

KEY ENVIRONMENTAL STRESS BIOMARKER CANDIDATES FOR THE OPTIMISATION OF CHEMOTHERAPY TREATMENT OF LEUKAEMIA

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Abstract. The impact of fluctuations of environmental parameters such as oxygen and starvation on the evolution of leukaemia is analysed in the current review. These fluctuations may occur within a specific patient (in different organs) or across patients (individual cases of hypoglycaemia and hyperglycaemia). They can be experienced as stress stimuli by the cancerous population, leading to an alteration of cellular growth kinetics, metabolism and further resistance to chemotherapy. Therefore, it is of high importance to elucidate key mechanisms that affect the evolution of leukaemia under stress. Potential stress response mechanisms are discussed in this review. Moreover, appropriate cell biomarker candidates related to the environmental stress response and/or further resistance to chemotherapy are proposed. Quantification of these biomarkers can enable the combination of macroscopic kinetics with microscopic information, which is specific to individual patients and leads to the construction of detailed mathematical models for the optimisation of chemotherapy. Due to their nature, these models will be more accurate and precise (in comparison to available macroscopic/black box models) in the prediction of responses of individual patients to treatment, as they will incorporate microscopic genetic and/or metabolic information which is patient-specific.

Keywords: leukaemia, environmental stress, hypoxia, autophagy, chemotherapy, cellular biomarkers

1 Introduction

Leukaemia is a severe cancer of the haematopoietic system characterised by the incapability of blood progenitors to mature normally, leading to the accumulation of immature white blood cells, the so-called blasts, in the bone marrow (BM) (Beutler et al., 2001). Alternatively, this disease can be viewed as the formation of an abnormal haematopoietic tissue, the initiation of which is the result of the function of a small amount of Leukaemic Stem Cells (LSCs) (Passegué et al., 2003). Depending on the decrease of the normal blood cell populations, the disease symptoms can consist of fatigue, haemorrhage, infections and fever. The health condition of the patient depends on the amount of normal blood cells compared to the amount of those that are leukaemic. According to Cancer Research U.K. (2014), 8,616 people in the U.K. were diagnosed with leukaemia in 2011, with 4,503 deaths from this disease occurring in 2010. Moreover, 82,300 new cases of leukaemia were diagnosed in 2012 within the European Union. Leukaemia can be divided into different types, depending on the haematopoietic lineage in which the proliferation disorder occurs. More specifically, *myeloid leukaemia* occurs in the myeloid lineage and *lymphocytic leukaemia* occurs in the lymphoid lineage. Depending on the speed of evolution of the disease, leukaemia can be divided into *acute*, whereby the number of blasts increases rapidly, leading to a faster disease evolution, and *chronic* i.e. the progress of the disease is slower as there is production of partly mature but not fully functional white blood cells. Based on this categorisation, the following four general types of leukaemia can be found: *Acute Myeloid Leukaemia (AML)*, *Chronic Myeloid Leukaemia (CML)*, *Acute Lymphocytic Leukaemia (ALL)* and *Chronic Lymphocytic Leukaemia (CLL)*.

Acute Myeloid Leukaemia (AML) is one of the most aggressive types of leukaemia. According to Cancer Research U.K. (2014), approximately 2,921 cases of AML occurred in 2011 in the U.K.

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Received: 24.07.2014 - Revised: 25.09.2014 - Accepted: 14.10.2014
- Published: 17.12.2014

AML is a type of leukaemia which is characterised by an accumulation of immature blasts in the myeloid lineage and, as a consequence, insufficient blood cell production. These blasts have low proliferation capability. However, a small population of cells with higher proliferation capacity and self-renewal potential, the LSCs, are the key component for maintenance of the disease (Bonnet & Dick, 1997). AML is usually the result of somatic mutations in either a pluripotent haematopoietic stem cell or in a slightly differentiated progenitor cell, leading to a deregulation and/or inhibition of normal haematopoiesis due to space restrictions and inhibitory and clonal factors specific to the disease (Lowenberg, Downing & Burnett, 1999; Lichtman, 2001; Panoskaltsis, Reid & Knight, 2003).

