH_3; R_1 = H ;n=1

I I Ib: R=C_2H_5; R_1 = H ;n=1

I I Ic: R=CH_3 ; R_1 = H ;n=2

I I Id: R=CH_3;R_1=CH_3 ;n=1

I I Ie: R=CH_3-CH-CH3; R_1 = H ;n=1

5-Hydroxy-azaisatin-3-hydrazone derivative (V)

Va :R=CH_3; R_1 = H ;n=1

Vb:R= C_2H_5; R_1 = H ;n=1

Vc:R=CH_3 ; R_1 = H ;n=2

Vd:R=CH_3;R_1=CH3 ;n=1

Ve :R=CH_3-CH-CH_3; R_1 = H ;n=1

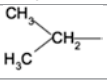
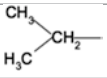
Scheme 1 Synthesis of 5-[N,N-dialkylamino alkoxy] azaindole 2-one,3- thiosemicarbazones and 5-[N,N-dialkylamino alkoxy] azaindole 3-hydrazone, 2-ones

(Ar). 1H NMR (300 MHz, DMSO-d6):13.3 (s, 1H, OH), 10.36 (s,1H,-CONH), 6.65-7.29 (m, 3 H, Ar-H).

Mass spectrum of compound III showed molecular ion (M+) base peak at m/z (164.1).

Compound (IIIa) showed characteristic IR peaks at 3368.41(NH2), 3282.52(CONH), 1708(C=O), 1576(Ar C=C), 1263(C-O), 1085(C=S), 1576(C=N), and 883.85 (Ar C-C). 1H NMR (300 MHz, DMSO-d6): 11.36(s,

Table 1 Physical data of 5-[2(3)-dialkyl amino alkoxy] Azaindole 2-one, 3-thiosemicarbazones (IIIa-IIIe) and 5-[2(3)-dialkyl amino alkoxy] Azaindole 3-hydrazone-2-ones(Va-Ve)

S.No	Compound	R	R	N	X	M.F	% YEILD	M.P	M.Wt
1	IIIa	CH_3	H	1	$NNHCSNH_2$	$C_{13}H_{17}N_5O_2S$	91%	280	307
2	IIIb	C_2H_5	H	1	$NNHCSNH_2$	$C_{15}H_{21}N_5O_2S$	86%	272	336
3	IIIc	CH_3	H	2	$NNHCSNH_2$	$C_{14}H_{19}N_5O_2S$	93%	283	353
4	IIId	CH_3	CH_3	1	$NNHCSNH_2$	$C_{14}H_{19}N_5O_2S$	85%	264	353
5	IIIe		H	1	$NNHCSNH_2$	$C_{16}H_{24}N_2O_2S$	81.8%	258	365
6	Va	CH_3	H	1	NNH_2	$C_{17}H_{27}N_5O_3$	92%	293	248
7	Vb	C_2H_5	H	1	NNH_2	$C_{14}H_{20}N_4O_2$	83%	269	276
8	Vc	CH_3	H	2	NNH_2	$C_{13}H_{18}N_4O_2$	92%	261	294
9	Vd	CH_3	CH_3	1	NNH_2	$C_{13}H_{18}N_4O_2$	86%	252	294
10	IVe		H	1	$NNHCONH_2$	$C_{13}H_{26}N_4O_2$	82%	248	306

1H,CONH),7.29(s,2H,NH_2),7.03(s,1H,Ar-H),7.20(d,1H,Ar-H), 7.94(d, 1H,Ar-H), 3.2(t,2H, O-CH_2), 2.9(t,2H,N-CH_2),1.36(s, 6H,N-$(CH_3)_2$).

Mass spectrum of compound IIIa showed molecular ion (M+) base peak at m/z 307. The mass spectrum shows its base peak at m/z 93 (100%), and it may be due to the fragmentation of the thiosemicarbazone from the molecule ion.

Compound (Va) showed characteristic IR peaks at 3450.13(NH2), 146.46(CONH), 1708(C=O), 1268 (C-O- C), 1085(C=S), and 1528(C=N). 1H NMR (300 MHz, DMSO-d6): 11.36(s,1H,CONH),7.29(s,2H,NH_2) 7.03(s,1H,Ar-H),7.20(d,1H,Ar-H),7.94(d,1H,Ar-H), 3.2(t,2H, O-CH_2), 2.9(t,2H, N-CH_2),1.36(s, 6H,N-$(CH_3)_2$).

Mass spectrum of compound Va showed molecular ion (M+) base peak at m/z 248 (100%). It also shows peak at m/z (71) which may be due to the fragmentation of the alkyl chain from the molecule ion.

3 Pharmacology/Anticonvulsant Activity

3.1 Maximal Electroshock Seizure (MES) Method and Neurotoxicity Study (Schlumpf et al. 1974)

The antiepileptic activity was studied by MES-induced convulsion method (Vogel 2002) by using electro-convulsometer. Healthy albino rats (150–220 g) were fasted overnight and divided into groups of six animals each. The test compounds suspended in (1% w/v SCMC, 1 ml/100 g) were administered at a dose of 100 mg/kg body weight i.p. The control group animals received only vehicle (1% w/v SCMC, 1 ml/100 g). The test started 30 min after i.p. injection. Maximal seizures were induced by the application of electrical current to the brain via corneal electrodes.

Table 2 Antiepileptic and neurotoxicity study of 5-[2(3)-dialkyl amino alkoxy] Azaindole-2-one, 3- thiosemicarbazone s(IIIa-IIIe) and 5-[2(3)-dialkyl amino alkoxy] Azaindole 3-hydrazone-2-ones(Va-Ve)

S.No	Compound	MES induced convulsions (%protected)	Skeletal muscle relaxantactivity (Neurotoxicity)(%)
1	IIIa	75.54 ± 0.341	6 ± 0.373
2	IIIb	68.61 ± 0.142	12 ± 1.234
3	IIIc	58.42 ± 0.151	6.7 ± 0.673
4	IIId	51.5 ± 1.234	3.4 ± 1.095
5	IIIe	47.46 ± 0.342	5.6 ± 2.384
6	Va	75.18 ± 0.436	8.2 ± 1,345
7	Vb	76.68 ± 0.234	3.7 ± 0.234
8	Vc	64.76 ± 0.763	5.8 ± 0.567
9	Vd	48.18 ± 1.236	6.8 ± 1.378
10	Ve	37.44 ± 1.451	7.4 ± 1.567
11	Phenytoin	100	–
12	Control	0	2
13	Diazepam	–	78 ± 0.256

Number of animals $n = 6$, the compounds were tested at a dose of 100 mg/kg (b.w)

The stimulus parameter for mice was 50 mA in a pulse of 60 Hz for 200 ms. Abolition of the hind limb tonic extensor spasm was recorded as a measure of antiepileptic activity. The neurotoxicity (Schlumpf et al. 1974) was studied by the rotarod method, using diazepam as a standard. Results are presented in Table 2.

5-[2-dimethyl amino ethoxy] Azaindole 3-hydrazone,2-one(IIIa) and 5-[2-dimethyl amino ethoxy] Azaindole 2-one,3-thiosemicarbazone(IVa) showed good anticonvulsant activity when compared with standard drug phenytoin and other compounds. These two compounds showed less neurotoxicity when compared with standard drug diazepam.

The antiepileptic effect of 5-[2-Dimethyl amino ethoxy] Azaindole 3-hydrazone, 2-one (Va) and 5-[2-Dimethyl amino ethoxy] Azaindole 2-one,3-thiosemicarbazone(IIIa) on the brain has not been experimentally confirmed. Therefore, the aim of the present investigation was to evaluate the effect of 5-[2-Dimethylamino ethoxy] Azaindole 3-hydrazone,2-one(V) and 5-[2-Dimethyl amino ethoxy] Azaindole 2-one, 3-thiosemicarbazone(IIIa) in rat brain after induction of epilepsy by MES in albino Wistar rats.

4 Biogenic Amines Estimation by Fluorimetric Micromethod

4.1 Experimental Design for Biogenic Amine Estimation

Albino Wistar rats were divided into four groups, each consisting of six animals. Group I received vehicle control (1% w/v SCMC, 1 ml/100 g), group II received standard drug (phenytoin, 25 mg/kg) i.p, and group III and IV received

5-[2-Dimethylamino ethoxy] Azaindole 3-hydrazone,2-one(V) and 5-[2-Dimethyl amino ethoxy] Azaindole 2-one, 3-thiosemicarbazone(IIIa) (100 mg/kg) i.p., respectively, for 14 days. On the 14th day, seizures were induced to all the groups by using an electro-convulsometer. The duration of various phases of epilepsy was observed.

4.2 A Fluorimetric Micromethod for the Simultaneous Determination of Serotonin, Noradrenaline, and Dopamine

On the 14th day after observing the convulsions, all rats were sacrificed, and their whole brain was dissected, and the forebrain was separated. Weighed quantity of tissue was homogenized in 0.1 ml hydrochloric acid–butanol (0.85 ml of 37% hydrochloric acid in 1 liter n-butanol for spectroscopy) for 1 min in a cool environment. The sample was then centrifuged for 10 min at 2000 rpm. 0.08 ml of supernatant phase was removed and added to an Eppendorf reagent tube containing 0.2 ml of heptane (for spectroscopy) and 0.025 ml 0.1 M hydrochloric acid. After 10 min of vigorous shaking, the tube was centrifuged under the same conditions to separate two phases. Upper organic phase was discarded, and the ethanol phase (0.02 ml) was used for estimation of serotonin, noradrenaline, and dopamine assay.

4.3 Noradrenaline and Dopamine Assay

The assay represents a miniaturization of the trihydroxide method. For the 0.02 ml of HCl phase, 0.05 ml 0.4 M and 0.01 ml EDTA/sodium acetate buffer (pH 6.9) were added, followed by 0.01 ml iodine solution (0.1 M in ethanol) for oxidation. The reaction was stored after 2 minutes by addition of 0.01 ml Na_2SO_3 in 5 M NaOH. Acetic acid was added 1.5 minutes later. The solution was then heated to 100 °C for 6 min. When the sample again reached room temperature, excitation and emission spectra were read in the micro cuvette as with 5-HT: in some cases, the readings were limited to the excitation maxima. 395–485 nm for NA and 330–375 nm for DA uncorrected instrument values (Applegate et al. 1986).

4.4 Serotonin Assay

As mentioned earlier, some modifications in reagent concentration together with changes in the proportions of the solvent became necessary; in order to obtain a good fluorescence yield with reduced volume for 5-HT determination, the

o-phthaldialdehyde (OPT) method was employed. From the OPT reagent, 0.025 ml were added to 0.02 ml of the HCl extract. The fluorophore was developed by heating at 100 °C for 10 min. After the samples reached equilibrium with the ambient temperature, excitation/estimation spectra or intensity readings at 360–470 nm were taken in the micro cuvette (Corcoran 1988).

5 Results and Discussions

Synthesis and characterization of 5-[2-dimethyl amino ethoxy] Azaindole 3-hydrazone(Va), 2- one and 5-[2-dimethyl amino ethoxy] Azaindole 2-one,3-thiosemicarbazone(IIIa) were done by standard methods.

Effect of 5-[2-dimethyl amino ethoxy] Azaindole 3-hydrazone,2-one (Va) and 5-[2-dimethyl amino ethoxy] Azaindole 2-one,3-thiosemicarbazone(IIIa) showed good antiepileptic activity in seizure-induced rats by MES. These compounds were specifically evaluated for levels of biogenic amines in rat brain.

A new series of five 5-[N,N-dialkyl amino alkoxy] Azaindole 2,3 dione derivatives were synthesized by reacting 5-hydroxyAzaindole 2,3 dione with 2-N,N di alkylamino alkyl halides. Evaluation of these compounds' anticonvulsant and skeletal muscle relaxant activity revealed that the compounds Va($R = CH_3$), Vb($R = C_2H_5$), IIIa($R = CH_3$), and IIIb($R = C_2H_5$) with a dimethyl and diethyl amino ethyl chain derivatives were found to be relatively superior in anticonvulsant activity followed by the compounds (IIIc, Vc, IIId, Vd, IIIe, Ve). All the compounds showed reduced neurotoxicity compared to diazepam**.**

5.1 Statistical Analysis

The data were expressed as mean ± standard error mean (SEM). The significance of differences among the group was assessed using one-way and multiple-way analysis of variance (ANOVA). The test was followed by Dunnett's test. p values less than 0.05 were considered as significance.

5.2 Noradrenaline

In MES model, noradrenaline levels significantly ($p < 0.01$) decreased in the forebrain of epileptic control animals. 5-[2-Dimethyl amino ethoxy] Azaindole 3-hydrazone,2-one (Va) and 5-[2-Dimethyl amino ethoxy] Azaindole 2-one,3-thiosemicarbazone(IIIa) at doses of 100 mg/kg, standard drugs phenytoin- and diazepam-treated animals showed a significant ($p < 0.05$ & $p < 0.01$) increase in noradrenaline levels in the forebrain of rats (Table 3).

Table 3 Effects of 5-[N,N-dialkyl amino alkoxy] Azaindole 2-one,3-thiosemicarbazone(III) and 5-[N,N-dialkyl amino alkoxy] Azaindole 2-one,3-hydrazone(V) on neurotransmitter levels in rat brain after MES-induced epilepsy

Group	Design of treatment	Noradrenaline	Dopamine	Serotonine
I	Vehicle control(SCMC 1ml/100gm)	762.25 ± 1.12	640.46 ± 4.17	132.39 ± 2.15
II	MESV(SCMC 1ml/100gm)	419.27 ± 2.21[a] **	589.17 ± 1.22[a] **	95.68 ± 2.15[a] **
III	Phenytoin 25mg/kg,i.p	648.18 ± 2.61[b] **	705.27 ± 2.64[b] **	15.27 ± 2.19[b] **
IV	5-[2-dimethyl amino ethoxy] Azaindole 2-one,3-thiosemicarbazone(IIIa) 100mg/kg	624.51 ± 2.12[b] **	665.28 ± 4.42[b] **	92.14 ± 2.15[b] **
V	5-[2-dimethyl amino ethoxy] Azaindole 3-hydrazone, 2-one(Va) 100mg/kg	774.16 ± 2.17[b] *	554.33 ± 1.41[b] *	78.19 ± 1.287[b]

Values are expressed as mean ± SEM of six observations. Comparison between (a) group I vs group II and (b) group III vs group IV and group V. Statistical significance test for comparison was done by ANOVA, followed by Dunnett's test $*p < 0.05$; $** p < 0.01$; units = pg/mg of wet tissue

5.3 Dopamine

In MES model, dopamine levels significantly ($p < 0.01$) decreased in the forebrain of epileptic control animals. Phenytoin- and diazepam-treated animals showed a significant ($p < 0.05$ & $p < 0.01$) increase in dopamine levels in the forebrain of rats (Table 3).

5.4 Serotonin

In MES model, serotonin levels significantly ($p < 0.01$) decreased in the forebrain of epileptic control animals. 5-[2-Dimethyl amino ethoxy] Azaindole 3-hydrazone,2-one(Va)and5-[2-Dimethylaminoethoxy]Azaindole2-one,3-thiosemicarbazone(IIIa) at doses of 100 mg/kg, standard drugs phenytoin- and diazepam-treated animals showed a significant ($p < 0.05$ & $p < 0.01$) increase in serotonin levels in the forebrain of rats (Table 3).