Chronic Myeloid Leukaemia (CML), alternatively known as Chronic Granulocytic Leukaemia (CGL), is a stem cell disease - of the myeloid lineage - characterised by an exaggerated granulocytosis, anaemia, granulocytic immaturity, basophilia and splenomegaly. There is an extreme cellular accumulation in the BM. From a genetic point of view, 90% of cases show a reciprocal translocation between chromosomes 9 and 22, the so-called *Philadelphia (Ph) chromosome translocation*. In many cases, the disease can progress into a very high speed phase, resembling AML (Lichtman, 2001). CML is a rather rare cancer of the blood. According to Cancer Research U.K. (2014), 675 patients were diagnosed with CML in 2011 in the U.K.

Acute Lymphocytic Leukaemia (ALL) (alternatively known as Acute Lymphoblastic Leukaemia) is a type of leukaemia which mostly affects children. Approximately 4,000 cases of ALL are reported on an annual basis in the U.S.A., of which two thirds are children (Pui & Evans, 2006). According to Cancer Research U.K. (2014), 654 cases of ALL were diagnosed in 2011 in the U.K. ALL is a leukaemia in which the cellular deregulation occurs in the lymphoid lineage of the haematopoietic system. More specifically, an abnormality in the lymphocytes takes place, leading to an accumulation of non-functional white blood cells (blasts) in the BM as a result of abnormal cellular proliferation, blocking of cellular differentiation and increased resistance to apoptosis i.e. cell death (Pui, 2009).

Chronic Lymphocytic Leukaemia (CLL) is a type of leukaemia which is characterised by an accumulation of immature lymphocytes (of the B-cell lineage) in the human BM, peripheral blood and the lymphoid tissues (Kipps, 2001). The progress of CLL is much slower than that of ALL and it affects older adults i.e. people over 51 years of age. According to the National Health Service (NHS) U.K., about 2,400 people in the U.K. are diagnosed with CLL on an annual basis and the disease affects 2.7 persons per 100,000 in the U.S.A. (Kipps et al., 2001). According to Cancer Research U.K. (2014), 3,233 people were diagnosed with CLL in the U.K. in 2011.

The most common treatment for all types of leukaemia is *chemotherapy* (Cancer Research U.K., 2014). A variety of chemotherapy drugs are generally used, depending on the type of leukaemia. The most commonly used drugs for treatment of leukaemia are *cytarabine* (cytosine arabinoside or ara-C), which is an antimetabolite targeting to block the DNA/RNA replication by attacking the cells that are in the S-phase of the cell cycle, and the *anthracycline* drugs (such as fludarabine) which attack cells that are in the G1-phase of the cell cycle (American Cancer Society, 2013). Current chemotherapy treatment protocols are designed on the basis of pre-clinical animal experiments and empirical clinical trials, as well as the acquired experience of specialist physicians. The design parameters for these protocols consist of the patient BM aspirate examination (blasts percentage, immunophenotype, cytogenetic and molecular analysis) and physiological patient characteristics (height, weight) for the normalisation of the dose applied on the body surface area (BSA).

2 Towards a Personalised Chemotherapy Treatment: integrating *in vivo*, *in vitro* and *in silico* knowledge

As mentioned in the previous section, traditional clinical diagnosis and further treatment of leukaemia focuses on each patient's clinical symptoms and signs, as well as characteristics (such as sex and family history) and laboratory imaging evaluation. This process is a reactive approach to the disease, initiating after the disease symptoms appear. Moreover, in the past, drug development by pharmaceutical industries was based on empirical observations. However, nowadays, with significant progress constantly taking place in the areas of genomics, proteomics and metabolomics, it is believed that specific information related to the genetic characteristics and proteomic and metabolomic profile of an individual patient could be used for tailored medical care, or *personalised medicine*. Some of the challenges in the delivery of personalised medicine lie in (a) the *fidelity and validity of current experimental, i.e. in vitro, systems* used to investigate human disease, (b) the *integration of patient-specific and disease-specific datasets i.e. in silico* and (c) the application of these models in clinical practice to identify *simple targets and more efficient, yet less toxic, therapies* for a specific condition, i.e. *in vivo*. **Therefore, 'closing the loop' from in vivo to in vitro and in silico is a first step towards optimisation and consequently, personalisation of chemotherapy treatment.** Moving in that direction, an appropriate platform has been developed at the Centre for Process Systems Engineering at Imperial College London, consisting of three main blocks i.e. *in vivo-in vitro-in silico* which, upon completion, will allow the prediction of optimal drug dosages for each individual, based on specific (personalised) characteristics (Velliou et al., 2014a). The main advantage of this platform is the dynamic interaction of its three robust building blocks. As opposed to existing computational or experimental studies, our platform consists of the interaction of both: data from our *in vitro* platform are used as an input for the development of mathematical tools. These mathematical tools are further validated with patient data. Figure 1 describes the different parts of the integrated platform that is currently being designed at the Centre for Process Systems Engineering at Imperial College London (Velliou et al., 2014a). Development and optimisation of the *in vitro* and *in silico* blocks will eventually bridge the gap between laboratory experimentation, mathematical modelling and *in vivo* optimal chemotherapy treatment for a specific individual.