6 Conclusion

A new series of azaindole derivatives were synthesized by reacting 5-hydroxyazaindole 3- hydrazone,2-one /5-hydroxyazaindole 3-hydrazone,2-one schiff bases with 2-N,N di alkyl-amino alkyl halides. The role of biogenic amines in epileptogenesis and in recurrent seizure activity is well- documented. Spontaneous

and experimentally induced deficiencies in noradrenaline (NA), dopamine (DA), and/or serotonin (5-hydroxy- tryptamine or 5-HT) have been implicated in the onset and perpetuation of many seizure disorders. Several experimental procedures designed to increase monoaminergic activity have proven antiepileptic properties (Corcoran 1988; McIntyre and Edson 1989; Pelletier and Corcoran 1993; Yan et al. 1995; Zis et al. 1992). In the present study, the established antiepileptic drugs such as phenytoin restored the monoamine levels on the brain (Applegate et al. 1992). Similarly, compound (IIIa) and compound (Va) significantly ($p < 0.05$ & $p < 0.01$) increased monoamines levels in the forebrain of rats. Many drugs that increase the brain contents of GABA have exhibited anticonvulsant activity against seizures induced by MES (McIntyre 1989). MES is probably the best validated method for assessment of anti- epileptic drugs in generalized tonic-clonic seizures.

In conclusion biogenic amines participate in the control of MES-induced seizure in rat model. Our findings support the hypothesis that decreased the monoamines levels in rat brain after induction of the seizure. In compound (IIIa) and compound (Va) treated rats, monoamines such as NA, DA & 5-HT levels were significantly restored on forebrain. Thus compound (IIIa) and compound (Va) increase the seizure threshold and decrease the susceptibility to MES induced seizure in rats. Hence, we suggest that new azaindole derivatives possess antiepileptic properties that may be able to restore the biogenic amines in rat brain.

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Genetic Counseling for Adult-Onset Spinal and Bulbar Muscular Atrophy (Kennedy Syndrome): Multiple Cases of Prenatal Testing in a Family

Christos Yapijakis, Achilleas Laskaratos, Antonia Angelopoulou, and Costas Voumvourakis

1 Introduction

X-linked spinal and bulbar muscular atrophy (SBMA), also known as Kennedy syndrome, is a late-onset neurodegenerative disorder characterized by slowly progressive muscle atrophy and fasciculation, dysarthria, dysphagia, weakness of the limbs, and hand tremor (Finsterer 2009; Fratta et al. 2014; Kennedy et al. 1968; Stefanis et al. 1975). Patients also exhibit male-specific endocrinological defects such as androgen insensitivity, gynecomastia, reduced fertility, testicular atrophy, impotence, azoospermia, and oligospermia (Finsterer 2009; Fratta et al. 2014). SBMA usually appears in middle-aged individuals and gradually leads to reduced mobility and ultimately to death, often due to respiratory failure (Finsterer 2009; Fratta et al. 2014). The prevalence of SBMA is commonly referred as 1 in 40,000 males; however, it could be higher due to the disease being underdiagnosed since some patients receive an incorrect diagnosis (Finsterer 2009).

SBMA is caused by an abnormal expansion of a polymorphic CAG trinucleotide repeat in the first exon of the gene encoding the androgen receptor on Xq12 (La

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Spada et al. 1991). The normal range is 9–36 CAG repeats in healthy individuals, while >37 repeats cause SBMA in male patients, with the repetition range being inversely correlated to the age of onset (Finsterer 2009; Fratta et al. 2014; Syrrou et al. 2001). Under normal conditions, the androgen receptor acts as a transcription regulator that mediates the actions of testosterone and 5a-dihydrotestosterone, while the mutant androgen receptor contains an expanded polyglutamine tract that results in insufficient response to androgens, hence leading to endocrinological abnormalities. Additionally, mutant androgen receptor molecules accumulate in the nuclei of the spinal and bulbar motor neurons resulting in their degeneration, thus causing neuromuscular impairments (Beitel et al. 2013).

SBMA is a rather severe neurodegenerative disorder; therefore, prenatal testing offered to couples at risk is justified. Nevertheless, several ethical issues related to prenatal testing for adult-onset conditions should be considered during genetic counseling (Hercher et al. 2016). The first two cases worldwide of prenatal DNA testing for SBMA were realized by our group two decades ago in Greece (Yapijakis et al. 1996). Since then, only one more case of fetal DNA testing in Germany has been reported in the literature (Jedele et al. 1998). In all three cases, chorionic villi were tested, and male fetuses were found to have the SBMA mutation allele; therefore, after extensive genetic counseling, the parents decided to terminate the pregnancies (Jedele et al. 1998; Yapijakis et al. 1996).

2 Methods and Results

Here we present a Hellenic family that originates from an Aegean island in Southern Greece. The extended pedigree that included 35 individuals of 5 generations contained 3 males with SBMA and 5 female carriers. A portion of the family is shown in Fig. 1. Informed consent was obtained from all individuals for every molecular testing reported in the study.

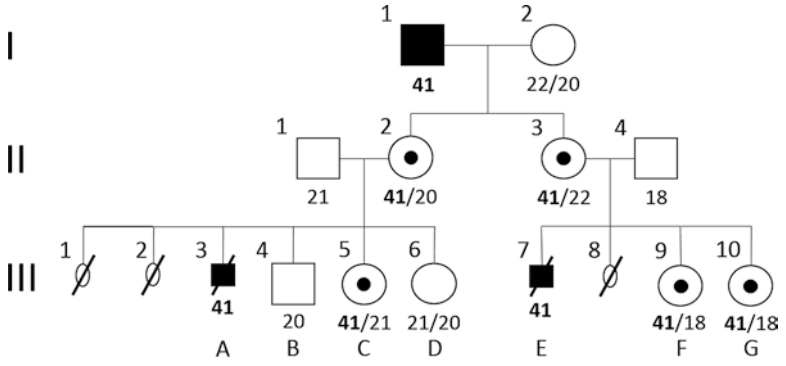

Fig. 1 Pedigree of the studied SBMA family. The CAG repeats alleles observed in each individual as well as in prenatal tests A, B, C, D, E, F, and G are shown

The index case (patient I-1, Fig. 1) was clinically diagnosed with SBMA at the age of 53, based on the presence of atrophy and weakness of the limbs and bulbar muscles, tremor, fasciculation of perioral, tongue and trunk muscles, muscle cramping, gynecomastia, and impotence. Molecular examination of a blood DNA sample by PCR amplification of the androgen receptor gene region containing the trinucleotide repeat, as previously described (Yapijakis et al. 1996), confirmed an expanded allele of 41 CAG repeats. His two daughters (28-year-old individual II-2 and 25-year-old individual II-3) as expected were heterozygous carriers of the expanded allele with 41/20 and 41/22 repeats, respectively (Fig. 1). After genetic counseling, both young women and their normal partners (II-1, II-4) asked for prenatal testing for X-linked SBMA.

The female carrier II-2 had a previous history of two spontaneous abortions during the first trimester of gestation (III-1, III-2). She underwent four prenatal tests of chorionic villi samples in a period of 6 years (Fig. 1). Her sister II-3 had three prenatal tests for SBMA in addition to a spontaneous abortion (III-8) over a period of 4 years (Fig. 1).

We performed all seven prenatal tests during the 9th–12th weeks of the pregnancies after chorionic villi sampling. Fetal DNA was extracted from the chorionic villi, and the CAG tandem repeats were amplified by PCR and analyzed for the precise determination of the repeat copy numbers with standard methodology (Yapijakis et al. 1996). In all cases, simultaneous karyotype testing of the fetus for chromosomal abnormalities revealed normal results.

The first prenatal testing (A) for SBMA in the first couple (I-1 and carrier II-2) revealed a male fetus (III-3) carrying a pathogenic allele of 41 repeats (Fig. 1). After extensive genetic counseling, the couple decided to terminate the pregnancy. For the next three consecutive pregnancies B, C, and D, favorable prenatal testing results were obtained (Fig. 1), and as a consequence three healthy children were born: a male carrying a normal allele (III-4) and two females, one heterozygous carrier with 41/21 repeats (III-5) and the other homozygous for the normal allele (III-6).

The first prenatal testing (E) in the second couple (II-3 and carrier II-4) detected a male fetus (III-7) carrying the SBMA allele of 41 repeats. This pregnancy was also terminated after genetic counseling and a firm parental decision. In the next two pregnancies, prenatal tests F and G revealed two carrier female fetuses (III-9, III-10) both bearing 41/18 repeats; hence, the pregnancies continued, and two healthy babies were born.

3 Discussion

Seven prenatal tests for SBMA were conducted in the reported family, with five favorable results and continued pregnancies and two terminated pregnancies because of unfavorable findings. Two years after the last prenatal testing, during a follow-up conversation, the two couples described their overall experience as challenging but rewarding since it allowed for stronger bonding of the family members

and deep appreciation of each child birth. The two sisters barely remembered their first trimester miscarriages which as far we know, most probably, had no association with SBMA.

SBMA is considered to be a rare syndrome, probably because of misdiagnosis of some patients with other motor syndromes, mainly amyotrophic lateral sclerosis (Finsterer 2009). Hence the motor neuron manifestations and the endocrinological disturbances reported together with the X-linked pattern of inheritance should lead a physician to molecular testing for SBMA (Yapijakis et al. 1996). The molecular confirmation of SBMA is a prerequisite for the successful prenatal diagnosis.

The presented seven prenatal testing cases for SBMA, in one family, illustrate the fact that several couples at risk choose prenatal testing as the chief method for prevention of this neurodegenerative disorder in their offspring. Molecular investigation for SBMA during pregnancy is rather straightforward and easier than other trinucleotide expansion disorders, since somatic instability is rare in SBMA (Jedele et al. 1998). The CAG repeat number of the mutant allele was observed to be consistent amongst all studied fetal tissues and chorionic villi samples in a prenatal diagnosis for SBMA, after termination of the pregnancy, thus ensuring the enhanced accuracy of the genetic testing (Jedele et al. 1998).

Since 2001, preimplantation genetic diagnosis (PGD) for SBMA has become available as an alternative to prenatal testing (Georgiou et al. 2001). Nevertheless, prospective parents at risk should bear in mind that by choosing the conventional prenatal testing, the chance of giving birth to healthy offspring is 75%, while the success rate of PGD is significantly lower. Each technique has both advantages and disadvantages. For instance, in case of a positive prenatal test, the woman might face the stressful consequences of a pregnancy termination during the third or fourth month of gestation. In PGD the woman will have to take hormones to provide oocytes for fertilization and implantation; however, the need for an unwanted pregnancy termination is usually bypassed. Therefore, each couple at risk has the right to be informed for both alternatives to be able to choose independently according to their own subjective perceptions.

The reported family case indicates that the severity of SBMA and the morbidity it causes are high enough so that the individuals who had a parent suffering from the syndrome finally chose the conduction of several prenatal tests and the termination of two pregnancies. Deciding on terminating those pregnancies was not a simple issue for these couples; therefore, extensive genetic counseling was needed before reaching a decision. One dilemma was whether it would be ethical to knowingly give birth to person(s) that would be certainly suffering from SBMA as they would grow up. On the other hand, the hope that a possible cure for the disease might be discovered in the future decades made the parents' decision even more puzzling. The prospective parents received genetic counseling before deciding to initiate a pregnancy, in order to have enough time to accept the situation and come to terms with it, so as to avoid any premature decision, in case of a positive prenatal test result. The choice of chorionic villi sampling instead of amniocentesis for prenatal testing was made because possible termination of a pregnancy is much easier in earlier months of

gestation both for the mother who at that period has lesser emotional bond with the fetus and for the obstetrician who has an easier procedure to perform.

During genetic counseling, it was clarified that a positive prenatal test result would not render the termination of the pregnancy obligatory, since all decision would continue to be deliberate. However, the possible non-termination of pregnancy would raise some ethical issues as it would result in a predictive test, denying the right of the child to genetic ignorance. For the same reason, considering that no cure has been found yet, we suggest that SBMA prenatal testing should not be performed in late stages of pregnancy when termination is no longer an option. In these cases, we recommend that the male child be born but not informed about the condition until he reaches adulthood. Only then should the individual be informed and tested presymptomatically, if he chooses to do so. Otherwise, the rights of that person to knowledge or ignorance regarding his future would be violated.

Prenatal diagnosis for SBMA is a procedure with high fidelity that aids in preventing births of affected offsprings that would otherwise suffer later in life. According to the father of medicine, Hippocrates, "prevention is better than cure" (Yapijakis 2009). Therefore, since there is currently no cure for SBMA, prenatal testing may be a good choice for couples at risk that hope for the best possible future of their offspring.

Conflict of Interest Authors declare that they have no conflict of interest.

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Transcriptomics and Metabolomics in Amyotrophic Lateral Sclerosis

Marios G. Krokidis

1 Introduction

Amyotrophic lateral sclerosis (ALS) is a complex and multifactorial disease characterized by the progressive degeneration of motoneurons. ALS is a highly heterogeneous disorder. 5–10% of ALS patients have the familiar form, associated with dominant mutations or deletion of the cytosolic Cu/Zn superoxide dismutase 1 gene (Rosen et al. 1993), while 90% of them are sporadic (Renton et al. 2014). Additional ALS-associated genes as risk factors are TAR DNA-binding protein (TAR-DBP) (Buratti and Baralle 2001; Sreedharan et al. 2008), fused in sarcoma protein (FUS) (Deng et al. 2014), ALS2/alsin (Yang et al. 2001), angiogenin (Greenway et al. 2006), ubiquilin 2 (Deng et al. 2011), optineurin (Maruyama et al. 2010) and chromosome 9 open reading frame 72 (C9ORF72) (DeJesus-Hernandez et al. 2010). ALS aetiology is not clearly clarified; as a consequence, a completed treatment that can relieve the disease burden does not exist (Bucchia et al. 2015). Riluzole, a glutamate-mediated excitatory neurotransmission blocker, does not erase the disease but slows progression and extends survival with modest effects (Cheah et al. 2010). Only recently, a new drug, edaravone, an antioxidant free radical scavenger, was approved by the Food and Drug Administration (FDA) for the treatment of ALS (Brooks et al. 2018; Takei et al. 2017). Omics approaches, such as genomics, transcriptomics and metabolomics, are high-throughput techniques that can assemble large amounts of data about human genome sequence or a large collection of metabolites, providing a more comprehensive understanding of the human biological states (Hoffman 2017). This review summarizes general aspects from gene expression profiling studies in amyotrophic lateral sclerosis, examines molecular signatures related to

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the pathogenesis of the disease, describes potential therapeutic targets and states recent findings from metabolic abnormalities in ALS that may contribute to unique biomarkers' determination.

2 Transcriptomics Studies in ALS

ALS is a complex and multifactorial disease characterized by the involvement of several pathological processes. Gene expression profiling studies using powerful microarray technologies provide the opportunity to analyse disease mechanisms as a significant method employed for genome-wide transcriptome profiling. The significant role of the astroglial glutamate transporter EAAT2 in motor neuron degeneration was determined, revealing the impairment in EAAT2 activity as part of the molecular mechanism in inherited ALS (Foran et al. 2014). Using Affymetrix GeneChip Mouse Genome 430A 2.0. arrays, *Eaat2*, *Fus/Tls*, netrin-1 and nestin were examined. The results showed statistically significant modifications in astrocytes of $SOD1^{G93A}$ mutant mice. Netrin-1, which was indicated as the top upregulated gene among the secreted differentially expressed genes, is linked with axon migration involvement (Foran et al. 2011). Yu et al. (2013) analysed the spinal cords of ALS transgenic $SOD1^{G93A}$ mice at different stages and found numerous of canonical and non-canonical Wnt signaling molecules such as Wnt1, Wnt7b and Wnt8b. The same pattern was also indicated at protein level (Yu et al. 2013). Heath et al. (2013) summarized in depth the utilization of the transcriptomic technology in order to consider alterations among ALS tissues. Gene expression was performed at the level of the tissue and at individual cellular types in both sporadic and familial forms of the disease in order to further understand the mechanisms implicated in motor neurons' death (Heath et al. 2013). Microarray human gene expression profiling studies in mixed-cell samples, laser capture microdissection cell samples and peripheral tissue have been executed, revealing distinctive key molecules associated with RNA splicing, neuroinflammation and cytoskeleton involvement (Cooper-Knock et al. 2012). These studies confirmed the significance of previously described disease pathways and investigated the role of novel molecules in the pathological process.

The consequential role of cytoskeleton-related genes in motor neurons and Schwann cells in the pre-symptomatic stages of ALS was observed using Agilent Whole Mouse Genome Oligo 4 × 44 K (Maximino et al. 2014). Altered expressed genes from 40- and 80-day-old $SOD1^{G93A}$ mice were found in the spinal cord, whereas altered genes from 60-day-old mice were found in the sciatic nerve associated with microtubule cytoskeleton, actin cytoskeleton and microfilament cytoskeleton. Gene expression profiling indicated differential regulation of *Kif1b* in the sciatic nerve Schwann cells and spinal cord motor neurons of 40-day-old pre-symptomatic $SOD1^{G93A}$ mice (significant existence in ALS pathogenesis). Furthermore, data from a second microarray analysis, using Whole Mouse Genome

Oligo 4x44 K from Agilent Technologies, revealed molecular alterations at the pre-symptomatic stage (40 and 80 days) in the lumbar spinal cord of transgenic SOD1^{G93A} mice (de Oliveira et al. 2014). The said alterations included regulation of glutamatergic synapse (*Gnai1*, *Slc17a6*), endocytosis (*Wwp1*, *Cxcr4*, *Acap2*), ubiquitin-mediated proteolysis (*Wwp1*, *Nedd4*, *Ubr5*), oxidative phosphorylation (*Ndufb11*, *Ndufb8*) and chemokine signaling pathway (*Cxcr4*, *Pik3r1*, *Wasl*), indicating that early neuromuscular abnormalities precede motor neuron death in ALS.

Distinct mechanisms of neuronal death under oxidative stress or excitotoxic stress were indicated in two separated cell cultures of primary cortical neurons of SOD1^{G93A} animals or nontransgenic, in response to the cellular stress induced by the NMDA or hydrogen peroxide, using Mouse Genome 430 2.0. array from Affymetrix (Boutahar et al. 2011). Performing analysis of SOD1^{G93A} neurons subjected to hydrogen peroxide, microarray analysis revealed altered transcriptional profiling in genes related to Wnt signaling pathway regulators, controlling actin-associated cytoskeletal remodeling and exogenesis, trophic factors or ion transport. Moreover, the same group using Affymetrix GeneChip Drosophila Genome 2.0. arrays investigated the cell-specific expression of mutant SOD1 in neuronal cells of flies, utilizing young and old flies with SOD1^{G85R} expression in motoneurons and glia (Kumimoto et al. 2013). Different altered pathways are associated with oxidative stress, lipid metabolism, development of nervous system and signaling genes. Microarray data from ALS, acute quadriplegic myopathy, mitochondrial encephalomyopathy, polymyositis, lactic acidosis as well as stroke-like episodes and dermatomyositis have been evaluated, in order to elucidate exclusive molecular markers for each human muscular disease (Gupta et al. 2014). Performing the analysis of ALS patients' data using Affymetrix HG-U133A Platform GPL96, myofibril genes like nebulin, alpha F-actin, tropomyosins and troponins were significantly downregulated, while different families such as actin-capping proteins like *Capza1*, *Capzb* and *Tmod1* were shown to be upregulated. Moreover, data analysis from whole genome expression profiles of ALS patients' motor cortex samples indicated characteristic alterations in specific genes involved in cell cycle phases, iron regulation homeostasis, synaptic plasticity molecular pathways and cytoskeleton structure development (Aronica et al. 2015).

A widespread comparison of gene expression profiles of laser captured motor neurons using two separate SOD1^{G93A} mouse strains with different phenotypes like C57-SOD1^{G93A} mice and 129v-SOD1^{G93A} has been performed, utilizing GeneChip Mouse Genome 430 2.0. (Affymetrix). Data analysis indicated transcriptional changes in mitochondrial regulation, protein degradation and axonal transport pathways (Nardo et al. 2013). Agilent whole mouse genome microarrays have been utilized for post-mortem analysis of human material in addition to SOD1^{G93A} ALS and P20L Tau frontotemporal dementia mouse models, in order to unravel common molecular pathways related to motor neuron degeneration (Kudo et al. 2010). A potent overlap between blood and spinal cord gene expression profile in the SOD1^{G93A} mouse model has been indicated by performing whole genome expression profile studies of the lumbar spinal cord with peripheral blood and tibialis

anterior muscle in SOD1^{G93A} mice at pre-symptomatic and early symptomatic stages (Saris et al. 2013). Utilizing Affymetrix GeneChip Mouse Gene 1.0. ST arrays on C57BL/6 J mouse brain, TDP-43 target genes' connection with synaptic function and development were indicated, based on their localization at the presynaptic membrane of axon terminals (Narayanan et al. 2012). Working on GMR-Gal4/UAS-TDP-43 transgenic Drosophila model, a deep microarray analysis on the brain of these flies revealed numerous modified molecules involved in cellular oxidative homeostasis and cell cycle regulation. More specifically, *Ucp4b* has been shown to be upregulated in transgenic flies, while notch genes associated with prion disease have also been observed to be upregulated, indicating that TDP-43 investigates changes influencing Notch neuronal regulation and the intercellular communication pathways in ALS pathogenesis (Zhan et al. 2013). Differences in gene expression and modified splicing profiles of TDP-43-silenced primary cortical neurons have been observed by comparing *Fus*-silenced neurons profiles using Affymetrix GeneChip Mouse Exon 1.0. ST Array. The results indicated that both TDP-43 and FUS proteins may in parallel regulate downstream RNA-regulated cascades which may potentially be implicated in ALS mechanisms (Honda et al. 2013).

3 Metabolomics Studies in ALS

The application of a wide variety of metabolomics approaches has been reported in ALS research. Metabolomics methodologies are based on modern approaches aiming to provide a global understanding of metabolites in living systems as well as to investigate diagnostic biomarkers and monitor therapeutic responses (Wang et al. 2010). Mass spectrometry and nuclear magnetic resonance spectroscopy are the two main platforms for performing metabolomics studies (Bingol 2018). Patin et al. (2016) determined the importance of the IL-6 pathway in a mouse model treated with a pharmacological antagonist of IL-6, named MR 16-1, revealing that the antagonist mainly affected branched chain amino acid, lipid, arginine and proline metabolism, while it negatively affected body weight, despite a moderated anti-inflammatory effect (Patin et al. 2016). In an outstanding work by Rozen et al. (2005), 300 metabolites in blood plasma from 28 patients with motor neuron diseases and 30 healthy controls were analysed, finding a distinctive signature of highly correlated metabolites in a set of 4 patients and 12 compounds that were significantly elevated in patients receiving riluzole (Rozen et al. 2005). In another study, serum urate levels in 132 ALS patients were compared with 337 age-/sex-matched controls, indicating that urate levels were lower in bulbar-onset ALS, compared to limb-onset ALS, but it was unclear whether it was related to the malnutrition induced by ALS (Zoccolella et al. 2011).

Blasco et al. (2010) analysed the CSF of patients with ALS by ^{1}H-NMR spectroscopy in order to investigate biomarkers in the early stages of the disease. They also evaluated characterized biochemical factors involved in the disease's

pathophysiology. Analysing CFS samples, 17 particular metabolites were observed including amino acids, organic acids and ketone bodies, indicating that CFS screening may be an important tool for early ALS diagnosis (Blasco et al. 2010). Choi et al. (2009) utilized in vivo and in vitro ^{1}H magnetic resonance spectroscopy (MRS) to monitor the progression of the disease using the SOD1^{G93A} mouse model. No alterations were indicated in the cerebellum as a control region, while at early time points starting around 80 days of age, there were elevated levels in brain glutamate. Brain creatine levels were found elevated in cerebellum followed by the medulla and then the cortex, revealing the ordering of creatine kinase activity. Diminished levels in N-acetylaspartylglutamate (NAAG) and N-acetyl aspartate (NAA) and elevated levels in Glu and Tau have also been detected (Choi et al. 2009). The same animal model has been selected by Niessen et al. (2007) to perform metabolomics signature in extracts of the cerebellum, cortex, brainstem and spinal cord. This comprehensive study revealed that glutamine and gamma-aminobutyric acid concentrations were significantly decreased, while significantly lower levels of N-acetyl aspartate have been noted in the spinal cord of SOD1^{G93A} mice (Niessen et al. 2007).

4 Conclusions

ALS is a multifactorial disease, rapidly progressive and extremely variable among patients, without reliable prognostic tools. Proposed pathogenic mechanisms have been implicated including mitochondrial dysfunction, endoplasmic reticulum stress, abnormal neurofilament function, protein aggregation and glutamate-mediated excitotoxicity, defining important key features (Krokidis and Vlamos 2018; Heath et al. 2013; Babu et al. 2008). Dysregulations in RNA metabolism, a strong aspect of ALS at multiple levels, lead to exacerbation of the disease's effects, including alterations in miRNA biogenesis, spliceosome integrity and RNA editing (Droppelmann et al. 2014; Buratti and Baralle 2008). In ALS, energy metabolism is modified and corresponds to disease progression. On account of this, targeting metabolism serves a reasonable strategy for ALS treatment. Some approaches select either the electron transport chain because of the cellular source of oxidative stress or the mitochondrial function (Smith et al. 2019). It is essential to clearly understand the metabolic biology of amyotrophic lateral sclerosis in order to unravel aberrant biochemical pathways and design prognostic protocols. Multi-omics methodologies offer the opportunity to elucidate the flow of information that underlies the disease. The potential of transcriptomics and metabolomics analysis helps to define candidate genes, diagnostic tools for future investigation and novel therapeutic targets' discovery. Omics-based approaches contribute in unravelling the disorder's pathophysiology in a more defined way and in better understanding the metabolic biology of ALS, indicating comprehensible targets for pharmacological response.

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Ocimum Sanctum Linn: A Potential Adjunct Therapy for Hyperhomocysteinemia-Induced Vascular Dementia

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1 Introduction

Dementia a common and collective term for progressive loss in cognitive and intellectual functioning. According to WHO statistics 35.6 million people were known to suffer from dementia, worldwide in 2010. This number was expected to increase by almost twofold every 20 years, i.e., to 65.7 million in 2030 and 115.4 million in 2050 (Prince et al. 2013). Vascular dementia (VaD) is second most common form of dementia after Alzhiemer's dementia. Vad occurs as a result of ischemic or hemorrhagic insult's that injure innermost regions of the brain such as the cortex or the hippocampus which play an important role in functions like memory, cognition,

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and behavior and functional abilities (Ekonomou et al. 2011; Román 2003). Acute oral administration of L-methionine or subcutaneous administration of DL-homocysteine in rats induce hyperhomocysteinemia (HHcy), which further leads to conditions like vascular dementia (VaD) (Hemanth Kumar et al. 2016, 2017; Boyina et al. 2018). Various studies suggest that there is a positive dose-dependent relationship between the increase in plasma total Hcy concentrations and neurodegenerative diseases like VaD and stroke (Nilsson et al. 2010; Obeid and Herrmann 2006). Serum homocysteine levels more than 15 μM are termed as HHcy (Seshadri et al. 2002). HHcy was previously reported for inhibiting the expression of antioxidant enzymes, and causing complex changes in blood vessels that include endothelial dysfunction (ED), oxidative stress, and pro-inflammatory effects such as expression of tumor necrosis factor (TNF) and inducible nitric oxide (NO) synthase (Hankey and Eikelboom 1999).

Ocimum sanctum (OS) (Lamiaceae) generally known as "holy basil," commonly found in India, is used as an important component in Ayurveda for the treatment of skin diseases, asthma, malaria, dysentery, chronic fever, hepatic diseases, and microbial infections. Previous literature on *Ocimum sanctum* reveals that leaves contain various bioactive constituents such as flavonoids and essential oils. These active constituents are known for their diverse biological activities and are responsible for neuroprotective effects against hypoperfusion-induced cognitive deficits and ischemia-induced oxidative stress (Kelm et al. 2000; Yanpallewar et al. 2004). Vascular dementia is known to be associated with cognitive deficits, oxidative stress, and endothelial dysfunction, but there are no reports suggesting the neuroprotective role of OS in VaD animal models. So, the present study was designed to evaluate the potential effect of ethanolic extract of *Ocimum sanctum* (EEOS) as an adjunct therapy for hyp0 erhomocysteinemia-induced vascular dementia and oxidative stress in rats and to predict the active chemical constituents responsible for this potential effect.