A very innovative *in vitro* tool has been developed and is being used for the *ex vivo* long-term patient sample cultivation at the Centre for Process Systems Engineering (Mortera-Blanco et al., 2010). This tool is a three-dimensional highly porous polyurethane-based matrix coated with collagen type I, therefore mimicking the porosity as well as the extracellular matrix present in the human BM. This three-dimensional system allows the long-term (up to 6 weeks) cultivation of patient cells in an environment that mimics the *in vivo* one.

An *in silico* tool, or mathematical model, which enables the estimation/design of chemotherapy protocols for a specific individual based on the personalisation of the drug schedule has been developed by Pefani et al. (2013). This model consists of a Pharmacokinetic (PK) and a Pharmacodynamic (PD) part. The PK part consists of mass balances of the drug distribution in different body organs such as the heart, the liver and the BM. The PD part calculates the effect of the drug on both normal and abnormal cells in the BM, which is the location of the tumour. The model input is the treatment inflow, which is calculated based on the drug administration route and the injection rate. The latter is a function of body characteristics such as height and weight.

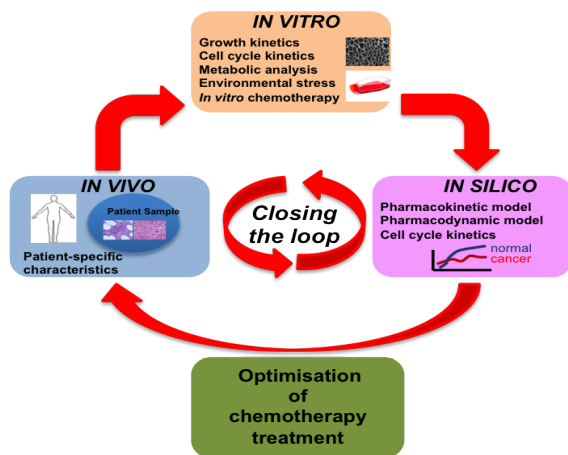


Figure 1: Towards optimisation/personalisation of chemotherapy for leukaemia treatment (adapted from Velliou et al., 2014a).

For cases in which the initial drug dose proposed clinically failed to eliminate the cancerous population, an optimisation process was followed in order to re-determine the optimal drug dose on the basis of clinically relevant constraints. Remarkably, the model output treatments suggest drug dosages similar to those used clinically but, for example, the scheduling is different. The optimised treatment would have much better outcomes with respect to the elimination of the tumour (Pefani et al., 2014). Furthermore, a more detailed cell cycle model based on the work of García-Münzer et al. (2013, 2014) has been developed, consisting of a multi-stage population balance model (MS-PBM) (Fuentes-Garí et al., 2014). This model is distributed on cell cycle progress-related events i.e. cyclin or DNA expression (different cyclins are produced at different phases of the cell cycle and DNA is produced at the S-phase of the cell cycle). Cell cycle kinetics are tracked not only across different cell cycle phases but also within each phase, thus allowing a more efficient monitoring of the evolution of the leukaemic population throughout the cell cycle. The latter is of high importance for chemotherapy optimisation since, as mentioned previously, most chemotherapeutic drugs are cell cycle-specific, targeting cells that are present in specific phases of the cell cycle. This PBM has been successfully validated with experimental data (Fuentes-Garí, 2014).