2 Materials and Methods

Male Wistar rats weighing 150–200 g were used in the present study. All animals were maintained under standard husbandry conditions. The rats were randomly assigned into six groups ($n = 6$). The experimental protocol was duly approved by the Institutional Animal Ethics Committee (Reg. No, I/IAEC/LCP/027/2013/WR-36). Fresh leaves of *Ocimum sanctum* were collected locally and were dried under shade and powdered, and the leaf ethanolic extract was prepared. This method involves extraction by percolation at room temperature using 70% ethanol. The yield of the final product during the extraction procedure was 17.32% (w/w). L-methionine (1.7 g/kg/day, p.o.) was administered for 4 weeks to induce hyperhomocysteinemia-associated vascular dementia and endothelial dysfunction in rats (Hemanth Kumar et al. 2016, 2017). EEOS, 100 mg/kg; EEOS, 200 mg/kg; EEOS, 400 mg/kg; and donepezil, 0.1 mg/kg p.o. were administered to L-methionine

(1.7 g/kg/p.o.) treated animals starting from the 14th day to 32nd day of the study. Animal grouping and experimental design were shown in Table 1 and Fig. 1, respectively. The spatial learning and working memory of all animals were assessed by Y-maze and Morris water maze (MWM) tasks (Hemanth Kumar et al. 2016, 2017). Y-maze was used for assessing spatial working memory and MWM test is used to assess hippocampal-dependent learning and memory. In MWM test an acquisition trial was conducted from 28th to the 31st day of the study followed by a retrieval trial on the 32nd day by using a video tracking system. After behavioral analysis, blood samples were collected for the estimation of serum biochemical parameters. Serum Hcy was determined according to the method described earlier using chemiluminescent microparticle immunoassay (Kemse et al. 2014) (Abbott Laboratory, Abbott Park, Chicago, IL). Serum nitrite was determined using the earlier described

Table 1 Grouping of animals

Animal groups	Treatment
Group I	0.5% carboxymethyl cellulose (10 ml/kg, p.o.) for 32 days
Group II	L-methionine (1.7 g/kg/p.o.) for 32 days
Group III	L-methionine (1.7 g/kg/p.o.) + EEOS low dose (100 mg/kg, p.o.) from 14th day to 32nd day
Group IV	L-methionine (1.7 g/kg/p.o.) + EEOS mid dose (200 mg/kg, p.o.) from 14th day to 32nd day
Group V	L-methionine (1.7 g/kg/p.o.) + EEOS high dose (400 mg/kg, p.o.) from 14th day to 32nd day
Group VI	L-methionine (1.7 g/kg/p.o.) + Donepezil (0.1 mg/kg, p.o.) from 14th day to 32nd day

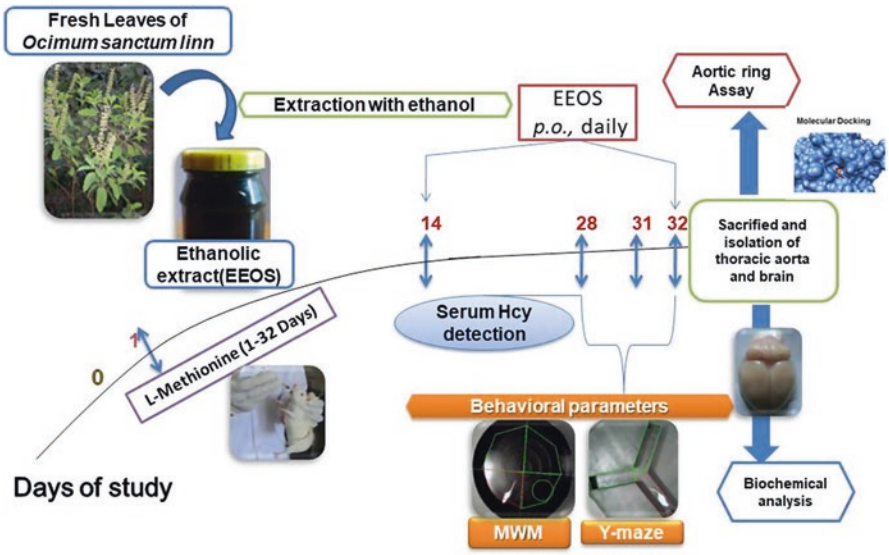


Fig. 1 Experimental Design

method of Sastry et al. (2002) with slight modifications. Rats with serum homocysteine levels of >15 μM were considered to be hyperhomocysteinemic. All animals were later sacrificed under anesthesia for isolation of thoracic aorta and brain tissues for biochemical estimations. Thoracic aorta was used for aortic ring assay, (Joseph and Nair 2013) and the clear supernatant of brain homogenate obtained was used for estimation of brain biochemical parameters like brain acetylcholinesterase, lipid peroxidation, reduced glutathione, and superoxide dismutase (SOD) levels. All biochemical procedures were carried out according to our previously published studies.

(Vinitha et al. 2014, Hemanth Kumar et al. 2016, 2017) All results were mentioned as mean ± SEM and analyzed using one-way ANOVA, followed by Tukey's multiple range tests. The results for aortic ring preparation were statistically analyzed by using repeated measure analysis of variance (ANOVA), followed by Newman-Keuls test. The statistical significance of difference was taken as $P < 0.05$. All the study results were statistically analyzed using GraphPad Prism 5.0 software.

At the end of this study, molecular docking studies were carried out for *Ocimum sanctum* leaf active constituents,(Joseph and Nair 2013) viz., apigenin, ascorbic acid, β-carotene, β-caryophyllene, luteolin, eugenol, carvacrol, methyl eugenol, Molludistin, and orientin. These chemical constituents were selected for docking in order to predict the possible binding interactions with S-adenosyl homocysteine hydrolase (SAH), which is an enzyme responsible for the metabolism of methionine (Obeid and Herrmann 2009). The crystal structure of SAH (PDB: 1LI4) was retrieved from the protein data bank (http://www.rcsb.com). The docking was carried out using default settings of the MOE program by initially preparing ligand and protein before docking (Radhakrishnam et al. 2019).

3 Results and Discussion

Plant polyphenols play a key role in controlling the levels of reactive oxygen species (ROS) and thus help in maintaining normal cell function. Considerable epidemiological data has been collected to recommend an association between consumption of fruits or leaves containing antioxidants and a reduced risk of certain chronic diseases (Kaur and Kapoor 2001; Pandey and Rizvi 2009). For the first time, this study reveals the neuroprotective effect of EEOS on hyperhomocysteinemia-induced vascular dementia and oxidative stress in rats. The MWM and Y-maze tests used in the current study were one of the generally accepted behavioral models for assessment of spatial learning memory ([22]. The percentage alternation of L-methionine-treated group (Group II) was significantly ($P < 0.001$) lowered and prevented L-methionine-induced decrease in percentage alternations compared with L-methionine-treated group ($p < 0.05$, $p < 0.01$ and $p < 0.001$, and $p < 0.001$) (Fig. 2a). The change in the behavior of rats in Y-maze indicates an assessment of spatial working or short-term memory.23.

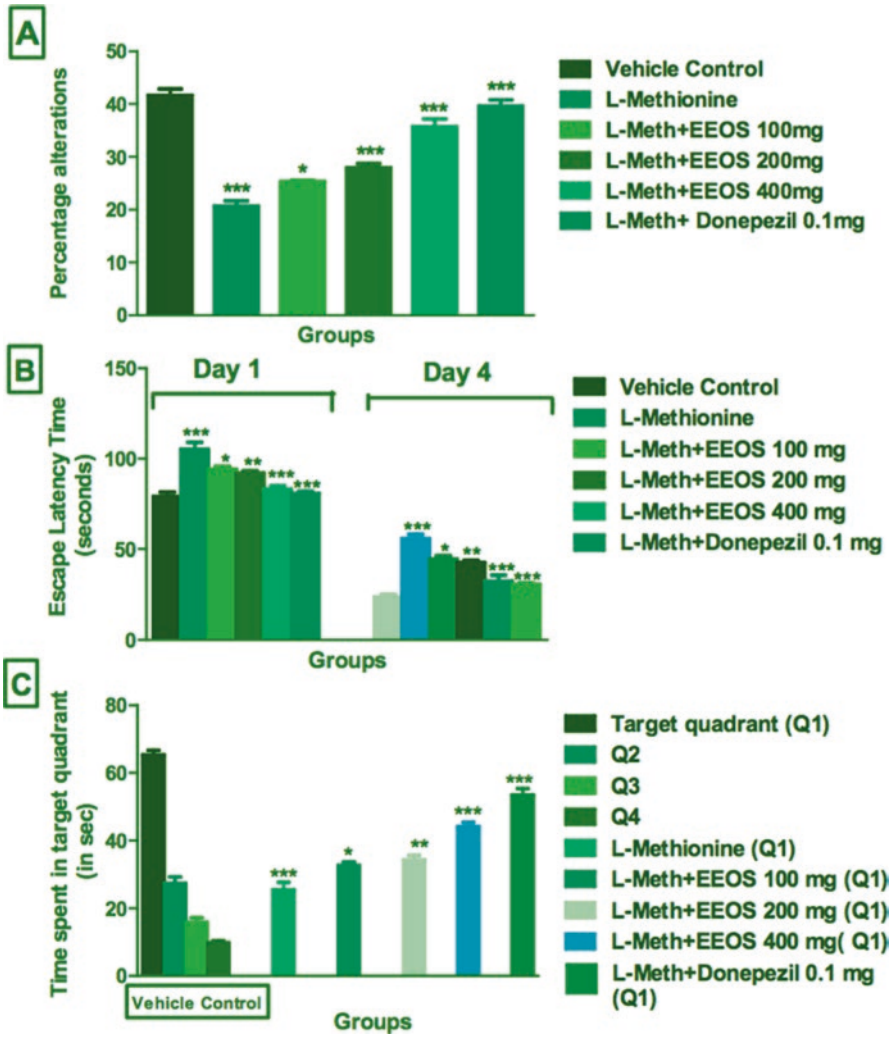


Fig. 2 Effect of EEOS on Y-maze test performance and ELT and TSTQ of Morris water maze test. All the results are expressed as mean ± SEM of $n = 6$ animals. The significance was defined as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; comparison of treated groups, control groups, and negative control groups

In the current study during Y-maze test, rats showed a decline in percentage alternations, which reveals the index of behavioral neurotoxicity after the administration of L-methionine. The main mechanism behind this could be due to HHcy and its effect on impaired function of N-methyl-D-aspartate receptor (NMDA) mediated through glutamate-NO/cyclic GMP pathway in the hippocampus, which is also known to be involved in the various forms of cognition and memory function (Obeid and Herrmann 2006). A significant increase in the percentage of alternation

in drug-treated animals during the Y-maze test was observed indicating EEOS role in the improvement of spatial working memory. These results were in concurrence with the results derived from testing of various natural dietary antioxidants and plant extracts published from our lab.

In the MWM test, the vehicle control-treated rats showed a decreased tendency in ELT of MWM. There was a significant fall in day 4 ELT when compared to day 1 ELT of these rats ($p < 0.001$), indicating their normal learning ability. In addition, on day 5, a significant increase in TSTQ was noted, when compared to the time spent in other quadrants ($p < 0.001$) that indicates a normal retrieval. However, L-methionine-treated rats showed a significant increase in day 4 ELT when compared to day 4 ELT of vehicle control animals ($p < 0.001$), also indicating impairment of acquisition. However, L-methionine administration also produced a significant decrease in day 5 TSTQ when compared to day 5 TSTQ of vehicle control ($p < 0.001$; indicating impairment of memory as well). Administration of EEOS (low, mid, and high dose) and donepezil significantly prevented L-methionine-induced rise in day 4 ELT ($P < 0.05$, $P < 0.01$, and $P < 0.001$) and $P < 0.001$, indicating reversal of L-methionine-induced impairment of acquisition. Further treatment also attenuated L-methionine-induced decrease in day 5 TSTQ in a significant manner ($P < 0.05$, $P < 0.01$ and $P < 0.001$, and $P < 0.001$), indicating a reversal of L-methionine-induced impairment of memory (Fig. 2b, c). The significant fall in escape latency time (ELT day 4) of control animals during acquisition trials represents the normal acquisition of memory. An increase in time spent in the target quadrant (TSTQ), in search of the invisible platform during retrieval, represents improvement of memory. In this present study, EEOS showed a significant effect in spatial learning and memory which was evident from the increase in the time spent in the target quadrant and the decrease in escape latency time on MWM, compared to L-methionine-treated rats. Treatment with three different doses of EEOS and donepezil had improved spatial recognition memory as evidenced by improved performance in the Morris water maze and the Y-maze. These results were in concurrence with the results derived from testing of various natural dietary antioxidants and plant extracts published from our lab (Hemanth Kumar et al. 2016, 2017; Boyina et al. 2018; Singh et al. 2015; Vinitha et al. 2014). HHcy has been shown to induce endothelial dysfunction by decreasing the bioavailability of nitric oxide (NO) and development of vascular oxidative stress. The reduced NO level has been shown to contribute to the pathogenesis of vascular dementia. Consequently, the serum nitrite concentration has been selected as a definitive marker of endothelial dysfunction (Abahji et al. 2007). In the current study, endothelial dysfunction was assessed by measuring the relaxation response of acetylcholine and sodium nitroprusside on precontracted aortic ring preparation of rats from different groups.

In L-methionine-treated rats, we observed a significant decline in acetylcholine-induced endothelium-dependent relaxation; however, SNP-induced relaxation was similar in animals of all groups, indicating that the capacity of the vascular smooth muscle to relax in response to exogenous NO was not impaired. Moreover, we observed an increase in vasoconstrictor responses to phenylephrine, and the mag-

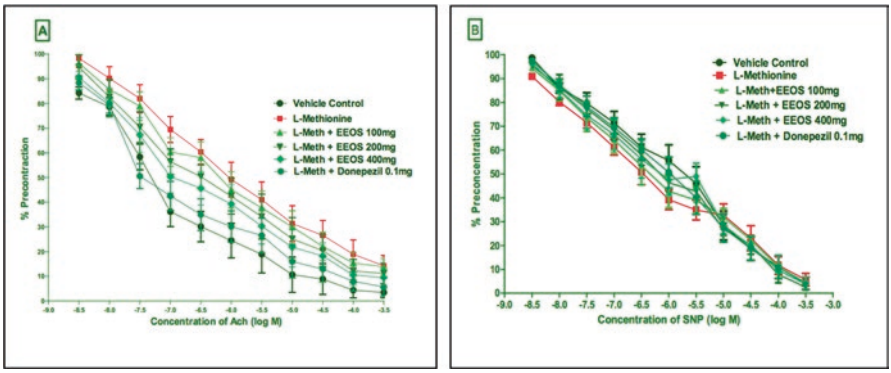


Fig. 3 Effect of EEOS on acetylcholine-induced endothelium-dependent relaxation and SNP-induced endothelium-independent relaxation. All the results are expressed as mean ± SEM of $n = 6$ animals. Responses are expressed as a percentage of precontraction induced by 3×10^{-6} M phenylephrine. The repeated measure (ANOVA) was performed, followed by Newman-Keuls test. (**a**) $p < 0.05$ versus vehicle control, (**b**) $p < 0.01$ versus L-methionine-treated group

nitude of the response to phenylephrine was lower in the aortic rings of L-methionine-treated rats. Compared with the vehicle control rats, the endothelium-dependent relaxation, induced by acetylcholine, was strong ($p < 0.05$), and a significant decrease in the negative control group (L-methionine) was noticed (Fig. 3a). However; it did not affect SNP-induced endothelium-independent relaxation (Fig. 3b). This dysfunction in the aorta was improved by treatment with EEOS (low, mid, and high dose), and donepezil administration significantly prevented the effect of L-methionine on endothelial relaxation ($p < 0.01$). Hence, our results recommend that the increased vascular reactivity to phenylephrine and the relative reduction of endothelial changes associated with decreased acetylcholine reactivity suggest that the treatment with L-methionine reduces NO bioavailability. EEOS demonstrated a protective role on endothelium by increasing the bioavailability of NO in a blood vessel and by decreasing the serum homocysteine levels, resulting in the improvement of the vascular dementia condition. Excess reactive oxygen species cause cell injury by damaging lipids, proteins, and DNA in the cell (Mattson and Shea 2003). Although an association between HHcy and oxidative stress has been reported, the mechanisms by which Hcy causes VaD remain poorly understood (Yang et al. 2003). In the present study, administration of L-methionine produced a significant increase in serum Hcy, brain TBARS, and depleted reduced glutathione, SOD, and catalase activities, suggesting oxidative damage. Treatment with EEOS (low, mid, and high dose) and donepezil has shown a significant effect on L-methionine and significantly reduced AChE and TBARS and attenuated the fall in SOD, GSH, serum Hcy, and nitrite levels in Group III, IV, V, and VI when compared with L-methionine-treated group (Group II) ($p < 0.05$, $p < 0.01$ and $p < 0.001$, and $p < 0.001$) (Table 2) demonstrating their antioxidant-like effect which is in

concurrence with our previous reports on L-methionine-induced HHcy (Hemanth Kumar et al. 2016, 2017).

Acetylcholinesterase inhibitors are the major class of drugs which are often used for the treatment of cognitive impairment. Donepezil is a cholinesterase inhibitor which is commonly used in the management of Alzheimer's disease (AD). Donepezil is known to show its therapeutic action by inhibiting AChE enzyme, thereby provoking a rise in Ach levels in the synapse. Donepezil also demonstrated to have antioxidative and neuroprotective actions (Koladiya et al. 2008). EEOS and donepezil at different doses show their neuroprotective effect by decreasing the raised levels of brain AChE.

We tried to predict the potential of *Ocimum sanctum* by docking its active chemical leaf constituents into the SAH active site, using the docking tool of MOE. The X-ray crystallographic structure of SAH complexed with neplanocin (PDB: 1LI4) was used for the docking calculations (Yang et al. 2015). The protein structure was optimized using structure preparation, and energy minimization was carried out using MOE default settings. The active site was determined using the "Site Finder" tool of the program. The binding free energy dG and the ligand-protein interactions with distance and energy were calculated and are shown in Table 3. The energy data showed that molludistin (IX) exhibited highest binding energy (dG) against SAH. The most active compounds IX and VII showed the highest dG values of −8.98 and − 7.08 kcal/mol, respectively, whereas crystal ligand has shown lesser binding energy with dG value −8.68 kcal/mol compared with IX. The results of 2D and 3D interactions were shown in Fig. 4. By this molecular docking study, it indicates that molludistin (IX) could be a specific chemical constituent for treatment as an adjunct therapy against hyperhomocysteinemia-induced vascular dementia. A further study is needed to explore the mechanism of molludistin against HHcy. EEOS in various studies has been reported to exert antioxidative, potential anti-neuroinflammatory actions and have neuroprotective and cognition-enhancing activity (Giridharan et al. 2011; Sampath et al. 2015; Suanarunsawat et al. 2009). Therefore, with support from earlier research and our study data, it may be proposed that EEOS mediates neuroprotective effect in L-methionine-induced vascular dementia and oxidative stress, which is due to its multiple effects including antioxidant, anticholinesterase, and memory-enhancing activity.

In conclusion, results show that the EEOS possess neuroprotective effect on HHcy-induced neurotoxicity and VaD. EEOS has incredibly restored cognitive impairments, learning, and memory. It also diminished the levels of AChE and has shown the protective role of endothelium by increasing the bioavailability of NO, thus decreasing the vascular oxidative stress. Further research on EEOS and its active chemical constituents especially flavonoids like molludistin is needed, for the lucid understanding of its molecular mechanism and in order to elucidate the important pathways that play a pivotal role in the process of neurodegeneration and vascular dysfunction.

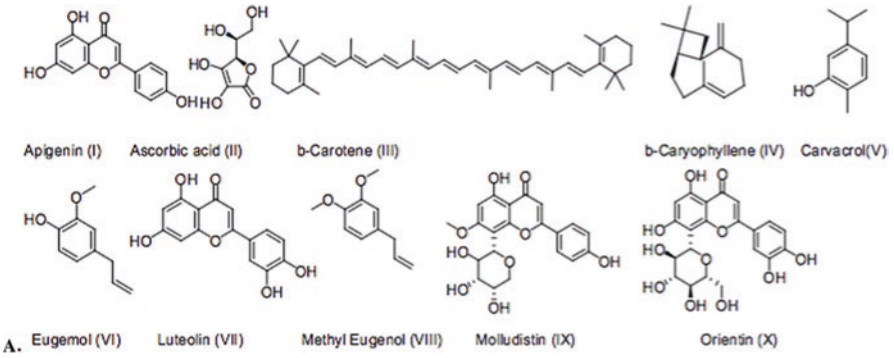
Table 2 Effect of EEOS on brain and serum biochemical parameters

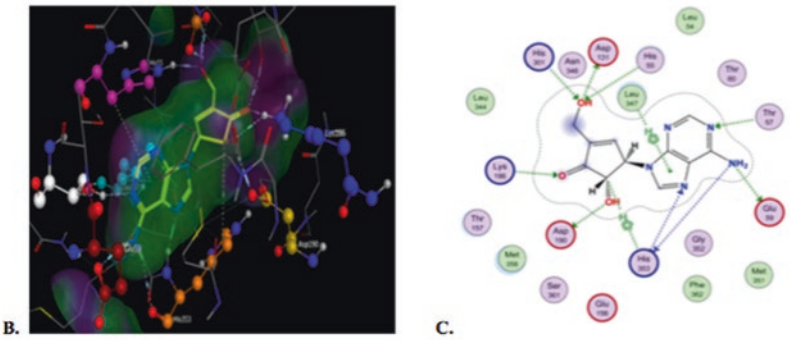
Biochemical parameters	Vehicle control	L-Methionine (1.7 g/kg)	L-Meth + EEOS (100 mg/kg)	L-Meth + EEOS (200 mg/kg)	L-Meth + EEOS (400 mg/kg)	L-Meth + donepezil (0.1 mg/kg)
Brain AchE (u/mg protein)	45.52 ± 1.76	72.06 ± 1.64[c]	69.08 ± 0.76[a]	68.38 ± 1.14[b]	54.56 ± 1.49[c]	47.27 ± 1.52[c]
Brain TBARS (nM /mg protein)	11.67 ± 1.63	43.00 ± 2.96[c]	38.33 ± 1.21[a]	37.33 ± 2.33[b]	25.17 ± 3.54[c]	15.50 ± 1.51[c]
Brain SOD (U/min/mg protein)	1.88 ± 0.06	0.81 ± 0.06[c]	0.96 ± 0.05[a]	1.15 ± 0.10[a]	1.51 ± 0.06[c]	1.76 ± 0.08[c]
Brain GSH (μM/mg protein)	9.46 ± 0.82	4.22 ± 0.40[c]	4.46 ± 0.36	5.60 ± 0.34[b]	7.26 ± 0.59[c]	8.67 ± 0.52[c]
Serum nitrite (μM/L)	13.78 ± 0.45	4.25 ± 0.53[c]	5.25 ± 0.31[a]	6.10 ± 0.536[c]	10.37 ± 0.86[c]	12.89 ± 0.49[c]
Serum homocysteine levels (μM/L)	4.56 ± 0.25	21.71 ± 0.68[c]	20.53 ± 1.15[a]	20.03 ± 0.53[b]	13.39 ± 0.48[c]	9.04 ± 0.30[c]

Data were analyzed using one-way ANOVA, followed by Tukey's multiple range tests. All the results are expressed as mean ± SEM of $n = 6$ animals. Significance was defined as follows: [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$; comparison of treated groups, control groups and negative control groups

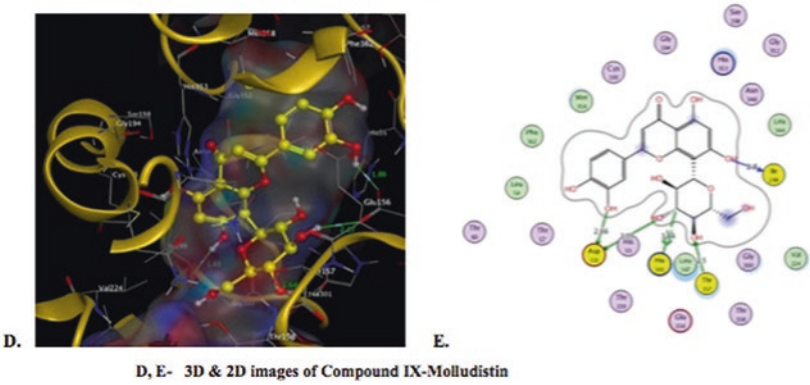
Table 3 The binding free energy dG (kcal/mol), ligand-protein interactions with distance, and energy of crystal ligand and *Ocimum sanctum* leaf chemical constituents

S. no	Compound	Type of bond, energy, distance, and interacting amino acid residues			Binding score
					dG
		H-donor	Distance	Energy	(kcal/mol)
1	Crystal ligand (neplanocin)	ASP-131	2.71	−2.4	−8.68
		ASP-131	2.93	−2.4	
		ASP-190	2.66	−3.5	
		GLU-59	2.94	−2.7	
		HIS-353	3.26	−1.2	
2	Molludistin (IX)	ASP-131	3.03	−2.4	−8.98
		ASP-131	2.66	−2.6	
		HIS-301	3.44	−0.6	
		ILE-299	2.80	−1.5	
		Thr-157	2.50	−0.5	
3	Apigenin (I)	ASN-346	3.16	−1.9	−6.55
		LYS-186	2.71	−7.4	
4	Ascorbic acid (II)	ASN-346	2.86	−2.3	−4.04
		LYS-186	3.88	−0.8	
5	β-Carotene (III)	–	–	–	−7.08
6	β-Caryophyllene (IV)	HIS-353	3.77	−1.1	−4.36
7	Carvacrol (V)	LEU-347	4.02	−0.8	−3.96
8	Eugenol (VI)	LEU-347	4.00	−0.7	−3.47
9	Luteolin (VII)	ASN-346	3.03	−2.5	−6.62
		HIS-55	3.10	−0.9	
		LYS-186	2.66	−9.2	
10	Methyl eugenol (VIII)	ASP-190	3.12	−0.8	2.89
11	Orientin (X)	ASP-190	2.55	−0.8	−5.53
		MET-358	3.37	−1.3	
		GLU-156	2.34	6.1	
		LYS-186	3.08	−4.9	
		LEU-347	4.06	−0.7	





B, C- 3D & 2D images of Crystal Ligand-Neplanocin



D, E- 3D & 2D images of Compound IX-Molludistin

Fig. 4 (**a**) *Ocimum sanctum* leaf chemical active constituents. (**b–e**) 2D and 3D docked structure of IX and crystal ligand (ball and stick) at SAH active site; hydrogen bond (green)

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Alzheimer's Disease: The Role of Mutations in Protein Folding

Eleftheria Polychronidou, Antigoni Avramouli, and Panayiotis Vlamos

1 Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder and the commonest form of dementia. It is clinically characterized by cognitive decline, memory loss, and visuospatial and language impairment. The disease is histopathologically confirmed post-mortem, by the presence of neurofibrillary tangles and amyloid plaques. Regions of the brain that are involved in short-term memory and learning such as the temporal and frontal lobes are impaired as a result of neuronal loss and the breakdown of the neuronal synaptic connections. Currently, there is no effective therapy, while the greatest risk factor for contracting the disease is advancing age (Vieira et al. 2013). Age 65 is often used to classify patients into early-onset Alzheimer's disease (EOAD) and late-onset Alzheimer's disease (LOAD). These categories (reviewed in Tellechea et al. 2018) are known as the familial and sporadic form of the disease, respectively. About 10% of AD patients have been diagnosed with EOAD. In these patients the first symptoms appear between the ages of 30 and 65, with a more frequent range of age diagnosis between 45 and 60 years. Although several studies record a heavier form of the disease or more prevalent histopathological features in younger patients, overall the pathological characteristics in patients with EOAD or LOAD are largely similar. This fact indicates that in the final stage of the disease, it is difficult to distinguish between the two categories with any other criterion except that of its onset (Katzman 1976).

The different molecular pathways and the abundance of genetic loci involved in the development and progression of AD lead to the view that it is not a distinct disease but a set of disorders with common characteristics. The word "Alzheimer" yields 7600 results (August 2018) in the GeneCards database containing all genes

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that have been implicated in AD (http://www.genecards.org/). These genes are associated with inflammation, oxidative stress, vascular regulation, immune function, and the function of specific proteases (a complete list is available at http://www.alzgene.org/). In this study, PSEN1 gene that is most related to the familial form of the disease and its sporadic appearance is studied (Hardy 2006; Selkoe and Hardy 2016). We asked the question how single changes in amino acid sequence affect the tertiary structure of the presenilin 1 and their relationship with the disease.

2 Related Work

Scientific literature establishes the pivotal role of the protein three-dimensional (3D) structure in functional characterization. Computational structural biology aims to determine the most efficient methodology on the prediction of the three-dimensional protein structures from amino acid sequences (Carmali et al. 2017). Hence when an unknown structure is present, alternative approaches of comparative modeling, structural clustering and classification, can provide a useful 3D model for a protein. Greater stability is achieved when the protein is related to at least one experimentally determined protein structure (Webb and Sali 2002).

To reduce the distance between the increasing numbers of proteins with known sequences and the proteins with experimentally characterized structure, several approaches were implemented. Protein families and superfamilies formation and multiple structural alignment (MSA) approaches follow direction (Skwark et al. 2014). The former is related to the classification based on proteins evolution relationships, while the latter focuses on the similarity-based and evolution-based structure prediction. The most comprehensive examples of protein families formation are CATH (Greene et al. 2007) and SCOP (Andreeva et al. 2008).

As was previously described, the classification threshold in these databases is the evolving relationship between proteins. A substantial similar domain, a family relationship, is established when proteins' three-dimensional structures reveal a significant similarity. Superfamilies are further grouped together if their members share a similar structural fold. FSSP database (Holm and Sander 1994) classifies structural alignments of proteins in the Protein Data Bank (PDB) based on homology. Recently Pfam (Finn et al. 2016) demonstrated the use of hidden Markov models (HMM) using the HMMER software in order to create a sustainable scoring system in protein families.

Toward similarity-based prediction, methods such as Clustal, Muscle, MAFFT, and T-Coffee were developed (Sievers and Higgins 2018). Clustal is based on progressive pairwise alignment. Muscle added a step in the guide tree that determines the order that sequences are added to a growing MSA. MAFFT follows a similar operation, involving fast Fourier transformations to approximate the pairwise alignments, and recently was updated with progressive and iterative refinement methods. T-Coffee evolves the method by implementing a more sophisticated scoring function. On the other hand, evolutionary methods mainly involve processes connected

to sequence evolution. Insertion, substitution, and deletion are the key features for this type of analysis, and they are depicted as an explicit phylogenetic tree. These methods are correlated to protein families (analyzed above).

Conclusively, focusing on the 3D structure similarity, several metrics exist, which measure the structural similarity of two protein models. Structural similarity is defined by the positional deviations of equivalent atoms upon rigid-body superimposition. Aligners were implemented with the ability to identify similarities between proteins with large conformational changes. Highlighted aligners in this category are RMSD, SAS score and GSAS score, TM-score, S score, STRUCTAL score, and Q-score (Hitomi and Holm 2009).

3 Methods

3.1 Data Consolidation

EOAD is usually autosomal dominant inherited, and amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2) proteins are regarded as major factors. Among these, mutations in PSEN1 are by far the most frequent (Jamal et al. 2017). Although EOAD barely constitutes 1–2% of AD compared to LOAD, it is much more multifaceted, genetically, due to the involvement of genetic, epigenetic, and environmental factors.

PSEN1 gene is located at chromosome 14q24.3 encoding presenilin 1, a large multi-pass transmembrane protein (Fig. 1), which along with presenilin 2 participate in the formation of the γ-secretase complex. This complex subserves plenty of cell signaling functions by way of regulated intramembrane proteolysis.