Moving towards the delivery of optimal chemotherapy protocols for each individual, it is important to understand and further incorporate in the mathematical models the impact of environmental stress. More specifically, the effect of alterations of environmental parameters (oxygen, temperature, nutrients) on the evolution and further response of leukaemia to chemotherapy have to be incorporated (Velliou et al., 2014a, b). Fluctuations of those parameters can take place either across different patients or within the same patient. For example, glucose levels in the blood can differ across a diabetic, a hypoglycaemic and a normal patient. During chemotherapy treatment, it is possible that a patient may have fever; high body temperature can be experienced as a temperature shock by the cancerous population. Oxygen levels can fluctuate in different body compartments e.g. the BM and peripheral blood. The impact of those environmental factors on the leukaemic evolution is further discussed in the following section.

2.1 Environmental Stress and Leukaemia

Fluctuations in the micro-environmental conditions of the BM i.e. oxygen concentration, composition and concentration of nu-

trients such as glucose, cytokines or other growth factors, may be experienced as an environmental stress. As a consequence, these fluctuations can highly affect the normal and abnormal haematopoietic proliferation, metabolic activity as well as drug resistance and further evolution. For example, several researchers have shown that oxidative stress i.e. the increase in the concentration of Reactive Oxygen Species (ROS), leads to activation of survival pathways and is a key factor that promotes progression of cancerous stem cells as well as resistance to chemotherapy (see as examples Adbal Dayem et al., 2010; Fruehauf & Mayskens, 2013; Liu et al., 2009; Lyu et al., 2008).

Especially in the case of the abnormal haematopoietic situation of leukaemia, alterations of the oxygen and glucose concentration in the different body compartments e.g. in the BM and the peripheral blood or the liver, and, on the other hand, between patients, i.e. individual cases of hypoglycaemia or hyperglycaemia, may lead to a different stress adaptation of the leukaemic population. The latter will most likely affect the cancer growth and inactivation kinetics, as well as the response to a chemotherapeutic drug *in vivo*.

A variety of research studies have revealed the strong relation between resistance i.e. longer survival and increased proliferation of haematopoietic and/or leukaemic cells and/or resistance to chemotherapy, with (1) **oxygen** or (2) **starvation stress** *in vitro* and/or *in vivo*. For example, Fecteau et al. (2013) observed an increased *in vitro* survival of cells from BM aspirates of patients with CLL in 5% O₂ compared to 20% O₂. This increased survival under hypoxic conditions was a result of the Mesenchymal Stromal Cells (MSCs') increased proliferation and the production of soluble pro-survival factors i.e. CXCL12. Interactions between CLL and (increased) MSCs lead to enhanced CLL resistance.

Wilkinson, Tome and Briehl (2012) state that chronic oxidative stress may contribute to increased resistance of lymphoma patients to chemotherapy. This is due to the fact that oxidative stress leads to alteration of the mitochondria i.e. release of inter-membrane proteins, which leads to an increased permeabilisation of the outer mitochondrial membrane and further resistance to apoptosis.

Zhou et al. (2010) pointed out the possible relation between the relapse of AML and increased oxidative stress *in vivo*. Specifically, parameters related to oxidative stress e.g. activities of adenosine deaminase and xanthine oxidase, antioxidant capacity (T-AOC), levels of human thioredoxin (TRX) and indoleamine 2,3-dioxygenase, as well as expression of specific genes related to oxidative stress, were monitored in patients with AML for a time period between a primary and a relapsed status. Low T-AOC and up-regulated TRX expression led to a relapse of the disease, indicating a strong correlation between oxidative stress and AML development and relapse.

Lodi et al. (2011) observed that hypoxia is a key factor that affects metabolic activity i.e. adaptation of phospholytic and glycolytic metabolism, and evolution of KG1a and K562 leukaemic cell lines. Mitochondrial respiration remained unaltered for both cell lines, indicating the ability of these leukaemic cell lines to increase their resistance under oxidative stress.

Giuntoli et al. (2011) studied the effect of the level of glucose on the growth and proliferation of K562 cell lines, U937 cell lines or primary CML cells under hypoxic conditions i.e. 0.1% O₂, as well as under normoxia i.e. 21% O₂. Although in general, slower growth was observed for lower glucose concentrations in hypoxia as well as in normoxia, glucose shortage in hypoxia led to increased size of the leukaemic population compared to the normal haematopoietic one.