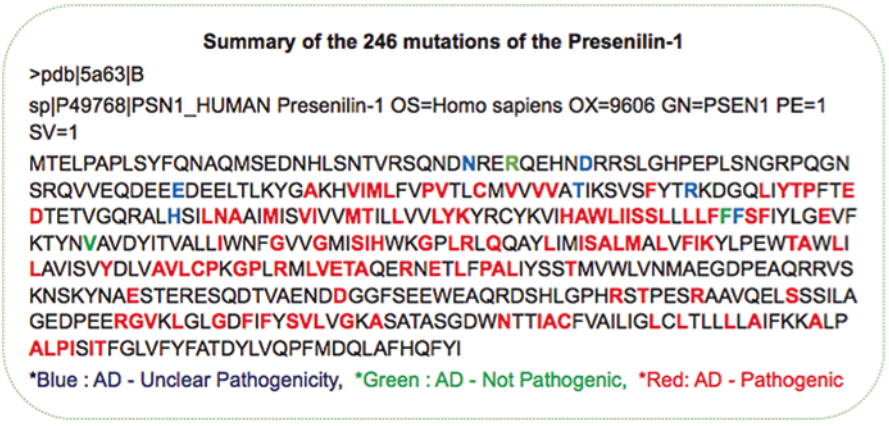


Fig. 1 Mutations that are reported in PSEN1 protein. The mutations are grouped into three categories

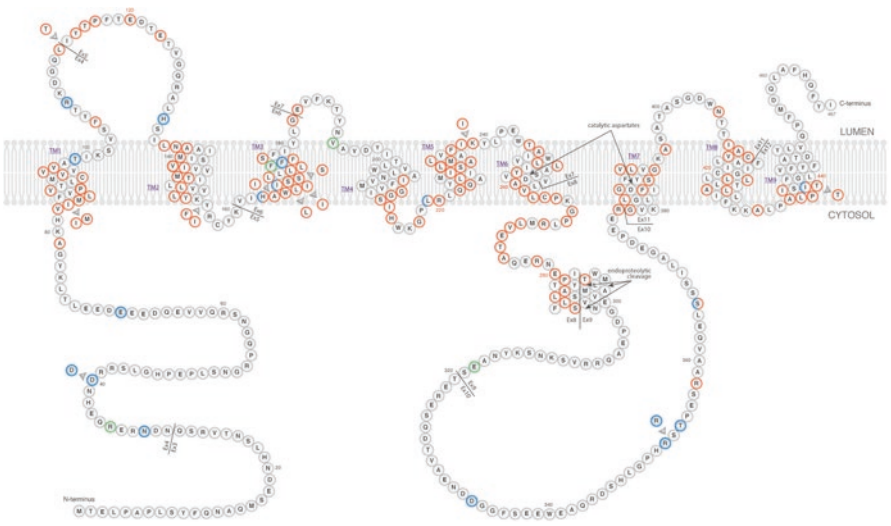


Fig. 2 Presenilin 1 is a large multi-pass transmembrane protein which participates in the formation of the γ-secretase complex (https://www.alzforum.org/mutations/psen-1)

PSEN1 mutations cause severe AD with clinical symptoms occasionally arising as soon as 30 years of age, demonstrating their importance for understanding the pathogenesis of AD (Cruts et al. 2012). The mechanism by which PSEN1 mutations lead to neurodegeneration and dementia in EOAD remains strongly debated. Currently more than 240 distinct mutations in PSEN1 gene are related with severe EOAD and thus are of central interest to the etiology of AD (https://www.alzforum.org/mutations/psen-1) (Fig. 2). PSEN1, as the catalytic subunit of γ-secretase, produces β- amyloid peptides of variable length, and the mutations tend to increase the produced Aβ42/Aβ40 ratio (Borchelt et al. 1996; Murayama et al. 1999). Despite extensive research, the molecular reasons for the pathogenesis of these mutations remain unknown. A possible explanation is that the mutations alter the distribution of Aβ isoforms to produce relatively more toxic Aβ42, susceptible to aggregation, as supported by recent versions of the amyloid hypothesis (Greene et al. 2007; Pauwels et al. 2012).

Mutations that cause loss of protein function could also serve as a reason (Cacquevel et al. 2012; Woodruff et al. 2013). A recent study correlated the Aβ42/Aβ40 ratio and PSEN1 mutations with the age of clinical symptom onset (Somavarapu and Kepp 2016). They found statistically significant correlations between age of onset and relative Ab42 levels, but more interestingly they also found significant correlations with specific changes in fundamental chemical properties of the mutants, associated with increased polarity and reduced protein stability. However, further reanalysis of the data provided controversial results (Sun et al. 2017; Tang and Kepp 2018).

Table 1 Selected mutations and mapping to code number and pathogenicity

Mapping	Mutation	Pathogenicity
V1	No	Normal
V2	R35Q	AD: Unclear pathogenicity
V3	I83T	AD: Pathogenic
V4	I83_M84del	AD: Pathogenic
V5	L113Q	AD: Pathogenic
V6	E184D	AD: Pathogenic
V7	E184G	AD: Pathogenic
V8	E120K	AD: Pathogenic
V9	E123K	AD: Pathogenic
V10	PSEN1 S290C;T291_S319del (ΔE9Finn)	AD: Pathogenic
V11	T291P	AD: Pathogenic
V12	R377M	AD: Pathogenic
V13	L381F	AD: Pathogenic
V14	L381V	AD: Pathogenic
V15	R358Q	AD: Pathogenic
V16	G384A	AD: Pathogenic
V17	G378V	AD: Pathogenic
V18	F386I	AD: Pathogenic
V19	G378E	AD: Pathogenic
V20	F386S	AD: Pathogenic
V21	F388L	AD: Pathogenic
V22	S390I	AD: Pathogenic
V23	S390N	AD: Pathogenic

In this study, some of the most representative mutations of PSEN1 gene were selected for further analysis through protein 3D structure. The mutations were mainly AD pathogenic as this type outnumbers the other mutation types and they also belong to the coding regions of the gene (Table 1).

3.2 Protein Structure Prediction and Methodologies Evaluation

Computational protein structure modeling techniques were selected to predict the protein structures of mutated proteins. The protein structure servers that were utilized for this study followed for the CAMEO-3D, Protein Structure 1-year Performance evaluation. The results of this analysis were used as prediction software benchmarking (Haas et al. 2018).

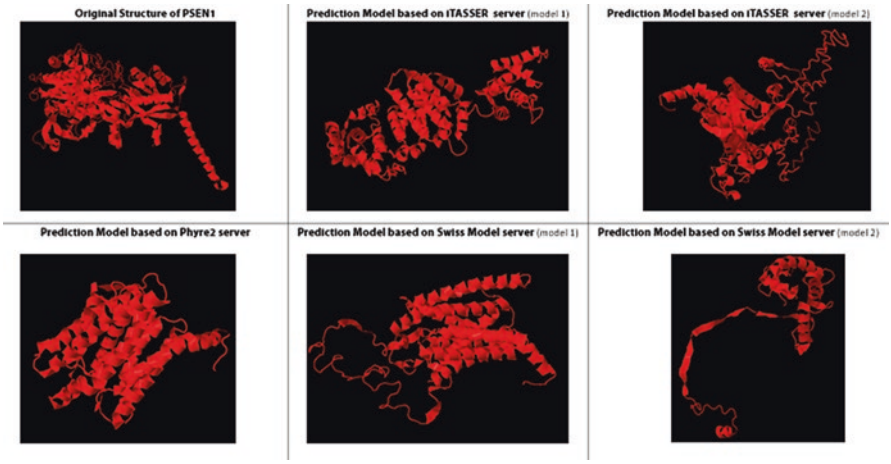


Fig. 3 Models of predictive structures from the selected methodologies. Original structure of PSEN1 was retrieved by PBDe (5a63: Cryo-EM structure of the human gamma-secretase complex at 3.4 angstrom resolution)

3.2.1 Prediction Software Benchmarking

The predicted models were compared against the corresponding experimental structure through the TM-align algorithm, and a benchmarking of the structural predictive tools was accomplished. Comparison results revealed that the I-TASSER software reached the highest accuracy between predicted and experimental structure (Zhang 2008; Roy et al. 2010; Yang et al. 2015). In Fig. 3, the visualization of the predicted 3D model of PSEN1 protein is presented. I-TASSER, Phyre, and Swiss Model are the list of servers that presented the highest accuracy on predicting the experimental predicted structure.

3.3 Structural Classification

Once the dataset was translated into tertiary protein structures, a comparison between the obtained models was implemented. The aforementioned phase was conducted with two methods. The first is related to a pure bioinformatics approach, while the second describes an innovative method that relied on 3D descriptors' extraction. The complete methodology is described in (Polychronidou El 2018).

3.3.1 Mutation Footprint

The final step of the analysis includes the association of the protein's tertiary structures to the established mutations. The changes in the original 3D structure occurring from the mutation were translated into a footprint percentage, and it was

correlated with the type of the mutation and the pathogenicity. The proteins that were correlated with mutations and footprint coefficients were grouped together to be further examined as disease biomarkers.

4 Results

In this study we used I-TASSER server, which performs comparative and ab initio prediction of protein structure for mutated forms of presenilin 1. This analysis can provide an essential knowledge toward a protein's functionality in structure-based drug design. Interestingly, the analysis of a small sample of single nucleotide alterations and some larger deletions, such as ΔE9Finn, revealed a high percentage alteration compared to the normal protein (Table 2). Those with a TM-score < 0.5 are mainly not in the same fold (V4, I83_M84del; V10, PSEN1 S290C;T291_S319del, ΔE9Finn; V17, G378 V; V23, S390 N) compared with the normal protein. V4, I83_M84del; V10, PSEN1 S290C;T291_S319del, ΔE9Finn; and V17, G378 V, are well-known mutations related to early onset of the disease. The first two deletions are also associated with cotton-wool plaques. S390 is a highly conserved residue (between PSEN1 and PSEN2 and across species), and S390 N mutation is predicted to be damaging by most prediction tools. Interestingly the predicted ligand binding site residues change, mostly in the ones with higher percentage of altered structure, but the results need further elucidation.

The proteins were clustered based on their structural differences. The distance between the structures was revealed by the 3D descriptors described in Sect. 3. In the following dendrograms, a visualization of the distance between the protein structures is presented. Each diagram corresponds to a different 3D descriptor, with the last one corresponding to the combination of all the above. The distribution of proteins along the dendrogram was analyzed for different groups of proteins belonging to different descriptors. In cladograms based on different descriptors, it is high-

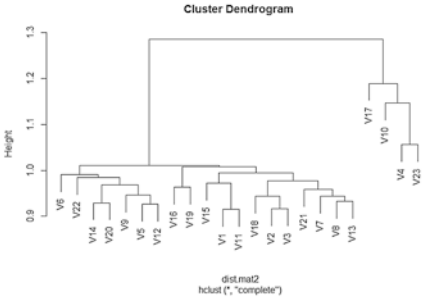
Table 2 Percentage of structure alterations due to mutation

%	Mutation	%	Mutation
5,58	R35Q	8,13	L381F
4,91	I83T	8,39	L381 V
52,55	I83_M84del	7,19	R358Q
8,69	L113Q	9,32	G384A
5,45	E184D	59,41	G378 V
7,78	E184G	6,06	F386I
6,79	E120K	6,09	G378E
8,26	E123K	5,49	F386S
71,04	S290C;T291_S319del	9,29	F388 L
5,09	T291P	10,35	S390I
6,46	R377M	52,65	S390 N

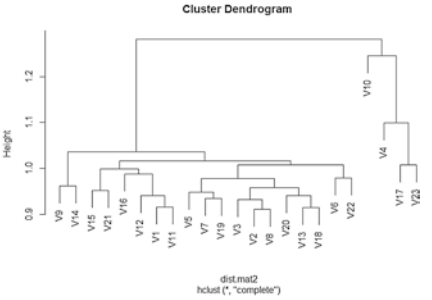
lighted that the mutations with the highest structure alteration are classified separately. We identify two main sub-clusters reflecting the isoforms' altered structure. The combined distances were finally selected as the optimal approach to perform the structure clustering.

Three different approaches of clustering were applied to the structures, hierarchical clustering, kmeans, and dbscan. The method that proved to be more accurate in biological terms was the hierarchical clustering.

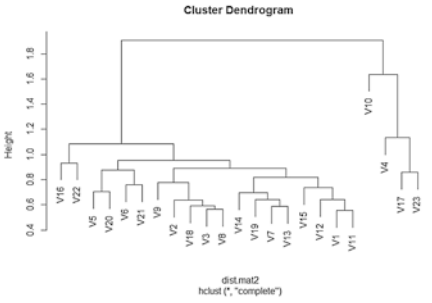
Dendrogram based on 3DSC Descriptors



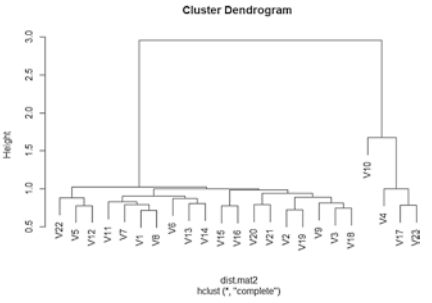
Dendrogram based on FPFH Descriptors

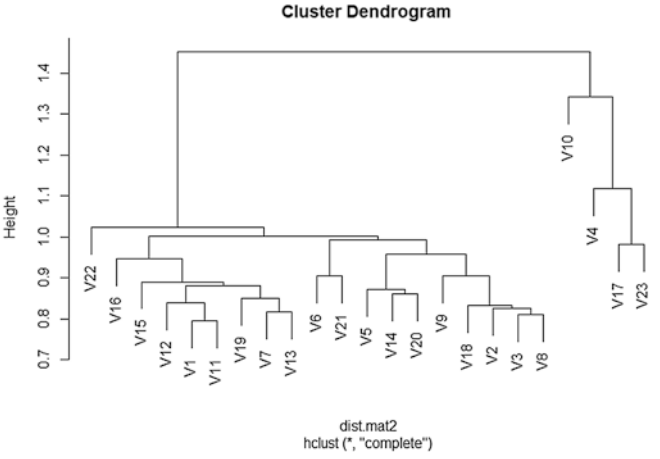


Dendrogram based on RSD Descriptors



Dendrogram based on VFH Descriptors





The last part of the structure analysis was to perform the comparison of the structures based on classic bioinformatics approaches. TM-align is an algorithm for sequence-independent protein structure comparisons. For two protein structures of unknown equivalence, TM-align first generates optimized residue-to-residue alignment based on structural similarity using dynamic programming iterations. An optimal superposition of the two structures, as well as the TM-score value which scales the structural similarity, will be returned. TM-score takes values of (0,1), where 1 indicates a perfect match between two structures.

5 Conclusion

In this computational analysis work, we presented preliminary results of our study on PSEN1 mutations and compared these changes to available clinical data for PSEN1 variants known to cause AD. To the best of our knowledge, this is the first study of its kind, investigating comparative and ab initio prediction of protein structures for mutated forms of presenilin 1. We hope that our supplementary findings will be of value in the development of more accurate disease models that can assist as a tool of personalized medicine, most notably in the diagnosis and strategic planning of patients with FAD. The experimental results verify that the use of 3D descriptors can be effectively applied to distinguish structural differences of proteins.

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Regulatory Role of MicroRNAs in Brain Development and Function

Christos Yapijakis

1 Introduction

According to the central dogma of Biology, which was developed in the early period of its molecular phase, the deoxyribonucleic acid (DNA) makes ribonucleic acid (RNA) which in turn makes protein. Later research revealed additional roles of RNA molecules, with some of them adding paths to the central dogma, e.g., reverse transcription of RNA to DNA, and others playing auxiliary or regulatory roles although they were not coding for protein sequences. The first discovered non-coding RNA molecule (ncRNA) was a yeast Alanine-tranferRNA (Ala-tRNA) characterized about half a century ago (Holley et al. 1965). Since then, a wide range of RNA molecules has been discovered including long ncRNAs (>200 nucleotides, nts) and short ncRNAs (<200 nts).

The short ncRNAs comprise a spectrum of molecules varying in size and function, including microRNA (miRNA), Piwi-interacting RNA (piRNA), small interfering RNA (siRNA), small nucleolar RNA (snoRNAs), small nuclear RNA (U-RNA), tRNA-derived small RNA (tsRNA), small rDNA-derived RNA (srRNA), etc. (Mari-Alexandre et al. 2016). The identification of novel untranslated sequences was greatly assisted by the release of the ENcyClopedia of DNA Elements (ENCODE) in 2003 (www.genome.gov/encode/).