Herst et al. (2011) have pointed out a possible relation between the level of glycolytic metabolism of AML blasts and resistance to chemotherapy. Analysis of 26 BM aspirates showed that AML cells with higher glucose consumption were more tolerant to *in vitro* apoptosis caused by ATRA and/or ATO drug.

All previously mentioned studies point out that there is a very strong relation between oxidative or starvation stress and the leukaemic evolution i.e. growth, kinetics or resistance to chemotherapy. Therefore, the possible oxidative and starvation cellular stress should be systematically followed and/or quantified experimentally (*in vitro*) and eventually further incorporated in mathematical models (*in silico*). Efficient monitoring of the level of oxidative and metabolic stress and further adaptation can take place via the selection and further quantification of specific biomarker(s) i.e. intracellular molecules, the expression and/or concentration of which may alter depending on the fluctuation of oxygen and glucose in the system. In order to select 'stress' biomarker(s), an in-depth investigation of possible mechanisms of oxidative and starvation stress cellular responses is needed (Vellou et al., 2014a). Hereafter, autophagy, a crucial mechanism which is activated in the absence of nutrients and low oxygen levels, is described and potential biomarker molecules related to the cell stress response are summarised.

2.2 (Macro-)Autophagy: the cellular response to metabolic stress and hypoxia

Autophagy is a cellular mechanism which aims at the maintenance of homeostasis of a normal cell, via degradation of organelles and cellular components by the lysosomes (Banerji & Gibson, 2012; Choi, Ryter & Levine, 2013; Kongara & Karantza, 2012; Levine & Kroemer, 2008; Lozy & Karantza, 2012). Autophagy is activated as a result of exposure to a stress factor and most probably in the absence of nutrients i.e. glucose starvation, as well under hypoxic conditions (Lum et al., 2005; Scherz-Shouval et al., 2007). Degradation of damaged mitochondria as well as aggregation of proteins and other cellular organelles via the autophagic mechanism protect the cells from apoptotic signalling (Jin & White, 2007; Moore, Allen & Sommerfield, 2006). Autophagy may be important in the regulation of cancer development and in the determination of the response of cancer cells to chemotherapy (Degenhardt et al., 2006; Hippest, O'Toole & Thorburn, 2006; Wilkinson, Tome & Briehl, 2012).

Several researchers have shown that autophagy plays a crucial role in maintenance of normal haematopoiesis and function of Haematopoietic Stem Cells (HSCs) (Kundu et al., 2008; Warr et al., 2013). Others have shown that autophagy may lead to an increased resistance to chemotherapy and survival of several cancers, including haematological malignancies. For example, Mortensen, Watchon & Simon (2011) observed that loss of autophagy resulted in loss of normal function of murine HSCs, leading to the expansion of a progenitor cell population in the BM which has as a consequence a severe myeloproliferation. This myeloproliferation strongly resembled human AML, indicating a possible link between maintenance of autophagy and avoidance of malignancies such as AML. Wallington-Beddoe et al. (2011) showed that activation of autophagy (induced by the FTY720 drug) leads to increased survival of ALL cells.

2.3 Biomarker Candidates for the Prediction of the Disease Evolution under Stress

2.3.1 (Autophagic) Biomarker candidates

For 'switching on' and maintenance of the autophagic response, a variety of genes are over-expressed and proteins, mainly kinases, are activated and/or de-activated, depending on whether they have a positive or negative regulatory role in autophagy. Therefore, in order to monitor autophagy in an *in vitro* system, many different biomarkers of genomic and/or protein level can be considered (see Table 1 for an overview).

Table 1. Stress biomarker candidates for leukaemia

Biomarker	Genomic	Protein
LKB1-AMPK		+
AMPK		+
ULK1		+
P53	+	
PTEN	+	
Atg7	+	
HIF		+
mTOR kinase		+
FOXO3A	+	
FUMH		+