MicroRNAs (micro, μικρό meaning small in Greek) are very short non-coding RNA molecules of about 20–22 nucleotides that function as posttranscriptional repression agents (Sun and Shi 2015; Mari-Alexandre et al. 2016). The first miRNA discovered was a *C. elegans* gene repressor in 1993 during investigation of an interesting

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worm mutant (Lee et al. 1993). Although the miRNAs have a wide range of nucleotide sequence, they all share a common functional mechanism. After their posttranscriptional maturation, miRNAs are loaded into the ribonucleoprotein complex RISC and modulate gene expression by binding to the 3′ untranslated region of their target messenger RNAs (mRNAs) through base-pairing, which in turn triggers mRNA degradation or translational inhibition. There is mounting evidence that miRNAs regulate various biological processes, including cell proliferation, differentiation, growth control, and apoptosis (Lang and Shi 2012). Several studies have shown that miRNAs play an important role in neurogenesis and brain development (Sun and Shi 2015).

2 Structure and Function of MicroRNAs

The study of structure and function of miRNAs may be effected with a range of molecular techniques, including screening with next-generation RNA sequencing (RNAseq) and miRNA microarrays, validation with quantitative PCR or real-time PCR, as well as functional analysis, luciferase assays with in situ hybridization, and live cell miRNA detection (Mari-Alexandre et al. 2016). Usually, first the miRNA sequence is identified during tissue screening or database analysis, and then the expression profile of the target miRNA is compared between groups of patients and matched healthy controls. Upon interesting findings, the next step relates to the validation of the expression profiles of selected miRNAs in larger sample cohorts. The goal is to develop functional assays in order to detect translational regulation of the target mRNAs by specific miRNAs. There are several bioinformatics tools for analysis of RNA primary or secondary structure, a good example being bpRNA (Danaee et al. 2018).

The primary structure of miRNAs is a short sequence of about 20–22 nts, but their respective encoding miRNA genes (*miR*) are much larger. In human and other mammalian cells, most *miR* genes are transcribed by RNA polymerase II to transcripts of primary miRNAs (pri-miRNAs) containing one (monocistronic) or several (polycistronic) 5′-capped and 3′-polyadenylated miRNA precursors within hundreds to thousands nucleotides that form hairpin loop structures (Fig. 1). The pri-miRNA structures are recognized and processed in the nucleus by microprocessor complex Drosha-DGCR8 (DiGeorge syndrome Critical Region gene 8) to release precursor miRNAs (pre-miRNAs) of about 70 nucleotides with stem-loop structures, which are then exported into the cytoplasm by exportin 5 (Fig. 1). In the cytoplasm each pre-miRNA is diced by the complex of Dicer and transactivation-responsive RNA binding protein (TRBP), and thus a miRNA duplex is produced (Fig. 1). The unwinding of the miRNA duplex results in the production of two strands, one being the mature miRNA of about 20 nucleotides which is incorporated with Dicer, TRBP, and Argonaute-2 (AGO2) in the RNA-induced silencing complex (RISC), while the other strand is degraded (Sun and Shi 2015; Mari-Alexandre et al. 2016). The miRNA-containing RISC complex binds to the complimentary 3′ region of the target mRNA and either blocks its translation or promotes its degradation (Fig. 1).

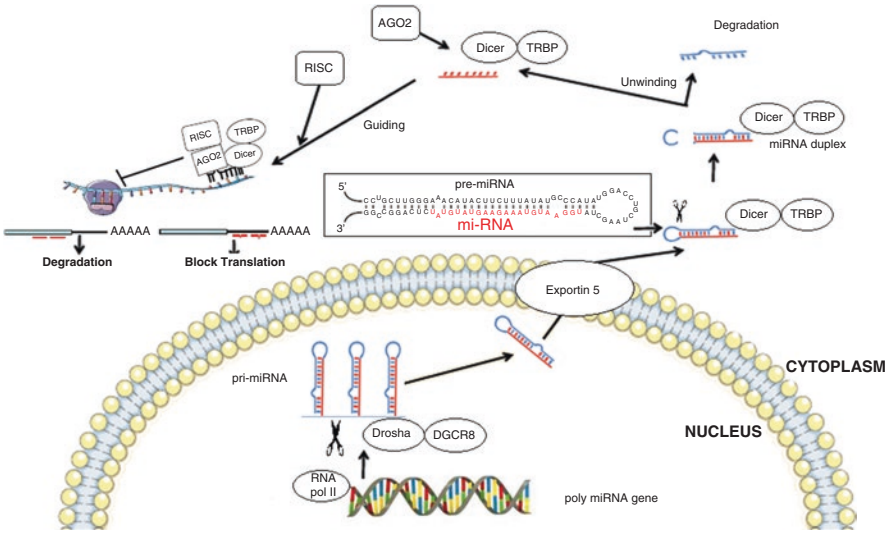

Fig. 1 Biogenesis and function of miRNAs

The miRNAs play a fundamental role of gene expression regulation in many biological processes by significantly repressing translation of hundreds of target mRNAs through specific recognition of complimentary sequence (Eulalio et al. 2008; Sun and Shi 2015; Mari-Alexandre et al. 2016). In particular, precise regulation of normal brain development is effected by miRNA expression, while dysregulation of miRNA expression and function has been implicated in neurodevelopmental disorders and craniofacial syndromes, as well as neurodegenerative and psychiatric diseases (Lang and Shi 2012; Dwivedi 2014; Sun and Shi 2015; Schoen et al. 2017).

Mutations or polymorphisms such as single nucleotide variants in miRNAs or in their target mRNA sites may affect miRNA function and may be the primary cause of congenital diseases, including neurodevelopmental disorders and craniofacial syndromes (Lang and Shi 2012; Schoen et al. 2017; Sun and Shi 2015). In addition, several agents may affect the level of miRNA expression, such as mutations in genes encoding regulators of miRNA biogenesis pathway, epigenetic changes, and environmental factors, leading to complex neurodegenerative and psychiatric diseases (Dwivedi 2014; Sun and Shi 2015).

3 Role of MicroRNAs in Neurodevelopment

The end result of synaptic architecture of the adult brain is a very complex developmental process which is occurring in a stepwise manner influenced by a combination of genetic and epigenetic influences in addition to relying to activity-dependent remodeling capacity in response to environmental conditions.

The organization of the assembly of neural circuits based on synaptic plasticity requires complex molecular regulation by involving several levels of gene expression. In addition to transcriptional regulation by a variety of cis and trans acting DNA elements, protein factors, and epigenetic influences on chromatin structure, the intracellular destiny of mRNA transcripts may be determined by ribonucleoprotein complexes and small non-coding RNAs such as miRNAs (Lang and Shi 2012; Hollins and Cairns 2016). An exceptional example is miR-124 which is specifically expressed in the central nervous system regulating neuronal development by suppressing several pathways associated with neurogenesis, neuronal differentiation, and synaptic plasticity (Sun and Shi 2015). The expression of miR-124 mostly occurs in post-mitotic neurons and increases during brain development (Akerblom et al. 2012). It targets various transcripts of genes involved in neuronal differentiation such as small CTD phosphatases 1, ephrin-B1, integrin beta 1, laminin gamma 1, and SRY-box containing gene 9 (Sun and Shi 2015). Furthermore, miR-124 targets various transcripts of genes involved in neuronal maturation such as cAMP response element-binding protein (CREB), LIM-related homeobox protein 2, and the small GTPase Ras homolog growth-related (Sun and Shi 2015). In addition, it regulates synaptic transmission by inhibiting the expression of early growth response gene 1 (Yang et al. 2012).

During the second trimester of fetal development, neural stem cells in the human brain produce about 2500 new neurons every minute (Miranda 2012). This tremendous rate of cell proliferation and differentiation renders the developing brain highly vulnerable to early minor disruptions of the maternal-fetal-environment (e.g., by hormonal deficiency or environmental teratogens) that may result in amplified consequences altering the structure and function of the adult brain (Prezioso et al. 2018). For example, exposure to ethanol during that period of fetal development is known to have teratogenic effects on proper production and function of several miRNAs causing aberrant neural stem cell proliferation and attenuation of terminal neuronal maturation (Miranda 2012).

Several miRNAs, which may not be specifically expressed in neurons, play nevertheless important roles in brain development. For example, miR-126 and miR-132 promote vasculogenesis and angiogenesis by interfering with multiple signal pathways that are vascular endothelial growth factor activity-dependent (Lang and Shi 2012; Sun and Shi 2015). Although their expression is not tissue-specific, their role in regulating neural stem cell proliferation, differentiation, and neuronal maturation appears to be very important (Zhuang et al. 2016; Chen et al. 2018).

Multiple studies indicate that defective neuronal plasticity and function due to mutations or functional polymorphisms in genes encoding miRNAs may play a significant role in the etiology of neuropsychiatric disorders as well (Sun and Shi 2015). Several miRNA genes have been associated with major psychoses like schizophrenia and bipolar disorder, including miR-9, miR-124, miR-132, miR-137, miR-195, miR-219, and miR-17 family members and miR-200 family members (Sun and Shi 2015). The most prominent association was found by the largest genome-wide association study (GWAS) of more than 40,000 people. It showed that the most significant association of a single nucleotide polymorphism

with schizophrenia involved an intron locus within the putative primary transcript for miR-137, while five other miR-137 target genes achieved genome-wide significance (Ripke et al. 2011).

Interestingly, a well-known entity with craniofacial and variable psychiatric characteristics (schizophrenia, schizoaffective disorder, or anxiety disorder) that appears to be associated with miRNA-related pathology is velocardiofacial syndrome/DiGeorge syndrome (Forstner et al. 2013; Sun and Shi 2015). DiGeorge syndrome is caused by a hemizygous microdeletion of 1.5–3 Mb on the long arm of chromosome 22 (22q11.2) that encompasses at least 35 genes in the minimal deleted region, including two genes implicated in microRNA-mediated dysregulation (Karayiorgou et al. 2010). These genes are *DGCR8* (DiGeorge syndrome Critical Region gene 8) and *MIR185* (Karayiorgou et al. 2010). As mentioned before, DGCR8 protein is a key part of a microprocessor complex together with Drosha that recognizes pri-miRNA structures and processes them into pre-miRNAs in the nucleus (Fig. 1); therefore its reduced amount seems to compromise miRNA biogenesis in important developmental stages when miRNA-related gene expression regulation is mostly needed. On the other hand, *MIR185* gene encodes miR-185, which was the top-scoring downregulated microRNA in both the prefrontal cortex and the hippocampus, the two brain areas which are mainly associated with schizophrenia (Karayiorgou et al. 2010). It appears that the decreased miR-185 expression does not regulate sufficiently its two target mRNAs (cell morphology and cell cycle-related GTPases RhoA and Cdc42); thus it contributes to deficits of dendritic and spine development in hippocampal neurons (Forstner et al. 2013).

In recent years, several studies have indicated that mutations in RNA binding proteins and changes in miRNA expression profiles in certain brain areas are significantly altered during the progression of neurodegenerative disorders, such as (among many others) miR-9 and miR-107 in Alzheimer's disease, miR-34b and miR-34c in Parkinson's disease, miR-124 in Huntington's disease, and miR-155 in amyotrophic lateral sclerosis (Rajgor 2018). The accumulating evidence suggests that certain miRNAs may be contributing factors toward neurodegeneration; therefore they may be possibly key targets for exploring future therapeutic treatments for various neurodegenerative disorders (Rajgor 2018).

4 Role of MicroRNAs in Craniofacial Development

Neural crest cells originating at fetal dorsal neural folds migrate to future facial region and populate it giving rise to facial ectomesenchyme, which generates the face after a complex morphogenetic process. Mutations in genes encoding regulators of any step of morphogenesis including miRNAs that target important transcription factors may be the genetic causes of congenital craniofacial malformations (Lang and Shi 2012; Schoen et al. 2017; Sun and Shi 2015). As previously mentioned, velocardiofacial syndrome/DiGeorge syndrome has craniofacial and psychiatric characteristics (schizophrenia, schizoaffective disorder, or anxiety disorder),

while its pathology is a miRNA-related hemizygous microdeletion of 1.5–3 Mb on 22q11.2 (Forstner et al. 2013; Sun and Shi 2015).

There are other known examples of craniofacial syndromes with miRNA involvement. A de novo deletion of 258 kilobases affecting the miR-873/miR-876 cluster was associated with a phenotype of craniofacial abnormalities including macrocephaly and hypertelorism (Koufaris et al. 2015). It turned out that miR-873 is involved in the regulation of Hedgehog signaling, which is an important pathway of cranial bone development, patterning, and differentiation (Koufaris et al. 2015).

Another syndrome with craniofacial, cardiovascular, and brain development defects is the fetal ethanol spectrum disorder (Miranda 2012). As mentioned before, maternal ethanol consumption during pregnancy has teratogenic effects on proper production and function of several miRNAs causing a constellation of brain defects including microencephaly, loss of corpus callosum, heterotopias of neuronal aggregates, and mental retardation (Miranda 2012). Although many miRNAs are ethanol-sensitive at different stages of neurodevelopment, it appears that the miR-9 family in particular is greatly vulnerable (possibly though epigenetic modification at its chromosome locus) across multiple stages of neural stem cell proliferation and neuronal maturation (Miranda 2012; Lussier et al. 2017).

MicroRNAs play critical roles in multiple processes of skeletal growth including differentiation and proliferation of growth plate chondrocytes that drive the endochondral bone development (Papaioannou et al. 2013), In an analogous manner, proper skull bone architecture requires the regulatory involvement of several miRNAs. Loss of certain functional miRNAs has been associated with macrocephaly and megalencephaly that correspond to increased head circumference in children at least two standard deviations above the age-related mean (Pavone et al. 2017). Macrocephaly is characterized by increased orbitofrontal head circumference as a result of various causes, including a bone skull anomaly, a subdural fluid collection, hydrocephalus, an intracranial mass, and an arterial or venous malformation. For example, a patient with macrocephaly reportedly had haploinsufficiency of the miR-873/miR-876 cluster (Koufaris et al. 2015). On the other hand, megalencephaly is characterized by enhanced growth of cerebral structures as a result of dysfunctions during various phases of brain development, including the proliferation and/or migration of neurons or even postnatal neurodevelopmental abnormalities (Pavone et al. 2017). The disorders associated with megalencephaly are classically defined into three groups: (a) idiopathic or benign disorders with no neurological impairments; (b) metabolic disorders with neurological impairment, such as metabolic leukoencephalopathies and lysosomal storage diseases; (c) anatomic disorders with neurological impairment, such as megalencephaly related syndromes achondroplasia, Bannayan-Riley-Ruvalcaba, and FG (Pavone et al. 2017). So far, miRNAs have been implicated only in syndromic cases with macrocephaly, but it is predictably only a matter of time that miRNA-involvement will be reported for syndromic cases with megalencephaly, as well (Koufaris et al. 2015; Papaioannou et al. 2013; Pavone et al. 2017).