A possible candidate-biomarker is the serine threonine kinase **ULK1**, which is a key initiator of autophagy. Its activation is essential for clearance of cellular mitochondria and ribosomes (Kundu et al., 2008). This kinase is activated under growth factor deprivation and leads to activation of the glycogen synthesis kinase 3 (GSK-3). The latter phosphorylates the acetyltransferase TIP60 which in turn acetylates and activates ULK1 (Lin et al., 2012). Another crucial kinase which is directly related to autophagy is the **mTOR kinase** which, under normal nutrient concentrations, binds and phosphorylates the ULK1, therefore suppressing autophagy. Under nutrient deprivation, the activation of the **P53 gene** enables activation of the ULK1 and autophagy (Feng et al., 2005). Another possible candidate biomarker is the **Atg7** gene, which is an essential gene for activation of autophagy and further regulation of HSC maintenance (Kundu et al., 2008; Mortensen et al., 2011; Mortensen & Simon, 2010). It has been shown that deleting this gene in murine HSC leads to death as a result of an accumulation of mitochondria and ROS, increased proliferation and DNA damage (Mortensen et al., 2011; Mortensen & Simon, 2010). **FOXO3A** can also be a possible biomarker candidate. It has been found to have a critical role in autophagy induced in mice in a cytokine-free environment (Warr et al., 2013a).

Several researchers have reported a tumour suppressor role of autophagy. More specifically, it has been shown that activation of the AMPK pathway has a suppressor role in AML. **AMPK** is a protein kinase which regulates protein and energy homeostasis at an intracellular level via autophagic recycling of intracellular components. Practically, AMPK acts as a metabolic sensor of alteration of the intracellular lipid composition and restores energy by maintaining the balance ATP vs AMP, through the LKB1-AMPK activation. **LKB1-AMPK** is a tumour suppressor in AML (Green et al., 2010). Activation of the P53/PTEN genes has been shown to have a tumour suppressive role as these allow initiation of autophagy via inhibition of the activity of the mTOR kinase (Feng et al., 2005).

2.3.2 (Non-autophagic) Stress biomarker candidates

A biomarker related to oxidative stress is the Hypoxia Induced Factor, HIF, which is the central regulator of oxygen homeostasis. More specifically, HIF1- α regulator is overexpressed in many cancer types and **HIF proteins** mediate cell adaptation to hypoxia (Birner et al., 2000; Talks et al., 2000; Warr & Passagué, 2013b; Zhong et al., 1999).

A possible cell biomarker related to starvation stress is the metabolic enzyme **fumarate hydratase (FUMH)** which converts fumarate to malate. This enzyme highly controls the intracellular levels of fumarate, with silencing of the expression of FUMH leading to fumarate intracellular accumulation (Ratcliffe, 2007).

3 Conclusion

The impact of environmental stresses such as oxidative and starvation stress on the leukaemic cell evolution i.e. growth and/or resistance to drugs, has been discussed in depth. It is clear that the effect of these factors on the cancer evolution should be taken into consideration for the accurate prediction of optimal chemotherapy protocols for specific individuals. Furthermore, several cellular components that are related to the cell response to the stress have been analysed. Quantitative information on these key biomarkers could serve as an appropriate input for the construction of more detailed predictive models for the *in silico* description of the leukaemic evolution. More specifically, from a detection/quantification point of view and as a first step, application of techniques such as next generation sequencing (NGS) or deep sequencing will enable the selection of biomarker candidates that could be linked to *in vitro* drug resistance. These data will enable an accurate model development. As a second step, screening of patients' genetic profile through NGS in BM biopsies, will enable a cross-link of *in vivo* with *in vitro* biomarkers. Consequently, the developed *in silico* tool will indicate appropriate and different chemotherapy treatment options in a closely personalised manner.

Overall, quantification of appropriate (intra-) cellular biomarkers that are related to the leukaemic *in vitro* kinetics can enable the combination of macroscopic kinetics with microscopic information, leading to the construction of more detailed models. The (micro) cellular information that these models account for will allow a much more accurate prediction of the cancer response, eventually leading to delivery of personalised chemotherapy protocols for the treatment of patients with leukaemia.

This review article was compiled as part of the invited seminar by Dr E.G. Velliou titled 'A framework for the personalisation/optimisation of the treatment of Acute Myeloid Leukaemia (AML)', which was held at the Faculty of Health Sciences, University of Malta on 4 April 2014.

4 Funding

This work is supported by ERC-BioBlood (no. 340719), ERC-Mobile Project (no. 226462), the EU 7th Framework Programme [MULTIMOD Project FP7/2007-2013, no. 238013] and the Richard Thomas Leukaemia Research Fund. Ruth Misener is further thankful for a Royal Academy of Engineering Research Fellowship.

5 Conflicts of Interest

The authors report no conflicts of interest.

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