The most common craniofacial congenital defect is cleft palate. It may occur on its own in about 70% of all cases, in combination with a cleft lip or as part of a

genetic syndrome (Mossey and Modell 2012). Minor perturbations of precise spatiotemporal regulation of gene expression during palatogenesis may result in cleft palate. Recent evidence indicates that miRNAs play key roles regulating a complex network of transcription factors both in normal palatogenesis and in cleft palate formation (Schoen et al. 2017). The involvement of miRNAs in mammalian cranial and palate development was first shown in knockout mice with deleted *Dicer* and *Dgcr8* genes (Graves and Zeng 2012) that encode two important proteins in miRNA biogenesis (Fig. 1). While homozygotic deletion of either gene in mice results in severe growth retardation and embryonic lethality, conditional knockout of miRNAs in the cranial neural crest-derived mesenchyme or oral ectoderm indicated that miRNAs are essential for palatogenesis in mice (Schoen et al. 2017). Microarray analysis has shown that a crucial role for normal palatogenesis plays a specific and regulated spatiotemporal pattern of several miRNAs, including miR-140, miR-200b, the miR-17-92 cluster, as well as at least 40 more miRNAs (Schoen et al. 2017).

5 Regulatory Role of MicroRNAs in Brain During Stress

Biological systems including the human brain are usually characterized by homeostasis defined as "the maintenance of a constant internal environment." Nevertheless, an inherent component of the natural world is a condition of stress which forces virtually all biological systems away from a physiological steady state. There are three different aspects of physiological stress that challenge the homeostasis of a living organism: environmental stress, intrinsic developmental stress, and aging stress (Kagias et al. 2012). Accumulated evidence has disclosed that miRNAs play an important role in response mechanisms to almost all types of stressful conditions and the maintenance of homeostasis (Kagias et al. 2012). The implication of miRNAs in biological mechanisms adjusting to stress is so old in evolutionary terms that exists also in plants (Lv et al. 2016).

In the current decade, several observations have revealed the involvement of certain neuronal miRNAs in various animals as a response to various stress conditions: miR-9 was associated with rat neuronal adaptation to alcohol stress by upregulating transcription of a voltage-activated potassium channel, which increases alcohol tolerance (Pietrzykowski et al. 2008); miR-7 was shown to significantly regulate stability under temperature stress during development of a sensory organ in *Drosophila* (Li et al. 2009); increased expression of miR-130 was observed in primary rat hippocampal neuronal cells under hypoxia leading to regulation of oxygen homeostasis by decreasing DDX6 protein levels and releasing HIF-1a mRNA from P-bodies (Saito et al. 2011); different panels of miRNAs were noticed to be upregulated in rat cell cultures of neurons or astrocytes as a response to ischemia (Ziu et al. 2011); and miR-71 was associated with the long-term survival of *C. elegans* during starvation and also associated with increased lifespan through the DAF-16/FOXO transcription factor in the nervous system of that nematode (Zhang et al. 2011; Boulias and Horvitz 2012).

In analogous ways to other animals, human neuronal circuits respond to several types of stress, including developmental stress, hypoxia, increased temperature, radiation effects on DNA, and aging (Kagias et al. 2012; Lang and Shi 2012). Human neuronal-responsive mechanisms include respectively spatiotemporal regulation of development, activation of latent respiratory pathway, heat shock response, DNA damage response, and some anti-aging repair mechanisms, although long-term deterioration seems inevitable. Neurons face stress by homeostasis regulation and increased expression of transcription factors, miRNAs, and other signaling molecules (Kagias et al. 2012). In addition, in order to cope with stressful conditions, the human brain uses the preconditioning approach, i.e., stored experience from prior exposure to different stresses, and the plasticity approach, i.e., remodeling of the synaptic and neuronal network (Kagias et al. 2012).

Several studies in mammals have observed that miRNAs regulate gene expression rapidly and reversibly as a dynamic response to stress conditions via neuronal plasticity, via the immune system, via the hypothalamus-pituitary-adrenal (HPA) cortex axis, etc. (Kagias et al. 2012; Dwivedi 2014; Hollins and Cairns 2016; Taouis 2016). For example, in hypothalamus, miR-7b may play a significant role in controlling homeostasis of osmolarity, while miR-103, miR-200a, and miR-488 possibly regulate energy homeostasis (Taouis 2016). The expression of certain miRNAs has been associated with prenatal stress conditions including maternal anxiety, maternal infection, alcohol consumption, etc. It has also been observed that miRNA expression may be epigenetically and transgenerationally affected by stress of parents, i.e., fetal hypoxia, pesticide vinclozolin in utero, paternal stress, etc. (Ma and Zhang 2015; Hollins and Cairns 2016). In addition, it is possible that alteration of expression of certain miRNAs as a response to oxidative stress may occur during craniofacial development (Driessen et al. 2013; Ma and Zhang 2015; Sakai and Trainor 2016).

Altered miRNA expression has been associated with acute psychological or physiological stress, environmental chemicals, etc. (Kagias et al. 2012; Dwivedi 2014; Hollins and Cairns 2016; Taouis 2016). Both acute and chronic stress may modify the morphology and function of several glial cell types in the brain (astrocytes, oligodendroglia, microglia) through involvement of miRNAs (Luarte et al. 2017). Any strong experience (skill learning or psychological trauma) may also alter the morphology and function of several glial cell types in the brain, while miRNAs are involved (Grossman et al. 2003). This change in miRNA expression may be epigenetically effected by stress (Gerhardt 2017; Lussier et al. 2017; Ma and Zhang 2015).

6 Conclusions

Accumulated evidence of recent years has revealed the key regulatory role of microRNAs in brain development and function:

(a) MiRNAs have various important regulatory functions in many biological processes by significantly repressing translation of target mRNAs through specific recognition of complimentary sequence.

(b) MiRNAs regulate various stages of neurodevelopment.
(c) Mutations affecting functions of miRNAs have been associated with neurodevelopmental and craniofacial syndromes.
(d) MiRNAs regulate homeostasis and cell response to stress.
(e) MiRNAs regulate response to stress in neurons and in glial cells.
(f) MiRNAs play a role in alteration of morphology and function of neurons and glial cells in response to experience stimuli.
(g) MiRNAs seem to be associated to psychopathology and neurodegeneration.

It appears that miRNAs are central regulatory players in the interface between genomic information, initial brain cell structure and function, environmental stimuli, adaptation of brain cell structure and function. Future research findings regarding the role of miRNAs in brain function are expected to be both exciting and unexpected, whereas our species may unravel the very essence of our existence.

Acknowledgments This paper is dedicated to the pioneer of RNA research Professor Harry F. Noller and the pioneer of stress research Professor George P. Chrousos.

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The Misfolding of Proteins

Agathi Argyrou

1 Introduction

Proteins are organic biomolecules crucial for the formation of the cell. They serve as a catalyst for biochemical reactions. Chemical reactions and the structural element inside the cell are supported and organized by proteins. Every gene contains the code for a unique protein. The three-dimensional structure of a protein has a special significance for the cell.

1.1 Protein Folding Definitions

Medical Definition Proteins are long polymers consisting of 20 amino acids. Proteins exist in the cells in a three-dimensional layout. This conformation allows proteins to exploit their low-energy status to perform their functions. As mentioned above, the folding of proteins is the next step in clarifying the genetic code.

Biophysics Definition The biophysical aspect of protein folding is based on the state of equilibrium that occurs under the influence of free energies in the protein population. The structure of the protein is determined by the folding structure. The thermodynamic stability that offers the greatest energy potential is the one that determines the state of equilibrium in each protein. In the Anfinsen theory, the protein receives the least possible free Gibbs energy under normal conditions. It is important to note that the equilibrium situation is likely to reflect the lowest energy available for the protein (National Research Council (US) 2005; Guarnera et al. 2009).

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1.2 Structure of Proteins

It is known that any gene present in cellular DNA is encoded by a single protein. The end product of the cell processes (replication, transcription, translation) is the protein. Proteins have a particularly important role as they participate in the biochemical processes of the cell. They consist of 20 different amino acids that are bonded together and then arranged with a variety of layouts in space. The sequence of amino acids has been shown to directly determine the refolding of the protein in space (Anfinsen theory). The arrangement of these linear, non-branch polymers has four levels of organization:

Level 1: Primary Structure
The amino acids of a polypeptide of a molecular subunit constitute its primary structure. The base pairs of the gene encoding a protein directly affect said protein.

Level 2: Secondary Structure
The normal folding and rotation of a single polypeptide chain in a variety of shapes constitute the secondary structure of a polypeptide. From weak bonds to polypeptides, hydrogen bonds and electrostatic bonds result in the secondary structure. The α-helix is one type of secondary structure, which is located in regions of many polypeptides. The number and extent occupied by α-helices vary between proteins. The second kind of secondary structure is the β-sheets. The sections of the β-pleated surface are folded in a zigzag shape and are arranged in parallel with each other, connected by hydrogen bonds. The proteins may contain both α-helix and β-sheets.

Level 3: Third-Party Structure
The way a single polypeptide chain wraps in space is the tertiary structure of proteins. Alternatively, when referring to the tertiary structure, we use the steroid conformation of the polypeptide. The chemical properties of the amino acid sequence are those that lead to the tertiary structure. The distribution of the R groups along the polypeptide chain is directly related to the three-dimensional structure.

Level 4: Quaternary Structure
The level of protein configuration in space concerns only the proteins that have more than one polypeptide chain (Alberts et al. 2002; Guarnera et al. 2009).

1.3 Function of Proteins

The protein population of a cell plays a key role in its efficient functioning. The function of proteins varies, and it includes aspects of cell function such as internal organization, maintenance, cell shape, etc. Also, they receive stimuli from outside of the cell and activate the cellular response. It is known that proteins take different shapes and sizes (small or large) (Alberts et al. 2002; Guarnera et al. 2009).

1.4 Energy and Kinetics of Proteins

The structure of the genes is one dimensional. This means that the linear nucleotide sequence encodes a linear amino acid sequence. It has been proven that translation has the ability to transfer the information from the linear genetic code to a three-dimensional protein structure. According to Anfinsen, all the information that a protein needs to fold into its three-dimensional structure is contained in the amino acid sequence.

The experiment conducted by Anfinsen involves the application of extreme chemical conditions for the folding of an enzyme. He used substances such as urea, which at high concentrations disturbs the peptide bonds between the molecules but also disturbs the disulfide bonds. The result was the folding of proteins. But when he switched to normal cellular conditions, he noticed that the amino acid structure automatically returned to its original form. He concluded that the physical configuration of the protein takes a particular shape because it is thermodynamically more stable in the intracellular environment (Anfinsen). Alternatively, it can be said that amino acids are packaged in such a way that the free energy of the molecule is minimal.

Amino acids have different side chains that give them different properties. The size differs, whether they are hydrophilic or hydrophobic, as well as the load of the side chains differs. In a well-packed protein, the hydrophobic amino acids are close together to protect themselves from the water molecules, while the hydrophilic ones are exposed to the surface of the protein and come into contact with cytoplasm water. This conformation helps to reduce the free energy of the protein.

The human body synthesizes about 30,000 different types of proteins. We know that newly synthesized proteins are properly folded with the appropriate minimum energy, so they can function properly.

The ability of the proteins to explore the possible layouts and take the appropriate structure within seconds is described as the Levinthal paradox. It suggests that the protein quickly folds because of the amino acids that interact locally, consequently reducing the exploration margin and at the same time taking the most stable structure that is feasible.

Most proteins follow the right path, but some proteins can fold into different ones with unwanted structures. In this case, there are specific protein chaperones that help these proteins achieve their normal structure.

2 Misfolding of Proteins

2.1 Chaperones

Proteins that have a particularly complex or unstable configuration may have difficulty achieving their natural state. In such cases, there are specialized protein "escorts," called chaperones that help them reach their natural working order.

Laskey observed that the nucleoplasm, a protein in the cell of the nucleus, has the ability to bind histones. Histones are proteins that interact with DNA and form structures such as nucleosomes. Laskey's discovery was that the nucleus functioned as a chaperone by monitoring the action of histones and blocking unfair interactions. However, the term "chaperones" was extended by Ellis to describe proteins that help other proteins to fold or create complex molecules. The importance of the final protein formation lies in the fact that the complex configuration determines the functional activity and is particularly important.

2.2 Protein Types

It is known that there are stable and unstable proteins. Proteins that receive their natural structure are synthesized in a healthy cell where usually the function is smooth, but our genome encodes proteins that have instability because they have the property of folding into alternative structures with different minimal energy. Some of these proteins are functional and useful for the cell, but the majority is useless or toxic. Chaperones will help unstable proteins fold in a desirable way, but even then, certain proteins will fold in an undesirable order.

Misfolding proteins are usually insoluble; they are also called toxic formulations and usually form linear or fibrous sets known as amyloid deposits. The way that the amino acids of a protein interact can have effective effects on this protein.

2.3 The Configuration of Proteins and the Concept of Misfolding

The protein arrangement follows a common pattern that is the α-helix. When a protein becomes toxic, an extensive structural change takes place, which is the β-sheet helix. The β-sheet helix configuration exists in many functional protein assemblies but is characteristic in amyloid.

The abnormal change of α-helix layout on a β-sheet helix exposes the hydrophobic sequences and promotes the aggregation of proteins. It is a fact that most of the time only the natural configuration of the proteins is produced in the cell during the life of a human being, but after the millions of copies, some may be toxic. The frequency of this change is more likely to be felt in molecules such as polyglutamine having repetitive amino acid sequences. One fact that requires particular attention is the ability of toxic devices to catalyze their change in the toxic situation. They are called infectious conformations. This activity expands until the cell is finally killed or disrupted. Prion proteins are a prime example of such activity (Fändrich and Dobson 2002; Reynaud 2010).

3 Computational Models for Protein Prediction

In order to predict protein folding, their stability, mobility, and structure are taken into account. So far, two models of protein prediction have been confirmed:

Diffusion-Collision Model: This model initially forms the core and subsequently the secondary structure, then the secondary structures collide with one another and are tightly packed together.

Nucleation-Condensation Model: In this model, the secondary and quaternary structures are made at the same time [6].

4 Neurodegenerative Diseases

Misfolding of proteins can cause neurodegenerative diseases. Common diseases due to the accumulation of unwanted protein foldings are amylopathies. The most prevalent is Alzheimer's; it affects a large proportion of the population. Alzheimer's is followed by Parkinson's and Huntington's disease. These diseases can be inherited or appear without a family history. Irrespective of the type of illness, the risk of getting it grows with age. The reason is that as people age the processes of synthesis and folding become more sensitive so then the misfolded proteins aggregate. The environmental factors that contribute to the development of neurodegenerative diseases are exposure to substances that affect mitochondria. Still, there are genetic factors that also play a role. We know that a person with a copy of the defective gene will develop the disease in light form, while a person with two copies will develop severe forms of the disease. Genetic factors may be particularly complex, since mutations of the same gene may have different risk levels (Choi et al. 2010; Anfinsen 1972).

5 Non-neurological Diseases

Accumulation of proteins causes diseases affecting the peripheral tissues of the central nervous system. Generally, the genes and protein products involved in such diseases are called amyloidogenic. Type 2 diabetes, inherited cataracts, some forms of atherosclerosis, and others are included in the said diseases. The common pattern in all these disorders is the accumulation of proteins due to misfolding. Accumulation can occur by chance, by mutations that make the protein unstable or from Prion proteins (Choi et al. 2010; Anfinsen 1972).

6 Conclusion

The final shape of a protein correlates directly with its function. Misfolding of proteins results in serious neurodegenerative and non-neurological diseases.

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In the original version of this book, Chapter 30 was inadvertently published by including one additional author Dinesh Kumar Bharatraj. His name has now been removed in this revised version of the book.

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Index

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